UNIVERSIDADE FEDERAL DO RIO GRANDE – FURG
INSTITUTO DE OCEANOGRAFIA
PROGRAMA DE PÓS-GRADUAÇÃO EM AQUICULTURA

ADIÇÃO DE CLORETO DE SÓDIO OU CARBONATO DE CÁLCIO NA ÁGUA COMO ALTERNATIVAS PARA MINIMIZAR A TOXICIDADE DO NITRITO EM JUVENIS DE PACU *Piaractus mesopotamicus*

GABRIEL CARDOSO NEVES

Rio Grande, 2019

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Tese apresentada como parte dos requisitos para obtenção do grau de Doutor em Aquicultura pelo Programa de Pós-Graduação em Aquicultura da Universidade Federal do Rio Grande - FURG.

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“Talvez não tenha conseguido fazer o melhor, mas lutei para que o melhor fosse feito. Não sou o que deveria ser, mas graças a Deus, não sou o que era antes”

Martin Luther King Jr

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LISTA DE SIGLAS E ABREVIATURAS

% – Por cento

µL – Microlitros

ACAP – Capacidade antioxidante

AOB – Bactérias amônia oxidantes

ATP – Adenosina trifosfato

Ca^{2+} – Cálcio

$CaCO_3$ – Carbonato de cálcio

CAT – Catalase

Cl^- – Cloreto

cm – Centímetros

CO_3^{-2} – Carbonato

DNA – Ácido desoxirribonucleico

EDTA – Ácido etilenodiamino tetra-acético

ERN – Espécies reativas ao nitrogênio

ERO – Espécies reativas ao oxigênio

Fe – Ferro

g – Grama

GP – Ganho de peso;

GST – Glutationa-S-transferase

H^+ – Ìon Hidrogênio

H_2O – Molécula de água

Hb – Hemoglobina

HCO_3^- – Bicarbonato

HHI – Hipotálamo-hipófise-interrenal

Hm – Hemácias

HNO_2 – Ácido nítrico não ionizado

Ht – Hematócrito

K^+ – Potássio

L – Litro

ln – Logaritmo natural

LPO – Lipoperoxidação

MCHC – Concentração de hemoglobina corpuscular média

MCH – Hemoglobina corpuscular média

MCV – Volume corpuscular médio

MDA – Malondialdeído

MetHb – metahemoglobina

mg – miligrama

$mg.L^{-1}$ – Miligrama por litro

Mg^{2+} – Magnésio

mm – Milímetro

N – Nitrogênio

Na^{+} – Sódio

NaCl – Cloreto de sódio

$NaNO_2$ –Nitrito de sódio

NH_3 – Amônia não ionizada

NH_4^+ – Amônio

NO_2^- – Nitrito

NO_3^- – Nitrato

O_2 – Oxigênio

ºC – Grau Celsius

PB – Proteína bruta

pH – Potencial hidrogeniônico

RAS – Sistema de recirculação de água

SO_4^2 – Sulfato

SOD – Superóxido dismutase

TCA – Taxa de conversão alimentar

TBARS - Substâncias reativas ao ácido tiobarbitúrico

TCE – Taxa de crescimento específico

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RESUMO

O pacu *Piaractus mesopotamicus* é uma espécie com grande potencial para a criação em sistemas intensivos. Entretanto, o aumento da densidade de estocagem pode levar ao acúmulo de compostos nitrogenados tóxicos como nitrito. Certas estratégias de manejo como o aumento das concentrações de cloreto de sódio e cálcio na água podem reduzir a toxicidade deste composto para os peixes. Sendo assim, o objetivo da presente tese foi verificar se a adição de cloreto de sódio (1 g $NaCl.L^{-1}$) ou carbonato de cálcio (150 mg $CaCO_3.L^{-1}$) na água reduz a toxicidade do nitrito para juvenis de pacu utilizando parâmetros zootécnicos, sanguíneos e de estresse oxidativo como biomarcadores. Foram demonstrados diferentes efeitos, tempo e concentração dependentes, nos peixes expostos ao nitrito, além de variáreis de respostas de estresse oxidativo entre os diferentes tecidos avaliados. O nitrito induziu o aumento da atividade da enzima glutationa-S-transferase e nos níveis de peroxidação lipídica (LPO), além de reduzir a capacidade antioxidante total (ACAP). Dentre os parâmetros sanguíneos, ocorreram alterações na contagem total de eritrócitos, hematócrito, hemoglobina total e nos índices hematimétricos. O aumento da taxa de conversão alimentar (TCA) também foi atribuído como um dos efeitos relacionados ao nitrito. O uso do NaCl foi efetivo na prevenção da LPO hepática e a redução da ACAP muscular. Já a adição do $CaCO_3$ evitou o aumento nos níves de LPO nas brânquias e no músculo, e o comprometimento da TCA. Ambos os compostos também atenuam alterações nos parâmetros sanguíneos decorrentes de exposições de curto período. Concluindo, a adição de NaCl ou $CaCO_3$ nas quantidades propostas foram capazes de reduzir a toxicidade do nitrito em juvenis de pacu, podendo ser uma estratégia interessante de manejo da qualidade da água, especialmente em sistemas de produção intensivos.

Palavras-chave: composto nitrogenado; dezempenho zootécnico; peroxidação lipídica; parâmetros sanguíneos

ABSTRACT

Pacu *Piaractus mesopotamicus* is a species with great potential for intensive breeding systems. However, increased stocking density may lead to the accumulation of toxic nitrogen compounds such as nitrite. Management strategies such as increased sodium chloride and calcium concentrations in the water can reduce the toxicity of this compound to fish. Thus, the aim of the present thesis was to verify if the addition of sodium chloride (1 g $NaCl.L^{-1}$) or calcium carbonate (150 mg $CaCO_3.L^{-1}$) in the water reduces nitrite toxicity to pacu juveniles using zootechnical, blood and oxidative stress parameters as biomarkers. Different time and concentration dependent effects were demonstrated on fish exposed to nitrite. In addition, oxidative stress responses were tissue specific. Nitrite induced increased glutathione-S-transferase activity and lipid peroxidation (LPO) levels and reduced total antioxidant capacity (ACAP). Among the blood parameters, changes in total erythrocyte count, hematocrit, total hemoglobin and hematimetric indices were reported. Increased feed conversion rate (FCR) was also attributed as one of the effects related to nitrite. The use of NaCl was effective in preventing hepatic LPO and reducing muscle ACAP. The addition of $CaCO_3$ prevented the increase in LPO levels in the gills and muscle, and the impairment of FCR. Both compounds also had a beneficial effect in attenuating changes in blood parameters resulting from short-term nitrite exposures. In conclusion, the addition of NaCl or $CaCO_3$ in the proposed quantities was able to reduce nitrite toxicity in pacu juveniles, and may be an interesting strategy for water quality management, especially in intensive production systems.

Keywords: nitrogen compound; zootechnical performance; lipid peroxidation; blood parameter

1. INTRODUÇÃO GERAL

A produção aquícola em 2016 foi de 110 milhões de toneladas, sendo o setor de produção de alimentos que mais cresce, contribuindo cada vez mais para o fornecimento de pescado para o consumo humano. Estima-se que em 2030 a aquicultura será responsável por mais de 60% da produção mundial de pescado para consumo humano (FAO, 2018).

O Brasil contempla boas condições para o desenvolvimento da aquicultura de forma sustentável, e o pacu, *Piaractus mesopotamicus*, um dos peixes de água doce nativos mais importantes da América do Sul (Schenone et al., 2011) e a quinta espécie mais produzida no Brasil, possui várias características favoráveis para o cultivo, evidenciando um grande potencial de produção em sistemas intensivos de criação (Urbinati et al., 2013).

A produção intensiva, no entanto, pode levar ao aumento de compostos nitrogenados, entre eles o nitrito (NO_2^-), que pode prejudicar o rendimento produtivo durante a criação (Baldwin et al., 2010). Por isso, é indispensável conhecer os limites de tolerância de cada espécie em relação ao NO_2^- em sistemas de criação intensiva (Campos et al., 2012).

Em sistemas de criação com baixa densidade, a concentração de NO_2^- é geralmente baixa (0,03 - 0,1 mg $N\text{-}NO_2^-.L^{-1}$) (Sipaúba-Tavares et al., 1999), no entanto, em alguns sistemas, como nos sistemas de recirculação de água (RAS) a produção em altas densidades de estocagem, associada a presença de bactérias nitrificantes, pode favorecer o acúmulo de NO_2^-, trazendo vários efeitos negativos para o cultivo, como a redução do desempenho zootécnico, indução ao estresse, e até a morte dos animais (Jensen, 2003; Hargreaves, 1998; Lewis and Morris, 1986).

O NO_2^- é encontrado em ecossistemas como um componente natural do ciclo do nitrogênio (Jensen, 2003) e existe na água sob duas formas, o ácido nítrico (HNO_2) e o nitrito ionizado (NO_2^-). O ácido nítrico se difunde livremente nas brânquias, enquanto o NO_2^- é transportado através da membrana branquial pelo co-transportador Cl^-/HCO_3^-, competindo com

o Cl^-. O NO_2^- normalmente é descrito como um fator limitante para a sobrevivência e o crescimento dos organismos aquáticos (Ostrensky et al., 1991; Tomasso, 1994; Bianchini et al., 1996) mesmo em baixas concentrações, devido a sua toxicidade (Baldisserotto, 2013) sendo, portanto, de extrema importância o seu monitoramento e controle durante a criação desses animais.

O principal e mais descrito mecanismo de toxicidade do NO_2^- é a oxidação do Fe^{2+} da hemoglobina em Fe^{3+}, resultando na formação de metahemoglobina (metHb), que é incapaz de se ligar e transportar oxigênio (Tilak et al., 2007; Lefevre et al., 2011) podendo resultar em hipóxia tecidual ou anóxia (Jensen, 2003; Tilak et al., 2007; Arana, 2010; Baldisserotto, 2013).

Além disso, a exposição ao NO_2^- pode induzir uma grande variedade de outros efeitos toxicológicos nos peixes, como alteração do metabolismo energético, regulação hormonal, desequilíbrio iônico (Jensen, 2003), indução do estresse oxidativo (Maltez et al., 2018), afetando a homeostase do organismo, levando a uma redução na taxa de crescimento (Ciji et al., 2014), aumentando a vulnerabilidade à doenças (Jia et al., 2016) e até a morte (Wuertz et al., 2013).

A absorção de NO_2^- em peixes de água doce ocorre principalmente através da membrana branquial e está relacionada às taxas de captação branquial de Cl^- (Jensen, 2003) porque o NO_2^- substitui o Cl^- no cotransportador Cl^-/HCO_3^- (Tomasso & Grosell, 2005). Sendo assim, o aumento da concentração de Cl^- na água pode reduzir as taxas de absorção e consequentemente a toxicidade do NO_2^- (Yanbo et al., 2006; Boudreaux et al., 2007; Kroupova et al., 2008), pois aumenta a competição pelo mesmo cotransportador (Huey et al., 1980; Arana, 2010). A exposição de peixes ao NO_2^- e a relação com diferentes concentrações de salinidade foram relatadas em alguns estudos (Weirich & Riche, 2006a, b; Sampaio et al., 2007; Costa et al., 2008).

A dureza da água é de suma importância para a aquicultura, e sua interação com diferentes parâmetros é relatada por diversos autores (Wurts & Durborow 1992; Townsend &

Baldisserotto, 2001; Baldisserotto, 2011), pode afetar o desempenho de várias espécies (Silva et al., 2005) e é determinada pelo conteúdo de sais de Ca^{2+} e Mg^{2+} (Wuertz, 2013). Os vertebrados são dependentes do Ca^{2+} para formação do esqueleto, coagulação sanguínea e outras funções celulares (Lovell, 1989; Coote et al., 1996). Além disso, o Ca^{2+} tem papel fundamental na regulação iônica por reduzir a permeabilidade das membranas biológicas e, consequentemente, o fluxo difusivo de íons para o meio aquático (Gonzal et al., 1987; Gonzalez, 1996; Wood and Mcdonald, 1988**;** Tomasso et al., 1980; Baldisserotto, 2013), melhorando a resistência dos peixes a substâncias tóxicas presentes na água (Perschbacher & Wurts, 1999), como o NO_2^-.

A entrada do Ca^{2+} da água se dá através de difusão pela formação do gradiente osmótico da bomba Na^+/Ca^{2+} (Flik & Verbost, 1993). A captação através das brânquias é um processo ativo mais ou menos contínuo e relativamente dependente dos níveis de Ca^{2+} na água (Flik et al., 1993). Vários estudos já foram feitos avaliando níveis ideais de dureza para diferentes espécies e estágios de vida (Silva et al., 2005). A faixa desejável de dureza da água para peixes de água doce é de 50 a 150 mg $CaCO_3.L^{-1}$ (Stone & Thomforde, 2004; Kasiri et al., 2011), valores inferiores a 20 mg $CaCO_3.L^{-1}$ causam estresse (Bhatnagar & Devi, 2013).

A toxicidade do NO_2^- é específica e muito pouco estudada para o pacu (Moraes et al., 2006). Além disso, a avaliação de estratégias de manejo de qualidade de água como a adição de NaCl e $CaCO_3$ na água para reduzir a toxicidade do NO_2^- em juvenis de pacu ainda é desconhecida.

2. REVISÃO BIBLIOGRÁFICA

2.1. A espécie: pacu (*Piaractus mesopotamicus*)

O pacu, *Piaractus mesopotamicus* (figura 1), é um dos peixes de água doce nativos mais importantes da América do Sul (Schenone et al., 2011), e a quinta espécie mais produzida no

Brasil (Figura 1). Possui hábito alimentar onívoro, rápido crescimento e boa aceitação no mercado consumidor pela excelente qualidade da carne, evidenciando um grande potencial de produção em sistemas intensivos de criação (Urbinati et al., 2013). Contudo, a produção do pacu enfrenta alguns problemas relacionados a condições inadequadas de criação e manejo que podem induzir o estresse nos animais afetando negativamente a produtividade (Barcellos et al., 2004).

Figura 1: Exemplar de juvenil de pacu (*Piaractus mesopotamicus*) (Fonte: arquivo pessoal).

2.2. Resíduos nitrogenados

A qualidade da água desempenha um papel importante no crescimento e na sobrevivência de organismos aquáticos, embora cada fator desempenhe seu papel individual, é a interação entre vários parâmetros que determina a obtenção de produtividades ideais na aquicultura (Suman et al., 2017; Surnar et al., 2018).

Os peixes em sistemas de cultivo normalmente recebem alimento com altos níveis de proteína. Parte é assimilada pelo animal e depositada no corpo como proteína, contudo, em caso de excesso desse componente, a porção que contém carbono é convertida em carboidratos, corpos cetônicos ou ácidos graxos, e o nitrogênio pode ser excretado como amônia (NH_3), que é a principal forma de excreção de nitrogênio dos peixes. Além disso, a amônia também se acumula como resultado da decomposição da matéria orgânica no ambiente aquático. Uma vez

no meio aquático, a amônia é oxidada por bactérias amônia oxidantes (AOB) e transformada em NO_2^- (Baldisserotto, 2013), conforme equação abaixo:

$$NH_4^+ + 1^{½}\, O_2 \rightarrow NO_2^- + 2H^+ + H_2O$$

O NO_2^-, por sua vez, é oxidado por bactérias nitrito oxidantes (NOB) e transformado em nitrato (NO_3^-), de acordo com a equação abaixo:

$$^{½}O_2 + NO_2^- \rightarrow NO_3^-$$

A presença desses compostos nitrogenados em níveis elevados pode gerar vários problemas fisiológicos, com alterações nas estruturas de órgãos importantes para a sobrevivência desses animais (Cavero et al., 2004).

2.3. Nitrito

O NO_2^- é encontrado em ecossistemas como um componente natural do ciclo do nitrogênio (Jensen, 2003) e existe na água em duas formas, o ácido nítrico (HNO_2) e o nitrito ionizado (NO_2^-), sendo que a concentração das duas formas é dependente do pH (Baldisserotto, 2013; Tomasso, 1994), onde em pH > 5,5, só é encontrada na forma ionizada. O ácido nítrico se difunde livremente nas brânquias, enquanto o NO_2^- é transportado através da membrana branquial pelo co-transportador Cl^-/HCO_3^-, competindo com o Cl^- (Jensen, 2003).

Os efeitos tóxicos do NO_2^- dependem da concentração e do tempo de exposição (Kroupova et al., 2008), e o principal mecanismo tóxico é a oxidação do Fe^{2+} da hemoglobina em Fe^{3+}, resultando na formação de metahemoglobina (metHb), que é incapaz de se ligar e transportar oxigênio (Tilak et al., 2007; Lefevre et al., 2011) dando uma coloração marron ao sangue e outros tecidos (Figura 2), podendo resultar em hipóxia funcional ou anóxia (Jensen, 2003; Tilak et al., 2007; Arana, 2010; Baldisserotto, 2013) induzindo a uma série de alterações hematológicas (Morgan & Iwama, 2011). Além disso, o NO_2^- também pode induzir a formação de espécies reativas de oxigênio (ERO) e espécies reativas de nitrogênio (ERN) e/ou comprometer o sistema antioxidante de peixes (Ciji et al., 2012; Sun et al., 2011, 2012; Lin et

al., 2018), provocar alterações hepáticas e ter efeito vasodilatador (Costa, 2004) podendo levar à morte do peixe (Tomasso, 1994).

A toxicidade do NO_2^- varia entre as espécies ou até mesmo entre diferentes populações de peixes. Essa variação pode ser devido a diferentes taxas de captação de Cl^- nas brânquias. Peixes com alta taxa de captação de Cl^- pelas brânquias são menos sensíveis ao NO_2^- do que os que têm baixa taxa de captação (Baldisserotto, 2013). A quantidade de metahemoglobina necessária para reduzir o crescimento ou matar os peixes varia de acordo com a espécie e com as condições ambientais. De um modo geral, concentrações em torno de 50% de metahemoglobina no sangue são consideradas críticas para os peixes (Bowser et al., 1983).

Figura 2: Coloração marrom no fígado homogeneizado de juvenil de pacu *Piaractus mesopotamicus* expostos a 15 (esquerda) e 0,03 (direita) mg $N\text{-}NO_2^-.L^{-1}$ (Fonte: arquivo pessoal).

2.4. Respostas fisiológicas dos peixes

O estresse pode ser definido como um conjunto de respostas fisiológicas de um organismo na presença de agentes estressores (Mazeaud et al., 1977; Wedemeyer & Mcleay, 1981) e tem grande importância como predisponente a doenças e como causador de redução de desempenho zootécnico e reprodutivo.

A resposta ao estresse compreende uma série de alterações fisiológicas coordenadas pelo eixo neuroendócrino hipotálamo-hipófise-interrenal (HHI), para promover o

restabelecimento da condição de homeostase. Inicialmente os peixes respondem ao fator adverso, chamado de estressor, elevando os níveis sanguíneos de hormônios, como as catecolaminas e o cortisol, sendo estas alterações hormonais chamadas de respostas primárias. O cortisol é o principal parâmetro da avaliação da resposta ao estresse em peixes. Esses hormônios provocam alterações e respostas hematológicas, metabólicas, hidrominerais entre outras, que são chamadas respostas secundárias (Barcellos et al., 2003).

O efeito dessas alterações em nível individual e populacional é chamado de alterações terciárias, que compreendem impactos negativos no sistema imunológico, no crescimento e na reprodução (Barcellos et al., 2001).

Em termos gerais, as respostas ao estresse podem ser vistas como um aumento das taxas de catabolismo, permitindo a utilização de reservas energéticas indisponíveis em condições normais (Pickering, 1981). Na aquicultura, é frequente a exposição dos peixes a procedimentos estressores, tais como captura, transporte e alterações em parâmetros de qualidade de água, incluindo níveis tóxicos de NO_2^-.

2.5. Parâmetros sanguíneos

Alterações nas características sanguíneas estão entre as primeiras respostas observadas nos peixes submetidos a situações de estresse e podem refletir o comprometimento de diversas funções biológicas (Heath, 1995). Diversos parâmetros sanguíneos podem ser utilizados para identificar uma condição de estresse (Segner et al., 2012; Dal'Bó et al., 2015), e são frequentemente relatados em peixes em resposta à exposição a níveis subletais de NO_2^- (Jensen, 2003; Kroupova et al., 2008; Ciji et al., 2012; Neves et al., 2017). Dessa forma, a utilização dos parâmetros sanguíneos são ferramentas úteis, pois fornecem informações relevantes sobre as condições fisiológicas e a saúde do indivíduo ou de sua população (Centeno et al., 2007; Tavares-Dias and Moraes, 2007). Além disso, a coleta de sangue (Figura 3) é um procedimento não letal e as análises fornecem resultados rápidos (Satheeshkumar et al., 2012).

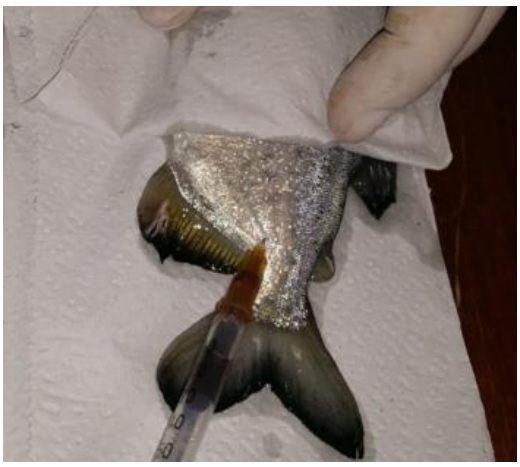

Figura 3: Coleta de sangue via veia caudal em juvenil de pacu *Piaractus mesopotamicus* (Fonte: arquivo pessoal).

2.6. Estresse oxidativo

O estresse ocasionado pelo NO_2^- pode levar os peixes a induzir a formação de espécies reativas de oxigênio (EROs) e espécies reativas de nitrogênio (ERNs) (Halliwell & Gutteridge, 2001; Sun et al., 2014). As EROs são geradas como subprodutos do metabolismo oxidativo e sua alta produção e acumulação pode resultar em um desequilíbrio a favor dos pró-oxidantes, caracterizando a condição de estresse oxidativo (Droge, 2003). Esse desequilíbrio pode resultar em danos a proteínas, lipídios, RNA e DNA (Nogueira, 2013), pois as EROs causam lipoperoxidação (LPO) das membranas celulares, sendo a LPO considerada um dos mecanismos mais comuns de dano celular (Halliwell & Gutteridge, 2015; Lushchak, 2011). A ação das EROs também resulta na formação de resíduos químicos como o malondialdeído (MDA), e dessa forma, a quantificação da peroxidação lipídica pelo método de TBARS (Lushchak et al., 2009) é determinante por ter uma importância como biomarcador para o estresse oxidativo (Lackner, 1998; Lushchak, 2011). Para minimizar os danos ocasionados pelo estresse oxidativo, os peixes possuem um sistema de defesa antioxidante que age na tentativa de eliminar excesso e reparar danos causados pelas EROs (Hermes-Lima et al., 2015). Para que isso ocorra o organismo apresenta as defesas antioxidantes enzimáticas, tais como, superóxido

dismutase (SOD), catalase (CAT), glutationa-S-transferase (GST); e não-enzimáticos, como vitaminas e minerias (Livingstone, 2001; Valavanidis et al., 2006; Belló et al., 2000; Kelley et al., 2010; Lushchak, 2011). A glutationa-S-transferase (GST) apresenta um importante papel neste sistema antioxidante, e sua atividade está envolvida na detoxificação celular de xenobióticos e metabólitos endógenos, incluindo produtos de dano oxidativo (Blanchette et al., 2007). No entanto, em condições de estresse mais severas, como a exposição a concentrações mais elevadas de NO_2^-, pode haver falha no sistema de defesa antioxidante, resultando, por exemplo, na redução da atividade dessas enzimas (Jia et al., 2015; Li et al., 2016). Desta forma, alterações no sistema de defesa antioxidante e a ocorrência de danos oxidativos em proteínas e lipídios de tecidos como brânquias, fígado e músculo, podem ocorrer em resposta a exposição ao NO_2^- (Ciji et al., 2012; Sun et al., 2014), comprometendo as funções fisiológicas dos animais (Livingstone, 2003; Valavanidis et al., 2006), levando a alterações na qualidade da carne (Zhang et al., 2016), o que afeta o principal produto final da aquicultura.

2.7. Mecanismos para minimizar as rotas de absorção do NO_2^-

O NO_2^- é transportado através da membrana branquial pelo anteporte Cl^-/HCO_3^-, competindo com o Cl^- (Baldisserotto, 2013), dessa forma, sempre que o NO_2^- encontra-se presente na água, uma parte da absorção de Cl^- será deslocada, do anteporte, para a absorção de NO_2^- (Jensen, 2003). Por conta desta competição, o aumento da salinidade na água pode reduzir a toxicidade deste composto para os peixes (Wuertz et al., 2013), pois diminuem a sua captação através da membrana branquial (Huey et al., 1980; Baldisserotto, 2013; Arana, 2010).

A dureza da água também pode afetar o desempenho de várias espécies (Silva et al., 2005) e é determinada pelo conteúdo de sais de Ca^{2+} e Mg^{2+}, os quais podem ser abundantes em águas dulcícolas, e estão ligados aos íons carbonato (CO_3^{-2}) e bicarbonato (HCO_3^-) ou a sulfato (SO_4^2), cloreto (Cl^-) e outros ânions de acidez mineral (Arana, 2010). O Ca^{2+} tem papel fundamental na regulação iônica por reduzir a permeabilidade das membranas biológicas e,

consequentemente, o fluxo difusivo de íons para o meio aquático (Gonzal et al., 1987; Gonzalez, 1996; Wood & Mcdonald, 1988**;** Tomasso et al., 1980; Baldisserotto, 2013), melhorando a resistência dos peixes a substâncias tóxicas presentes na água (Perschbacher & Wurts, 1999), provavelmente por diminuir a permeabilidade da membrana branquial a estas substâncias (Tomasso et al., 1980; Baldisserotto, 2013).

Os peixes de água doce podem absorver Ca^{2+} e Mg^{2+} diretamente da água pelas brânquias ou da alimentação via intestino (Wurts & Durborow, 1992; Baldisserotto & Mimura, 1995; Bijevelds et al., 1998; Flik et al., 1993). A captação através das brânquias é um processo ativo mais ou menos contínuo e relativamente dependente dos níveis de Ca^{2+} na água (Flik et al., 1993).

A entrada do Ca^{2+} se dá através de difusão pela formação do gradiente osmótico da bomba Na^{+}/ Ca^{2+} (Flik & Verbost, 1993) (Figura 4).

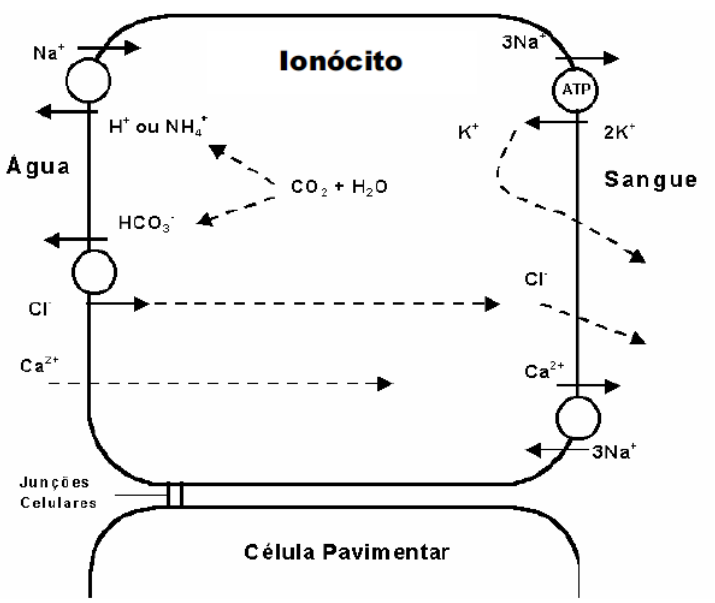

Figura 4: Modelo de célula branquial para captação de íons em peixes adaptados a ambientes hipotônicos (Fonte: Adaptado de Flik & Verbost, 1993).

Como a concentração de Ca^{2+} na água é variável conforme o ambiente, os peixes desenvolveram um sistema de regulação de Ca^{2+} que reage rapidamente às variações do meio (Kaneko & Hirano, 1993). Krous et al. (1982) afirmam que altas concentrações de Ca^{2+}

geralmente reduzem a perda de Cl^- através das brânquias, isto por sua vez diminui o requisito para captação de NO_2^-.

A determinação dos limites de tolerância ao NO_2^- para as diferentes espécies é importante e pode ser verificada a partir de alterações no desempenho zootécnico, distúrbios fisiológicos e de estresse oxidativo causados nos peixes. A escassez de informações acerca da toxicidade do NO_2^- e os efeitos do aumento da concentração de NaCl e $CaCO_3$ na água para minimizar a toxicidade deste composto para o pacu (*Piaractus mesopotamicus*) justificam a realização do presente trabalho.

3. OBJETIVOS

3.1. Objetivo geral

- Avaliar os efeitos letais e subletais da exposição ao NO_2^- e a utilização do NaCl e $CaCO_3$ como estratégia para minimizar a toxicidade deste composto em juvenis de pacu *Piaractus mesopotamicus* utilizando o desempenho zootécnico e os parâmetros sanguíneos e de estresse oxidativo como biomarcadores.

3.2. Objetivos específicos

- Verificar as concentrações letais de NO_2^- e NaCl em juvenis de pacu ao longo de 96 h de exposição;

- Avaliar os efeitos da exposição ao NO_2^- e sua interação com NaCl e $CaCO_3$ no desempenho zootécnico em juvenis de pacu;

- Avaliar os efeitos da exposição ao NO_2^- e sua interação com NaCl e $CaCO_3$ no sistema de defesa antioxidante e nos níveis de dano oxidativo no fígado, músculo e brânquias em juvenis de pacu;

- Avaliar os efeitos da exposição ao NO_2^- e sua interação com NaCl e $CaCO_3$ nos parâmetros sanguíneos em juvenis de pacu.

4. HIPÓTESES

- Concentrações subletais de NO_2^- induzem alterações nos parâmetros sanguíneos no sistema de defesa antioxidante e nos níveis de dano oxidativo, bem como podem afetar o desempenho zootécnico em juvenis de pacu;

- A adição de NaCl e $CaCO_3$ na água diminui a captação de NO_2^-, reduzindo a sua toxicidade e seus efeitos no desempenho zootécnico, nos parâmetros sanguíneos, no sistema de defesa antioxidante e de dano oxidativo em juvenis de pacu.

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CAPÍTULO 1

Sodium chloride addition to the water attenuates nitrite induced oxidative stress responses in juvenile pacu *Piaractus mesopotamicus*

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Abstract

The aim of this work was to verify if the addition of NaCl in water reduces the nitrite (NO_2^-) toxic effects in pacu *Piaractus mesopotamicus* juveniles, using survival, growth, oxidative damage and antioxidant responses as biomarkers. Firstly, two trials were performed for determining the Lethal Concentrations ($LC_{50\text{-}96h}$) of NaCl and NO_2^-. After determining the $LC_{50\text{-}96h}$, the fish were exposed for 60 days to six treatments consisting by the interaction of two NaCl (0 and 1 g $NaCl.L^{-1}$) and three NO_2^- (0, 5 and 15 mg $N\text{-}NO_2^-.L^{-1}$) concentrations. At the beginning and the end of the experiment the fish were sedated with benzocaine hydrochloride for biometric measurements. In addition, liver, muscle and gills samples were collected from nine fish per treatment at the end of exposure for measure the total protein content, glutathione-S-transferase activity (GST), lipid peroxidation (TBARS) and total antioxidant capacity against peroxyl radicals (ACAP). The $LC_{50\text{-}96h}$ for NaCl and NO_2^- concentration for pacu is 13.08 mg $NaCl.L^{-1}$ and 107.5 mg $N\text{-}NO_2^-.L^{-1}$, respectively. The results demonstrated that stress caused by the concentration from 5 mg $N\text{-}NO_2^-.L^{-1}$ led to a reduction in protein content and affect the antioxidant defense system, resulting in enhanced GST activity and reduced ACAP. The exposure to higher NO_2^- concentration (15 mg $N\text{-}NO_2^-.L^{-1}$) without the NaCl addition was also able to induced enhanced levels of liver TBARS. However, the increase in NaCl concentration prevented the increase of liver lipid peroxidation and the reduction in muscle ACAP related to nitrite exposure, and better results of weight gain, biomass and specific growth rate compared to the control treatment without the salt addition. In conclusion, the increasing of NaCl concentration in water can improves growth performance and reduce the NO_2^- induced oxidative stress in juvenile *P. mesopotamicus*.

Keywords: nitrogen compound; salt; zootechnical performance; antioxidants; lipid peroxidation

1 Introduction

The pacu *Piaractus mesopotamicus* is a native freshwater fish from South America (Schenone et al., 2011), being one of the most produced species in continental aquaculture. The success of this species in the activity is related to its omnivorous food habit, fast growth and good acceptance by the consumer market due to the excellent meat quality, and therefore show a great potential of production in intensive breeding systems (Urbinati et al., 2013).

The recirculation aquaculture systems (RAS) is a technology that allows the intensification of production. However, high stocking density and an inefficient nitrification process may lead to the build up of toxic nitrogen compounds, among them NO_2^-, which can affect negatively the production (Kroupova et al., 2005). Therefore, it is indispensable to know the tolerance limits of each species to NO_2^- (Campos et al., 2012), once the toxicity of this compound is specie-specific (Kroupova et al., 2005). The effects of NO_2^- to pacu is still poorly described (Moraes et al., 2006) and more information are necessary to establish appropriate levels to the species.

The main and most described toxic mechanism of NO_2^- is the oxidation of hemoglobin Fe^{2+} to Fe^{3+} resulting in the formation of methemoglobin (metHb), which is unable to bind and carry oxygen to the tissues (Tilak et al., 2007). As a consequence, higher levels of metHb compromise the oxygen uptake (Lefevre et al., 2011) and can result in functional hypoxia or anoxia (Jensen, 2003; Tilak et al., 2007).

In addition, exposure to NO_2^- may also induce a variety of other toxicological effects on fish, such as altered energy metabolism, hormone regulation, disruption of ionic balance (Jensen, 2003), induction of oxidative stress (Maltez et al., 2018) affecting the functioning and homeostasis of the physiological system, leading to a reduction in the growth rate (Ciji et al., 2014), increase vulnerability to diseases (Jia et al., 2016), and even death (Wuertz et al., 2013).

Some studies have shown that the stress caused by NO_2^- exposure can lead to induce reactive oxygen species (ROS) and reactive nitrogen species (RNS) formation and/or compromise the antioxidant system of fish (Ciji et al., 2012; Sun et al., 2011, 2012;Jensen and Hansen 2011; Jensen et al., 2015; Lin et al., 2018). This led to an imbalance in favor of the pro-oxidants agents, characterizing the condition of oxidative stress, that can result in enhaced oxidative damage to macromolecules such as proteins, lipids and DNA, which can have their biological functions lost or compromised (Halliwell and Gutteridge, 2015).

The NO_2^- uptake in freshwater fish occurs mainly through the gill membrane and it is related to branchial Cl^- uptake rates (Jensen, 2003) because NO_2^- competes with Cl^- in the Cl^-/HCO_3 cotransporter (Tomasso and Grosell, 2005). Consequently, the increase of waterborne Cl^- levels (NaCl) can reduce NO_2^- toxicity (Kroupova et al.,2005; Yanbo et al., 2006; Boudreaux et al., 2007) as demonstrated for different species (Sampaio et al., 2002; Weirich and Riche, 2006a, 2006b; Costa et al., 2008; Wuertz et al., 2013).

Therefore, the objective of the present study was to verify if the addition of NaCl in the water can be used as an alternative to reduce the long-term toxicity related to the NO_2^- exposure, evaluating survival, growth and oxidative stress parameters in juveniles of *P. mesopotamicus*.

2 Material and Methods

This study was performed in the Laboratório de Aquacultura Continental (LAC) of the Universidade Federal do Rio Grande - FURG. Juvenile *P. mesopotamicus* (35.8 ± 0.4 g and 12.2 ± 0.1 cm) were obtained from a commercial fish farm located in Rio Grande do Sul state, southern Brazil. Fish were maintained for acclimation in nine tanks (250 L useful volume) consisting of three RAS with biological and mechanical filters. Throughout acclimation and experimental period the diet was offered twice a day (9:00 am and 4:00 pm) until the apparent satiety with extruded commercial feed (Supra Aquafeed® with 46% of Crude Protein).

2.1 Acute Toxicity of NaCl and NO_2^-

Two trials were performed for determining the acute toxicity ($LC_{50\text{-}96h}$) of NaCl and NO_2^- for *P. mesopotamicus*. Fish were exposed to six concentrations for NaCl (0, 6, 12, 13, 14 and 15 g $NaCl.L^{-1}$) and NO_2^- (0, 60, 90, 120, 150 and 200 mg $N\text{-}NO_2^-.L^{-1}$), in triplicate. Tested concentrations were obtained with NaCl and $NaNO_2$ dilution in the water. For each experiment, a total of 90 *P. mesopotamicus* juveniles were randomly distributed in eighteen tanks (80 L), five fish per tanks. The experimental were conducted in semi-static systems with water quality parameters controlled and maintained in suitable conditions to the pacu, except the NaCl and NO_2^- concentrations, which were adjusted to meet the experimental design. During the experiment the mortality was recorded every 12 hours. Fish with no opercular movement and no response to mechanical stimuli were considered dead and removed from the tanks.

2.2 NaCl and NO_2^- Exposure

For the development of this experiment the fish (22 per tank) were kept in 18 tanks (80 L useful volume), distributed in six RAS without the biological filter, with suitable water quality parameters for pacu, except to mg NaCl and NO_2^- concentration. After acclimation (15 days) in these systems, fish were submitted to six treatments consisting by the interaction of two NaCl (0 and 1 g $NaCl.L^{-1}$) and three NO_2^- (0, 5 and 15 mg $N\text{-}NO_2^-.L^{-1}$) concentrations, in triplicate, for 60 days. The NaCl and NO_2^- concentrations were chosen based on the $CL_{50\text{-}96h}$ and safe level results obtained from the experiment described in item 2.1. The safe level of both compounds were estimated by the multiplication of the LC_{50} by the application factor (0.1), according to Sprague (1971). Concentrations of nitrite below and above of safe level were chosen and one

NaCl concentration below the safe level was used. The desired concentrations were obtained by the addition of NaCl and $NaNO_2$ to the water, or water exchange, when required.

2.3 Water quality parameters

The water quality parameters were monitored daily (pH, temperature, dissolved oxygen, NaCl concentration, ammonia and NO_2^-) or twice a week (alkalinity, water hardness) and kept as follows: pH (7.53 ± 0.04) (pH meter FE 20-FiveEasy TM, Mettler Toledo), dissolved oxygen (7.46 ± 0.23 $mg.L^{-1}$) and temperature (27.3 ± 0.03 °C) (digital oximeter YSI EcoSense® DO200A), total ammonia nitrogen (0.32 ± 0.12 mg $TAN.L^{-1}$) (UNESCO, 1983), un-ionized ammonia (0.03 ± 0.02 $mg.L^{-1}$) (Colt, 2002), total alkalinity (79.31 ± 3.91 mg $CaCO_3.L^{-1}$) (Eaton et al., 2005) and water hardness (64.12 ± 4.57 mg $CaCO_3.L^{-1}$) (Adad et al., 1982). During the experimental period the NaCl concentration (0.0 ± 0.0 and 1.0 ± 0.01 g $NaCl.L^{-1}$) (handheld refractometer model RBX2862) and NO_2^- concentrations (0.02 ± 0.01; 5.42 ± 0.17 and 14.87 ± 0.95 mg $N\text{-}NO_2^-.L^{-1}$) (Bendschneider and Robinson, 1952) were altered and maintained according to the experimental treatments described before. Photoperiod was fixed at 12 h light/12 h dark.

2.4 Biochemical parameters

After 60 days of exposure, nine fish from each treatment (24 hours fasting feed) were randomly captured and immediately euthanized with a lethal concentration of benzocaine hydrochloride (250 ppm). Samples of liver, gills and muscle tissues were collected, placed in microtubes (2 mL), immediately frozen in liquid nitrogen, and stored in an ultrafreezer (-80 ºC) until homogenization. The tissues were homogenized according Da Rocha et al (2009). The supernatants resulting from the centrifugation of the homogenates (10.000×g, 20 min, at 4 °C)

were kept in an ultra-freezer at -80 °C until the analysis. The total protein content of the homogenized samples was determined using a commercial kit (Doles®) based on the Biuret assay (550 nm). The total protein content and all other analyzes were performed using a spectroflorimeter (Biotek®, Synergy HT)

2.4.1 *Total antioxidant capacity against peroxyl radical*

All samples were diluted with homogenization buffer to 2.0 mg of protein mL^{-1} and the total antioxidant capacity against peroxyl radicals (ACAP) was determined according to the method described by Amado et al. (2009). The ACAP values (expressed as a relative area) were calculated using the expression proposed by Monserrat et al. (2014). For interpretation purpose, a higher relative area means a lower antioxidant capacity.

2.4.2 *Glutathione-S-transferase activity*

Glutathione-S-transferase activity (GST) (nanomole of CNDB-GSH conjugate min^{-1} mg wet $tissue^{-1}$ was determined according to Habig et al. (1974) and Habig and Jakoby (1981).

2.4.3 *Lipid peroxidation*

The lipid peroxidation (LPO) levels were measured according to Oakes and Van Der Kraak (2003). This method quantifies the malondialdehyde levels (MDA), a byproduct of LPO, by the measurement of thiobarbituric acid reactive substances (TBARS). The results were expressed as nmol TMP mg wet $tissue^{-1}$, where TMP stands for tetramethoxypropane (ACROS Organics), employed as standard.

2.5 Growth parameters

Twenty-two fish from each tank (n = 66 per treatment) were randomly sampled and were then sedated with 50 ppm of benzocaine hydrochloride before final biometric (day 60). Growth parameters evaluated were: Survival (S in %) = number of fish at the end of each analyzed period/initial fish number x 100; Weight gain (WG in g) = final weight (g) - initial weight (g); Biomass (B in g) = average weight (g) x number of fish at the end of each analyzed period; Specific growth rate (SGR in %) = 100 x [(ln final weight (g) - ln initial weight (g)/time (days)]; Food intake (FI in g): quantity of food consumed (g)/number of fish at the end of each analyzed period; Food conversion rate (FCR in g) = food provided (g)/weight gain (g).

2.6 Statistical analysis

The homogeneity of variances and normality were verified by Levene and Kolmogorov-Smirnov's test, respectively. Data were analyzed by two-way ANOVA (NaCl x NO_2^- concentrations) followed by the Tukey test ($p<0.05$). Data were presented as mean ± standard error. The mean lethal concentrations (NaCl and NO_2^-) and its confidence interval (CI 95%) were estimated by the Spearman-Karber (Hamilton et al. 1977).

2.7 Ethic

The methodology applied in this study was approved by the Ethics Committee and Animal Welfare Committee of the Universidade Federal do Rio Grande - FURG (process number P095/2016).

3 Results

The LC_{50} of NO_2^- at 72 and 96 hours were estimated in 138.5 mg $N\text{-}NO_2^-.L^{-1}$ (confidence interval: 107.0 – 179.3 mg $N\text{-}NO_2^-.L^{-1}$) and 107.5 mg $N\text{-}NO_2^-.L^{-1}$ (confidence interval: 95.7 – 120.8 mg $N\text{-}NO_2^-.L^{-1}$), respectively. The LC_{50} of NaCl at 42, 72 and 96 hours were estimated in 13.64 g $NaCl.L^{-1}$ (confidence interval: 13.41 – 13.88 g $NaCl.L^{-1}$), 13.21 g $NaCl.L^{-1}$ (confidence interval: 12.94 – 13.49 g $NaCl.L^{-1}$) and 13.08 g $NaCl.L^{-1}$ (confidence interval: 12.83 – 13.34 g $NaCl.L^{-1}$), respectively. In 24 and 48 hours (NO_2^-) and 24 hours (NaCl) it was not possible to estimate the LC_{50} because 50% mortality was not reached in any of the treatments (Table 1). The safe level of NO_2^- and NaCl were estimated at 10.7 mg $N\text{-}NO_2^-.L^{-1}$ and 1.30 g $NaCl.L^{-1}$, respectively.

Table 1: Cumulative mortality (%) of *Piaractus mesopotamicus* submitted to acute exposure to different NO_2^- (mg $N\text{-}NO_2^-.L^{-1}$) and NaCl (g $NaCl.L^{-1}$) concentrations and estimated $LC_{50\text{-}96h}$ with respectively confidence intervals.

Hours	NO_2^- (mg $N\text{-}NO_2^-.L^{-1}$)						$LC_{50\text{-}96h}$ (IC)
	0	60	90	120	150	200	
24	0	0	0	0	0	0	-
48	0	0	0	0	15	20	-
72	0	5	15	45	55	65	138.5 (107.0 – 179.3)
96	0	5	35	55	80	100	107.5 (95.7 – 120.8)
	NaCl (g $NaCl.L^{-1}$)						
	0	6	12	13	14	15	
24	0	0	0	0	20	33.3	-
48	0	0	0	6.6	73.3	66.3	13.64 (13.41 – 13.88)
72	0	0	0	33.3	93.3	100	13.21 (12.94 – 13.49)
96	0	0	0	40	100	100	13.08 (12.83 – 13.34)

No statistical differences was observed between treatments for survival and feed conversion rate. However, fish maintained at 5 mg $N\text{-}NO_2^-.L^{-1}$ present significantly lower weight gain and biomass in relation to 15 mg $N\text{-}NO_2^-.L^{-1}$ and control treatment in 1 g $NaCl.L^{-1}$. Fish maintained at 0 mg $N\text{-}NO_2^-.L^{-1}$ /1 g $NaCl.L^{-1}$ presented significantly higher weight gain, biomass and specific growth rate in relation to the control treatment (0 mg $N\text{-}NO_2^-.L^{-1}$/0 g $NaCl.L^{-1}$). Fish maintained at 5 mg $N\text{-}NO_2^-.L^{-1}$ presented significantly lower specific growth

rate in relation to the control treatment in 1 g $NaCl.L^{-1}$. Fish maintained at 5 mg $N\text{-}NO_2^-.L^{-1}$/1 g $NaCl.L^{-1}$ presented significantly lower feed intake than those kept at 0 mg $N\text{-}NO_2^-.L^{-1}$/1 g $NaCl.L^{-1}$ and at 15 mg $N\text{-}NO_2^-.L^{-1}$/1 g $NaCl.L^{-1}$ (Table 2).

Table 2: Survival and growth parameters of *Piaractus mesopotamicus* juveniles exposed to different NO_2^- and NaCl concentration in water for 60 days.

Treatments (NO_2^-+NaCl)	Survival (%)	WG (g)	Biomass (g)	SGR (%)	FI (g)	FCR
0 NO_2^- + 0	100±0.0	36.57±4.28Ab	944.6±71Ab	1.14±0.09Ab	49.39±2.98Aa	1.25±0.05
5 NO_2^- + 0	100±0.0	35.60±4.84Aa	931.4±65Aa	1.13±0.11Aa	44.15±2.95Aa	1.26±0.09
15 NO_2^- + 0	100±0.0	40.60±2.10Aa	1018.6±19Aa	1.25±0.04Aa	52.75±1.81Aa	1.27±0.03
0 NO_2^- + 1	100±0.0	50.50±1.33Aa	1124.3±18Aa	1.46±0.02Aa	54.24±0.93Aa	1.07±0.01
5 NO_2^- + 1	100±0.0	33.97±1.85Ba	903.83±29Ba	1.11±0.04Ba	39.59±1.93Ba	1.16±0.02
15 NO_2^- + 1	100±0.0	46.66±1.41Aa	1070.9±45Aa	1.39±0.02ABa	51.84±2.34Aa	1.11±0.08

WG= weight gain; SGR= specific growth rate; FI= food intake; FCR= food conversion rate. Different lowercase letters in the same column indicate statistically significant differences ($p<0.05$) between NaCl at the same NO_2^- concentration. Different capital letters in the same column indicate statistically significant differences ($p<0.05$) between NO_2^- at the same NaCl concentration (Two-way ANOVA and Tukey test, $p< 0.05$, $n = 66$).

3.1 *Total protein content*

The total protein content in liver of the fish maintained at 5 and 15 mg $N\text{-}NO_2^-.L^{-1}$ present significantly lower values in relation to the control treatment in 1 g $NaCl.L^{-1}$ (Figure 1A). In muscle of the fish maintained at 5 and 15 mg $N\text{-}NO_2^-.L^{-1}$ was significantly lower in relation to the respective control treatment in both NaCl (Figure 1B). There were not observed significant differences between treatments in gill (Figure 1C).

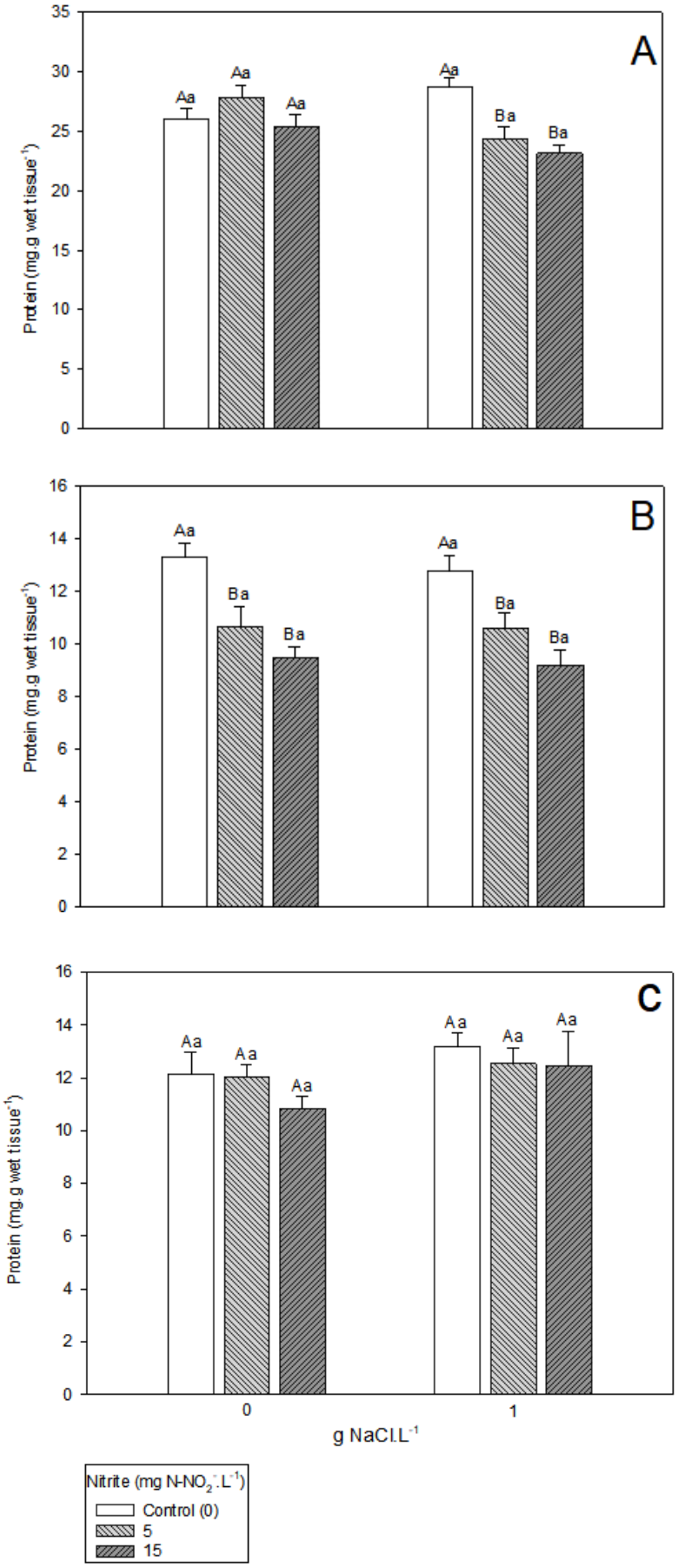
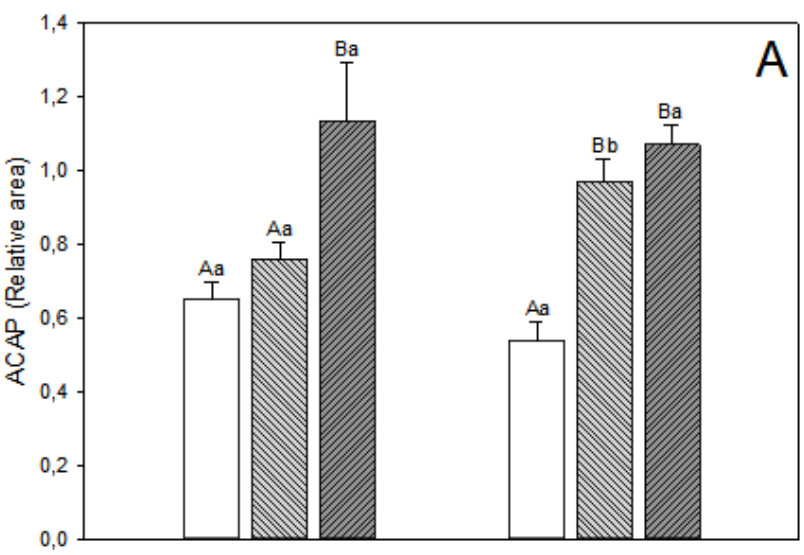

A
B
C
Protein (mg.g wet tissue-1)
Aa
Ba
0
1
g NaCl.L-1
Nitrite (mg N-NO2-.L-1)
Control (0)
5
15

Figure 1. Total protein content (mg g wet tissue^{-1}) in liver (A), muscle (B) and gill (C) of *Piaractus mesopotamicus* juveniles exposed to different NO_2^- and NaCl concentration in water for 60 days. Different lowercase letters indicate statistically significant differences (P<0.05) between NaCl at the same NO_2^- concentration. Different capital letters indicate statistically significant differences (P<0.05) between NO_2^- at the same NaCl concentration (Two-way ANOVA and Tukey test, $p < 0.05$, $n = 9$).

3.2 *Total antioxidant capacity against peroxyl radicals*

The ACAP in liver were lower in fish maintained at 15 mg N-NO_2^-.L^{-1} in relation to fish maintained at 5 mg N-NO_2^-.L^{-1} and the control treatment in 0 g NaCl.L^{-1}. Reduced in ACAP was observed in fish maintained at 5 and 15 mg N-NO_2^-.L^{-1} in relation to fish maintained at 0 mg N-NO_2^-.L^{-1} /1 g NaCl.L^{-1} (Figure 2A). Reduced in ACAP was observed in muscle of fish maintained at 15 mg N-NO_2^-.L^{-1} in relation to the control treatment (Figure 2B). Due to the particularities of the tissue itself, it was not possible to generate data on gill samples with the applied methodology.



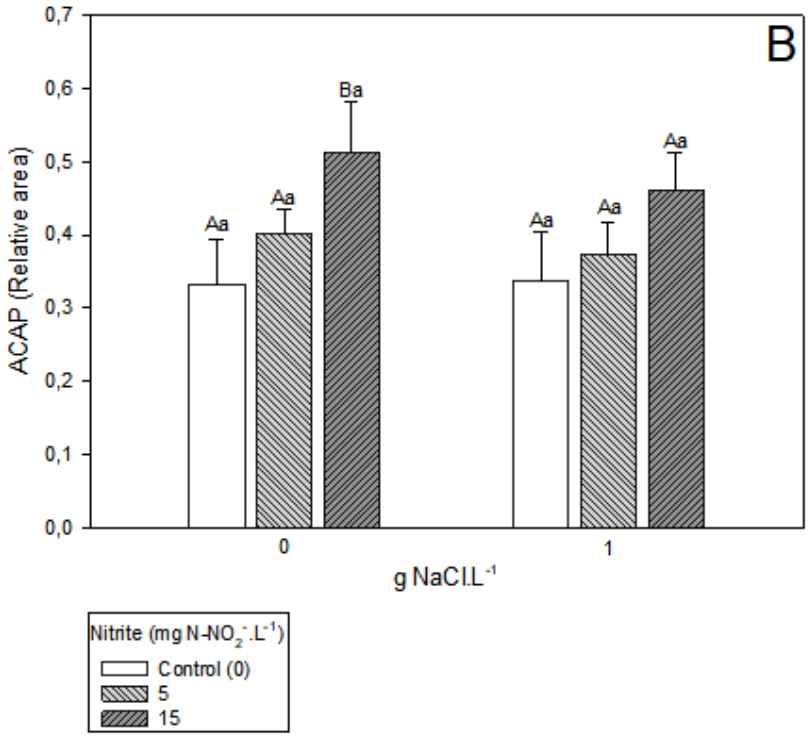
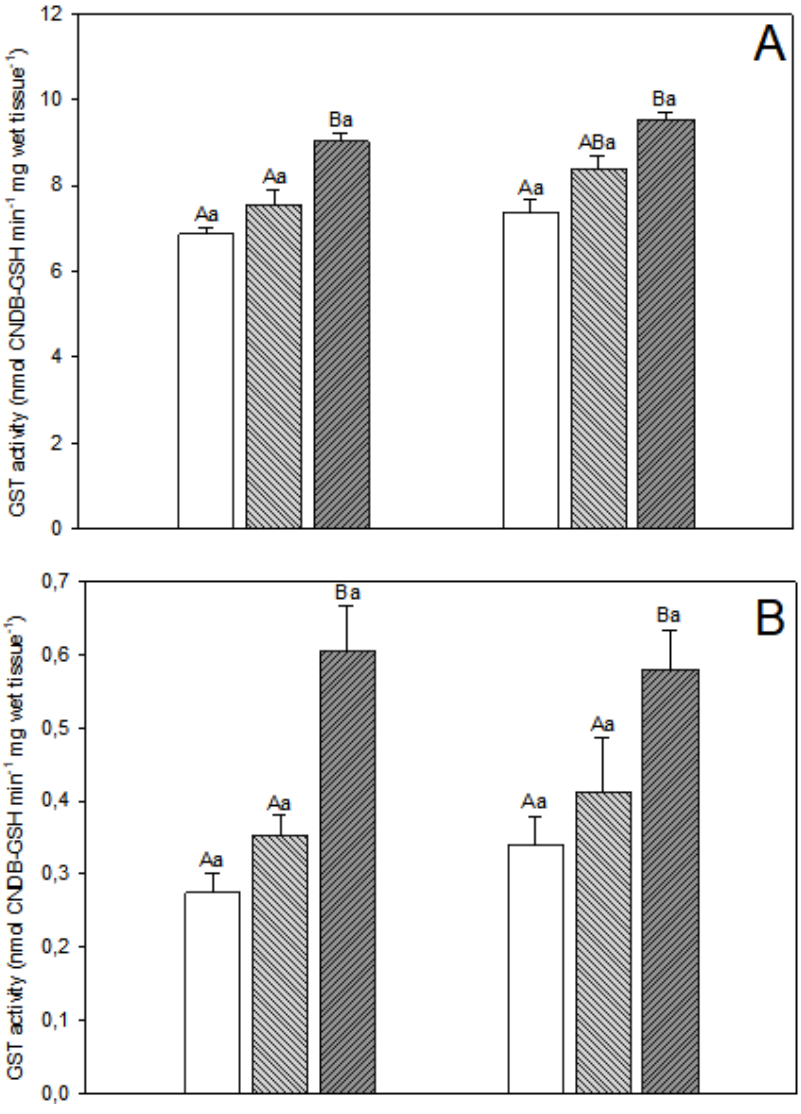


Figure 2. Total antioxidant capacity against peroxyl radicals (ACAP) in liver (A) and muscle (B) of *Piaractus mesopotamicus* juveniles exposed to different NO_2^- and NaCl concentration in water for 60 days. Different lowercase letters indicate statistically significant differences ($P<0.05$) between NaCl at the same NO_2^- concentration. Different capital letters indicate statistically significant differences ($P<0.05$) between NO_2^- at the same NaCl concentration (Two-way ANOVA and Tukey test, $p < 0.05$, $n = 9$).

3.3 *Glutathione-S-transferase activity*

The GST activity in liver of the fish maintained at 15 mg $N\text{-}NO_2^-.L^{-1}$ presented significantly highest activity in relation to fish maintained at 5 mg $N\text{-}NO_2^-.L^{-1}$ and the control treatment in 0 g $NaCl.L^{-1}$. Fish maintained at 15 mg $N\text{-}NO_2^-.L^{-1}$ /1 g $NaCl.L^{-1}$ presented significantly highest activity compared to fish exposed at 0 mg $N\text{-}NO_2^-.L^{-1}$ /1 g $NaCl.L^{-1}$ (Figure 3A). In muscle of the fish maintained at 15 mg $N\text{-}NO_2^-.L^{-1}$ presented significantly highest activity in relation to the control treatment. Fish maintained at 15 mg $N\text{-}NO_2^-.L^{-1}$/1 g $NaCl.L^{-1}$ presented significantly highest activity in relation to fish maintained at 0 mg $N\text{-}NO_2^-.L^{-1}$/1 g

NaCl.L^{-1} (Figure 3B). There were not observed significant differences between treatments in the GST activity in gills (Figure 3C).



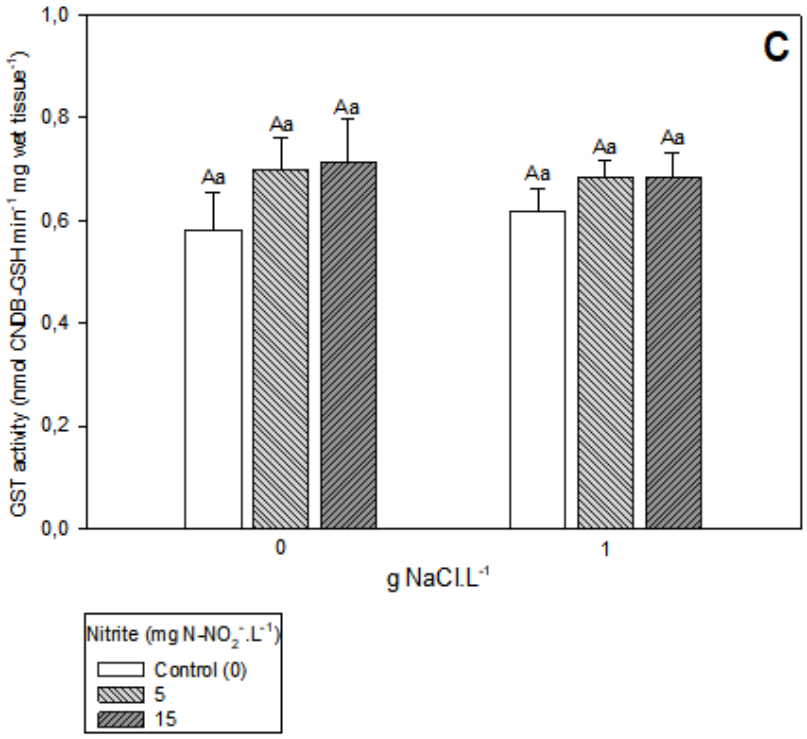


Figure 3. Glutathione-S-transferase (GST) activity in liver (A), muscle (B) and gill (C) of *Piaractus mesopotamicus* juveniles exposed to different NO_2^- and NaCl concentration in water for 60 days. Different lowercase letters indicate statistically significant differences ($P<0.05$) between NaCl at the same NO_2^- concentration. Different capital letters indicate statistically significant differences ($P<0.05$) between NO_2^- at the same NaCl concentration (Two-way ANOVA and Tukey test, $p < 0.05$, $n = 9$).

3.4 *Lipid Peroxidation – TBARS*

The TBARS levels in liver of fish maintained at 15 mg $N\text{-}NO_2^-.L^{-1}$ presented significantly higher results in relation to fish maintained at 5 mg $N\text{-}NO_2^-.L^{-1}$ and the control treatment in 0 g $NaCl.L^{-1}$ (Figure 4A). In muscle of fish maintained at 15 mg $N\text{-}NO_2^-.L^{-1}$ presented significantly higher results in relation to fish maintained at 5 mg $N\text{-}NO_2^-.L^{-1}$ and the control treatment in 0 g $NaCl.L^{-1}$, and fish maintained at 15 mg $N\text{-}NO_2^-.L^{-1}$/1 g $NaCl.L^{-1}$ presented significantly higher results in relation to fish maintained at 5 mg $N\text{-}NO_2^-.L^{-1}$/1 g $NaCl.L^{-1}$ and 0 mg $N\text{-}NO_2^-.L^{-1}$/1 g $NaCl.L^{-1}$ (Figure 4B). In gills there were not observed significant differences between treatments (Figure 4C).

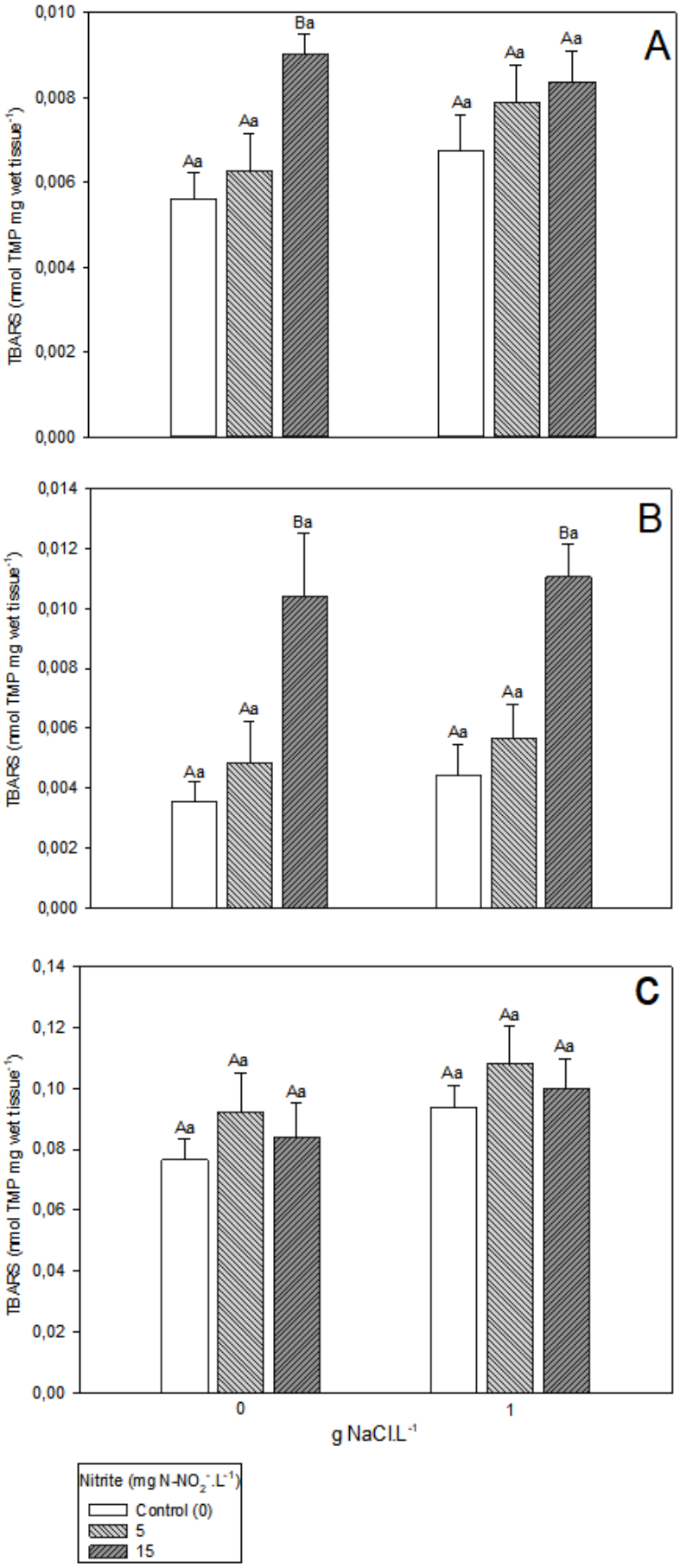

A
B
C
TBARS (nmol TMP mg wet tissue-1)
0,010
0,008
0,006
0,004
0,002
0,000
0,014
0,012
0,010
0,008
0,006
0,004
0,002
0,000
0,14
0,12
0,10
0,08
0,06
0,04
0,02
0,00
Aa
Ba
0
1
g NaCl.L-1
Nitrite (mg N-NO2-.L-1)
Control (0)
5
15

Figure 4. Thiobarbituric acid reactive substances (TBARS) content (nmol TMP mg wet tissue^{-1}) in liver (A), muscle (B) and gill (C) of *Piaractus mesopotamicus* juveniles exposed to different NO_2^- and NaCl concentration in water for 60 days. Different lowercase letters indicate statistically significant differences ($P<0.05$) between NaCl at the same NO_2^- concentration. Different capital letters indicate statistically significant differences ($P<0.05$) between NO_2^- at the same NaCl concentration (Two-way ANOVA and Tukey test, $p < 0.05$, $n = 9$).

4 Discussion

Besides the main objective of the present study is to evaluate the use of NaCl to reduce nitrite toxicity, the results obtained can assist also in the delimitation of safe levels of this compound to pacu juvenile. Lethal effects were observed from 60 mg $N\text{-}NO_2^-.L^{-1}$ and the $LC_{50\text{-}96h}$ was estimated in 138.5 mg $N\text{-}NO_2^-.L^{-1}$, suggesting that the species can be considered tolerant to nitrite, mainly for a freshwater fish that in general are less tolerant (Baldisserotto, 2013). The safe level was estimated in 12.08 mg $N\text{-}NO_2^-.L^{-1}$ based on Sprague (1971). However, other evaluated parameters shown that concentration of 5 mg $N\text{-}NO_2^-.L^{-1}$ was enough to induce a stress condition, led ta changes in the protein metabolism and the antioxidant defense system. These results evidencing that this kind of assessment based only mortality data is unreliable to establish security limits of toxic compounds in production systems or natural environments.

The reduction in total protein content in liver and muscle of fish kept at may indicate an inhibition of synthesis or increased protein degradation (Firat et al., 2011; Oruc, 2011). The stress caused by nitrite can result in several physiological adjustments to supply increased energy demand, which can affect fish protein metabolism (Barton 2002).

Oxidative stress induced by environmental contaminants in fish is a result of increased ROS and RNS production and / or weakening of the antioxidant system (Lushchak, 2016), and its effect was demonstrated in fish exposed to NO_2^- (Ciji et al., 2012, Sun et al., 2012, 2014;

Jensen et al. 2015; Maltez et al., 2018). In the present study, changes in antioxidant system were demonstrated by the reduction of ACAP and the enhanced in GST activity.

The antioxidant system comprises enzymatic and non-enzymatic defenses in stressful situations such as NO_2^- exposure, this system can be positive modulated or in even cases, the failure or the commitment of some components may occur due to the high stress degree (Halliwell and Gutteridge, 2015). The increase in GST activity in liver and muscle of fish maintained at 15 mg $N\text{-}NO_2^-.L^{-1}$ can be related to an increased ROS production, triggering an antioxidant response, since this group of enzymes plays an important role in the detoxification of xenobiotics and endogenous metabolites, including LPO end products (Blanchette et al., 2007). Ciji et al. (2012) and Lin et al. (2018) working with *Labeo rohita* and *Aristichthys nobilis* (48.6 mg $N\text{-}NO_2^-.L^{-1}$) exposed to NO_2^- for 96h, also observed an increase in GST activity in the gills and liver of the fish. Jia et al. (2015) observed an increase in enzymatic and non-enzymatic antioxidant defenses activity in juvenile turbot (*Scophthalmus maximus*) gills exposed by 96 hours similar NO_2^- concentrations (5.60 and 11.20 mg $N\text{-}NO_2^-.L^{-1}$).

The reduction in ACAP suggests the compromised production or the use of antioxidant components to combat the increased production of peroxyl radicals, which involves the use of different enzymatic and non-enzymatic antioxidant components measured by the methodology proposed by Amado et al. (2009). Maltez et al. (2018) using NO_2^- concentrations similar to the present study also found a reduction in ACAP in *Paralichthys orbignyanus*.

The increase of oxidative damage levels were also induced by higher nitrite exposure in the present study, suggesting an intensification of pro-oxidant condition with the enhanced of nitrite concentration. The higher TBARS values were observed in liver and muscle of fish maintained at 15 mg $N\text{-}NO_2^-.L^{-1}$ indicating increased levels of LPO, which consist of a chain reaction, initiated by a hydroxyl radical that leads to oxidation of polyunsaturated fatty acids (PUFA) (Halliwell and Gutteridge, 2015). The main effect of LPO is a decrease in membrane fluidity due to PUFA oxidation, affecting various biological processes (Halliwell and

Gutteridge, 2015) and causing a variety of adverse health and disease effects on organisms (Livingstone, 2003; Valavanidis et al., 2006). Our results are consistent with previous work that has demonstrated NO_2^- induced oxidative damage to macromolecules, including lipids in fish tissue (Sun et al., 2014; Jia et al., 2015; Maltez et al., 2018).

The safe NaCl concentration was estimated at 1.30 g $NaCl.L^{-1}$, thus, we propose the use of 1 g $NaCl.L^{-1}$ for reduce NO_2^- toxicity. The beneficial effects of NaCl addition to the water at the proposed concentration (1 $g.L^{-1}$) were evidenced by the prevention of increased liver LPO and reduced muscle ACAP, which were related in fish exposed to NO_2^- at 0 g $NaCl.L^{-1}$. In treatments with NaCl addition, these two biomarkers remain at basal levels even at higher NO_2^- concentration. Thus, the prevention of lipid peroxidation in liver avoid negative effects related to oxidative damage that can compromise its vital important functions, including detoxification process. In addition, changes in muscle antioxidant capacity aren't desirable either, because can make you more the main final aquaculture product more susceptible to oxidative damage, and consequently the meat quality can also be affected (Zhang et al. 2016).

Prolonged exposure to environmental stressors can result in tertiary stress responses, which can negatively affect reproduction, immune system and the growth of organisms (Barton, 2002). This kind of stress responses were reported for some authors related to nitrite toxicity in fish. Rainbow trout (*Oncorhynchus mykiss*) exposed to 1.0 mg $N\text{-}NO_2^-.L^{-1}$ showed no change in growth, but at 3.0 mg $N\text{-}NO_2^-.L^{-1}$, growth was reduced, and 65% mortality was found after 28 days of exposure (Kroupova et al., 2008). Furthermore, channel catfish (*Ictalurus punctatus*) showed reduced growth at 1.6 mg $N\text{-}NO_2^-.L^{-1}$ and mortality started at 3.71 mg $N\text{-}NO_2^-.L^{-1}$ (Colt et al., 1981). Otherwise, in our study, no evident changes were observed in survival and growth of pacu associated to nitrite exposure up to concentrations of 15 mg $N\text{-}NO_2^-.L^{-1}$. However, fish kept at 0 mg $N\text{-}NO_2^-.L^{-1}$/1 g $NaCl.L^{-1}$ showed better results of weight gain, biomass and specific growth rate compared to the control treatment (0 mg $N\text{-}NO_2^-.L^{-1}$/1 g $NaCl.L^{-1}$), indicating that the use of 1 g $NaCl.L^{-1}$ can also be beneficial to improve growth performance.

5 Conclusions

In conclusion, the addition of 1 g $NaCl.L^{-1}$ to the water improves performance and reduce the NO_2^- induced oxidative stress in juvenile pacu *P. mesopotamicus*.

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CAPÍTULO 2

Enhanced waterborne by calcium carbonate addition reduces nitrite toxic effects in juvenile of pacu *Piaractus mesopotamicus*

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Abstract

The aim of this study was to verify if the addition of calcium carbonate ($CaCO_3$) to increase water hardness reduces the long-term nitrite (NO_2^-) toxicity in juvenile pacu *Piaractus mesopotamicus*. Fish were exposed for 60 days to six treatments comprised by the interaction of three NO_2^- concentrations (0, 15 and 30 mg $N\text{-}NO_2^-.L^{-1}$) and two water hardness (60 and 150 mg $CaCO_3.L^{-1}$), all in triplicate. The zootechnical indices, and blood and oxidative stress parameters were evaluated at the end of experimental period. The results showed that at lower hardness, NO_2^- exposure led to enhanced levels of lipid peroxidation (LPO) of muscle and gills, and compromise the food conversion rate. Otherwise, the LPO levels were maintained at basal condition and no changes were found in zootechnical parameters at higher hardness treatments. Thus, the increase in hardness to 150 mg $CaCO_3.L^{-1}$ using $CaCO_3$ may reduce the NO_2^- toxic in juveniles of pacu, and can be an interesting strategy of water quality management, especially in intensive production systems.

Keywords: freshwater fish; nitrogen compound; lipid peroxidation; zootechnical performance

1- Introduction

Aquaculture production is the fastest growing food production sector reaching a production of 110 million tons in 2016, being responsible for a growing contribution to meeting the demand for fish for human consumption. It is estimated that by 2030 the aquaculture will account for over 60% of world fish production (FAO, 2018). However, the intensification of production and the development of technologies that make it more sustainable are fundamental for the activity to continue growing (Klinger and Naylor, 2012). The pacu *Piaractus mesopotamicus* (Characidae) is considered one of the most important native freshwater fish reared in South America's (Schenone et al., 2011), and presents a great potential for intensive aquaculture (Urbinati et al., 2013).

In intensive systems, nitrogen compounds can accumulate reaching toxic levels that can negatively affect growth, survival and susceptibility to diseases, and then compromising the production. Therefore, a special attention should be given to the monitoring and maintenance of these compounds at appropriate levels (Boyd and Tucker, 2012). The decomposition of the organic matter and fish excretion led to the accumulation of ammonia, which can be oxidize to NO_2^-, and subsequently to nitrate by nitrification process (Blancheton et al., 2013). Some intensive aquaculture systems, as recirculation aquaculture system (RAS), for example, are dependent of this process to cope with nitrogen compounds. However, an inefficient bacterial processes may lead to the build-up of NO_2^- in the environment of production.

The most described toxic mechanism of NO_2^- is the red blood cells permeation with the consequent iron oxidation, transforming hemoglobin to methemoglobin, which does not bind oxygen (Kroupova et al., 2008; Wuertz et al., 2013). However, the exposure to NO_2^- may also induce a variety of other toxicological effects on fish (Jensen, 2003; Maltez et al., 2018; Ciji et al., 2014; Jia et al., 2016).

Changes in hematological indices and blood biochemical parameters were frequently reported in fish in response to theses effects related to the exposure to sublethal levels of NO_2^- (Jensen, 2003; Kroupova et al., 2008; Ciji et al., 2012). Thus, the use of blood parameters is a useful tool to provide relevant information about the physiological conditions and health of fish (Centeno et al., 2007; Tavares-dias and Moraes, 2007).

Some studies have shown that the stress caused by NO_2^- exposure can also lead to induce reactive oxygen species (ROS) and reactive nitrogen species (RNS) formation and/or compromise the antioxidant system of fish (Ciji et al., 2012; Sun et al., 2014; Lin et al., 2018) resulting in an imbalance in favor of the pro-oxidants, characterizing the condition of oxidative stress. This imbalance may result in enhanced oxidative damage to macromolecules such as proteins, lipids and DNA, which may have their biological functions lost or compromised (Halliwell and Gutteridge, 2015).

Water hardness is specified by Ca^{2+} and Mg^{2+} content (Wurts, 1993) and is also a water quality important for aquaculture once can affect the production of several species (Silva et al., 2005).Vertebrates are dependent on Ca^{2+} for skeletal formation, blood coagulation and other cellular functions (Lovell, 1989; Coote et al., 1996). The interaction of water hardness with different parameters is related by several authors (Wurts, 1992; Townsend and Baldisserotto, 2001; Baldisserotto, 2011), including its effects on NO_2^- toxicity reduction (Neves et al., 2017).

The Ca^{2+} plays a key role in ion regulation by reducing the permeability of biological membranes and thus the diffusive flow of ions to water (Gonzal et al., 1987; Wood and Mcdonald, 1988; Gonzalez, 1996; Tomasso et al., 1980; Baldisserotto, 2013). Ca^{2+} enters through diffusion by the formation of the osmotic gradient of the Na^+/Ca^{2+} pump (Flik and Verbost, 1993), and the gill uptake is a continuous active process that is relatively dependent on Ca^{2+} levels in water (Flik et al., 1993). Thus, the enhanced levels of this ion in the water can

minimize the NO_2^- uptake, which occur through the gill in substitution to the Cl^- in Cl^-/HCO_3^- cotransporter (Jensen, 2003).

Therefore, the aim of this study was to verify if the increase of water hardness using $CaCO_3$ reduce the long-term NO_2^- toxicity in juvenile pacu *P. mesopotamicus* using blood and oxidative stress parameters, and the zootechnical performance of fish for this evaluation.

2- Material and Methods

This study was developed in the Continental Aquaculture Laboratory of the Universidade Federal do Rio Grande - FURG. Juvenile of *P. mesopotamicus* (25,3±0,6 g and 11,8±0,3 cm) were obtained from a commercial fish farm in Rio Grande do Sul state, Brazil.

2.1 Hardness ($CaCO_3$) and NO_2^- ($NaNO_2$) Exposure

The fish were randomly distributed in eighteen tanks (250 L useful volume) (22fish/experimental unit) consisting of six RAS without the biological filter. Throughout acclimation (15 days) and experimental period the diet was offered twice a day (9:00 am and 4:00 pm) until the apparent satiety with extruded commercial feed (Supra Aquafeed® with 46% of crude protein). Fish were submitted for 60 days to three concentrations of NO_2^- (0, 15 and 30 mg $N\text{-}NO_2^-.L^{-1}$) and two concentrations of $CaCO_3$ (60 and 150 mg $CaCO_3.L^{-1}$) totalizing six treatments, all performed in triplicate.

The NO_2^- levels (above the safe level) was chosen based on the results of the $CL_{50\text{-}96h}$ (Neves et al., unpublished data), and the $CaCO_3$ levels according to the ideal range found in the literature (Stone and Thomforde et al., 2004; Kasiri et al., 2011; Rajkumar et al., 2018). The desired NO_2^- and $CaCO_3$ levels was obtained by addition of sodium nitrite ($NaNO_2$) and calcium carbonate ($CaCO_3$) to the water, respectively, or water exchange, when required. Fish were

fasted for 24h and then sedated with 50 ppm of benzocaine hydrochloride to biometry at the beginning and end of experimental period.

2.2 Water quality parameters

The water quality parameters were monitored daily (pH, temperature, dissolved oxygen, ammonia and NO_2^-) or twice a week (alkalinity, water hardness) and kept within the expected values for tropical freshwater fish (Boyd and Tucker, 2012), except NO_2^- concentrations were adjusted according to the experimental treatments. The parameters were maintained as follows: pH (7.41 ± 0.03) (pH meter FE 20-FiveEasy TM, Mettler Toledo), dissolved oxygen (7.32 ± 0.19 $mg.L^{-1}$) and temperature (26.8 ± 0.02 °C) (digital oximeter YSI EcoSense® DO200A), total ammonia nitrogen (0.53±0.32 mg $TAN.L^{-1}$) (Unesco, 1983), un-ionized ammonia (0,02 ± 0.03 $mg.L^{-1}$) (Colt 2002) and total alkalinity (92.67 ± 3.56 mg $CaCO_3.L^{-1}$) (Eaton et al., 2005). The concentrations of NO_2^- (0.03 ± 0.01; 15.65 ± 1.34 and 29.58 ± 1.38 mg $N\text{-}NO_2^-.L^{-1}$) (Bendschneider and Robinson, 1952) and hardness (66.73 ± 2.76 and 152.31 ± 3.37 mg $CaCO_3.L^{-1}$) (Adad et al., 1982) were adjusted and maintained according to the experimental design described previously. Photoperiod was fixed at 12 h light/12 h dark.

2.3 Blood parameters

To verify hematological parameters, animals were anesthetized (50 ppm of benzocaine hydrochloride) and the blood collection was performed by caudal vein puncture. The blood samples were used for measure pH (pH meter FE 20-FiveEasy TM with appropriated probe, Mettler Toledo), glucose levels (Accu-check Performa - Roche®), hematocrit percentage (Goldenfarb et al., 1971) and hemoglobin concentration with a colorimetric kit (Doles®). The erythrocyte number was also counted using a Neubauer chamber in diluted blood (1:200) using

0.65% sodium chloride solution (Raninid-Paiva et al., 2013). The hematimetric parameters: mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated according to Wintrobe (1934).

2.4 Biochemistry analysis of antioxidant and oxidative stress responses

At the end of the experiment (60^{th} day), nine animals (three per tank) from each treatment were euthanized with a lethal dose of the same anesthetic (250 ppm). Samples of gill, liver and muscle were collected, immediately frozen in liquid nitrogen and then stored in an ultra-freezer at -80 °C. The organs were homogenized (100 mM Tris-HCl, 2 mM EDTA and 5 mM $MgC_{l2}.6H_2O$, pH 7.75) and the supernatants resulting from the centrifugation of the homogenates (10.000×g, 20 min, at 4 °C) were used for all analyses (Da Rocha et al., 2009). The total protein content of samples was determined using a commercial kit (Doles®) based on the Biuret assay (550 nm). The total antioxidant capacity against peroxyl radicals (ACAP) was determined according the protocol proposed by Amado et al. (2009). For ACAP determination, total protein (Biuret method) content of all samples were standardized at 2 mg/ml. ACAP results were expressed in relative area, in which a higher relative area means a lower antioxidant capacity and vice-versa. For ACAP determination on gills, a modification on the ABAP reagent (2,2'-Azobis(2-methylpropionamidine) dihydrochloride) concentration used was performed to achieve satisfactory results and some differences between curves generated by fluorimeter readings of samples with and without ABAP. The activity of glutathione-S-transferase (GST) (Habig, Pabst and Jakoby, 1974) and determination of lipid peroxidation levels by measuring thiobarbituric reactive substances (TBARS) (Oakes and Van Der Kraak, 2003) were also performed. A spectrofluorimeter (Biotek, Synergy HT®) was used for all analyzes.

2.5 Growth parameters

Twenty-two fish from each tank (n = 66 per treatment) were randomly sampled and were then sedated with 50 ppm of benzocaine hydrochloride before final biometric (60 days). Growth parameters evaluated were: Survival (S in %) = number of fish at the end of each analyzed period/initial fish number x 100; Weight Gain (WG in g) = final weight (g) - initial weight (g); Biomass (B in g) = average weight (g) x number of fish at the end of each analyzed period; Specific Growth Rate (SGR in %) = 100 x [(ln final weight (g) - ln initial weight (g)/time (days)]; Food Intake (FI in g): quantity of food consumed (g)/number of fish at the end of each analyzed period; Food Conversion Rate (FCR in g) = food provided (g)/weight gain (g).

2.6 Statistical analysis

The homogeneity of variances and normality were verified by Levene and Kolmogorov-Smirnov's test, respectively. Data were analyzed by two-way ANOVA (NO_2^- x $CaCO_3$ levels) followed by the Tukey test. The significance level was set at 5% in all cases ($p < 0.05$).Data were presented as mean ± standard error.

2.7 Ethic

The methodology applied in this study was approved by the Ethics Committee and Animal Welfare Committee of the Universidade Federal do Rio Grande - FURG (process number P095/2016).

3 - Results

Regarding blood parameters, no statistical differences were observed for glucose, pH, hematocrit, hemoglobin and MCHC among treatments. However, fish maintained at 15 mg N-NO_2^-.L^{-1} + 60 mg $CaCO_3$.L^{-1} presented significantly higher erythrocyte and lower MCV and MCH in relation to control treatment and 30 mg N-NO_2^-.L^{-1} + 60 mg $CaCO_3$.L^{-1} treatment. The same treatment (15 mg N-NO_2^-.L^{-1} + 60 mg $CaCO_3$.L^{-1}) presented significantly higher erythrocyte in relation at 15 mg N-NO_2^-.L^{-1} + 150 mg $CaCO_3$.L^{-1} treatment. The 0,05 mg N-NO_2^-.L^{-1} + 150 mg $CaCO_3$.L^{-1} and 30 mg N-NO_2^-.L^{-1} + 150 mg $CaCO_3$.L^{-1} treatment presented significantly lower MCV in relation to control treatment and 30 mg N-NO_2^-.L^{-1} + 60 mg $CaCO_3$.L^{-1} treatment, respectively (Table 1).

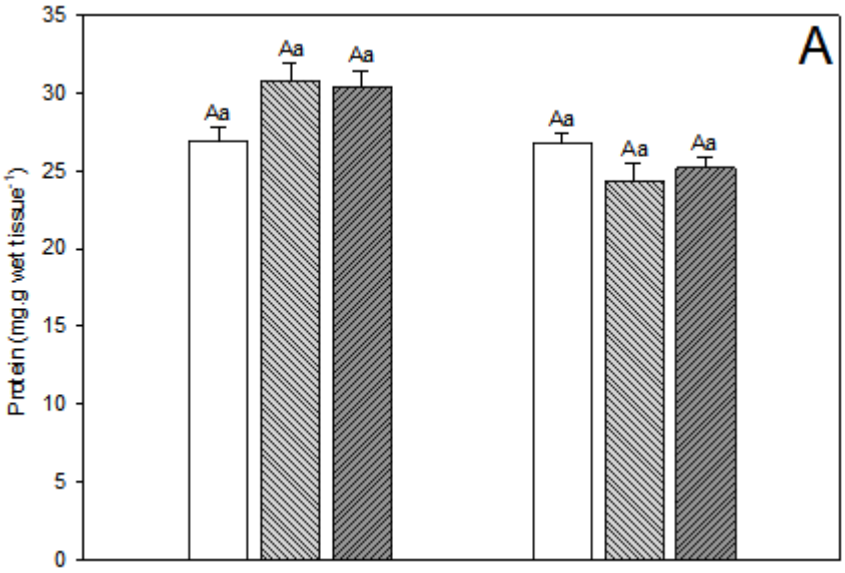
Table 1. Blood parameters results for *Piaractus mesopotamicus* juveniles exposed to different $CaCO_3$ and NO_2^- concentrations in water for 60 days.

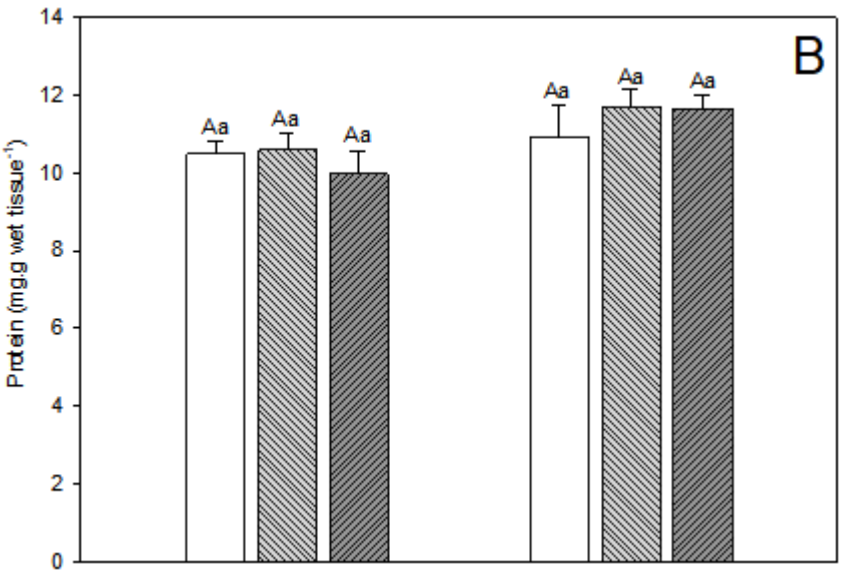
Parameters	Treatments					
	Control	15 NO_2^- + 60	30 NO_2^- + 60	0 NO_2^- + 150	15 NO_2^- + 150	30 NO_2^- + 150
Glucose (mg dL^{-1})	72.22±0.77	72.24±2,62	74.33±8.95	70.11±6.06	68.77±5.50	75.0±5.78
pH	7.55±0.09	7.48±0.07	7.48±0.01	7.50±0.01	7.54±0.01	7.54±0.05
Hematocrit (%)	32.33±0.50	31.55±1.30	32.11±0.80	30.77±0.40	30.44±1.63	29.11±1.63
Erythrocyte (10^6 μL^{-1})	1.23±0.07Aa	1.53±0.15Ba	1.26±0.09Aa	1.38±0.07Aa	1.34±0.03Ab	1.37±0.11Aa
Hemoglobin (g dL^{-1})	8.36±0.26	8.55±0.47	8.37±0.49	8.56±0.14	8.28±0.17	8.30±0.23
MCV	271.5±17.4Aa	211.36±15.3Ba	271.84±15.8Aa	226.69±9.9Ab	232.67±18.0Aa	214.78±8.2Ab
MCH	70.60±6.21Aa	56.92±2.88Ba	70.71±6.96Aa	63.49±4.94Aa	63.08±2.17Aa	61.74±3.59Aa
MCHC	25.90±0.50	27.16±0.72	26.09±2.11	28.01±0.87	27.47±1.73	28.94±1.05

MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration. Different lowercase letters in the same row indicate statistically significant differences ($P<0.05$) between $CaCO_3$ at the same NO_2^- concentration. Different capital letters in the same row indicate statistically significant differences ($P<0.05$) between NO_2^- at the same $CaCO_3$ concentration (Two-way ANOVA and Tukey test, $p < 0.05$, $n = 9$).

3.1 *Total protein content*

There was not observed significant differences between treatments in the total protein content in liver (Figure 1A), muscle (Figure 1B) and gill (Figure 1C).


A
Protein (mg.g wet tissue^{-1})
35
30
25
20
15
10
5
0
Aa
Aa
Aa
Aa
Aa
Aa


B
Protein (mg.g wet tissue^{-1})
14
12
10
8
6
4
2
0
Aa
Aa
Aa
Aa
Aa
Aa

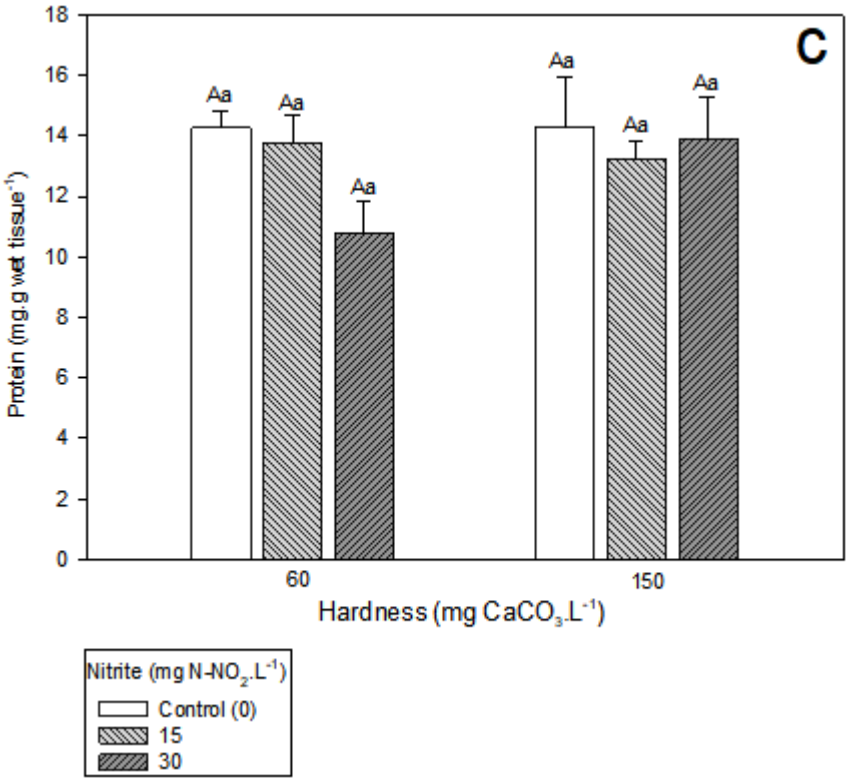
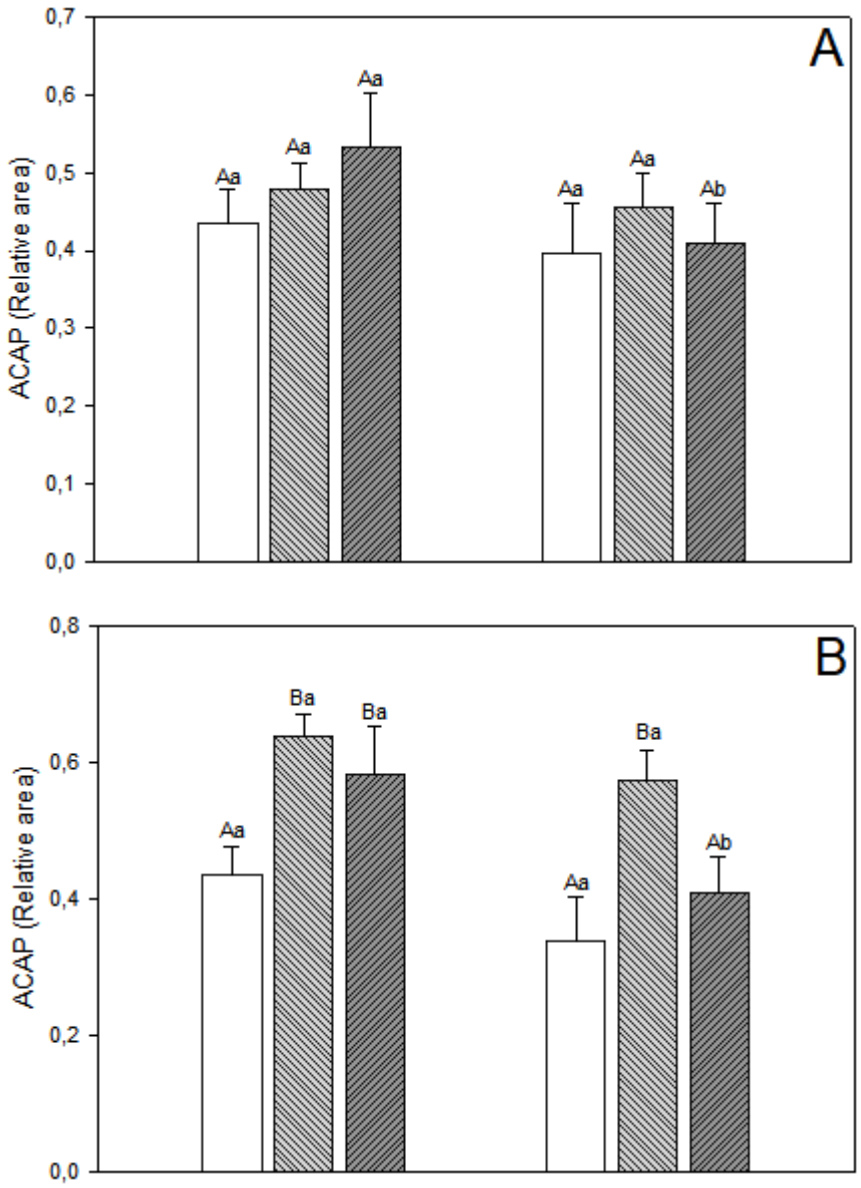


Figure 1. Total protein content (mg g wet tissue^{-1}) in liver (A), muscle (B) and gill (C) of *Piaractus mesopotamicus* juveniles exposed to different NO_2^- and $CaCO_3$ concentration in water for 60 days. Different lowercase letters indicate statistically significant differences ($P<0.05$) between $CaCO_3$ at the same NO_2^- concentration. Different capital letters indicate statistically significant differences ($P<0.05$) between NO_2^- at the same $CaCO_3$ concentration (Two-way ANOVA and Tukey test, $p < 0.05$, $n = 9$).

3.2 *Total antioxidant capacity against peroxyl radicals*

The total antioxidant capacity against peroxyl radicals in liver reduced were observed of fish maintained at 30 mg $N\text{-}NO_2^-.L^{-1}$ + 150 mg $CaCO_3.L^{-1}$ in relation to fish maintained at 30 mg $N\text{-}NO_2^-.L^{-1}$ + 60 mg $CaCO_3.L^{-1}$ (Figure 2A). Reduced antioxidant capacity was observed in muscle of fish maintained at 15 mg $N\text{-}NO_2^-.L^{-1}$ + 60 mg $CaCO_3.L^{-1}$ and 30 mg $N\text{-}NO_2^-.L^{-1}$ + 60 mg $CaCO_3.L^{-1}$ in relation to the control treatment. Fish maintained at 15 mg $N\text{-}NO_2^-.L^{-1}$ + 150 mg $CaCO_3.L^{-1}$ presented significantly higher results compared to fish exposed to 0,05 mg $N\text{-}NO_2^-.L^{-1}$ + 150 mg $CaCO_3.L^{-1}$ and 30 mg $N\text{-}NO_2^-.L^{-1}$ + 150 mg $CaCO_3.L^{-1}$. Fish maintained at 30 mg $N\text{-}NO_2^-.L^{-1}$ + 150 mg $CaCO_3.L^{-1}$ presented significantly lower results compared to

fish exposed to 30 mg $N\text{-}NO_2^-.L^{-1}$ + 60 mg $CaCO_3.L^{-1}$ (Figure 2B). There were not observed significant differences between treatments in the total antioxidant capacity against peroxyl radicals in gill (Figure 2C).



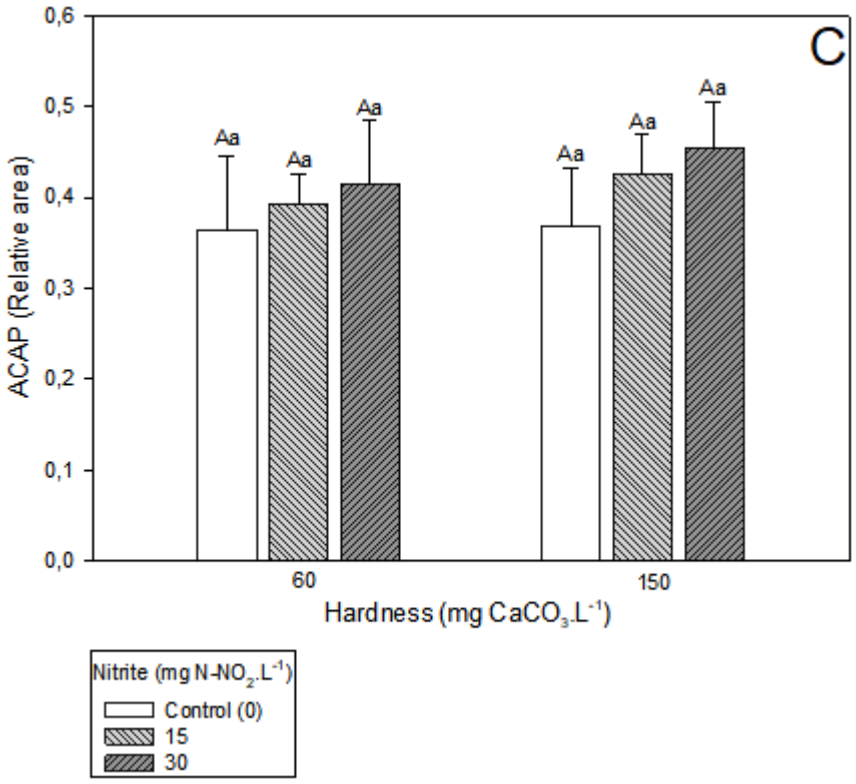
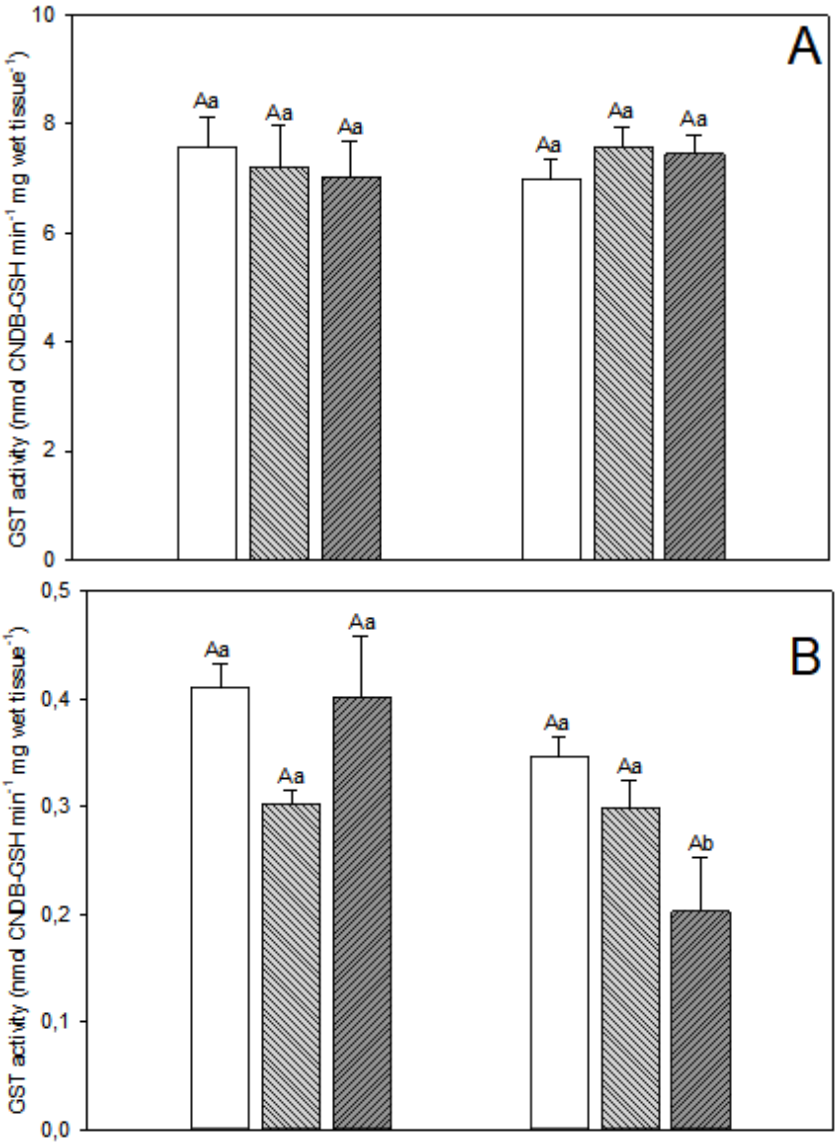


Figure 2. Total antioxidant capacity against peroxyl radicals (ACAP) in liver (A), muscle (B) and gill (C) of *Piaractus mesopotamicus* juveniles exposed to different NO_2^- and $CaCO_3$ concentration in water for 60 days. Different lowercase letters indicate statistically significant differences ($P<0.05$) between $CaCO_3$ at the same NO_2^- concentration. Different capital letters indicate statistically significant differences ($P<0.05$) between NO_2^- at the same $CaCO_3$ concentration (Two-way ANOVA and Tukey test, $p < 0.05$, $n = 9$).

3.3 *Glutathione-S-transferase activity*

There was not observed significant differences between treatments in the GST activity in liver (Figure 3A). The GST activity in muscle of the fish maintained at 30 mg $N\text{-}NO_2^-.L^{-1}$ + 150 mg $CaCO_3.L^{-1}$ was significantly lower in relation to fish maintained at 30 mg $N\text{-}NO_2^-.L^{-1}$ + 60 mg $CaCO_3.L^{-1}$ (Figure 3B). The GST activity in gill of the fish maintained at 0,05 mg $N\text{-}NO_2^-.L^{-1}$ + 150 mg $CaCO_3.L^{-1}$ presented significantly higher results in relation to control treatment. And the GST activity in gill of the fish maintained at 30 mg $N\text{-}NO_2^-.L^{-1}$ + 150 mg $CaCO_3.L^{-1}$ was significantly lower in relation to fish maintained at 0,05 mg $N\text{-}NO_2^-.L^{-1}$ + 150 mg $CaCO_3.L^{-1}$ and 30 mg $N\text{-}NO_2^-.L^{-1}$ + 60 mg $CaCO_3.L^{-1}$ (Figure 3C).


A
B
GST activity (nmol CNDB-GSH min-1 mg wet tissue-1)
10
8
6
4
2
0
0,5
0,4
0,3
0,2
0,1
0,0
Aa
Aa
Aa
Aa
Aa
Aa
Aa
Aa
Aa
Aa
Ab

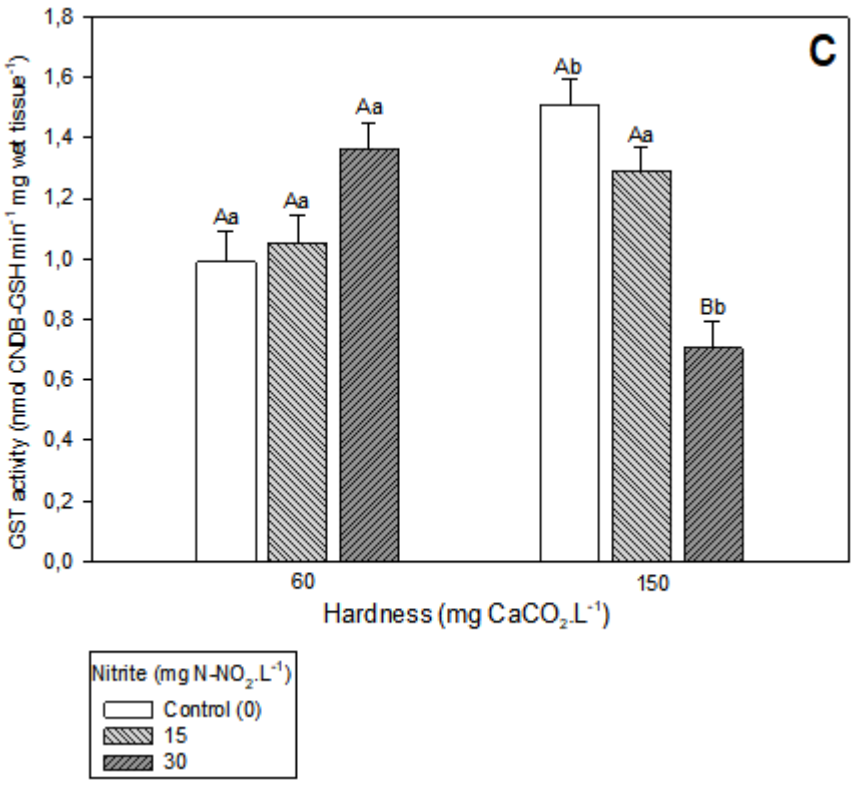
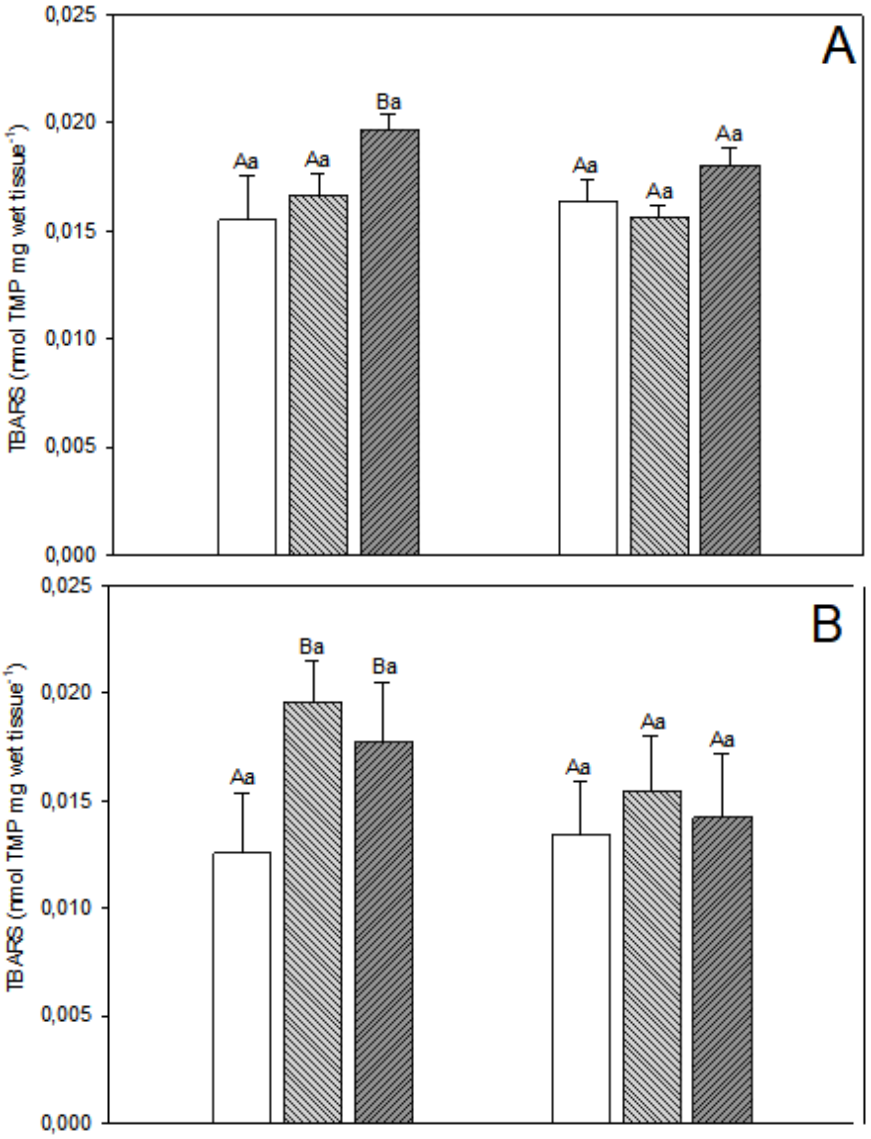


Figure 3. Glutathione-S-transferase (GST) activity in liver (A), muscle (B) and gill (C) of *Piaractus mesopotamicus* juveniles exposed to different NO_2^- and $CaCO_3$ concentration in water for 60 days. Different lowercase letters indicate statistically significant differences ($P<0.05$) between $CaCO_3$ at the same NO_2^- concentration. Different capital letters indicate statistically significant differences ($P<0.05$) between NO_2^- at the same $CaCO_3$ concentration (Two-way ANOVA and Tukey test, $p < 0.05$, $n = 9$).

3.4 *Lipid Peroxidation – TBARS*

The TBARS levels in liver of fish maintained at 30 mg $N\text{-}NO_2^-.L^{-1}$ + 60 mg $CaCO_3.L^{-1}$ was significantly lower in relation to fish maintained at 15 mg $N\text{-}NO_2^-.L^{-1}$ + 60 mg $CaCO_3.L^{-1}$ and control treatment (Figure 4A). The TBARS levels in muscle of fish maintained at 15 mg $N\text{-}NO_2^-.L^{-1}$ + 60 mg $CaCO_3.L^{-1}$, 30 mg $N\text{-}NO_2^-.L^{-1}$ + 60 mg $CaCO_3.L^{-1}$ and 0,05 mg $N\text{-}NO_2^-.L^{-1}$ + 150 mg $CaCO_3.L^{-1}$ was significantly lower in relation to control treatment (Figure 4B). There were not observed significant differences between treatments in the TBARS levels in gills (Figure 4C).


A
B
TBARS (nmol TMP mg wet tissue-1)
0,025
0,020
0,015
0,010
0,005
0,000
Aa
Aa
Ba
Aa
Aa
Aa
Aa
Ba
Ba
Aa
Aa
Aa

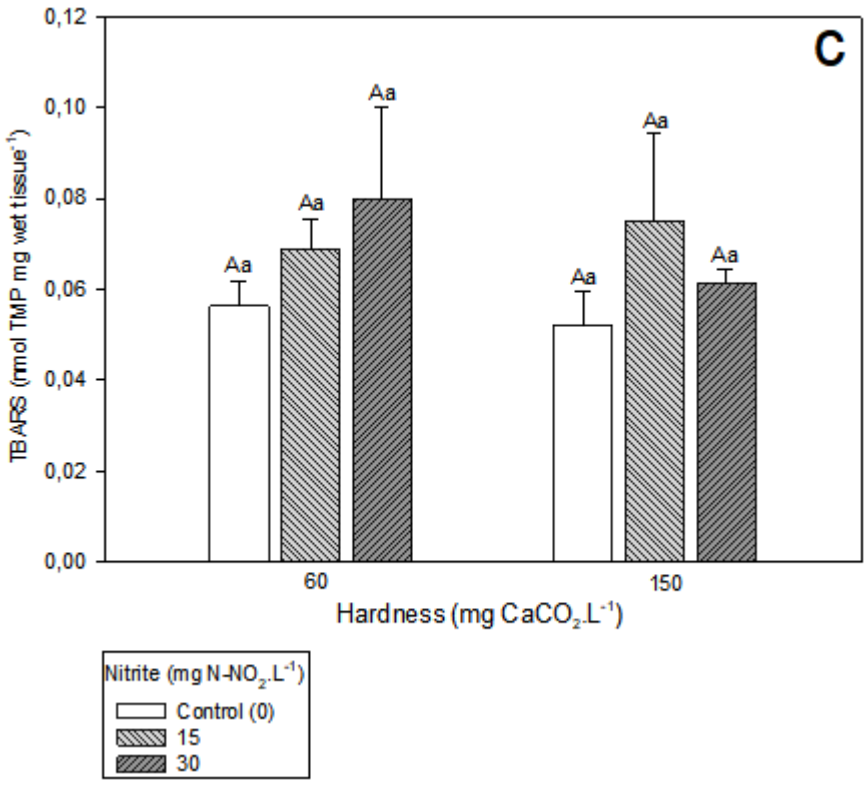


Figure 4. Thiobarbituric acid reactive substances (TBARS) content (nmol TMP mg wet tissue^{-1}) in liver (A), muscle (B) and gill (C) of *Piaractus mesopotamicus* juveniles exposed to different NO_2^- and $CaCO_3$ concentration in water for 60 days. Different lowercase letters indicate statistically significant differences ($P<0.05$) between $CaCO_3$ at the same NO_2^- concentration. Different capital letters indicate statistically significant differences ($P<0.05$) between NO_2^- at the same $CaCO_3$ concentration (Two-way ANOVA and Tukey test, $p < 0.05$, $n = 9$).

No statistical differences was observed between treatments for survival, weight gain, biomass and specific growth rate. However, fish maintained at 0,05 mg N-NO_2^-.L^{-1} + 150 mg $CaCO_3$.L^{-1} presented significantly lower feed intake than those kept at control treatment. The treatment with 15 mg N-NO_2^-.L^{-1} + 60 mg $CaCO_3$.L^{-1} and 30 mg N-NO_2^-.L^{-1} + 60 mg $CaCO_3$.L^{-1} presented significantly higher feed conversion rate in relation at control treatment, and the treatment 30 mg N-NO_2^-.L^{-1} + 150 mg $CaCO_3$.L^{-1} was significantly lower feed conversion rate in relation to 30 mg N-NO_2^-.L^{-1} + 60 mg $CaCO_3$.L^{-1} treatment (Table 2).

Table 2: Survival and growth parameters of *Piaractus mesopotamicus* juveniles exposed to different $CaCO_3$ and NO_2^- concentration in water for 60 days.

Treatments (NO_2^-+ $CaCO_3$)	Survival (%)	WG (g)	Biomass (g)	SGR (%)	FI (g)	FCR
Control (0+60)	100±0.0	43.33±4.48	807.20±39.50	1.47±0.15	57.68±1.50 Ab	1.35±0.09 Aa
15 NO_2^- + 60	100±0.0	34.44±5.10	689.05±49.52	1.28±0.17	55.22±3.60 Aa	1.64±0.13 Ba
30 NO_2^- + 60	100±0.0	33.06±3.69	645.19±44.28	1.25±0.10	58.13±4.82 Aa	1.77±0.06 Bb
0 NO_2^- + 150	100±0.0	34.07±1.00	709.13±18.75	1.43±0.03	40.61±2.13 Aa	1.19±0.07 Aa
15 NO_2^- + 150	100±0.0	35.40±3.75	636.55±48.37	1.38±0.09	43.31±1.45 Aa	1.24±0.08 Aa
30 NO_2^- + 150	100±0.0	39.43±3.14	693.20±39.14	1.42±0.08	44.98±2.66 Aa	1.14±0.05 Aa

WG= weight gain; SGR= specific growth rate; FI= food intake; FCR= food conversion rate. Different lowercase letters in the same column indicate statistically significant differences ($P<0.05$) between $CaCO_3$ at the same NO_2^- concentration. Different capital letters in the same column indicate statistically significant differences ($P<0.05$) between NO_2^- at the same $CaCO_3$ concentration (Two-way ANOVA and Tukey test, $p < 0.05$, $n = 9$).

4- Discussion

Water quality plays an important role in the growth and reproduction of aquatic organisms, although each factor plays its own influence, the interaction between various parameters should also be taken into account to aquaculture success (Suman et al., 2017; Surnar et al., 2018). NO_2^- exposure can induce a stress condition in fish affecting the homeostasis of the physiological system, leading to a reduction in growth rate (Ciji et al., 2014), increases vulnerability to disease (Jia et al., 2016) and even death (Wuertz et al., 2013). However, few studies seek alternatives to minimize the damage caused or reduce the toxicity of these compounds (Maltez et al., 2018; Neves et al., 2017). In this perspective, this study demonstrated that the increase of water hardness through the addition of $CaCO_3$ can decrease the NO_2^- toxicity to pacu.

Several studies with different species and life stages have been conducted to establish the optimal water hardness levels (Silva et al., 2005), which are usually between 50 and 150

mg $CaCO_3.L^{-1}$ (Stone and Thomforde et al., 2004; Kasiri, 2011), and values below 20 mg $CaCO_3.L^{-1}$ cause considered able to cause stress (Bhatnagar et al., 2004). In our study, the higher hardness concentrations (150 mg $CaCO_3.L^{-1}$) did not induce any negative effect on survival and growth of pacu. The blood and oxidative stress parameters in general also remain at basal levels, except for a reduction in the MCV, indicating the absence of an evident stress condition triggered by the $CaCO_3$ addition. Fish kept at higher hardness also showed reduced FI, but similar zootechnical performance, suggesting a lower energy demand in this condition. Copatti et al. (2011a, 2011b) showed that exposure of silver catfish up to 180 mg $CaCO_3.L^{-1}$ at pH 7.0 did not change growth compared to lower water hardness.

It is widely discussed in the literature that the toxic effects of NO_2^- induce changes in different blood parameters (Jensen, 2003; Kroupova et al., 2008; Neves et al., 2017) including hematocrit, total hemoglobin, number of red blood cells, among others (Avilez et al., 2004; Kroupova et al., 2008; Ciji et al., 2012). In the present study, the exposure even at the highest NO_2^- concentration did enough to induce alterations in the most of the evaluated blood parameters (pH, glucose, hematocrit, hemoglobin and MCHC) of juvenile pacu. Another possible explanation for the absence of changes may be the adaptation of fish to the stressor after a long period of exposure restoring your homeostasis. The only observed differences among treatments there was an increase in erythrocytes with led to consequent reduction in MCV and MCH in fish exposed to 15 mg $N\text{-}NO_2^-.L^{-1}$ and lower water hardness.

The oxidative stress induced by NO_2^- in fish is a result of enhanced ROS or RNS production and/or weakening of the antioxidant system (Jia et al., 2016; Ciji et al., 2014; Maltez et al., 2018). The antioxidants system comprises several enzymatic and non-enzymatic defenses and ACAP assessment sheds light on the overall antioxidant status of the fish. The ACAP reduction was observed in pacu muscle during NO_2^- exposure suggesting a pro-oxidant condition resulting in expenditure or inhibition of antioxidant components due to oxidative

damage. In addition, coping with stress involves increased energy demand (Barton, 2002), which can lead to a reduced antioxidant production by the organism.

GST represent the major group enzymes involved in detoxification of xenobiotics and endogenous metabolites including oxidative damage end-products, through the catalysis of nucleophilic attack of the sulfur atom of glutathione to electrophilic substrates providing cellular defense against chemically induced toxicity (Blanchette et al. 2007). Lin et al. (2018) observed an increase in GST enzyme activity in *Aristichthys nobilis* exposed to NO_2^- (48.6 mg $N\text{-}NO_2^-.L^{-1}$) for 96 h, evidencing the importance of this enzyme activity in NO_2^- induced oxidative stress. Otherwise, in the present study, the redution of GST activity was demonstrated in muscle and gill of fish maintained at 30 mg $N\text{-}NO_2^-.L^{-1}$ + 150 mg $CaCO_3.L^{-1}$.This result indicate that the group of enzymes was compromised or a less need for detoxification when the fish was exposed to interaction with higher NO_2^- concentrations and high hardness.

When the antioxidant system is unable to efficiently counteract the action of pro-oxidants as ROS and RNS, oxidative damage levels is intensified (Halliwell and Gutteridge 2015). Between possible oxidative damages in cells, lipid peroxidation occurs as a chain reaction, initiated by a hydroxyl radical that leads to oxidation of polyunsaturated fatty acids (PUFA) that could be measure by TBARS assay. Exposure to NO_2^- induced an increase in lipid peroxidation in the muscle of pacu at concentrations of 15 and 30 mg $N\text{-}NO_2^-.L^{-1}$ in lower hardness. Similarly, Maltez et al. (2018) also demonstrated NO_2^- induced enhanced levels of TBARS in gills and muscle of juvenile Brazilian flounder *Paralichthys orbignyanus*. The main effect of LPO is a decrease in membrane fluidity due to PUFA oxidation, affecting several biological processes (Halliwell and Gutteridge 2015), and causing a variety of adverse health effects and diseases in the organisms (Livingstone, 2003; Valavanidis et al., 2006). In addition, the impairment of flesh quality can be related to higher oxidative degradation in muscle, the main product of interest in aquaculture (Zhang et al. 2016).

The prolonged exposed to inadequate NO_2^- levels can result in tertiary stress responses affecting growth, reproduction and the immune system, alter behavioral patterns, and reduce the ability to tolerate subsequent or additional stressors (Barton, 2002). Saoud et al. (2014) demonstrated reduced growth and survival, besides changes in hematological parameters and gill histology, in Rabbitfish *Siganus rivulatus* exposed to NO_2^-. In the present study, NO_2^- exposure did not negatively affect the survival, WG, biomass, SGR and FI of pacu. However, fish exposed to both NO_2^- concentrations at low hardness concentrations (15 mg $N\text{-}NO_2^-.L^{-1}$ + 60 mg $CaCO_3.L^{-1}$ and 30 mg $N\text{-}NO_2^-.L^{-1}$ + 60 mg $CaCO_3.L^{-1}$) had higher FCR, suggesting a worst utilization of food consumption for weigh gain.

Summary, the result of the present study showed that at lower hardness, negative effects of NO_2^- were most evident, leading to enhanced levels of oxidative damage in lipids of muscle and gills, and higher FCR. Otherwise, at higher hardness despite the changes in the antioxidant defenses, the levels of lipid peroxidation were maintained at basal levels and no changes were found in zootechnical parameters. These results indicate that the increase in hardness to 150 mg $CaCO_3.L^{-1}$ may reduce the NO_2^- toxic action in juveniles of pacu, and can be an interesting alternative of water quality management, especially for intensive aquaculture of this species. Recently, Neves et al. (2017) founded that the increase of Ca^{2+} levels did not minimize NO_2^- toxicity in Silver catfish *Ramdia quelen*. However, it is important to emphasize that the authors used a lower Ca^{2+} concentration in relation to the used in the present study, which can explain the differences obtained between the studies. Thus, we suggesting that further studies should consider evaluating different levels of Ca^{2+} addition in water.

5- Conclusions

In conclusion, NO_2^- exposure induced the oxidative stress and compromise de FCR in juvenile *Piaractus mesopotamicus*, and the addition of $CaCO_3$ in the water was effective to minimize this long-term toxic effect.

6- Acknowledgements

The authors are grateful to the Conselho Nacional de Desenvolvimento Tecnológico (CNPq), Comissão de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Universidade Federal do Rio Grande/FURG.

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CAPÍTULO 3

Sodium chloride and calcium carbonate addition in the water reduces short-term nitrite toxicity and its effects on blood parameters of juvenile pacu *Piaractus mesopotamicus*.

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Abstract

The aim of the present work was to evaluate if the NaCl or $CaCO_3$ addition in the water reduces short-term NO_2^- toxicity and its effects on blood parameters of juvenile pacu *Piaractus mesopotamicus*. For each experiment, fish were submitted for five days to six treatments comprised by the interaction of two NaCl (0 and 5 g NaCl. L^{-1})(Experiment 1) or $CaCO_3$ (60 and 150 mg $CaCO_3^-.L^{-1}$)(Experiment 2), and three NO_2^- concentrations (0, 15 and 30 mg N-$NO_2^-.L^{-1}$). After 2 and 5 days of exposure, the fish were anesthetized and the blood was collected by caudal vein puncture to measure the pH, glucose levels, hemoglobin and erythrocyte concentration, and the hematimetric indices: mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). Exposure to NO_2^- induced different changes in blood parameters of pacu including enhanced erythrocyte concentration and MCHC values, and reduction in hematocrit, MCV and MCH. The NaCl or $CaCO_3$ addition led to no statistical differences between the control and the NO_2^- treatments at the end of exposure period (day 5) in both cases, while some parameters were altered in the treatments without the addition of compounds. In addition, more parameters presented changes from respective control at day 2 in the treatments without the addition than in treatments with $CaCO_3$. In conclusion, short-term NO_2^- exposure induces changes in blood parameters of juvenile pacu and the use of NaCl or $CaCO_3$ was effective in minimizing these toxic effects.

Keywords: freshwater fish; nitrogen compound; toxicity; hematimetric indices; hemoglobin

1- Introduction

The pacu *Piaractus mesopotamicus* (Characidae) is a omnivorous species from Plata basin, and one of the most important native freshwater fish to pisciculture in South America. It is a rustic species, well adapted to handling and rearing conditions, that presents good zootechnical performance and acceptance in the consumer market due to excellent meat quality. Due to these characteristics the species presents a great potential for intensive aquaculture (Urbinati et al., 2013).

One of the main goals of modern aquaculture is the intensification of production system allowing higher productitivy in a more environmentally sustainable manner, reducing land and freshwater use and minimizing wastewater production (Klinger and Naylor, 2012). However, higher stocking density requires greater attention to monitoring and controlling water quality parameters, avoiding undesirable conditions to produced animals (Boyd and Tucker, 2012).

In terms of water quality, one of the most limiting factors in intensive breeding systems is the build-up of toxic nitrogenous compounds. Among them, the NO_2^- is an intermediate product in bacterial nitrification and denitrification processes. In rearing systems, such as in recirculation aquaculture systems (RAS), imbalances of nitrification process may lead to NO_2^- build-up, which causes a potential risk to farmed fish. The main toxic mechanism related to NO_2^- is the oxidation of hemoglobin (Hb) to methemoglobin (metHb), which cannot bind to oxygen, compromising the tissue oxygenation (Kroupova et al., 2008; Wuertz et al., 2013). In addition, a number of other toxicological effects are also linked to NO_2^- toxicity in fish (Jensen, 2003; Maltez et al., 2018; Ciji et al., 2014; Jia et al., 2015; Wuertz et al., 2013). These physiological disorders can result in changes in hematological and blood biochemical parameters (Jensen et al., 2003, Kroupova et al., 2008, Ciji et al., 2012; Neves et al., 2017; Maltez et al., 2018), which be considered excellent biomarkers of the health status and welfare of fish.

Taking in account the high toxicity of NO_2^- to organisms and the risk of this compound reach toxic levels in the environment of production, the search for alternatives to minimize its deleterious effects NO_2^- becomes relevant.

Some studies demonstrated that the NO_2^- toxicity to fish can be reduced by the increased NaCl concentration (Sampaio et al., 2002; Weirich and Riche, 2006a, b; Costa et al., 2008). NO_2^- uptake in freshwater fish occurs mainly through the gill membrane and it is related to branchial Cl^- uptake rates (Jensen, 2003) because NO_2^- competes with Cl^- in the Cl^-/HCO_3 cotransporter (Tomasso and Grosell, 2005). Consequently, the increase of waterborne Cl^- levels can reduce NO_2^- toxicity (Kroupova et al.,2005; Yanbo et al., 2006; Boudreaux et al., 2007).

Enhanced water hardness through the addition of Ca^{2+} also can be assessed as a strategy to minimize NO_2^- toxicity. The Ca^{2+} plays a key role in ion regulation by reducing the permeability of biological membranes and thus the diffusive flow of ions to water (Wood and Mcdonald, 1988; Gonzalez, 1996). Furthermore, the increase of waterborne Ca^{2+} can then reduce Cl^- loss in freshwater fish and the activity of the Cl^-/HCO_3 cotransporter, reducing NO_2^- uptake and toxicity. Studies have demonstrated that an increase of waterborne $CaCl_2$ has a stronger effect on reducing acute NO_2^- toxicity than an increase of waterborne NaCl in some species (Tomasso et al., 1980; Kroupova et al., 2005).

Therefore, the aim of the present study was to verify if the addition of NaCl or $CaCO_3$ in the water reduces short-term NO_2^- toxicity and its effects on blood parameters of juvenile pacu *P. mesopotamicus*.

2- Material and Methods

This study was developed in the Laboratório de Aquacultura Continental of the Universidade Federal do Rio Grande - FURG. Juvenile *P. mesopotamicus* (42.6 ± 0.8 g and 14.4 ± 0.07 cm) were obtained from a commercial fish farm located in Rio Grande do Sul state,

southern Brazil. Throughout acclimation period the diet was offered twice a day (9:00 am and 4:00 pm) until the apparent satiety with extruded commercial feed (Supra Aquafeed® with 46% of crude protein). During the experimental period, the fish were not fed.

2.1 NaCl and NO_2^- Exposure

After acclimation, fish (6/tank) were randomly distributed in 18 tanks (80 L useful volume) kept in semi-static systems. Fish were submitted for five days to six treatments comprised by the interaction of two NaCl (0 and 5 g NaCl. L^{-1}) and three NO_2^- concentrations (0, 15 and 30 mg $N\text{-}NO_2^-.L^{-1}$). All treatments were performed in triplicate. The NO_2^- levels above the safe level estimated by Neves et al., (unpublished data) were chosen to perform the present study. The NaCl and NO_2^- concentrations were obtained by addition of NaCl and Sodium nitrite ($NaNO_2$) to the water, or water exchange, when required. During the experimental period the water quality parameters were maintained at suitable conditions for pacu, except the NaCl and NO_2^- concentration, which were modified according experimental treatments.

2.2 $CaCO_3$ and NO_2^- Exposure

The experimental design was similar to that described in item 2.1. However, in this experiment fish were exposed during five days to six treatments comprised by the interaction of two hardness (60 and 150 mg $CaCO_3^-.L^{-1}$) and three NO_2^- concentrations (0, 15 and 30 mg $N\text{-}NO_2^-.L^{-1}$), all performed in triplicate. The $CaCO_3$ levels according to the ideal range found in the literature (Stone and Thomforde, 2004; Kasiri et al., 2011; Rajkumar et al., 2018). The water hardness was adjusted by the addition of calcium carbonate ($CaCO_3$) to the water.

2.3 Blood parameters

After 2 and 5 days of exposure the fish were anesthetized (50 ppm of benzocaine hydrochloride) and the blood collection was performed by caudal vein puncture. The blood samples were used for measure pH (pH meter FE 20-FiveEasy TM with appropriated probe, Mettler Toledo), glucose levels (Accu-check Performa - Roche®), hematocrit percentage (Goldenfarb, Bowyer, Hall and Brosious, 1971) and hemoglobin concentration using commercial colorimetric kit (Doles®, Brazil). The erythrocyte number was counted in a Neubauer chamber in diluted blood (1:200) in 0.65% sodium chloride solution (Ranzini-Paiva et al., 2013). The hematimetric indices: mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were also estimated (Wintrobe, 1934).

2.4 Water quality parameters

The water quality parameters were monitored daily (pH, temperature, dissolved oxygen, NaCl concentration, ammonia and NO_2^-) or twice a week (alkalinity, water hardness) and kept within the expected values for tropical freshwater fish (Boyd and Tucker, 2012). The parameters were maintained as follows: pH (7.55 ± 0.04) (pH meter FE 20-FiveEasy TM, Mettler Toledo), dissolved oxygen (7.16 ± 0.14 $mg.L^{-1}$) and temperature (27.2 ± 0.01 °C) (digital oximeter YSI EcoSense® DO200A), total ammonia nitrogen (0.39±0.25 mg $TAN.L^{-1}$) (UNESCO, 1983), un-ionized ammonia (0.03 ± 0.01 $mg.L^{-1}$) (Colt, 2002) and total alkalinity (81.72 ± 1.37 mg $CaCO_3.L^{-1}$) (Eaton et al., 2005).

The concentrations of NO_2^- (0.01 ± 0.01; 14.29 ± 0.21 and 31.47 ± 0.74 mg $N\text{-}NO_2^-.L^1$) (Bendschneider and Robinson, 1952), NaCl concentration (0.0 ± 0.0 and 5.0 ± 0.01 g $NaCl.L^{-1}$) (handheld refractometer model RBX2862) and hardness (64.12 ± 0.54 and 148.82 ± 1.14 mg

$CaCO_3.L^{-1}$) (Adad et al., 1982) were altered and maintained according to the experimental treatments described before. Photoperiod was fixed at 12 h light/12 h dark.

2.5 Statistical analysis

The homogeneity of variances and normality were verified by Levene and Kolmogorov-Smirnov's test, respectively. For parameters that did not meet the data assumptions were transformed by applying the equation X = Arcsine √X% / 100 (hematocrit and erythrocyte). It was applied a multifactorial model to compare the blood parameters between treatments, having as predictor factors: (NO_2^- x NaCl) and (NO_2^- x $CaCO_3$). Differences of the mean was calculate using Bonferroni tests ($p<0.05$).

2.6 Ethic

The methodology applied in this study was approved by the Ethics Committee and Animal Welfare Committee of the Universidade Federal do Rio Grande - FURG (process number P095/2016). All fish used in the experiment were returned to acclimatization tanks for recovery.

3- Results

With two and five days, the erythrocyte concentration increased at fish maintained at 15 and 30 mg $N\text{-}NO_2^-.L^{-1}$. With five days, MCV and MCH decreased at 15 and 30 mg $N\text{-}NO_2^-.L^{-1}$ in relation to control treatment, however, this change was not observed with increasing NaCl concentration (Table 1).

Table 1. Blood parameters results for *Piaractus mesopotamicus* juveniles with two and five days of exposure to different NaCl and NO_2^- concentrations in water

Parameters	Treatments					
Day 2	Control	15 NO_2^- + 0	30 NO_2^- + 0	0 NO_2^- + 5	15 NO_2^- + 5	30 NO_2^- + 5
Glucose (mg dL^{-1})	71.88±5.79	78.66±11.10	77.88±4.83	57.77±10.06	69.77±13.84	80.22±16.78
pH	7.44±0.03	7.46±0.01	7.50±0.02	7.52±0.02	7.53±0.04	7.49±0.04
Hematocrit (%)	34.22±2.83	40.61±10.19	36.00±8.35	31.72±0.72	38.33±6.02	32.66±1.0
Erythrocyte (10^6 μL^{-1})	1.31±0.02Ba	1.59±0.13Aa	1.56±0.08Aa	1.39±0.06Ba	1.51±0.07Aa	1.53±0.04Aa
Hemoglobin (g dL^{-1})	7.98±0.06	7.61±0.16	7.84±0.49	7.77±0.25	7.19±0.31	6.94±0.22
MCV	260.49±25.4	251.99±107.4	233.40±59.0	200.81±22.8	259.80±55.2	217.40±15.0
MCH	61.02±1.59	49.21±5.18	50.85±4.68	56.45±1.47	47.85±4.11	45.44±1.18
MCHC	25.77±1.65	21.07±4.09	24.46±2.58	24.75±0.29	20.06±1.90	21.57±0.96
Day 5						
Glucose (mg dL^{-1})	58.77±8.26	52.77±1.86	71.88±2.43	57.11±5.07	47.88±7.07	64.33±6.55
pH	7.49±0.06	7.49±0.02	7.54±0.02	7.49±0.03	7.51±0.03	7.45±0.01
Hematocrit (%)	30.11±1.39	29.77±0.72	27.44±0.58	29.88±1.63	31.55±2.72	31.77±3.34
Erythrocyte (10^6 μL^{-1})	1.27±0.02Ba	1.64±0.09Aa	1.59±0.07Aa	1.39±0.06Aa	1.49±0.05Aa	1.44±0.04Aa
Hemoglobin (g dL^{-1})	8.42±0.17	7.91±0.27	8.72±0.28	8.41±0.06	8.35±0.20	7.91 ±0.37
MCV	238.46±8.9Aa	183.42±7.8Ba	173.66±6.7Ba	216.78±4.5Aa	212.43±13.8Aa	221.83±25.4Aa
MCH	66.71±2.53Aa	48.57±1.36Ba	55.43±2.31Ba	61.58±3.14Aa	56.39±1.25Aa	55.23±4.27Aa
MCHC	28.53±2.20	26.60±0.36	31.95±1.51	28.38±1.72	26.86±1.57	25.39±1.75

MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration. Different lowercase letters in the same row indicate statistically significant differences ($P<0.05$) between NaCl at the same NO_2^- concentrations. Different capital letters in the same row indicate statistically significant differences ($P<0.05$) between NO_2^- at the same NaCl concentrations (Two-way ANOVA and Tukey test, $p < 0.05$, $n = 9$).

In relation to fish exposure at different $CaCO_3$ and NO_2^- concentrations, with two days the percentage of hematocrit was lower at fish maintained at 30 mg $N\text{-}NO_2^-.L^{-1}$ + 60 mg $CaCO_3.L^{-1}$ in relation to control treatment and fish maintained at 15 mg $N\text{-}NO_2^-.L^{-1}$ + 150 mg $CaCO_3.L^{-1}$ was lower in relation to fish maintained at 0 mg $N\text{-}NO_2^-.L^{-1}$ + 150 mg $CaCO_3.L^{-1}$ and fish maintained at 15 mg $N\text{-}NO_2^-.L^{-1}$ + 60 mg $CaCO_3.L^{-1}$. The erythrocyte concentration increased at fish maintained at 15 and 30 mg $N\text{-}NO_2^-.L^{-1}$ in relation to control treatment, and in the same treatments, MCV decreased in relation to control treatment regardless of $CaCO_3$ concentration. MCH decreased and MCHC increased at fish maintained at 30 mg $N\text{-}NO_2^-.L^{-1}$ in relation to control treatment. MCHC decreased at fish maintained at 15 mg $N\text{-}NO_2^-.L^{-1}$ + 60

mg $CaCO_3.L^{-1}$ in relation to fish maintained at 15 mg $N\text{-}NO_2^-.L^{-1}$ + 150 mg $CaCO_3.L^{-1}$. With five days, the erythrocyte concentration increased at fish maintained at 15 and 30 mg $N\text{-}NO_2^-.L^{-1}$ in relation to control treatment. MCV decreased at fish maintained at 30 mg $N\text{-}NO_2^-.L^{-1}$ in relation to control treatment (Table 2).

Table 2. Blood parameters results for *Piaractus mesopotamicus* juveniles with two and five days of exposure to different $CaCO_3$ and NO_2^- concentrations in water

Parameters	Treatments					
Day 2	Control	15 NO_2^- + 60	30 NO_2^- + 60	0 NO_2^- + 150	15 NO_2^- + 150	30 NO_2^- + 150
Glucose (mg dL^{-1})	88.33±5.41	87.66±1.38	99.33±4.24	83.77±3.90	79.22±1.72	85.44±7.27
pH	7.69±0.02	7.81±0.04	7.93±0.04	7.71±0.01	7.78±0.07	7.83±0.08
Hematocrit (%)	33.44±0.61Aa	31.66±0.69Aa	25.77±2.02Ba	31.55±0.55Aa	26.44±0.90Bb	27.33±0.57ABa
Erythrocyte (10^6 μL^{-1})	1.37±0.04Aa	1.71±0.07Ba	1.67±0.04Ba	1.42±0.03Aa	1.46±0.05Aa	1.51±0.03Aa
Hemoglobin (g dL^{-1})	7.51±0.14	7.58±0.15	7.20±0.39	7.71±0.21	7.75±0.06	7.67±0.30
MCV	244.9±12.3Aa	189.7±4.4Ba	150.8±12.9Ba	224.6±6.4Aa	180.7±2.7Ba	182.3±8.5Ba
MCH	54.92±1.17Aa	45.60±1.11ABa	42.22±2.87Ba	54.77±0.28Aa	52.97±1.51Aa	51.32±3.21Aa
MCHC	22.50±0.80Ba	24.05±0.08Ba	28.15±1.06Aa	24.50±0.62Aa	29.94±0.88Ab	28.51±0.79Aa
Day 5						
Glucose (mg dL^{-1})	65.11±7.92	51.00±4.59	55.77±1.93	60.55±6.88	55.11±1.30	58.33±4.24
pH	7.69±0.05	7.72±0.01	7.77±0.03	7.66±0.02	7.69±0.02	7.72±0.03
Hematocrit (%)	34.72±2.07	33.44±1.92	31.77±0.29	31.44±1.68	29.33±0.66	29.11±0.80
Erythrocyte (10^6 μL^{-1})	1.39±0.03Ba	1.70±0.05Aa	1.72±0.02Aa	1.45±0.04Aa	1.53±0.02Aa	1.59±0.04Aa
Hemoglobin (g dL^{-1})	8.06±0.12	8.25±0.39	8.54±0.24	7.71±0.12	7.77±0.43	7.26±0.04
MCV	248.99±8.1Aa	197.67±17.1ABa	184.62±3.7Ba	220.14±16.0Aa	191.13±6.6Aa	184.51±8.8Aa
MCH	58.24±2.74	48.77±3.62	49.63±1.79	53.96±2.40	50.77±3.66	45.98±1.34
MCHC	23.44±1.67	24.83±0.60	26.90±0.54	25.29±1.53	26.90±1.41	25.06±0.83

MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration. Different lowercase letters in the same row indicate statistically significant differences ($P<0.05$) between $CaCO_3$ at the same NO_2^- concentrations. Different capital letters in the same row indicate statistically significant differences ($P<0.05$) between NO_2^- at the same $CaCO_3$ concentrations (Two-way ANOVA and Tukey test, $p < 0.05$, $n = 9$).

4- Discussion

NO_2^- is a toxic nitrogen compound that can be a potential problem in intensive aquaculture production systems that the nitrification process is present. In freshwater fish, the NO_2^- uptake occurs mainly through the gill and accumulate in blood and tissues causing several physiological disturbances in short and long-term (Jensen, 2003; Kroupova et al., 2005). In the present study, the NO_2^- induced effects on blood parameters of pacu were time and dose-depedent. In addition, the pattern of changes caused by NO_2^- were influenced by the addition of NaCl or $CaCO_3$ to the water.

The parameter that present major variation during the first experiment was the erythrocyte count. Higher values were observed in fish exposed to NO_2^- when compare to control values irrespective of NaCl addition at day 2, and in NO_2^- treatments without NaCl addition at day 5. The higher erythrocyte suggests the activation of the compensatory erythropoietic process as an attempt to ensure oxygen transport in the fish body submitted to hypoxic condition (Knudsen and Jensen, 1997). This result may an adaptive response to cope with NO_2^- induced methemoglobin formation and functional hypoxia (Jensen, 2003). Das et al. (2004) reported an initial increase in erythrocyte count for fingerling *Catla catla* during short-term exposure to sublethal levels of NO_2^-.

The reduction in MCV and MCH indicate the reduction in erythrocyte size and hemoglobin concentration, respectively. The entry of NO_2^- into the erythrocytes occurs by exchange with the K^+ causing an effect of red blood cells shrinkage to control hyperkalemia in the extracelular environment (Stormer et al. 1996; Maltez et al. 2019). Lower values of MCH can be explained by methemoglobin formation, then reducing hemoglobin concentration into the erythrocytes (Jensen, 2003).

This increased erythrocyte concentration in the blood can be responsible for maintaining total hemoglobin content and hematocrit levels at basal levels, even when MCV and MCH presented reduced values at the same sampling day. Otherwise, several works demonstrated that the NO_2^- has the capacity to reduce the hematocrit and hemoglobin concentration in blood. Martinez and Souza (2002) founded this pattern of response in study using *Prochilodus lineatus*. In species such as *Cyprinus carpio*, a decrease in hemoglobin concentrations has already been observed in a time interval of less than 96 hours with exposure to NO_2^- (Tilak et al. 2007). Thus, these results obtained in the present study suggesting that the pacu presented an effective mechanism to replace the hemoglobin transformed into methemoglobin as well as to compensate the red blood shrinkage. This serve as an evidence of the animal fitness in response stressors.

In the second experiment, NO_2^- induced changes were generally consistent with those found in the experiment 1, and including enhanced erythrocyte count, and reduced values of MCV and HCM. In addition, about MCHC, it was find an increase of this parameter pointing to the fact that this parameter is calculated by the relationship between hemoglobin and hematocrit, so an eventual decrease in hematocrit results in a higher MCHC values. In the present study, it was also possible to see effects related to the decrease in hematocrit due NO_2^- caused by the decrease of the MCV as it was explained this is a consequence of the output of K^+ from the erythrocyte as previously mentioned (Stormer et al. 1996; Maltez et al. 2019).

The use of NaCl or $CaCO_3$ addition result in positive effects in terms of reduces nitrite induced changes in blood parameters of pacu. It is evidenced by no statistical differences between the control and the NO_2^- treatments at the end of exposure period (day 5) in both cases, while some parameters remained altered in the treatments without the addition of

compounds. In addition, it's also pointing to a possible mechanism of homeostasis recovery with the prolongation of stress, once changes were observed in NaCl and $CaCO_3$ treatments at day 2 (Evans and Lambert 2015; Baldisserotto 2019) reinforcing pacu's ability to adapt to NO_2^- stress.

The addition of NaCl in the water can be a useful mechanism to prevent the toxic effects of NO_2^-, which depends on the increase of Cl^- in the water to limit the entry of the ion through the gills and potentially limiting the activity of the Cl^- / HCO_3^- exchanger. The indirect effect that arises from this is the reduction of NO_2^- income due to competition with Cl. Some authors also suggest that Na^+ could provide a synergistic effect together with Cl^- and the decrease in Cl^- / HCO_3^- activity may lead to excessive retention of HCO_3^- ion with potential alkalization effects of blood pH. Thus Na^+ when added in the water its entry is made in a facilitated way which ends up decreasing the activity of the Na^+ / H^+ exchanger causing the retention of the H^+ ion and compensating the alkalization generated by the retention of HCO_3^-. (Parks et al., 2009; Baldisserotto, 2019). This compensatory acid-base mechanism is supported by the present results, which no changes in pH were observed.

The addition of $CaCO_3$ to enhanced hardness attenuated gill permeability, then reducing stress induced by nitrite (Wood and Mcdonald 1988). Crawford and Allen (1977) demonstrated on chinook salmon (*Oncorhynchus tshawytscha*) that Ca^{2+} does not reduce the production of methemoglobin, the main toxic effect related to NO_2^- toxicity, but its effect as a protector can be attributed to the its capacity to limit the Cl^- uptake through the gills which could lead to a lower activity of the Cl^- / $HCO3^-$ exchanger (Baldisserotto 2019).

5- Conclusions

In conclusion, short term nitrite exposure is able to induce some changes in blood parameters of juvenile pacu and the use of NaCl or $CaCO_3$ was effective in minimizing these toxic effects.

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5. CONCLUSÕES GERAIS

As $CL_{50\text{-}96h}$ estimadas para NaCl e nitrito foram de 13.08 g $NaCl.L^{-1}$ e 107.5 mg $N\text{-}NO_2^-.L^{-1}$, respectivamente.

A exposição ao nitrito em longo prazo induziu efeitos como a ativação de respostas antioxidantes (aumento na atividade da GST), o comprometimento do sistema antioxidante (redução na ACAP) e o aumento nos níveis de LPO. Além disso, foi demonstrado efeito sobre os parâmetros de desempenho zootécnico, com o aumento da taxa de conversão de alimentar.

A exposição em curto período levou a alterações nos parâmetros sanguíneos, incluindo o aumento na concentração de eritrócitos e nos valores de CHCM, e redução no hematócrito e no VCM e HCM.

O uso do NaCl foi efetivo na prevenção da LPO hepática e a redução da ACAP muscular. Já a adição do $CaCO_3$ evitou o aumento nos níves de LPO nas brânquias e no músculo, e o comprometimento da TCA. Ambos os compostos também apresentaram efeito benéfico em atenuar alterações nos parâmetros sanguíneo decorrentes de exposições de curto período. Sendo assim, a adição de NaCl ou $CaCO_3$ nas quantidades propostas foram capazes de reduzir a toxicidade do nitrito em juvenis de pacu.

6. CONSIDERAÇÕES FINAIS

Os resultados obtidos na presente tese poderão contribuir para a compreensão dos mecanismos de toxicidade do nitrito para o pacu, bem como das respostas fisiológicas da espécie frente a níveis indesejáveis de nitrito.

Uma vez que foi demonstrada a atenuação dos efeitos tóxicos do nitrito, recomenda-se manter as concentrações de NaCl e $CaCO_3$ em 1 $g.L^{-1}$ e 150 $mg.L^{-1}$, respectivamente, nos sistemas de produção no caso de necessidade de lidar com níveis indesejáveis deste composto. Esta pode ser uma estratégia interessante de manejo da qualidade da água, especialmente em sistemas de produção intensivos.

Os dados gerados também são relevantes no estabelecimento de critérios de qualidade de água nos sistemas de produção, bem como no desenvolvimento de estratégias de manejo adequadas. Frente as alterações observadas na presente tese, concentrações a partir de 5 mg $N\text{-}NO_2^-.L^{-1}$ já são suficientes para induzir o estresse no pacu e, portanto, são prejudiciais para a espécie.