Universidade Federal do Rio Grande – FURG
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TOXICIDADE DA AMÔNIA E DO NITRITO EM JUVENIS DE LINGUADO *Paralichthys orbignyanus*

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"Você não pode mudar o vento, mas pode ajustar as velas do barco para chegar onde quer."

Confúcio

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RESUMO GERAL

O linguado *Paralichthys orbignyanus* é uma espécie que apresenta grande potencial para a aquicultura intensiva, onde o acúmulo de compostos nitrogenados pode ser um dos principais limitantes a produção. A amônia e o nitrito são os mais tóxicos dentre estes compostos, sendo capazes de induzir diversos distúrbios fisiológicos nos peixes, mesmo se em níveis subletais. Portanto, a presente tese teve como objetivo determinar os efeitos subletais da exposição à amônia e ao nitrito, e posterior recuperação em juvenis do linguado utilizando parâmetros sanguíneos, de estresse oxidativo e histopatologia como biomarcadores. Foram realizados dois experimentos, nos quais os peixes foram expostos a três concentrações de amônia (0,12; 0,28 e 0,57 mg NH_3-N.L^{-1}) ou nitrito (5,72; 10,43 e 15,27 mg NO_2-N.L^{-1}), além de um tratamento controle, durante 10 dias. Em seguida os animais foram mantidos por mais 10 dias em água livre ou com concentrações mínimas de amônia (0.00 mg NH_3-N.L^{-1}) ou nitrito (0.05 mg NO_2-N.L^{-1}) para avaliar a recuperação. Foram coletadas amostras de sangue, brânquia, fígado, músculo e cérebro de 9 peixes por tratamento após 1, 5 e 10 dias de exposição, e ao final da recuperação. Os resultados demonstram que os efeitos atribuídos à ação tóxica da amônia e do nitrito nos juvenis de linguado foram influenciados pelo tempo de exposição e concentração, e mesmo nas concentrações mais baixas testadas de ambos os compostos, houve perturbação da homeostase de alguns dos parâmetros. A análise dos parâmetros sanguíneos (glicose, Na^+, K^+, Ca^{++}, HCO_3^-, pCO_2, pH, perfil leucocitário) durante a exposição a amônia e nitrito indicaram diferentes distúrbios metabólicos, osmorregulatórios, ácido-base e na resposta imune dos peixes. A indução a uma condição pró-oxidante nas brânquias, fígado e músculo também foi demonstrada como mais um dos mecanismos de toxicidade destes compostos para a espécie. A inibição do sistema antioxidante (redução da capacidade antioxidante total e da atividade da enzima antioxidante glutationa-S-transferase) e/ou aumento nos níveis de dano oxidativo em lipídio e proteínas demonstram este efeito. Além disso, a ativação de diferentes defesas antioxidantes enzimáticas e não enzimáticas foi observada, indicando a um mecanismo adaptativo para combater ou minimizar o estresse oxidativo. Não foram evidenciadas alterações histopatológicas no cérebro, no fígado e nas brânquias dos peixes decorrentes da exposição à amônia ou ao nitrito. Após o período de recuperação,

apenas os peixes submetidos ao tratamento 5,72 mg NO_2-N.L^{-1} apresentaram todos os parâmetros sanguíneos e de estresse oxidativo dentro dos valores basais. Para os demais tratamentos do experimento com nitrito e nos peixes expostos a amônia, este restabelecimento ocorreu apenas em parte dos parâmetros. Como conclusão, concentrações a partir de 0,12 mg NH_3-N.L^{-1} e 5,72 mg NO_2-N.L^{-1} causam diversas alterações fisiológicas nos juvenis de linguado, portanto devem ser evitadas nos sistemas de produção. O período de recuperação de 10 dias foi capaz de levar ao restabelecimento parcial ou total da condição fisiológica dos peixes, dependendo do composto e concentração ao qual foram expostos. A recuperação completa foi demonstrada apenas nos peixes expostos a 5,72 mg NO_2-N.L^{-1}.

Palavras-chave: composto nitrogenado, estresse oxidativo, histologia, parâmetro sanguíneo, recuperação, toxicidade

ABSTRACT

The flounder *Paralichthys orbignyanus* is a species with high potential for intensive aquaculture, in which the accumulation of nitrogen compounds is one of the main limiting factors to production. Ammonia and nitrite are the most toxic among these compounds, being able to induce many physiological disturbances in fish, even when in sublethal levels. Therefore, the present thesis aimed to determine the sublethal effects of exposure to ammonia and nitrite and posterior recovery in juvenile flounder using blood and oxidative stress parameters and histopathology as biomarkers. Two experiments were performed, in which the fish were exposed to three concentrations of ammonia (0.12; 0.28 and 0.57 mg NH_3-N.L^{-1}) or nitrite (5.72; 10.43 and 15.27 mg NO_2-N.L^{-1}), plus a control, during 10 days. Then the animals were maintained for more 10 days in water with minimum concentrations of ammonia and nitrite to evaluate the recovery. Blood, gills, liver, muscle and brain were sampled from nine fish per treatment after 1, 5 and 10 days of exposure and at the end of recovery. The results demonstrate that the effects attributed to the toxic action of ammonia and nitrite in juvenile flounder were influenced by the exposure time and concentration. However, even in the lowest concentrations tested for both compounds, there was disturbance of the homeostasis of some of the parameters. The analysis of blood parameters (glucose, Na^+, K^+, Ca^{++}, HCO_3^-, pCO_2, pH, leukocyte profile) during the exposure to ammonia and nitrite indicated different metabolic, osmoregulatory and acid-base disturbances and immune responses of fish. Induction to a pro-oxidant condition in the gills, liver and muscle was also demonstrated as one of the toxicity mechanisms of these compounds to the species. The inhibition of the antioxidant system (reduction of total antioxidant capacity and of the activity of the antioxidant enzyme glutathione-S-transferase) and/or increase of oxidative damage levels in lipids and proteins demonstrate this effect. Furthermore, the activation of different enzymatic and non-enzymatic antioxidant defenses was observed, indicating an adaptive mechanism to combat or minimize the oxidative stress. Histopathological changes in the brain, liver and gills of fish exposed to ammonia or nitrite were not evidenced. After the recovery period, only the fish submitted to treatment 5.72 mg NO_2-N.L^{-1} presented all blood and oxidative stress parameters within the baseline values. For the other treatments of the nitrite experiment and

the fish exposed to ammonia, this restoration occurred only for some of the parameters. In conclusion, concentrations from 0.12 mg NH_3-N.L^{-1} and 5.72 mg NO_2-N.L^{-1} cause several physiological alterations in juvenile flounder, and should be avoided in production systems. The period of recovery of 10 days was able to induce partial or total reestablishment of the fish physiological condition, depending of the compound and concentration to which they were exposed. Only fish exposed to 5.72 mg NO_2-N.L^{-1} showed complete resumption of normal physiological condition after recovery.

Keywords: blood parameter, histology, nitrogen compound, oxidative stress, recovery, toxicity

1. INTRODUÇÃO

1.1. Aquicultura

Em um cenário global marcado pelo aumento populacional e uma crescente competição por recursos naturais, a produção de alimento para a humanidade será um dos maiores desafios para as próximas décadas. Diante deste panorama, a aquicultura tem despontado como uma alternativa viável para o uso dos recursos naturais disponíveis para produção de proteínas de alta qualidade, de modo econômico, social e ambientalmente sustentável (Troell et al., 2014; FAO, 2016).

A aquicultura é o setor de produção de alimentos de origem animal que mais cresce atualmente, o que se deve principalmente a um rápido aumento na demanda por pescado, enquanto a produção pesqueira permanece praticamente constante. No ano de 2014, a produção mundial de pescado foi de 167,2 milhões de toneladas, das quais 73,8 milhões de toneladas (44,1 %) foram provenientes da aquicultura. O Brasil encontra-se na 14ª posição no ranking dos países com maior produção aquícola no mundo, com uma produção total de 562,5 mil toneladas, sendo a piscicultura continental (474,3 mil toneladas) a principal responsável por esta produção (FAO, 2016).

Por outro lado, a piscicultura marinha ainda é incipiente no país (FAO, 2016), apesar do extenso litoral e de uma ictiofauna nativa rica em espécies com potencial para a aquicultura, que conferem ao Brasil múltiplas possibilidades para o crescimento da atividade. Nos últimos anos, a introdução do beijupirá *Rachycentron canadum* na piscicultura marinha brasileira não só motivou pesquisas, como iniciativas no setor produtivo, proporcionando ótimas perspectivas para o desenvolvimento da atividade e mudança deste cenário (Sampaio et al., 2010). Entretanto, a carência de insumos (ração, equipamentos, estruturas de produção) e serviços especializados (mão-de-obra, seguro aquícola), e a ineficiência nas questões legais para cessão de águas públicas e licenciamento ambiental ainda são os principais entraves para o crescimento da piscicultura marinha no Brasil (Cavalli et al., 2011). Outro ponto chave necessário para impulsionar a atividade é o desenvolvimento de pacotes tecnológicos adequados para a criação de outras espécies

nativas com potencial para aquicultura. O robalo-peva *Centropomus parallelus*, o peixe-rei marinho *Odontesthes argentinensis,* a tainha *Mugil liza* e o linguado *Paralichtys orbignyanus* são algumas dessas espécies (Baldisserotto & Gomes, 2010).

1.2. Linguado *Paralichthys orbignyanus*

O linguado *P. orbignyanus* (Valenciennes, 1839) (Figura 1) é uma espécie bentônica, carnívora, que pode atingir até 1 m de comprimento total e cerca de 10 kg. Habita águas costeiras e estuarinas, em geral até cerca de 20 m de profundidade, o que facilita a obtenção de reprodutores e a criação nestes ambientes (Bianchini et al., 2010). Sua distribuição se estende do estado do Rio de Janeiro, no Brasil, até Mar del Plata, na Argentina (Figura 2) (Figueiredo & Menezes, 2000), constituindo um importante recurso pesqueiro nestas áreas em que ocorre (Millner et al., 2005). A sua importância comercial é atribuída ao elevado valor de mercado devido a excelente qualidade da carne, além de apresentar um alto rendimento de filetagem (Robaldo et al., 2012).

Figura 1 – Exemplar de linguado *Paralichtys orbignyanus* (Fonte: arquivo pessoal).

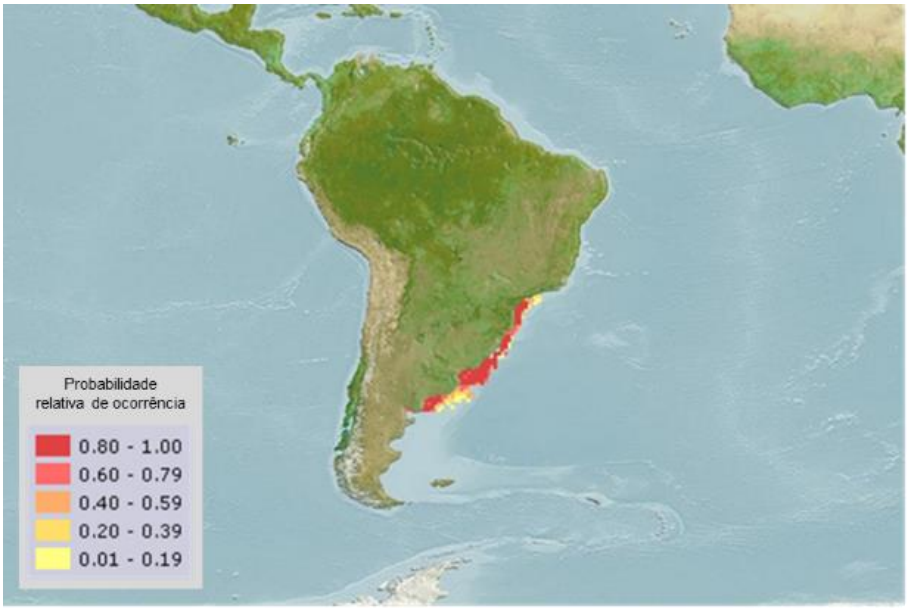

Figura 2 – Distribuição geográfica e probabilidade relativa de ocorrência do linguado *Paralichtys orbignyanus* (Fonte: FishBase – www.fishbase.org).

Esta espécie apresenta uma ampla tolerância aos fatores ambientais, como pH (Wasielesky et al. 1997), temperatura (Okamoto & Sampaio, 2012; Garcia et al. 2015), salinidade (Sampaio & Bianchini, 2002) e compostos nitrogenados (Bianchini et al. 1996), bem como a procedimentos de manejo, incluindo anestesia, transporte e captura (Bolasina et al., 2011; Benovit et al., 2012). Além disso, sua desova e larvicultura têm sido realizadas com sucesso em condições de laboratório, permitindo a produção de juvenis com altas taxas de sobrevivência (Sampaio et al. 2007; 2008).

Em virtude das características citadas, o linguado é uma das espécies consideradas promissoras para o desenvolvimento da piscicultura marinha no Brasil (Sampaio et al. 2007). Apesar da produção comercial ainda não ser uma realidade, a criação desta espécie de forma intensiva, utilizando sistemas de recirculação de água (SRA) vem sendo realizado em escala experimental no Laboratório de Piscicultura Estuarina e Marinha da FURG. Entretanto, ainda há necessidade de mais estudos que proporcionem informações relevantes para que o processo de criação do linguado se torne uma alternativa viável comercialmente nestes sistemas.

1.3. Sistema de recirculação de água

A intensificação dos sistemas de produção na aquicultura é importante para permitir o desenvolvimento sustentável da atividade, pois aumenta a produtividade e minimiza o uso de espaço e água e a produção de efluentes (Klinger & Naylor, 2012). Entretanto, o incremento da densidade de estocagem exige uma maior atenção ao monitoramento e controle dos parâmetros de qualidade de água, evitando que os organismos sejam mantidos em condições indesejáveis, que afetem o bem-estar e desempenho dos mesmos (Boyd & Tucker, 2012).

Dentre os parâmetros de qualidade da água, um dos maiores limitantes para a aquicultura intensiva, sobretudo em sistemas fechados com pequenas taxas de renovação de água, é o acúmulo de compostos nitrogenados, que pode ocorre devido principalmente à excreção dos animais e decomposição de matéria orgânica, tais como sobras de ração e fezes (Ebeling & Timmons, 2012).

Apesar de o nitrogênio ser indispensável aos organismos, o problema associado ao acúmulo deste nutriente no ambiente de cultivo está relacionado ao fato de que, alguns compostos nitrogenados, em especial a amônia e nitrito apresentam elevada toxicidade para os peixes (Randall & Tsui, 2002; Jensen, 2003). O uso do SRA (Figura 3a) é uma das alternativas para se intensificar a produção, sem que estes compostos atinjam concentrações indesejáveis. Nestes sistemas, a remoção da amônia e nitrito é assegurada pelo processo de nitrificação que ocorre no biofiltro (Figura 3b). Este processo envolve duas etapas, nas quais a amônia é oxidada a nitrito, e este por sua vez a nitrato, pela ação de bactérias como por exemplo, as dos gêneros *Nitrosomonas* e *Nitrobacter*, de acordo com as reações:

Nitrosomonas: $NH_4^+ + 1{,}5O_2 \rightarrow NO_2^- + 2H^+ + H_2O$

Nitrobacter: $NO_2^- + 0{,}5O_2 \rightarrow NO_3^-$

Como produto final da nitrificação, o nitrato tende a acumular em SRA (Ebeling & Timmons, 2012), entretanto este composto é menos tóxico que a amônia e o nitrito para os peixes, principalmente em água salgada, tornando-se um problema apenas em concentrações extremamente elevadas (Baldisserotto, 2013).

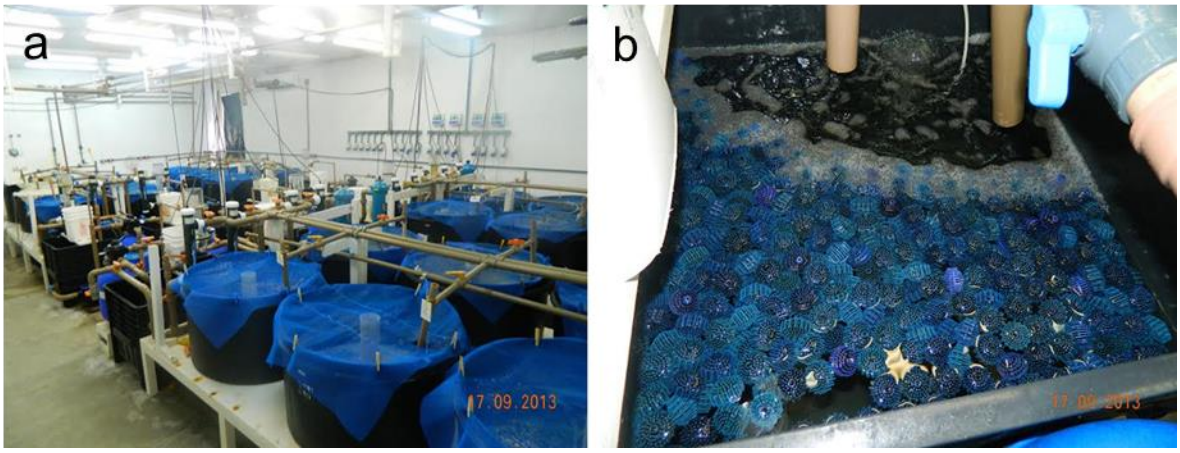

Figura 3 – Sistemas de recirculação de água (a) com biofiltro (b) do Laboratório de Piscicultura Estuarina e Marinha da FURG (Fonte: arquivo pessoal).

Apesar da comprovada eficiência do SRA no controle das concentrações de amônia e nitrito, para que isto ocorra o sistema deve ser planejado e construído adequadamente. Assim, deve-se levar em conta o tipo de cultivo a ser empregado (espécie, densidade de estocagem, manejo alimentar, critérios de qualidade da água), de modo que o processo de nitrificação seja eficiente (Ebeling & Timmons, 2012). Entretanto, em sistemas que não apresentem um design adequado, que o biofiltro não esteja devidamente maturado, ou que a ação das bactérias nitrificantes seja afetada negativamente por algum fator, estes compostos nitrogenados podem atingir níveis tóxicos para os organismos cultivados.

1.4. Amônia e nitrito: definições e toxicidade

No ambiente aquático, a amônia existe em sob duas formas: a amônia não ionizada (NH_3) e a amônia ionizada ou íon amônio (NH_4^+). A soma de ambas as formas é denominada de nitrogênio amoniacal total ou simplesmente amônia total (NAT), a qual pode ser mensurada através de técnicas analíticas. A proporção relativa de cada uma das formas depende da temperatura, salinidade e principalmente do pH da água. A fração NH_3

aumenta com o aumento da temperatura e pH, e com a redução da salinidade (Boyd & Tucker, 2012).

A toxicidade da amônia é usualmente descrita em termos das concentrações de NH_3 (Boyd & Tucker, 2012), o que leva a interpretações equivocadas de que esta forma realmente seja mais tóxica. O que acontece de fato é que a NH_3 por sua natureza lipofílica atravessa facilmente as membranas branquiais por difusão (Ip & Chew, 2010), e elevadas concentrações de NH_3 também comprometem a excreção, favorecendo o acúmulo de amônia nos tecidos dos peixes. A excreção da NH_3 por difusão ocorre pela via transcelular através das glicoproteínas Rhesus (Rhbg e Rhcg) devido ao gradiente sangue-água favorável, o qual pode ser reduzido ou invertido quando a concentração de NH_3 no ambiente é alta. Além disso, em espécies de água doce e algumas adaptadas a água salgada, a amônia também pode ser eliminada através das células de cloreto, onde é dependente ou facilitada pela excreção de H^+ pela V-ATPase e/ou pelo antiporte Na^+/H^+ (Baldisserotto, 2013). O mecanismo primário de toxicidade da amônia nos peixes está relacionado ao efeito despolarizante do íon NH_4^+ nos neurônios e músculo branco, o que leva a uma ativação excessiva de receptores glutamatérgicos do tipo NMDA (N-metil D-Aspartato). Esta ativação excessiva ocasiona a depleção de ATP e um aumento na concentração intracelular de Ca^{2+} ativando enzimas Ca^{2+}-dependentes que desencadeiam uma série de reações que levam a morte celular (Randall & Tsui, 2002).

Assim como a amônia, o nitrito também está presente em duas formas na água: ácido nítrico (HNO_2) e nitrito ionizado (NO_2^-), e o equilíbrio entre as duas formas depende do pH. Porém, em valores de pH da água acima de 5,5 apenas o NO_2^- está presente, portanto esta é a forma a ser considerada quando se fala em termos de toxicidade do nitrito nos sistemas de produção aquícola (Baldisserotto, 2013).

Em peixes de água doce, o nitrito é absorvido do ambiente através das brânquias, via antiporte Cl^-/HCO_3^-, competindo com o Cl^- (Jensen, 2003). Por conta desta competição entre o Cl^- e o nitrito pelo mesmo transportador, salinidades elevadas podem reduzir a toxicidade deste composto para os peixes (Wuertz et al., 2013). Entretanto, como a permeabilidade do epitélio branquial de peixes marinhos é relativamente alta, a entrada de nitrito pode ocorrer por difusão a favor do gradiente eletroquímico (Jensen, 2003; Baldisserotto, 2013). Outra importante via de absorção do nitrito também ocorre em peixes

marinhos ou adaptados a água do mar. Por serem hiposmóticos em relação ao meio, precisam beber água para compensar a perda por osmose, e o nitrito é absorvido principalmente no intestino, também em substituição ao Cl^- nos simportes $Na^+/K^+/2Cl^-$ e Na^+/Cl^- (Grossel & Jensen, 2000).

O nitrito que é absorvido durante a exposição tende a se acumular inicialmente no plasma (Hvas et al., 2016) e, posteriormente, se difunde para os eritrócitos, onde oxida o Fe^{2+} da hemoglobina a Fe^{3+}, formando a metahemoglobina que não é capaz de transportar oxigênio, ocasionando uma hipóxia funcional nos peixes (Jensen, 2003).

Além dos mecanismos de toxicidade primários descritos, a exposição à amônia e nitrito pode induzir uma ampla variedade de alterações fisiológicas (Baldisserotto et al., 2014; Jia et al., 2015), morfológicas (Saoud et al., 2014; Rodrigues et al., 2014) e até comportamentais (Schram et al., 2010; Roques et al., 2015) nos peixes. Os efeitos tóxicos de ambos os compostos são espécie-específicos e dependem da concentração e duração da exposição (Kroupova et al., 2005; USEPA, 2013), podendo em casos extremos levar até a morte dos animais (Medeiros et al., 2016; Pinto et al., 2016).

Entretanto, na aquicultura, a exposição a concentrações subletais pode ser um problema grave, pois muitas vezes seus efeitos não são detectáveis através da simples observação dos animais e de seu comportamento pelo produtor. Porém, o estresse causado nos peixes pode levar a uma redução no desempenho zootécnico (Paust et al., 2011; Ciji et al., 2014), ao comprometimento imunológico aumentando a susceptibilidade a doenças (Chen et al., 2011; Jia et al., 2016), e até a perda de qualidade do produto final (Veeck et al., 2013).

1.5. Estresse

Iwama et al. (2011) apontam que existe uma dificuldade em se estabelecer uma definição universal de estresse, diante da grande diversidade de definições já propostas até então por diferentes autores. Nesta tese, o termo estresse será aplicado para caracterizar um estado desencadeado por um agente estressor, no qual a homeostase ou condição biológica basal foi alterada (Barton & Iwama, 1991). Este desbalanço da homeostase pode resultar de

algum efeito direto desencadeado pelo estressor, como por exemplo, a formação de metahemoglobina induzida pela exposição ao nitrito. Além disso, o estresse pode estar relacionado a uma resposta adaptativa ou compensatória inespecífica, desencadeada pela ativação do sistema nervoso simpático. Este ajuste fisiológico permite aos organismos uma maior chance de sobrevivência diante de uma condição desfavorável, e pode ou não ser prejudicial dependendo da severidade e duração (Bonga, 1997; Barton, 2002).

As diferentes respostas ao estresse podem ser classificadas em primárias, secundárias e terciárias de acordo com o nível de organização biológica em que ocorrem. A resposta primária é neuroendócrina e envolve a liberação de catecolaminas (adrenalina e noradrenalina) e hormônios corticosteroides na corrente sanguínea. As respostas secundárias são caracterizadas por alterações em parâmetros metabólicos (glicose, lactato e glicogênio) e perfil hematológico, distúrbios osmorregulatórios e ácido-base, dentre outras. Por fim, as respostas terciárias acontecem em nível de indivíduos ou populacional, podendo afetar o crescimento, reprodução e sistema imune, alterar padrões comportamentais, além de reduzir a capacidade de tolerar estressores subsequentes ou adicionais (Bonga, 1997; Barton, 2002). Apesar da distinção entre os tipos de estresse, as respostas acontecem de forma integrada, e em certas circunstâncias, as respostas primárias e secundárias, acabam por desencadear as respostas secundárias e terciárias, respectivamente (Figura 4) (Barton, 2002).

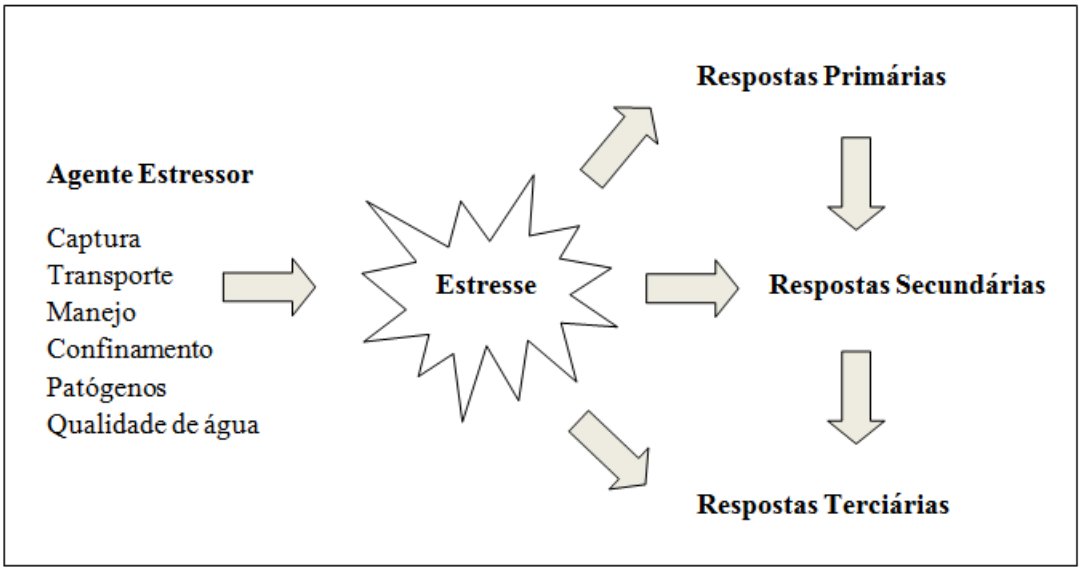

Figura 4 – Respostas (primárias, secundárias e terciárias) de estresse frente a um agente estressor e as possíveis relações entre os diferentes tipos de resposta (Fonte: Adaptado de Barton, 2002).

Considerando que o estresse relacionado a níveis elevados de amônia e nitrito nos sistema de produção é um risco em potencial, sobretudo na aquicultura intensiva, reconhecer as respostas biológicas envolvidas e os limites de tolerância do organismo cultivado é fundamental para estabelecer uma estratégia de manejo adequada, assegurando o bem-estar animal e a produção (Conte, 2004; Iwama et al., 2011). Neste sentido, a avaliação de respostas primárias e/ou secundárias de estresse é fundamental, pois fornece um diagnóstico precoce acerca dos efeitos tóxicos decorrentes da exposição a níveis indesejáveis destes compostos. Desta forma, o monitoramento de parâmetros que mensurem estas respostas deve ser uma prática rotineira na aquicultura, a fim de permitir a tomada das medidas corretivas necessárias (Satheeshkumar et al., 2012), evitando outros efeitos de longo prazo como a redução no desempenho zootécnico dos animais.

Diversos biomarcadores tem sido aplicados na avaliação do estresse desencadeado pela amônia ou nitrito nos peixes, dentro os quais se pode citar: os parâmetros sanguíneos (Clifford et al., 2015; Hvas et al., 2016) e de estresse oxidativo (Jia et al., 2015; Li et al., 2016) e análises histopatológicas (Wuertz et al., 2013; Banihashemi et al., 2016). Vale ressaltar que, quando viável, a utilização de múltiplos biomarcadores é recomendada, pois permite uma compreensão mais ampla e integrada dos efeitos tóxicos destes compostos.

1.5.1. Parâmetros sanguíneos

O perfil hematológico e de outros parâmetros bioquímicos e fisiológicos do sangue dos peixes está intimamente relacionado com as respostas fisiológicas frente a fatores ambientais (Roche & Bogé, 1996; Clauss et al., 2008). 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 (trocas gasosas, manutenção do equilíbrio iônico e ácido-base, eliminação de resíduos metabólitos, dentre outras) nas quais o sangue está envolvido (Heath, 1995). Além disso, a coleta de

sangue (Figura 5) dos animais é um procedimento não letal e as análises de modo geral, fornecem resultados rápidos (Satheeshkumar et al., 2012). Devido a estas vantagens, o monitoramento dos parâmetros sanguíneos é uma ferramenta que vem sendo amplamente utilizada na avaliação do estado de saúde e bem estar dos animais (Satheeshkumar et al., 2012; Segner et al., 2012; Dal'Bó et al., 2015).

Figura 5 – Coleta de sangue via veia caudal em juvenil de *Paralichtys orbignyanus* (Fonte: arquivo pessoal).

Diante das diferentes alterações fisiológicas decorrentes do estresse, uma grande diversidade de parâmetros sanguíneos pode ser utilizada para identificar esta condição nos peixes (Segner et al., 2012; Dal'Bó et al., 2015). Todavia, a escolha dos parâmetros adequados para esta avaliação é fundamental e deve ser feita de acordo com o estressor, sobre o qual os efeitos querem ser investigados. Dentre as alterações fisiológicas resultantes do estresse ocasionado pela exposição a níveis indesejáveis de amônia e nitrito, incluem-se distúrbios respiratórios, iônicos, ácido-base, metabólicos e imunológicos. Como consequência destes distúrbios, concentrações plasmáticas de glicose e íons (Na^+, K^+, Ca^{2+} e HCO_3^-), pressão parcial de gases (pO_2 e pCO_2), pH e o perfil leucocitário estão entre os parâmetros sanguíneos que podem ter a homeostase afetada, demonstrando a aplicabilidade dos mesmos como biomarcadores na avaliação dos efeitos tóxicos destes compostos

(Vosyliene & Kazlauskiene, 2004; Wood & Nawata, 2011; Saoud et al., 2014; Clifford et al., 2015; Jia et al., 2015; Hvas et al., 2016).

1.5.2. Estresse oxidativo

Durante o processo de produção de energia por via aeróbica, a redução tetravalente do oxigênio molecular (O_2) na mitocôndria resulta na formação de água. Entretanto, mesmo em condições fisiológicas normais dos organismos, em torno de 1 a 3% do oxigênio metabolizado nos diversos processos endógenos é apenas parcialmente reduzido, formando as espécies reativas de oxigênio (ERO) (Livingstone, 2003), tais como os radicais superóxido (O_2^-) e hidroxila (OH^-) e o peróxido de hidrogênio (H_2O_2). Esta produção de radicais livres dá origem ao termo "Paradoxo do Oxigênio", pois apesar de essencial para a vida dos organismos aeróbios, o oxigênio também pode ter efeitos prejudiciais inerentes ao seu metabolismo (Davies, 2016). Os efeitos deletérios das ERO estão relacionados à capacidade que possuem de oxidar e danificar biomoléculas, como proteínas, lipídeos, DNA e RNA, prejudicando ou levando a perda de suas funções biológicas (Halliwell & Gutteridge, 2015).

Os peixes, como todos os organismos aeróbios, desenvolveram evolutivamente um complexo sistema antioxidante composto de diversas defesas não enzimáticas e enzimáticas que atuam impedindo a formação e ação das ERO's, ou favorecendo o reparo e a reconstituição de moléculas que sofreram dano oxidativo (Halliwell & Gutteridge, 2015). Os antioxidantes não enzimáticos são representados por moléculas de baixo peso molecular, como o tripeptídeo glutationa (GSH), as metalotioneínas (MTs), o ácido ascórbico (vitamina C), o β-caroteno e o α-tocoferol, precursores das vitaminas A e E, respectivamente, os carotenóides, os flavonóides, além de minerais como o zinco, o cobre, o selênio e o magnésio.

Entre as principais enzimas envolvidas no sistema antioxidante temos a superóxido dismutase (SOD), a catalase (CAT) e a glutationa peroxidase (GPx), as quais são consideradas a primeira linha de defesa celular, devido a sua atuação direta na conversão em formas menos reativas ou na eliminação das ERO. A SOD catalisa a dismutação do O_2^-

em O_2 e H_2O_2, enquanto a CAT decompõe o H_2O_2 em O_2 e água. Já a GPx atua na detoxificação de H_2O_2 e hidroperóxidos orgânicos utilizando a GSH como cofator (Sevcikova et al., 2011). Entretanto, outras enzimas como a glutationa redutase (GR) e as glutationa-S-transferases (GST) também apresentam um importante papel neste sistema antioxidante. A GR é uma enzima auxiliar que tem como principal função reciclar a GSH a partir da sua forma oxidada (GSSG) (Hermes-Lima, 2004). Enquanto as GST são enzimas multifuncionais que catalisam o ataque nucleofílico da GSH a compostos que apresentem átomos eletrofílicos, e sua atividade está envolvida na detoxificação celular de xenobióticos e metabólitos endógenos, incluindo produtos de dano oxidativo (Figura 6) (Blanchette et al., 2007).

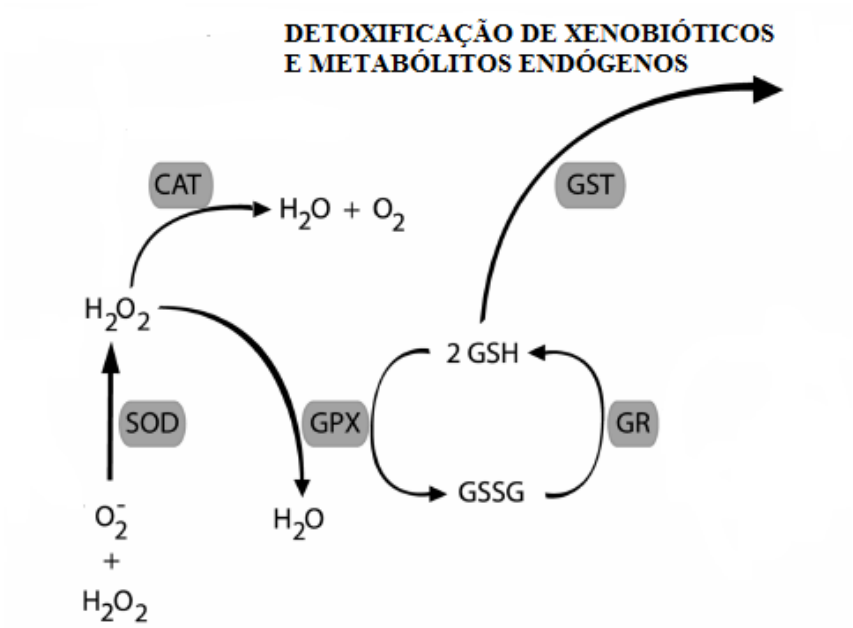

Figura 6 – Principais enzimas do sistema de defesa antioxidante atuando de forma integrada na proteção celular contra radicais oxidativos. SOD – superóxido dismutase; CAT – catalase; GPX – glutationa peroxidase; GR – glutationa redutase; GST – glutationa-S-transferases; GSH – glutationa reduzida; GSSG – glutationa oxidada (Fonte: adaptado de Hermes-Lima, 2004).

A atuação destas defesas antioxidantes e dos pró-oxidantes no organismo dos animais apresenta um equilíbrio em condições normais. Entretanto, em resposta a agentes estressores, incluindo alterações em variáveis ambientais ou durante a exposição a poluentes, pode haver a perturbação deste equilíbrio (Lushchak, 2011; Stoliar & Lushchak, 2012). O estresse oxidativo pode ser definido como aumento transitório ou crônico na concentração de ERO, quando este desequilíbrio acontece em favor dos pró-oxidantes, resultando em aumento nos níveis de dano oxidativo (Lushchak, 2011; Halliwell & Gutteridge, 2015).

Apesar de ser um tema ainda relativamente pouco estudado, trabalhos recentes tem demonstrado que a exposição à amônia ou ao nitrito pode induzir a um aumento na produção de radicais livres, incluindo ERO e espécies reativas de nitrogênio (ERN), nos tecidos de peixes (Sinha et al., 2014; Cheng et al., 2015; Jensen et al., 2015). A produção excessiva destes agentes oxidantes leva a ativação de fatores de transcrição redox-sensível (FoxO, Nrf2, p53, HIF-1α, NF-κB, etc.) e a modificação covalente de proteínas antioxidantes, resultando no aumento da expressão e atividade de enzimas antioxidantes (Hermes-Lima et al., 2015). Este tipo de mecanismo adaptativo para minimizar o estresse oxidativo já foi demonstrado para enzimas como a SOD, CAT, GPx, GST, dentre outras, durante a exposição de peixes à amônia (Sinha et al., 2014; Li et al., 2016) e ao nitrito (Ciji et al., 2012; Sun et al., 2014). Entretanto, em certas condições de estresse mais severas, como a exposição prolongada ou a concentrações mais altas destes compostos, pode haver falha do sistema de defesa antioxidante, resultando, por exemplo, em redução da atividade de enzimas e concentrações de GSH (Jia et al., 2015; Li et al., 2016).

Diante da capacidade que a amônia e o nitrito apresentam de elevar a produção de agentes oxidantes e de diminuir a eficácia do sistema antioxidante, o aumento nos níveis de dano oxidativo é esperado, e já foi demonstrado em lipídeos, proteínas e até DNA nos tecidos de peixes (Sinha et al., 2014; Sun et al., 2014; Cheng et al., 2015; Jia et al., 2015; Li et al., 2016; Lisser et al., 2017). Esta condição, além de comprometer as funções fisiológicas dos animais (Livingstone, 2003; Valavanidis et al., 2006), pode também causar alterações na qualidade da carne (Zhang et al., 2016), afetando negativamente a aquicultura.

1.5.3. Histopatologia

O aumento nos níveis de dano oxidativo, além de outras alterações fisiológicas e bioquímicas, desencadeadas pela exposição a um agente estressor, como contaminantes, pode resultar no aparecimento de histopatologias que comprometem a funcionalidade de diferentes órgãos dos peixes (Bernet et al., 1999; Simonato et al., 2008; Sun et al., 2014). Portanto, análises histopatológicas podem ser consideradas úteis para avaliar os efeitos de alterações de qualidade de água em um nível de organização biológica mais alto nos animais (Liebel et al., 2013).

Diversas metodologias são empregadas nos estudos histopatológicos, podendo ser realizadas análises descritivas (Rodrigues et al., 2014; Saould et al., 2014) ou quantitativas (abundância) das lesões observadas (Reiser et al., 2010), ou ainda aplicados índices que mensuram o grau de lesão do órgão (Bernet et al., 1999). Vários órgãos alvo são considerados adequados para este tipo de avaliação (Bernet et al., 1999), entretanto, o fígado e principalmente a brânquia são os mais aplicados para avaliar os efeitos da exposição dos peixes à amônia e nitrito (Park et al., 2007; Dong et al., 2013; Wuertz et al., 2013; Rodrigues et al., 2014; Saoud et al., 2014).

A avaliação das brânquias deve-se ao fato de que toda sua superfície está em contato direto e constante com a água e, consequentemente, com os contaminantes presentes (Bernet et al., 1999). Além disso, é um órgão que participa ativamente dos mecanismos relacionados à absorção e à eliminação de amônia e nitrito do organismo (Jensen, 2003; Ip & Chew, 2010).

Hiperplasia e hipertrofia de células epiteliais, hipertrofia de células de cloreto, fusão lamelar, descolamento do epitélio branquial e telangiectasia são algumas das alterações histopatológicas reportadas nas brânquias de peixes expostos a estes compostos (Rodrigues et al., 2014; Saoud et al., 2014; Medeiros et al., 2016). De acordo com Mallat (1985), a não especificidade destas alterações sugerem que as mesmas estejam relacionadas a um mecanismo de defesa, que visa aumentar a distância entre o meio externo e o meio interno. Entretanto, o que primariamente é um mecanismo de proteção contra o estresse

ambiental, pode acabar se tornando prejudicial para o organismo, dificultando trocas gasosas, excreção e processos osmorregulatórios.

O fígado apresenta funções metabólicas vitais, sendo o principal órgão envolvido nos processos de biotransformação e detoxificação de xenobióticos, e a presença de contaminantes rapidamente induz alterações estruturais, bioquímicas e moleculares, que podem resultar em lesões neste tecido dos peixes (Bernet et al.,1999). Dilatação sinusoidal, degeneração hidrópica, deposição lipídica nos hepatócitos, desintegração de hepatócitos e necrose são alguns dos exemplos de lesões hepáticas relacionadas à toxicidade da amônia ou do nitrito (Benli et al., 2008; Rodrigues et al., 2014; Thangam, 2014).

Rodrigues et al. (2014) demonstraram pela primeira vez que alterações histopatológicas como a proliferação de células de glia e espaço de Virchow-Robin indicando satelitose e edema perisvascular severo, respectivamente, também podem ocorrer no cérebro de peixes relacionadas ao potencial neurotóxico da amônia. Entretanto, com relação ao nitrito, não foram encontrados na literatura trabalhos que demonstrem o aparecimento de lesões no cérebro de peixes associadas aos feitos tóxicos deste composto.

1.6. Recuperação

Mesmo durante a exposição à amônia ou ao nitrito, os peixes são capazes de realizar ajustes fisiológicos adaptativos que podem restabelecer a homeostase de certos parâmetros, os quais acabam apenas sendo alterados transitoriamente (Martinez & Souza, 2002; Sinha et al., 2012). Em certas condições, entretanto, é necessário que o agente estressor seja eliminado para que o animal consiga restabelecer a sua condição fisiológica normal. Esta informação é corroborada por trabalhos prévios que demonstram que a transferência para água com concentração mínima ou livre de amônia e nitrito é suficiente para a recuperação dos animais (Knudsen & Jensen, 1997; Gisbert et al., 2004; Yang et al., 2010).

Esta recuperação esta relacionada à capacidade que os peixes naturalmente possuem em eliminar o excesso desses compostos dos organismos, a qual durante a exposição acaba se tornando comprometida ou insuficiente (Jensen, 2003; Clifford et al., 2015). Uma vez que são transferidos para uma água com boa qualidade, ocorre gradualmente a

detoxificação do excesso de amônia e nitrito que se acumularam no plasma e tecidos dos peixes (Knudsen & Jensen, 1997; Gisbert et al., 2004; Clifford et al., 2015), e as alterações fisiológicas decorrentes de seus efeitos tóxicos tendem a diminuir ou desaparecer com o tempo. Este tempo necessário para o restabelecimento da homeostase pode variar desde horas (Gisbert et al., 2004) até alguns dias (3-15), dependendo da espécie, do composto ao qual o peixe foi exposto e do parâmetro avaliado (Knudsen & Jensen, 1997; Gisbert et al., 2004;Yang et al., 2010).

Na aquicultura, o período de recuperação é fundamental após qualquer evento ou procedimento de manejo que atue como um estressor para os peixes, visando minimizar os efeitos de um estresse subsequente (Wedemeyer, 2012), e até a perda da qualidade do produto final, devido a uma condição estressante pré-abate (Terlouw et al., 2008; Veeck et al., 2013). Apesar da importância, as respostas fisiológicas durante a recuperação pós-exposição à amônia e nitrito ainda são relativamente pouco estudadas nos peixes e uma maior atenção deve ser dada neste sentido.

1.7. Toxicidade da amônia e nitrito no *Paralichthys orbignyanus*

Atualmente, o conhecimento acerca dos efeitos tóxicos da amônia e do nitrito para o linguado é limitado apenas à determinação das concentrações letais ($CL_{50\text{-}96h}$) em duas condições experimentais de salinidade e temperatura: “condição de inverno” (0 ‰ e 12°C) e “condição de verão” (30 ‰ e 25°C). Os valores obtidos para a $CL_{50\text{-}96h}$ foram maiores na "condição de verão" (0,67 mg NH_3-N.L^{-1} e 30,57 mg NO_2-N.L^{-1}) do que na "condição de inverno"(0,19 mg NH_3-N.L^{-1} e 24,01 mg NO_2-N.L^{-1}) para ambos os compostos (Bianchini et al., 1996). Estes resultados demonstram a influência de outros parâmetros ambientais na toxicidade da amônia e nitrito, e que, principalmente em água salgada e temperaturas elevadas, o linguado apresenta uma boa tolerância a estes compostos.

Entretanto, não existem trabalhos que demonstrem os efeitos do estresse ocasionado pela exposição do linguado a concentrações subletais destes compostos, as quais podem ser um fator limitante para sua produção em sistemas intensivos, incluindo SRA. A avaliação da recuperação dos animais em água com boa qualidade (concentrações mínimas ou livre

de amônia não-ionizada e nitrito) após um período de exposição também ainda não foi estudada para a espécie. Desta forma, as informações obtidas na presente tese poderão contribuir para a compreensão dos mecanismos de toxicidade da amônia e nitrito no linguado, bem como das respostas fisiológicas da espécie frente a níveis indesejáveis dos mesmos e durante a recuperação. Na perspectiva da aquicultura, os dados gerados irão auxiliar no estabelecimento de critérios de qualidade de água nos sistemas de produção, bem como no desenvolvimento de estratégias de manejo adequadas. Além disso, as respostas dos diferentes biomarcadores avaliados servirão como referência, quando da aplicação dos mesmos, para monitorar o estado fisiológico e bem estar dos animais e relacioná-los com as concentrações de amônia e nitrito na água.

2. OBJETIVOS

2.1. Objetivo geral

- Avaliar os efeitos subletais da exposição à amônia e nitrito, e posterior recuperação em juvenis do linguado *Paralichthys orbignyanus* utilizando parâmetros sanguíneos, de estresse oxidativo e análises histopatológicas como biomarcadores.

2.2. Objetivos específicos

- Determinar as alterações nos parâmetros sanguíneos (glicose, Na^+, K^+, Ca^{2+}, HCO_3^-, pO_2, pCO_2, pH e contagem diferencial de leucócitos) ocasionadas pela exposição dos juvenis de linguado a diferentes concentrações subletais de amônia e nitrito;

- Avaliar os efeitos da exposição a concentrações subletais de amônia e nitrito no sistema de defesa antioxidante e nos níveis de dano oxidativo no fígado, no músculo e nas brânquias em juvenis de linguado;

- Verificar se ocorrem alterações histopatológicas no fígado, brânquias e cérebro de juvenis de linguado submetidos a concentrações subletais de amônia e nitrito;

- Avaliar se a recuperação em água livre ou com concentrações mínimas de amônia e nitrito é suficiente para o restabelecimento da condição fisiológica normal dos juvenis de linguado após a exposição a diferentes concentrações destes compostos.

3. HIPÓTESES

- Concentrações subletais de amônia e nitrito são agentes estressores para os juvenis de linguado, o que resulta em alterações nos parâmetros sanguíneos, induz o estresse oxidativo e o aparecimento de histopatologias;

- As alterações nos parâmetros sanguíneos, de estresse oxidativo e histopatológicas decorrentes da exposição de juvenis de linguado à amônia ou ao nitrito dependem do tempo de exposição e concentração;

- A recuperação em água livre ou com concentrações mínimas de amônia e nitrito é capaz de reverter as alterações fisiológicas ocasionadas pela exposição a estes compostos nos juvenis de linguado;

- A recuperação dos juvenis de linguado é dependente da concentração de amônia ou nitrito a qual foram expostos.

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CAPÍTULO 1

Secondary stress responses in juvenile Brazilian flounder *Paralichthys orbignyanus* throughout and after exposure to sub-lethal levels of ammonia and nitrite

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Abstract

The aim of this study was to investigate the secondary stress responses in blood of juvenile Brazilian flounder *Paralichthys orbignyanus* throughout exposure to sub-lethal levels of ammonia and nitrite and after recovery in water with minimum concentrations of these compounds. Fish were exposed to three concentrations of ammonia (0.12; 0.28 and 0.57 mg NH_3-N.L^{-1}) or nitrite (5.72; 10.43 and 15.27 mg NO_2-N.L^{-1}), plus a control, for 10 days followed by the same time for recovery. Blood parameters (glucose, Na^+, K^+, Ca^{2+} and HCO_3^- concentrations, pO_2, pCO_2, pH and differential leukocyte count) were measured after 1, 5 and 10 days of exposure, and after recovery. Fish exposed to all ammonia concentrations presented lower glucose levels after 10 days of exposure and higher values after recovery. Although ammonia induced initial ionic disturbances (Na^+, K^+, Ca^{2+}), basal levels were restored still over the course of exposure. A metabolic blood alkalosis occurred from the 5th day of exposure and forward until the end of the experiment (day 20). Nitrite exposure caused hyperglycemia, increased plasma K^+ levels and respiratory alkalosis, whereas a metabolic acidosis was observed in fish after recovery. The differential leukocyte count revealed an increased proportion of monocytes and granulocytes, or only monocytes. Yet, reductions of lymphocytes in response to intermediate concentrations of ammonia (0.28 mg NH_3-N.L^{-1} at day 1) and nitrite (10.43 mg.L^{-1} at day 5), and after recovery in treatments 0.28 and 0.57 mg NH_3-N.L^{-1} were observed. Exposure to ammonia also decreased the proportion of granulocytes at day 5. In conclusion, exposure to sub-lethal levels of ammonia and nitrite for 10 days led to alterations in blood parameters of juvenile Brazilian flounder, and concentrations at 0.12 mg NH_3-N.L^{-1} and 5.72 mg NO_2-N.L^{-1} should be avoided to prevent physiological disorders that can compromise aquaculture production. Furthermore, only fish exposed to 5.72 mg NO_2-N.L^{-1} following a 10-days recovery period showed complete resumption of normal physiological condition.

Keywords: blood parameters; estuarine fish; homeostasis; nitrogen compounds; toxicity

1. Introduction

The Brazilian flounder *Paralichthys orbignyanus* inhabits estuaries and shallow coastal waters of the southwestern Atlantic Ocean, from Rio de Janeiro, Brazil to Argentinian Patagonia, representing an important fishery resource in South America (Millner et al., 2005). It is considered a promising species for the development of intensive marine aquaculture due to its wide tolerance to changes in water quality (Bianchini *et al.* 1996; Wasielesky *et al*. 1997; Sampaio and Bianchini, 2002; Okamoto and Sampaio, 2012) and handling (Bolasina, 2011). Spawning, larviculture and juvenile production have been successfully achieved in Brazilian flounder (Sampaio et al., 2007; Sampaio et al., 2008).

Build-up of nitrogen compounds is one of the most important limiting factors in intensive aquaculture. In recirculating aquaculture systems (RAS), ammonia and nitrite are removed via the nitrification process whereby bacteria inside the biofilter oxidize ammonia to nitrite, and subsequently to nitrate (Blancheton et al., 2013). However, imbalances within these bacterial processes may lead to increments in concentrations of these compounds, which are extremely toxic to fish (Randall and Tsui, 2002; Jensen, 2003).

The accumulation of ammonia in production systems occurs due to decomposition of the organic matter and fish excretion, being the main by-product of protein catabolism in most teleost species (Wilkie, 2002). Ammonia exists in ionized (NH_4^+) and un-ionized forms (NH_3), and its toxicity is mainly attributed to un-ionized form, which can readily diffuse across gill membranes because of its high solubility in lipids (Ip and Chew, 2010). High concentrations of environmental ammonia impair the blood-to-water gradient for NH_3 diffusion and therefore, ammonia build-up in blood and tissues is favoured by the decreased gill excretion (Wilkie and Wood, 1996). According to Randall and Tsui (2002), the main cause of ammonia toxicity is related to the depolarizing effect of the ion NH_4^+ in neurons, leading to excessive activation of N-Methyl-D-Aspartate (NMDA) type glutamate receptors. Over activation of these receptors leads to an influx of Ca^{2+} to cells, which in turn activates Ca^{2+}-dependent enzymes, being followed by a series of reactions that can ultimately cause cell death.

Environmental nitrite is taken up across the gill epithelium via the active chloride uptake mechanism, following further accumulation in the extracellular fluid (Bath and

Eddy, 1980). In marine fish, the intestinal epithelium is another important route for nitrite uptake (Grosell and Jensen, 2000). From the blood plasma, nitrite diffuses into red blood cells (RBCs) oxidizing hemoglobin Fe^{2+} to Fe^{3+} and thus giving rise to the formation of methemoglobin (metHb), losing its oxygen-binding capacity (Jensen, 2003).

In addition to the primary toxic mechanisms as aforementioned, exposure to ammonia and nitrite can also induce metabolic, ionic, acid-base and immune changes in fish, which led to alterations of some blood parameters as glucose, concentration of electrolytes, partial pressure of gases, pH and leukocyte profile (Vosyliene and Kazlauskiene, 2004; Wood and Nawata, 2011; Saoud et al., 2014; Jia et al., 2015; Hvas et al., 2016). These secondary stress responses are early signs of compromise to fish health, and therefore should not be overlooked, for they can provide relevant information to establish nontoxic thresholds for NH_3-N and NO_2-N concentrations. Maintaining safe thresholds for nitrogen compounds in RAS is critical to avoid stress, which, if linger on above safe levels, may eventually progress to tertiary responses (Barton et al., 2002) such as growth suppression (Paust et al., 2011; Ciji et al., 2014), diseases and fish death (Medeiros et al., 2016).

Information on the suitable time length to allow for ammonia and nitrite detoxification from plasma and tissues of fish is very scarce. Although some studies have shown resumption of normal physiological conditions during recovery in fish previously exposed to ammonia and nitrite (Gisbert et al., 2004; Yang et al., 2010), such similar results cannot be simply expected when different concentrations, time of exposure, or distinct species are used. Different species might require distinct time frames for resumption of homeostasis. Fish reared in intensive aquaculture systems that have been exposed to oscillations in nitrogen compounds should undergo an adequate recovery period in ammonia- and nitrite-free water, in order to minimize the risk of cumulative stress (Wedemeyer, 2012) and to decrease impairment of end-products due to pre-slaughter stress (Terlouw et al., 2008).

Studies dealing with ammonia and nitrite toxicity to the Brazilian flounder are limited to the determination of lethal concentration (LC_{50}-96h) in two experimental conditions of salinity and temperature: the "winter condition" (0‰ and 12°C) and the "summer condition" (30‰ and 25°C). Values for LC_{50}-96h were 0.67 mg NH_3-N.L^{-1} and

30.57 mg NO_2-N.L^{-1} in "summer condition", and 0.19 mg NH_3-N.L^{-1} and 24.01 mg NO_2-N.L^{-1} in "winter condition" (Bianchini *et al.*, 1996). Responses of Brazilian flounder exposed to sub-lethal levels of these compounds, following a recovery period in ammonia- and nitrite-free water are unknown. Thus, the aim of this study was to assess the effects of exposure to sub-lethal levels of un-ionized ammonia and nitrite on secondary stress responses of juvenile *P. orbignyanus*. Furthermore, responses of fish allowed to recover in ammonia- and nitrite-free water were also monitored.

2. Materials and methods

2.1. Fish and experimental conditions

Juvenile Brazilian flounders were produced and reared at the Laboratory of Marine Fish Culture of the Federal University of Rio Grande - FURG in southern Brazil. The experimental protocol was approved by the Committee on Animal Experimentation of FURG under the registration number 23116.001030/2016-15.

Two experiments were performed: 180 fish (107.3 ± 1.9 g and 21.2 ± 0.1 cm) were exposed to ammonia (Experiment 1 – E1), and another 180 fish (310.0 ± 5.6 g and 29.4 ± 0.2 cm) were exposed to nitrite (Experiment 2 – E2). Fish were randomly distributed in 12 tanks (250 L useful volume) consisting of four RAS for acclimation to experimental conditions during 20 days.

The juveniles were fed twice daily (3% of total biomass) with commercial diet (46% crude protein) throughout the acclimation period and experiments. Uneaten food and faeces were siphoned out after feeding and the water was replenished at the same experimental conditions.

After the acclimation period, fish were exposed for 10 days to three sub-lethal concentrations of ammonia (E1): 0.12 ± 0.01; 0.28 ± 0.02 and 0.57 ± 0.02 mg NH_3-N.L^{-1} or nitrite (E2): 5.72 ± 0.02; 10.43 ± 0.05 and 15.27 ± 0.04 mg NO_2-N.L^{-1}, plus a control (E1 - 0.05 ± 0.01 mg NH_3-N.L^{-1}; E2 - 0.08 ± 0.01 mg NO_2-N.L^{-1}), being each treatment in triplicate. Water flow was turned off and throughout the exposure period fish were kept in a

semi-static system. Concentrations tested were chosen based on the safe levels of un-ionized ammonia (0.067 mg NH_3-N.L^{-1}) and nitrite (3.06 mg NO_2-N.L^{-1}) that were estimated by Bianchini et al. (1996) in seawater. The desired ammonia and nitrite concentrations were obtained by the addition of ammonium chloride (NH_3Cl_4) or sodium nitrite ($NaNO_2$) (Synth, Brazil) solutions, or water exchanges. In both experiments, at the end of the exposure period, water was entirely renewed in all tanks, water recirculation was re-established and fish remained for another 10 days in a RAS for the evaluation of recovery (E1 – 0.00 ± 0.00 mg NH_3-N.L^{-1}; E2 – 0.05 ± 0.01 mg NO_2-N.L^{-1}).

Water quality parameters were measured daily before the first feeding: temperature and dissolved oxygen (oximeter YSI 55), salinity (refractometer), pH (pH meter, WTW 315i), total ammonia (UNESCO, 1983), un-ionized ammonia (Colt, 2002), nitrite (Bendschneider and Robinson, 1952), and total alkalinity (Eaton et al., 2005). Water quality was maintained as follows: salinity (E1 – 27.82 ± 0.06; E2 – 23.91 ± 0.05), temperature (E1 – 22.65 ± 0.07 °C; E2 – 21.68 ± 0.10 °C), dissolved oxygen concentration (E1 – 6.88 ± 0.01 mg O_2.L^{-1}; E2 – 7.34 ± 0.02 mg O_2.L^{-1}), pH (E1 – 8.20 ± 0.01; E2 – 8.17 ± 0.01), alkalinity (E1 – 168.85 ± 1.19 mg $CaCO_3$.L^{-1}; E2 – 232.19 ± 1.50 mg $CaCO_3$.L^{-1}). Photoperiod was fixed at 12 h Light: 12 h Dark.

2.2. Blood sampling and analyses

Feeding was discontinued 24 hours prior to each sampling day and three fish per tank (nine per treatment) were collected after 1, 5 and 10 days of exposure, and after the recovery period (day 20). After collection, fish were anaesthetized with benzocaine hydrochloride (50 ppm) and blood samples were obtained by caudal puncture using heparinized syringes.

The blood glucose was measured using a glucose meter (Accu-Chek Advantage; Roche Diagnosis®). Ions (Na^+, K^+ and Ca^{2+}) and bicarbonate concentration (HCO_3^-), partial pressure of gases (pCO_2 and pO_2) and pH and were measured with a portable i-STAT® clinical analyser using CG8+ cartridges (Abbott laboratories, Chicago, IL, USA). Values for pO_2, pCO_2, HCO_3 and pH were corrected for the water temperature with the assumption that water temperature and fish body temperatures were equivalent (Hanley et al., 2010).

Corrections were made according to the manufacturer's specifications. The accuracy of the gained data using an i-STAT device has been questioned when compared to data from conventional laboratory analyses, because some discrepancies can occur (Harter et al., 2014). However, when using i-STAT measurements, the focus should not be on attaining absolute values, but in other ways, allow for comparisons among experimental treatments (Calabrese et al., 2017) or assess conditions in which precision is more relevant than accuracy (Davidson et al., 2014). Beyond that, monitoring of blood parameters using i-STAT may be a useful and practical tool for a quick evaluation of stress in animals. Many recent studies have used i-STAT-based analyses on fish (Barbas et al., 2016; Hvas et al., 2016; Calabrese et al., 2017).

Blood smears were prepared and stained with May-Grünwald-Giemsa (Rosenfeld, 1947). Two thousand cells per slide were counted under a microscope (100x) for differential counting of leukocytes (monocytes, lymphocytes and granulocytes).

2.3. Statistical analysis

All data were expressed as means ± standard error (SEM). To verify the normality and homogeneity of variances, data were submitted to Kolmogorov-Smirnov and Levene tests, respectively. After assumptions were satisfied, one-way ANOVA and Tukey test were performed to check for differences among treatments (Ammonia/Nitrite concentrations) within the same sampling day. The significance level was set at $p < 0.05$ in all cases.

3. Results

The survival rate of juvenile Brazilian flounder was 100% in both experiments.

Glucose levels were significantly lower in juvenile Brazilian flounder irrespective of ammonia concentration when compared to control after 10 days of exposure, whereas after the recovery period, all treatments showed higher glycemia than control (Table 1).

The blood K^+ concentration was higher in fish exposed to ammonia, regardless of concentration, in relation to control on day 1. Ca^{2+} concentrations increased after one day of exposure in treatment 0.57 mg NH_3-N.L^{-1} compared to control. At the first day of exposure, Na^+ concentration was higher in treatment 0.57 mg NH_3-N.L^{-1} compared to treatment 0.12 mg NH_3-N.L^{-1} and control, and in the treatment 0.28 mg NH_3-N.L^{-1} compared to control. On day 5, concentration of Na^+ was lower in treatment 0.57 mg NH_3-N.L^{-1} compared to control (Table 1).

The pO_2 levels were higher in treatment 0.57 mg NH_3-N.L^{-1} compared to treatment 0.12 mg NH_3-N.L^{-1} at day 1. Fish exposed to 0.57 mg NH_3-N.L^{-1} for one day had a reduction in pCO_2 and HCO_3^- concentration in relation to all other treatments. On day 5, the HCO_3^- concentration was higher in treatment 0.57 mg NH_3-N.L^{-1} when compared to treatment 0.12 mg NH_3-N.L^{-1}. The pH increased significantly in treatments 0.28 and 0.57 mg NH_3-N.L^{-1} in relation to control at day 5. At the end of the exposure period (day 10), the HCO_3^- concentration was higher in fish exposed to 0.28 mg NH_3.L^{-1} compared to the other treatments. In the same day (day 10), the pH was higher in treatment 0.28 mg NH_3-N.L^{-1} when compared to the others, and in treatment 0.57 mg NH_3-N.L^{-1} in comparison with control. After the recovery period, the HCO_3^- concentration was higher in treatments 0.28 and 0.57 mg NH_3-N.L^{-1} than in control, being the pH also higher in these treatments in relation to control and treatment 0.12 NH_3-N.L^{-1} (Table 1).

Brazilian flounder exposed for one day to 0.28 mg NH_3-N.L^{-1} showed increased proportion of monocytes and reduced lymphocytes when compared to other treatments. The proportion of granulocytes was higher in treatment 0.28 mg NH_3-N.L^{-1} in relation to the treatment 0.57 mg NH_3-N.L^{-1}. Fish exposed to ammonia, irrespective of concentration showed decreased proportion of granulocytes in relation to control on day 5. After recovery, proportion of granulocytes increased in treatment 0.57 mg NH_3-N.L^{-1} in relation to control and treatment 0.12 mg NH_3-N.L^{-1}, and this proportion increment was also observed in treatment 0.28 mg NH_3-N.L^{-1} compared to control. There was a reduction in lymphocytes in treatments 0.28 and 0.57 mg NH_3-N.L^{-1} compared to control and 0.12 mg NH_3-N.L^{-1} after the recovery period (Table 2).

Blood glucose was higher in fish exposed to treatments 10.43 and 15.27 mg NO_2-N.L^{-1} compared to the other treatments at the end of the exposure period (day 10). On day

5, the K^+ concentration was higher in treatment 15.27 mg NO_2-N.L^{-1} compared to all other treatments, whereas on the 10th day it was higher in treatments 10.43 e 15.27 mg NO_2-N.L^{-1} than that of control. There was an elevation and a reduction in Na^+ concentrations in treatment 15.27 mg NO_2-N.L^{-1} compared to all other treatments at day 5 and after recovery, respectively. The pCO_2 levels were lower in all treatments exposed to nitrite as compared to control throughout the exposure period (1, 5 and 10 days). After five days of exposure, the HCO_3^- concentration was lower in treatment 15.27 mg NO_2-N.L^{-1} compared to all other treatments and in treatments 5.72 and 10.43 mg NO_2-N.L^{-1} when compared to control. Fish exposed to 15.27 mg NO_2-N.L^{-1} had a reduction in blood pH compared to 5.72 mg NO_2-N.L^{-1} and control on day 5. Blood pH increased significantly in all treatments compared to controls on days 1 and 10. After the recovery period, treatments 10.43 and 15.27 mg NO_2-N.L^{-1} had significantly lower HCO_3^- concentrations compared to treatment 5.72 mg NO_2-N.L^{-1} and control, whereas the pH values were lower in treatment 15.27 mg NO_2-N.L^{-1} in relation to all others treatments, and in treatment 10.43 mg NO_2-N.L^{-1} compared to control (Table 3).

Exposure to 10.43 mg NO_2-N.L^{-1} during five days led to increased percentage of monocytes and granulocytes, and a reduction in lymphocytes, compared to all other treatments. After recovery, the treatment 15.27 NO_2-N.L^{-1} showed a reduced proportion of lymphocytes and an increased proportion of granulocytes relative to treatment 10.43 NO_2-N.L^{-1} (Table 4).

4. Discussion

The build-up of toxic nitrogen compounds imposes a potential risk for intensive aquaculture, and exposure to certain levels of ammonia and nitrite can be lethal to fish (Medeiros et al., 2016). In the present study, the absence of mortality in fish exposed to concentrations of up to 0.57 mg NH_3-N.L^{-1} or 15.27 mg NO_2-N.L^{-1} for 10 days, is in agreement with the observations of Bianchini et al. (1996) when evaluating acute toxicity for *P. orbignyanus* in seawater (30‰ and 25°C) for 96 h. These authors demonstrated 100% of survival in fish exposed to concentrations around 0.54 mg NH_3-N.L^{-1} and 10 mg NO_2-N.L^{-1}, and mortality occurred only at concentrations 0.67 mg NH_3-N.L^{-1} and 20 mg

NO_2-N.L^{-1}or above. However, taking into account the different exposure time between the two studies, our results suggest that the species is capable of tolerating longer periods of exposure to these ammonia and nitrite concentration ranges, without mortality, which is an important feature of a candidate species for intensive aquaculture.

Despite the absence of mortality, evaluation of blood parameters revealed the triggering of several secondary stress responses, which were time and concentration dependent. The required physiological adaptations which increase the chances of survival under stress conditions, involve energy expenditure that is likely to impair other biological processes closely related to aquaculture productivity, as in the case of growth, reproduction and tolerance to additional stress (Bonga, 1997; Barton, 2002). Therefore, the knowledge on the ammonia- and nitrite-induced changes in blood, combined with a periodic monitoring of fish blood and water quality, allows for an early diagnosis of stress induced by deleterious levels of these compounds in the production system, therefore reducing the possibility of more severe or long-term effects.

Nitrogenous compounds can provoke the release of catecholamine and cortisol to the bloodstream as primary stress responses in fish. Elevation of plasma cortisol induces the mobilization of energy substrates, resulting in increased plasma glucose levels, which in turn, is related to carbohydrate metabolism, such as glycogenolysis and gluconeogenesis (Bonga, 1997). Increased glucose levels were observed in Brazilian flounder after recovery irrespective of ammonia treatment, and hyperglycemia was also caused by higher nitrite concentrations (10.43 and 15.27 mg NO_2-N.L^{-1}) after 10 days of exposure, indicating an increased metabolic demand in either cases. In contrast, reduced plasma glucose levels were observed after 10 days of exposure to ammonia. Similar response was also reported by Baldisserotto et al. (2014) for silver catfish *Rhamdia quelen* exposed to 0.18 and 0.50 mg NH_3-N.L^{-1} for five days. This reduction is presumably caused by the depletion of glycogen reserves in the liver and muscle during ammonia-induced stress (Miron et al., 2008).

Exposure to ammonia and nitrite caused osmoregulatory disturbances in Brazilian flounder, which led to alterations in plasma electrolyte concentrations. Fish exposed to ammonia (0.12 - 0.57 mg NH_3-N.L^{-1}) showed an initial elevation (day 1) of plasma K^+ concentration. This result was similar to those observed for Atlantic cod *Gadus morhua*

(Remen et al., 2008) and turbot *Scophthalmus maximus* (Person-Le Ruyet et al., 2003). The primary mechanism of ammonia toxicity concerns to the ability of NH_4^+ in replacing K^+ in neurons (Binstock and Lecar, 1969) and muscle (Beaumont et al., 2000). This could have decreased the concentration of K^+ in the intracellular fluids, as demonstrated in the muscle of rainbow trout *Oncorhynchus mykiss* (Vedel et al., 1998), and in turn, promoted an elevation of K^+ concentration in the blood plasma.

Elevation of plasma K^+ concentrations in juvenile Brazilian flounder exposed to 10.43 (day 10) and 15.27 mg NO_2-N.L^{-1} (days 5 and 10) was probably caused by the release of K^+ from RBCs and skeletal muscle. In RBCs, the K^+ efflux is related to the formation of metHb, which activates the K^+/Cl^- cotransport (Jensen, 1992). The transport mechanism involved in the K^+ losses from the muscle seems to be similar to that observed in RBCs (Knudsen and Jensen, 1997). Hyperkalemia has been demonstrated in several species during nitrite exposure (Grosell and Jensen, 2000; Gisbert et al., 2004; Ciji et al., 2012; Jia et al., 2015).

Increased plasma Ca^{2+} concentrations of fish exposed to 0.57 mg NH_3-N.L^{-1} (day 1) are likely a transient disturbance of calcium exchanges through the gill and intestine and/or come from internal pools (Person-Le Ruyet et al., 2003). Silver catfish exposed to ammonia (0.18 - 0.50 mg NH_3-N.L^{-1}) for 24 h also showed increased plasma Ca^{2+} concentrations, with subsequent restoration of basal levels after 5 days (Baldisserotto et al., 2014), resembling the results of the present study.

Juvenile Brazilian flounder showed increased plasmatic concentration of Na^+ when exposed to 0.28 and 0.57 mg NH_3-N.L^{-1} (day 1) and 15.27 mg NO_2-N.L^{-1} (day 5). Similar results were reported for European sea bass *Dicentrarchus labrax* (Sinha et al., 2015) and Atlantic Salmon *Salmo salar* in seawater (Knoph and Thorud, 1996) during ammonia exposure and for sea bass exposed to nitrite (Woo and Chiu, 1997). The rise in Na^+ plasma concentrations suggests a reduction of Na^+ excretion. This could be related to an inhibition of branchial Na^+/K^+ ATPase activity and/or Na^+/NH_4^+ exchange mechanism, which are involved in ammonia excretion (Weihrauch et al., 2009). In contrast, the Na^+ plasma concentration of fish subjected to 0.57 mg NH_3-N.L^{-1} was reduced on the 5th day. These results resemble those observed for turbot after seven days of exposure to 0.73 and 0.88 mg NH_3-N.L^{-1} (Person-Le Ruyet et al., 2003) and can be explained by different osmoregulatory

imbalances. More Na^+ is excreted across the gills due to an enhanced in Na^+/K^+-ATPase activity, which is triggered by endocrine stress responses (McCormick, 2001). Ionic disturbances between intra and extracellular fluids (Vedel et al., 1998) and reduction of Na^+ uptake due to competition for active transport between Na^+ and NH_4^+ (Schmidt-Nielsen, 1997) are also causes of lessened plasma Na^+ concentrations.

The ionic disturbances associated with ammonia toxicity were limited to the first 5 days of exposure. These results indicate an adaptation of Brazilian flounder to environmental ammonia, which involves physiological adjustments to recover and maintain normal osmoregulation, without requiring a recovery period in ammonia-free water. In the nitrite experiment, the 10 days of recovery sufficed to restore the basal levels of plasma K^+, which was the only altered ionic parameter at the end of the exposure.

Acid-base regulation is essential to maintain the physiological and biochemical functions and consequently, the whole animal performance. Fish can regulate an acid–base disturbance through modulations in ventilation rate to modify blood pCO_2 and mainly by ionic regulation across the gills (Evans et al., 2005; Perry and Gilmour, 2006). Brazilian flounder juveniles exposed to 0.57 mg NH_3-N.L^{-1} (day 1) and all nitrite concentrations (day 5) presented a reduction in pCO_2 and bicarbonate concentration, which indicates a compensatory mechanism to regulate the acid-base status. However, exposure to ammonia and nitrite also elicits changes in blood pH, which is an indication of failure in acid-base regulation.

Exposure to 0.28 and 0.57 mg NH_3-N.L^{-1} induced a slight metabolic alkalosis from day 5 and forward, throughout the exposure period, and even after recovery. Similarly, Nawata et al. (2015) reported a slight alkalosis and a significant rise in plasma bicarbonate of spiny dogfish shark *Squalus acanthias* during ammonia exposure. Clifford et al. (2015) also found a metabolic alkalosis characterized by a rise in plasma bicarbonate concentration and an unchanged plasma pCO_2 in Pacific hagfish *Eptatretus stoutii* exposed to ammonia. Rainbow trout acclimated to seawater experienced a gradual increase in blood pH as the ammonia exposure continued during 24 h without changes in plasma bicarbonate (Wilson and Taylor, 1992). Analysis of blood non-respiratory acid-base status of seawater fish exposed to ammonia revealed a metabolic acid consumption (base load) through the conversion of NH_3 to NH_4^+ and subsequently extrusion via NH_4^+/Na^+ exchange (Wilson

and Taylor, 1992; Clifford et al., 2015). For fish living in small volumes of water, as it occurs in intensive production systems, it is possible that acid excretions may have a significant impact on environmental pH. Water acidification and blood alkalosis reduce the concentration of NH_3 in the water and increase it in plasma, respectively, raising NH_3 plasma-water gradient and therefore its excretion (Chew et al., 2003). Thus, under certain conditions, fish can take advantage of the mechanisms of ionic and acid-base regulation to facilitate the detoxification of excessive ammonia.

Increased blood pH levels in fish exposed to all nitrite concentrations on days 1 and 10 indicate a respiratory alkalosis as a consequence of reductions in blood pCO_2, which in turn could be caused by hyperventilation (Williams et al., 1997). Metahemoglobin-related reductions in blood O_2 content during nitrite intoxication may be a trigger mechanism for hyperventilation (Aggergaard and Jensen, 2001). Another strategy observed in fish during nitrite exposure would be to reduce the metabolism to decrease the oxygen demand (Espina & Alcaraz, 1993), resulting in lower blood pCO_2 (Williams et al., 1997).

The period of recovery was sufficient for resumption of pCO_2 to baseline levels irrespective of treatment. The loss of nitrite to the environment across the gills and via the urine, recovery of functional hemoglobin levels due to the methaemoglobin-reductase system and the ability to oxidise nitrite to nitrate, are efficient mechanisms involved in the detoxification and recovery from nitrite exposure (Jensen, 2003). Thus, the return of pCO_2 values and plasma K^+ concentrations to baseline levels can be an indication of restored functional hemoglobin levels. However, fish exposed to 10.43 and 15.27 mg NO_2-N.L^{-1} (day 20) had a decreased concentration of bicarbonate and pH, indicating a metabolic acidosis. According to Clifford et al. (2015), the stepwise correction of the alkalosis was likely due to an increase in the addition of metabolic acid to the blood, resulting in a temporary acidosis during recovery. Metabolic acidosis was more pronounced in the highest nitrite concentration concomitantly with the decreased plasma Na^+. Since the excretion of H^+ is related to the uptake of Na^+ (Perry and Gilmour, 2006), this result suggests a possible reduction in Na^+/H^+ exchange mechanisms, leading to an acid load. Nevertheless, further studies are needed for a better understanding of the ionic and acid-base changes observed during recovery from nitrite exposure.

The use of leukocyte profiles to assess immune responses is also an important tool for the monitoring of stress in fish (Davis et al., 2008; Souza Neves et al., 2014; Zebral et al., 2015). Brazilian flounder exposed to intermediate concentrations of ammonia (0.28 mg NH_3-N.L^{-1} on day 1) and nitrite (10.43 mg NO_2-N.L^{-1} on day 5), and after recovery for those previously exposed to 0.28 and 0.57 mg NH_3-N.L^{-1}, presented increased proportion of monocytes and/or granulocytes, and reduced number of lymphocytes. This pattern is described as a common secondary stress response in fish (Davis et al., 2008) and represents an innate immune response (Miller et al., 1998). Increased proportion of granulocytes, and decreased lymphocytes were observed in rainbow trout (Vosyliene and Kazlauskiene, 2004) and common carp *Cyprinus carpio* (Dabrowska and Wlasow, 1986) exposed to sublethal levels of ammonia. Saoud et al. (2014) demonstrated an increased proportion of eosinophils and neutrophils, two types of granulocytes, during exposure of rabitfish *Siganus rivulatus* for 56 days to nitrite (up to 40 mg NO_2-N.L^{-1}). Immune response of Brazilian flounder exposed to intermediate concentrations revealed a protective mechanism response at these concentrations. However, at higher concentrations or longer exposure, this response was not activated probably due to the higher magnitude of stress (Svobodova et al. 1994), as observed in mrigal *Cirrhinus mrigala* (Das et al., 2004) and rabitfish *S. rivulatus* (Saoud et al., 2014) exposed to ammonia and nitrite, respectively.

Otherwise, irrespective of concentration, exposure to ammonia induced a decreased proportion of granulocytes at day 5, indicating an immunosuppressive effect of ammonia and/or a migration of these cells from blood stream to other tissues. The suppression of the immune system responses increases the susceptibility to diseases during stress (Nardocci et al., 2014), which can be a serious problem for aquaculture development, especially at high stocking densities.

Briefly, the alterations in secondary stress responses of Brazilian flounder were present even in fish exposed to the lowest concentration of either compounds (0.12 mg NH_3-N.L^{-1} and 5.72 mg NO_2-N.L^{-1}). Thus, these are defined as the lowest-observable-effect concentration for the species and provide relevant information for the establishment of safe thresholds of NH_3-N and NO_2-N concentrations in production systems. The findings of this study also demonstrated that recovery responses after transfer of Brazilian flounder to good quality water for a period of 10 days were dependent both on the concentration and toxic

compound to which the fish were exposed. The return of all parameters to basal levels was demonstrated in treatment 5.72 mg NO_2-N.L^{-1}. As for the other treatments, only some parameters were restored to normal levels (as in treatments 0.12 mg NH_3-N.L^{-1}; 10.43 and 15.27 mg NO_2-N.L^{-1}) or no recovery was observed (as in the case of 0.28 and 0.57 mg NH_3-N.L^{-1} treatments), suggesting that a longer recovery time is necessary, especially for fish exposed to ammonia.

5. Conclusion

Exposure to ammonia and nitrite caused metabolic, ionic and acid-base disturbances, with modulation of immune responses in juvenile Brazilian flounder. In this perspective, concentrations at 0.12 mg NH_3-N.L^{-1} and 5.72 mg NO_2-N.L^{-1} should be avoided in production systems in order to prevent physiological disorders, which are likely to negatively impact *P. orbignyanus* performance reared under intensive production systems. Furthermore, only fish exposed to 5.72 mg NO_2-N.L^{-1} following a 10-days recovery period showed complete resumption of normal physiological condition.

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Tables

Table 1 – Blood parameters (mean ± SEM) in juvenile Brazilian flounder *Paralichthys orbignyanus* subjected to ammonia exposure (1, 5 and 10 days) followed by a recovery period (day 20).

		Blood parameters							
		Glucose (mg dL^{-1})	**K^+ (mmol L^{-1})**	**Ca^{2+} (mmol L^{-1})**	**Na^+ (mmol L^{-1})**	**pO_2 (mm Hg)**	**pCO_2 (mm Hg)**	**HCO_3^- (mmol L^{-1})**	**pH**
Day 1	**Control**	30.22±1.79a	3.35±0.05b	1.31±0.04b	154.12±0.82c	18.27±0.84ab	4.10±0.07a	2.19±0.03a	7.33±0.01a
	0.12 mg NH_3-N.L^{-1}	26.78±0.62a	3.68±0.08a	1.37±0.03ab	154.89±0.75bc	15.87±1.02b	4.01±0.10a	2.13±0.06a	7.34±0.01a
	0.28 mg NH_3-N.L^{-1}	29.11±1.84a	3.63±0.03a	1.37±0.02ab	157.12±0.60ab	19.45±1.22ab	4.04±0.09a	2.14±0.07a	7.34±0.01a
	0.57 mg NH_3-N.L^{-1}	27.22±1.83a	3.75±0.10a	1.45±0.01a	158.33±0.60a	19.76±0.69a	3.63±0.07b	1.94±0.02b	7.34±0.01a
Day 5	**Control**	27.22±1.39a	3.42±0.11a	1.34±0.04a	154.11±0.42a	17.05±0.88a	4.00±0.11a	2.35±0.08ab	7.35±0.01b
	0.12 mg NH_3-N.L^{-1}	23.67±0.98a	3.52±0.07a	1.37±0.04a	153.11±0.63ab	19.01±0.99a	3.89±0.10a	2.04±0.06b	7.37±0.01ab
	0.28 mg NH_3-N.L^{-1}	25.44±2.02a	3.29±0.05a	1.35±0.03a	152.78±0.78ab	16.48±0.71a	3.81±0.16a	2.28±0.11ab	7.39±0.01a
	0.57 mg NH_3-N.L^{-1}	26.66±1.27a	3.54±0.08a	1.34±0.04a	151.11±0.95b	19.29±1.28a	4.11±0.11a	2.43±0.07a	7.40±0.01a
Day 10	**Control**	27.00±0.94a	3.52±0.11a	1.39±0.03a	154.78±0.72a	16.19±0.91a	4.00±0.14a	2.13±0.09b	7.31±0.01c
	0.12 mg NH_3-N.L^{-1}	21.11±1.34b	3.62±0.15a	1.44±0.03a	155.78±0.83a	15.07±1.28a	3.87±0.16a	2.09±0.07b	7.33±0.02bc
	0.28 mg NH_3-N.L^{-1}	22.00±1.03b	3.47±0.08a	1.34±0.02a	154.00±0.33a	17.71±1.40a	4.16±0.11a	2.85±0.12a	7.42±0.02a
	0.57 mg NH_3-N.L^{-1}	22.33±0.91b	3.64±0.10a	1.38±0.04a	156.44±0.73a	15.15±0.95a	3.75±0.15a	2.07±0.07b	7.36±0.01b
Day 20	**Control**	27.89±0.73b	3.45±0.09a	1.38±0.03a	153.89±1.05a	20.76±1.17a	5.01±0.17a	2.51±0.10b	7.30±0.02b
	0.12 mg NH_3-N.L^{-1}	33.25±0.75a	3.55±0.09a	1.37±0.05a	155.37±1.01a	20.15±1.61a	5.21±0.12a	2.60±0.09ab	7.30±0.01b
	0.28 mg NH_3-N.L^{-1}	36.22±1.60a	3.62±0.12a	1.40±0.02a	153.00±0.87a	17.67±2.38a	5.25±0.15a	2.95±0.13a	7.36±0.01a
	0.57 mg NH_3-N.L^{-1}	32.78±0.74a	3.60±0.07a	1.36±0.02a	153.77±0.77a	18.47±1.00a	5.17±0.06a	2.94±0.12a	7.36±0.01a

Different lowercase letters indicate significant differences among treatments within the same sampling day (Tukey's test, $p < 0.05$, $n = 9$).

Table 2 – Differential leukocyte count (monocytes, lymphocytes and granulocytes) (mean ± SEM) in juvenile Brazilian flounder *Paralichthys orbignyanus* exposed to ammonia (1, 5 and 10 days) followed by a recovery period (day 20).

		Monocyte (%)	**Granulocyte (%)**	**Lymphocyte (%)**
Day 1	**Control**	3.38±0.87b	3.16±0.86ab	93.45±0.86a
	0.12 mg NH_3-N.L^{-1}	1.79±0.49b	3.32±0.50ab	94.88±0.55a
	0.28 mg NH_3-N.L^{-1}	6.89±0.36a	5.83±1.18a	87.28±1.29b
	0.57 mg NH_3-N.L^{-1}	3.62±0.55b	1.94±0.55b	94.44±0.78a
Day 5	**Control**	2.69±0.82a	5.64±1.23a	92.80±1.59a
	0.12 mg NH_3-N.L^{-1}	2.97±0.91a	1.24±0.47b	95.80±1.27a
	0.28 mg NH_3-N.L^{-1}	4.27±0.69a	1.12±0.27b	94.61±0.78a
	0.57 mg NH_3-N.L^{-1}	5.13±1.06a	1.49±0.30b	93.38±0.94a
Day 10	**Control**	0.83±0.54a	1.48±0.56a	97.69±1.05a
	0.12 mg NH_3-N.L^{-1}	0.96±0.26a	0.00±0.00a	99.04±0.26a
	0.28 mg NH_3-N.L^{-1}	1.71±0.52a	0.99±0.44a	97.30±0.69a
	0.57 mg NH_3-N.L^{-1}	1.93±0.83a	0.19±0.16a	97.87±0.87a
Day 20	**Control**	1.23±0.39ab	0.74±0.30c	98.02±0.67a
	0.12 mg NH_3-N.L^{-1}	0.66±0.15b	1.32±0.15bc	98.01±0.26a
	0.28 mg NH_3-N.L^{-1}	1.47±0.40ab	4.33±1.35ab	94.19±1.58b
	0.57 mg NH_3-N.L^{-1}	3.55±1.18a	4.68±0.49a	91.77±1.04b

Different lowercase letters indicate significant differences among treatments within the same sampling day (Tukey's test, $p < 0.05$, $n = 9$).

Table 3 – Blood parameters (mean ± SEM) in juvenile Brazilian flounder *Paralichthys orbignyanus* exposed to nitrite (1, 5 and 10 days) followed by a recovery period (day 20).

		Blood parameters							
		Glucose (mg dL^{-1})	**K^+ (mmol L^{-1})**	**Ca^{2+} (mmol L^{-1})**	**Na^+ (mmol L^{-1})**	**pO_2 (mm Hg)**	**pCO_2 (mm Hg)**	**HCO_3^- (mmol L^{-1})**	**pH**
Day 1	**Control**	27.67±2.83a	3.61±0.05ab	1.27±0.05a	155.55±0.97a	12.62±0.97a	5.67±0.06a	3.10±0.10a	7.34±0.01b
	5.72 mg NO_2-N.L^{-1}	29.22±1.25a	3.52±0.09ab	1.13±0.05a	156.33±0.50a	11.41±0.83a	5.06±0.17b	3.24±0.11a	7.41±0.01a
	10.43 mg NO_2-N.L^{-1}	28.67±2.35a	3.38±0.04b	1.15±0.06a	154.22±0.76a	13.86±1.05a	4.91±0.13b	3.21±0.10a	7.43±0.01a
	15.27 mg NO_2-N.L^{-1}	28.44±1.24a	3.76±0.06a	1.22±0.07a	154.22±1.12a	12.18±1.26a	4.77±0.07b	3.10±0.08a	7.40±0.01a
Day 5	**Control**	25.89±1.54a	3.51±0.12b	1.12±0.04a	155.67±0.47b	13.43±1.21a	5.21±0.10a	3.24±0.07a	7.41±0.01a
	5.72 mg NO_2-N.L^{-1}	27.11±0.48a	3.44±0.07b	1.11±0.05a	155.55±0.41b	14.26±0.58a	4.46±0.18b	2.78±0.11b	7.40±0.02a
	10.43 mg NO_2-N.L^{-1}	28.44±1.51a	3.47±0.14b	1.19±0.03a	156.42±0.28b	15.17±0.51a	4.71±0.08b	2.81±0.13b	7.37±0.01ab
	15.27 mg NO_2-N.L^{-1}	26.67±1.30a	4.04±0.05a	1.07±0.04a	158.44±0.18a	16.43±0.99a	4.45±0.10b	2.38±0.07c	7.34±0.02b
Day 10	**Control**	23.67±0.75b	3.42±0.09b	1.05±0.4a	154.33±0.83a	12.68±1.19a	4.82±0.18a	2.77±0.12a	7.37±0.01b
	5.72 mg NO_2-N.L^{-1}	24.11±0.39b	3.57±0.05ab	1.19±0.07a	155.44±0.69a	12.65±0.68a	4.19±0.21b	2.87±0.12a	7.43±0.01a
	10.43 mg NO_2-N.L^{-1}	31.22±0.90a	3.73±0.07a	1.09±0.06a	154.44±0.47a	13.21±1.11a	4.20±0.13b	3.03±0.12a	7.46±0.01a
	15.27 mg NO_2-N.L^{-1}	33.11±0.89a	3.78±0.08a	1.21±0.05a	155.78±0.36a	13.96±0.71a	4.01±0.07b	2.74±0.06a	7.43±0.01a
Day 20	**Control**	25.44±1.48a	3.58±0.06a	1.19±0.05a	155.11±0.61a	15.28±1.08a	5.35±0.16a	3.17±0.05a	7.41±0.01a
	5.72 mg NO_2-N.L^{-1}	22.45±1.16a	3.58±0.13a	1.19±0.03a	155.56±0.44a	14.10±1.00a	5.51±0.10a	3.37±0.08a	7.38±0.01ab
	10.43 mg NO_2-N.L^{-1}	24.11±0.68a	3.68±0.09a	1.12±0.07a	155.55±0.71a	14.07±0.55a	5.08±0.16a	2.83±0.08b	7.36±0.01b
	15.27 mg NO_2-N.L^{-1}	26.77±1.15a	3.52±0.05a	1.00±0.05a	151.78±0.94b	13.96±0.75a	5.39±0.12a	2.87±0.06b	7.31±0.01c

Different lowercase letters indicate significant differences among treatments within the same sampling day (Tukey's test, $p < 0.05$, $n = 9$).

Table 4 – Differential leukocyte count (monocytes, lymphocytes and granulocytes) (mean ± SEM) in juvenile Brazilian flounder *Paralichthys orbignyanus* exposed to nitrite (1, 5 and 10 days) following a recovery period (day 20).

		Monocyte (%)	**Granulocyte (%)**	**Lymphocyte (%)**
Day 1	**Control**	3.40±1.74a	2.33±1.20a	93.67±2.73a
	5.72 mg NO_2-N.L^{-1}	2.29±0.33a	4.55±2.28a	93.16±2.45a
	10.43 mg NO_2-N.L^{-1}	3.62±1.45a	3.27±0.88a	93.11±2.09a
	15.27 mg NO_2-N.L^{-1}	2.50±1.50a	2.22±0.78a	95.28±2.25a
Day 5	**Control**	1.32±0.34b	1.31±0.33b	97.37±0.34a
	5.72 mg NO_2-N.L^{-1}	1.96±0.59b	1.93±0.54b	96.11±0.54a
	10.43 mg NO_2-N.L^{-1}	7.49±0.86a	14.62±2.67a	77.90±3.53b
	15.27 mg NO_2-N.L^{-1}	1.29±0.85b	0.65±0.65b	98.06±0.97a
Day 10	**Control**	1.64±0.86a	4.26±1.18a	94.11±2.04a
	5.72 mg NO_2-N.L^{-1}	2.22±0.85a	4.19±1.14a	93.55±1.36a
	10.43 mg NO_2-N.L^{-1}	2.99±0.58a	2.50±0.87a	94.51±1.45a
	15.27 mg NO_2-N.L^{-1}	2.39±0.44a	5.89±2.62a	91.71±2.04a
Day 20	**Control**	1.90±1.10a	1.58±0.83ab	96.52±0.82ab
	5.72 mg NO_2-N.L^{-1}	0.66±0.33a	2.30±1.30ab	97.05±1.50ab
	10.43 mg NO_2-N.L^{-1}	1.97±0.01a	4.61±1.20a	93.40±1.20b
	15.27 mg NO_2-N.L^{-1}	1.28±0.33a	0.31±0.32b	98.41±0.32a

Different lowercase letters indicate significant differences among treatments within the same sampling day (Tukey's test, $p < 0.05$, $n = 9$).

CAPÍTULO 2

Ammonia exposure and subsequent recovery trigger oxidative stress responses in juveniles of Brazilian flounder *Paralichthys orbignyanus*

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Abstract

It was evaluated the effects of ammonia exposure and recovery on oxidative stress parameters and histology of juvenile Brazilian flounder *Paralichthys orbignyanus*. The fish were exposed to 0.05 (control), 0.12, 0.28 and 0.57 mg NH_3-N L^{-1} for 10 days followed by the same recovery time in ammonia free-water. Gill, liver and muscle samples (n=9) were collected after 1, 5 and 10 days of exposure and after recovery for oxidative stress analysis (Antioxidant Capacity Against Peroxyl Radicals (ACAP); Glutathione-S-transferase (GST) activity; lipoperoxidation levels measured through thiobarbituric acid reactive substances (TBARS) content). For the histological assessment, it was collected gill, liver, and brain samples. Exposure to all NH_3-N concentrations induced different time- and concentration-dependent changes in oxidative stress parameters. Reduced antioxidant capacity of liver and muscle, and enhanced TBARS levels of gill and liver were demonstrated. Differently, high ammonia concentration elicited lower hepatic TBARS levels. Enhanced GST activity in all organs and increased antioxidant capacity of gills were also observed. No ammonia-induced histopathological effects were demonstrated. After recovery, most of the parameters (liver ACAP, GST activity in muscle and liver, and TBARS in gills) returned to baseline levels. However, liver TBARS and gills GST activity remained altered in treatment 0.57 mg NH_3-N L^{-1}. The recovery period also led to a decrease in gills antioxidant capacity and an increase in muscle antioxidant capacity. In conclusion, concentrations from 0.12 mg NH_3-N L^{-1} induce oxidative stress and antioxidant responses in juvenile Brazilian flounder. Moreover, 10-day recovery period was not sufficient to restore fish homeostasis.

Keywords: nitrogen compound; lipid peroxidation; antioxidant system; glutathione-S-transferase; histology

Introduction

Aquaculture is the fastest growing animal food production sector, mainly due to the stagnation of global capture fishery production and rapidly rising in demand for seafood (FAO 2016). The use of recirculating aquaculture system (RAS) is an interesting strategy for intensification of production in a more environmentally sustainable manner, reducing land and freshwater use and minimizing wastewater production (Klinger and Naylor 2012).

One of the most limiting factors in intensive aquaculture system is the buildup of nitrogenous compounds, mainly ammonia due to its high toxicity to fish (Randall and Tsui 2002). In RAS, ammonia is removed in biofilters by nitrification process, in which nitrifying bacteria oxidize ammonia into nitrite and nitrite into nitrate (Schreier et al. 2010). However, a system functioning in an inadequate way or not properly planned can lead to an insufficient nitrification process to oxidize the input of ammonia, which in turn accumulates in the water.

In aquatic environments, ammonia exists in ionized (NH_4^+) and un-ionized (NH_3) forms and the NH_3 form can readily diffuse across gill membranes. Furthermore, ammonia excretion is inhibited at high NH_3 due to reduced gradient for diffusive elimination (Wilkie and Wood 1996). Therefore, the buildup of ammonia in blood and tissues can lead to physiological, biochemical, morphological and behavioral changes in fish (Schram et al. 2010; Kolarevic et al. 2012; Dong et al. 2013; Baldisserotto et al. 2014; Cheng et al. 2015). These alterations can result in reduction of growth performance (Paust et al. 2011), increased susceptibility to diseases (Ackerman et al. 2006) and death (Medeiros et al. 2016), all factors that affect the production of aquaculture systems.

Oxidative stress is a biochemical response frequently triggered by environmental variables and pollutants in aquatic organisms (Monserrat et al. 2007; Lushchak 2011; Stoliar and Lushchak 2012). This condition is defined as a transiently or chronically enhanced concentration of reactive oxygen species (ROS) due to an unbalanced state between pro-oxidants and antioxidants, resulting in damage of cellular constituents, such as DNA, proteins, and lipids (Lushchak 2011; Halliwell and Gutteridge 2015). In recent years, studies have shown that exposure of fish to ammonia can induce oxidative stress by enhancing ROS production and/or by a decrease in antioxidant defenses (Ching et al. 2009;

Hegazi et al. 2010; Yang et al. 2010a; Sinha et al. 2014; Sun et al. 2014; Cheng et al. 2015). However, the response of oxidative stress parameters after recovery in ammonia free-water is still unknown.

The use of histopathological investigations is also a sensitive tool to assess health status of fish since tissue alterations of target organs may occur due to environmental stressors and related biochemical responses (Schwaiger et al. 1997; Rašković et al. 2013). Several studies demonstrate that exposure to environmental ammonia induces tissue injuries in different fish organs, such as gill, liver, and brain (Roumieh et al. 2013; Rodrigues et al. 2014). The recovery of damaged tissues was also demonstrated for fish kept in ammonia free water for 7 or 14 days (Ravindrababu and Neeraja 2012).

The Brazilian flounder *Paralichthys orbignyanus* occurs in estuaries and shallow coastal waters of the southwestern Atlantic Ocean, from Rio de Janeiro, Brazil to Mar del Plata, Argentina (Figueiredo and Menezes 2000; Millner et al. 2005). This species is an important fishing resource in these areas and presents a wide tolerance to water quality parameters (Bianchini et al. 1996; Wasielesky et al. 1997; Wasielesky et al. 1998; Sampaio and Bianchini 2002, Okamoto and Sampaio 2012; Garcia et al. 2015) and handling procedures (Bolasina 2011). In addition, the spawning, larviculture and juvenile production has been successfully performed (Sampaio et al. 2007; Sampaio et al. 2008). Thus, the Brazilian flounder is a species with a great potential to intensive aquaculture (Sampaio et al. 2007). However, limited information exists related to the toxicity of ammonia to this species (Bianchini et al. 1996) and more studies are necessary to evaluate the mechanisms of toxicity and defense strategies during exposure and recovery.

Therefore, the aim of the present study was to assess the effects of sub-lethal ammonia exposure and followed recovery on oxidative stress parameters (glutathione-S-transferase (GST) activity , total antioxidant capacity against peroxyl radicals (ACAP) and lipid peroxidation levels) and histology of different juvenile Brazilian flounder tissues.

Materials and methods

Fish and experimental conditions

The juveniles Brazilian flounder (107.3 ± 1.9 g and 21.2 ± 0.1 cm) were produced and reared at the Aquaculture Marine Station of the Universidade Federal do Rio Grande - FURG in Rio Grande do Sul, Southern Brazil. The experimental protocol and use of fish were approved by the Committee on Animal Experimentation of FURG under registration number 23116.001030/2016-15.

Fish (15 per tank) were distributed randomly in 12 tanks (250 L useful volume) in four recirculation system for acclimation to experimental conditions during 20 days. The fish were fed twice daily (3% of total biomass) with commercial diet (46% crude protein) throughout the acclimation and experimental period. Photoperiod was fixed at 12 h Light : 12h Dark.

After the acclimation period, the fish were exposed to 0.05 ± 0.01 (control), 0.12 ± 0.01, 0.28 ± 0.02 and 0.57 ± 0.02 mg NH_3-N L^{-1} in triplicate for 10 days in a semi-static system. The tested concentrations were chosen above the safety level (0.06 mg NH_3-N L^{-1}) obtained for this species in saltwater by Bianchini et al. (1996). The desired ammonia concentrations were obtained by adding ammonium chloride (NH_3Cl_4) (Synth, Brazil) solutions or water exchange of the tanks. After the exposure period, fish remained for 10 days in recirculation system for assessment of recovery in ammonia free-water.

Temperature and dissolved oxygen (oximeter YSI 55), salinity (refractometer), pH (pH meter, WTW 315i), total alkalinity (Eaton et al. 2005), total (Intergovernmental Oceanographic Commission 1983) and un-ionized ammonia (Colt 2002), and nitrite (Bendschneider and Robinson 1952) were measured daily before first feeding. All parameters were maintained as follows: temperature (22.65 ± 0.07 °C), dissolved oxygen concentration (6.88 ± 0.01 mg L^{-1}), salinity (27.82 ± 0.06), pH (8.20 ± 0.01), alkalinity (168.85 ± 1.19 mg $CaCO_3$ L^{-1}) and nitrite (0.92 ± 0.11 mg L^{-1}).

Organ sampling

Nine fish of each treatment (three per tank) were netted after 1, 5 and 10 days of exposure, and after recovery period (day 20). Fish were immediately euthanized with a lethal dose of hydrochloride benzocaine (500 ppm), and samples of gills, liver, brain, and muscle were collected. Feeding was ceased 24 h prior each sampling day.

Oxidative stress analyses

Organs homogenization

Samples of gill, liver and muscle tissues were maintained in an ultra-freezer (-80 ºC) and then homogenized (1:5; w/v) in a Tris–HCl (100 mM, pH 7.75) buffer with EDTA (2 mM) and Mg^{2+} (5 mM) (da Rocha et al. 2009). The supernatants resulting from the centrifugation of the homogenates (10000 x *g*, 20 minutes, 4ºC) were used for all analyses. The total protein content of homogenized samples was determined in a microplate reader (BioTek LX 800) using a commercial kit (Doles®) based on the Biuret assay (550 nm).

Glutathione-S-transferase activity

GST activity was determined following the conjugation of 1 mM glutathione (GSH) and 1 mM1-chloro-2,4-dinitrobenzene (CDNB) according to Habig et al. (1974) and Habig and Jakoby (1981). Potassium phosphate buffer (KH_2PO_4 – 0.05 M; K_2HPO_4 – 0.05 M; pH = 7.0) was used as reaction medium (previously heated at 25 ºC with a water bath). The absorbance (340 nm) was read in transparent 96 wells microplate using a spectrofluorometer (Victor 2, Perkin Elmer, MA, USA). The data were expressed as nmol of CNDB-GSH conjugate min^{-1}mg wet $tissue^{-1}$.

Total antioxidant capacity against peroxyl radicals

ACAP was determined through detection of ROS in samples treated or not with a peroxyl radical generator, according to the method described by Amado et al. (2009). All samples were previously diluted with homogenization buffer to 2.0 mg protein mL^{-1} and then exposed to peroxyl radicals generated by thermal (37°C) decomposition of 2,2′-azobis

(2 methylpropionamidine) dihydrochloride (ABAP, 4 mM). Peroxyl radicals reacted with a fluorescent substrate (2′,7′ dichlorofluorescein diacetate - H_2DCF-DA) and fluorometry (excitation: 485 nm; emission: 520 nm) was measured through a microplate reader (Victor 2, Perkin Elmer) with readings at every 5 minutes, during 35 minutes. The results were expressed as a relative area (the difference between the ROS area with and without ABAP relative to the without ABAP). For interpretation purposes of the results, a higher relative area means a lower antioxidant capacity.

Lipid Peroxidation

The lipid peroxidation levels were measured according Oakes and Van Der Kraak (2003). To determine the thiobarbituric acid reactive substances (TBARS) by the quantification of MDA (malondialdehyde), 20 µL of BHT solution (67 µM), 150 µL 20% acetic acid solution, 150 µL 0.8 % TBA solution, 50 µL Milli-Q H_2O, and 20 µL of 8.1% SDS were added to samples (gills: 40 µL; liver: 60 µL; muscle: 100 µL) before being heated at 95 ºC during 30 minutes. Thereafter, 100 µL of Milli-Q H_2O and 500 µL of n-butanol were added to the final solution. The remaining supernatant after centrifugation (3,000 x *g*, 10 minutes, 15 ºC) was used to determine the fluorescence (excitation: 520 nm; emission: 580 nm) and the results were expressed as nmol TMP mg wet tissue^{-1}, where TMP stands for tetramethoxypropane (ACROS Organics), employed as standard.

Histopathological analysis

The samples of gill tissues were fixed in Bouin's solution, and brain and liver samples were fixed in 10% buffered formalin. Samples were dehydrated using sequentially increasing concentrations of ethanol, cleared in xylene and embedded in Paraplast® with an automatic tissue processor (LUPETEC, PT 05, Brazil). The sections (5 µm) were prepared using a microtome (LUPETEC, MRP03, Brazil), stained with hematoxylin-eosin (H-E) and slides analyzed with an optical microscope (ZEISS, PRIMO STAR).

Statistical analysis

All data were expressed as means ± standard error (SEM) and analysed through analysis of variance (ANOVA). ANOVA assumptions (normality and variance homogeneity) were previously verified by Kolmogorov-Smirnov and Levene tests, respectively. Two-way ANOVA (factors: ammonia concentrations and exposure time) and Tukey tests were performed to check differences among treatments. For all analysis, the significance level was set at 5% ($\alpha = 0.05$).

Results

The fish survival rate was 100% throughout the period of exposure and recovery for all treatments.

Fish exposed to 0.28 and 0.57 mg NH_3-N L^{-1} showed higher antioxidant competence on gills compared to control and to the treatment 0.12 mg NH_3-N L^{-1} at day 1, and lower values compared to control after the recovery period (Fig. 1a). The ACAP on the liver were lower than the control in fish exposed to treatments 0.28 and 0.57 mg NH_3-N L^{-1} during the entire period of exposure (days 1, 5 and 10), and to treatment 0.12 mg NH_3-N L^{-1} only at day 1 (Fig. 1b). On the muscle, a lower ACAP were also demonstrated in treatment 0.57 mg NH_3-N L^{-1} compared to control at days 1 and 5, and in treatment 0.28 mg NH_3-N L^{-1} in relation to control and to treatment 0.12 mg NH_3-N L^{-1} at day 5. The muscle ACAP was higher for all NH_3-N treatments compared to control after the recovery period (Fig. 1c).

The GST activity was higher on the gills of fish exposed to all ammonia concentrations when compared to control at day 1, and in treatments 0.28 and 0.57 mg NH_3-N L^{-1} in relation to control and to treatment 0.12 mg NH_3-N L^{-1} at the end of the exposure period (day 10). After recovery, only treatment 0.57 mg NH_3-N L^{-1} remained higher than the control (Fig. 2a). Fish exposed to 0.28 and 0.57 mg NH_3-N L^{-1} presented higher GST activity of liver compared to control and to treatment 0.12 mg NH_3-N L^{-1} at days 1 and 10 (Fig. 2b). An enhanced GST activity was also observed on the muscle of fish exposed to 0.28 or 0.57 mg NH_3-N L^{-1} when compared to control at day 10 (Fig. 2c).

The TBARS levels were higher in relation to control and to 0.12 mg NH_3-N L^{-1} on the gills of fish exposed to 0.28 and 0.57 mg NH_3-N L^{-1} at day 10 (Fig. 3a). The values for

this parameter are significantly lower on the liver of fish exposed to treatment 0.57 mg NH_3-N L^{-1}, compared to control and to all the other treatments, at days 1 and 5, respectively. At day 10, the levels of TBARS presented higher values in treatment 0.28 mg NH_3-N L^{-1} in relation to the others, and lower values in treatment 0.57 mg NH_3-N L^{-1} compared to control. After recovery, the values were lower on the liver of fish exposed to the treatment 0.57 mg NH_3-N L^{-1} in relation to control and treatment 0.12 mg NH_3-N L^{-1}, and in treatment 0.28 mg NH_3-N L^{-1} compared to treatment 0.12 mg NH_3-N L^{-1} (Fig. 3b). No significant differences were registered among treatments for TBARS levels on the muscle (Fig. 3c).

The histological analysis did not reveal any morphological alteration in brain, gills, and liver of flounder juveniles resulting from the experimental treatments.

Discussion

Under normal physiological conditions, around 0.1% of the oxygen consumed in aerobic metabolism is converted in the mitochondria into ROS (Fridovich 2004), such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO^\bullet$), which can oxidize and damage several biomolecules (Halliwell and Gutteridge 2015). Despite a not complete understanding of the mechanisms involved, previous evidences indicate that ammonia can trigger a pro-oxidant scenario in different fish organs (Hegazi et al. 2010; Sinha et al. 2014; Cheng et al. 2015; Li et al. 2016). In the present study, changes in oxidative stress parameters of Brazilian flounder juveniles were found depending on the exposure time and concentration, and reflect the toxic effects of this compound at biochemical level and possible adaptive responses.

Fish, like other aerobic organisms, present a complex antioxidant system with several enzymatic and non-enzymatic defences to protect tissues against oxidative damage (Halliwell and Gutteridge 2015). The analysis of total antioxidant capacity evaluates the overall antioxidant status, without distinguishing between enzymatic and non-enzymatic components of the antioxidant system. The basal total antioxidant capacity of Brazilian flounder tissues was higher in gill, followed by the liver and muscle, in this order,

indicating variability in antioxidant competence among different organs. The values found in the liver and gill were like those reported for *Micropogonias furnieri, Pimelodus pintado, Loricariichthys anus* and *Parapimelodus nigribarbis* using the same technique (Da Rocha et al. 2009). In contrast, the muscle presented an extremely low antioxidant capacity, differently than demonstrated for other fish species (Amado et al. 2009; Da Rocha et al. 2009; Enamorado et al. 2015). This is possibly related to the very low swimming activity of the species (Wilhelm Filho et al. 1993), which results in reduced metabolism and consequently little generation of ROS in this organ, as demonstrated by the TBARS levels that show the same pattern (gill > liver > muscle) found for total antioxidant competence. Therefore, reduced antioxidant defences are sufficient to maintain tolerable levels of oxidative damage in this organ.

Besides the differences in antioxidant competence among organs, the response to a stressor is also organ-specific. Exposure to ammonia lead to a reduction in the total antioxidant capacity in liver (0.12 mg NH_3-N L^{-1} on day 1; 0.28 and 0.57 mg NH_3-N L^{-1} on days 1, 5 and 10) and muscle (0.28 mg NH_3-N L^{-1} on day 5; 0.57 mg NH_3-N L^{-1} on days 1 and 5) of Brazilian flounder. Previous studies have reported that ammonia-induced oxidative stress is at least in part related to inhibition of antioxidant defences (Yang et al. 2011; Li et al. 2016), resulting in increased susceptibility to suffer oxidative damage. The gill showed a different response with an initial induction of antioxidant defence mechanisms demonstrated by an increase in antioxidant capacity of fish exposed to 0.28 and 0.57 mg NH_3-N L^{-1} on day 1. Other authors also demonstrate various antioxidants responses, such as increased synthesis of glutathione and induced activity of various antioxidant enzymes as an attempt to alleviate ammonia-induced oxidative stress in fish organs (Ching et al. 2009; Hegazi et al. 2010; Yang et al. 2010a; Sinha et al. 2014).

The glutathione S-transferases (GSTs) are a family of multifunctional enzymes that play an important role as antioxidant defence. These enzymes catalyse the nucleophilic attack of reduced glutathione (GSH) on electrophilic groups and are involved in detoxification of xenobiotic and endogenous metabolites including products of oxidative damage (Blanchette et al. 2007), and the determination of their activity is a useful biochemical biomarker of environmental stress (Carvalho-Neta and Abreu-Silva 2013). In the present study, ammonia exposure led to an initial (day 1) increased GST activity in the

gills (0.12, 0.28 and 0.57 mg NH_3-N L^{-1}) and liver (0.28 and 0.57 mg NH_3-N L^{-1}) of fish, followed by a return to baseline levels on day 5. GST activity also showed an increase in all organs (gill, liver, and muscle) of fish exposed to 0.28 and 0.57 mg NH_3-N L^{-1} at the end of the exposure period (day 10), indicating that the activity of this enzyme is part of an adaptive response against a pro-oxidant condition induced by ammonia exposure at different times. The enhanced ROS formation during an environmental stress situation causing the activation of redox-sensitive transcription factors (Nrf2, for example) and ROS-mediated covalent modification of antioxidant proteins led to enhancement of antioxidant defences (Hermes-Lima et al. 2015). Similar patterns of GST activity were reported for Nile tilapia *Oreochromis niloticus* (Hegazi et al. 2010), rainbow trout *Oncorhynchus mykiss* (Farzad et al. 2015) and rockfish *Sebastes schlegelii* (Kim et al. 2015) exposed to ammonia. Additionally, the increased GST activity can also suggest a role of glutathione system in the detoxification of ammonia (Farzad et al. 2015; Kim et al. 2015). This could explain the high ammonia tolerant capability of this species point out by Bianchini et al. (1996). In further studies, GSH is an important parameter that should also be evaluated to a better comprehension of these biochemical mechanisms against ammonia toxicity.

The compromise of the antioxidant system and/or the ROS overproduction during ammonia exposure (Sinha et al. 2014; Cheng et al. 2015) can result in enhanced oxidative damage of proteins, lipids and DNA as demonstrated in other fish species (Hegazi et al. 2010; Sinha et al. 2014; Sun et al. 2014; Cheng et al. 2015). In present study, this condition was demonstrated by the increased TBARS contents in gills (0.28 and 0.57 mg NH_3-N L^{-1}) and liver (0.28 mg NH_3-N L^{-1}) on day 10. The TBARS assay quantifies by-end products of lipid peroxidation (LPO), which is a chain reaction, initiated by the action of a hydroxyl radical that led to an oxidative degradation of polyunsaturated fatty acids (PUFA). The main effect of LPO is the decrease in membrane fluidity due to PUFA oxidation, affecting several biological processes (Halliwell and Gutteridge 2015). According to the classification of oxidative stress based on its intensity proposed by Lushchak (2014), in the present study, ammonia exposure induce a low intensity (mild) oxidative stress in liver and gill of Brazilian flounder, which is characterized by a slightly enhanced level of oxidative damage and enhanced activity of antioxidant enzymes (as observed for GST). Although the results found for ACAP and GST activity also suggest a potential pro-oxidant condition

in the muscle, the TBARS levels were kept in baseline levels indicating less harmful effects of ammonia in this organ.

Sun et al. (2014) exposed juvenile bighead carp *Hypophthalmichthys nobilis* to 0.053, 0.106, 0.159 and 0.212 mg NH_3-N L^{-1} for six weeks and reported an increase in TBARS levels only in the two lower concentrations. These same authors suggest that at higher concentrations of ammonia, a lack of increase in TBARS levels may indicate more severe effects, possibly related to a decreased PUFA levels. This may be a possible explanation for the reduction in liver TBARS levels of the fish exposed to 0.57 mg NH_3-N L^{-1} throughout the experimental period in the present study. Results of Li et al. (2014) support this possibility, since they showed an enhance in saturated fatty acids content and lower levels of PUFA in juvenile dark barbel catfish *Pelteobagrus vachelli* exposed to 0.12 mg NH_3-N L^{-1} for 14 days. Gottschalk and Zwingmann (2009) also demonstrates that ammonia caused alterations in the metabolism and composition of fatty acids, leading to increased mono and decreased polyunsaturated fatty acids levels in cultured astrocytes.

In the context of aquaculture, the oxidative stress and the energy cost involved in antioxidant responses can result in negative implications for the health and performance of animals affecting productivity, and led to the impairment of final product (Zhang et al. 2016). The inclusion of antioxidants in the diet may be an interesting strategy to minimize the ammonia-induced oxidative stress (Pan et al. 2011; Kütter et al. 2014; Enamorado et al. 2015) and can be the subject of future studies with Brazilian flounder.

The transfer of fish to ammonia free-water after a period of exposure can allow the recovery of the basal physiological conditions as demonstrated by Yang et al. (2010b), assessing haematological parameters and gill Na^+/K^+ ATPase activity of juvenile crucian carp *Carassius auratus*. However, this is the first study that evaluates the effects of ammonia recovery on oxidative stress parameters of fish and the results demonstrate that recovery capacity is variable, depending on the exposure concentration, the parameter evaluated and organ. After recovery, most of the parameters (liver ACAP, GST activity in muscle and liver, and TBARS in gill) that differ from the control at the end of the exposure period returned to baseline levels, regardless of treatment, indicating that the 10-day period was long enough for the recovery of these parameters. Differently, as in the case of liver TBARS and gill GST activity, the recovery of normal levels was demonstrated only in fish

exposed to 0.28 mg NH_3-N L^{-1}, remaining altered at the highest concentration (0.57 mg NH_3-N L^{-1}). The recovery period also triggered new changes in antioxidant defenses, including a decrease in ACAP of the gills of fish exposed to 0.28 and 0.57 mg NH_3-N L^{-1}, and an increase in antioxidant capacity of muscle in all concentrations of ammonia. The decrease in antioxidant capacity combined with increased GST activity suggests a pro-oxidant scenario in the gills during recovery, possibly related to the process of ammonia detoxification and/or oxidative damage products removal. In muscle, the response observed may be an adaptive antioxidant response to prevent possible enhanced oxidative damage during recovery. Based on our findings, we suggest that a longer time is required to restore the complete normal physiological conditions, however, further studies should be conducted for a more accurate determination of the required time.

Besides the recovery in ammonia-free water, it is worth mentioning that certain biochemical adjustments can occur in fish even during exposure period restoring the homeostasis, as shown in treatment 0.12 mg NH_3-N L^{-1}. From the fifth day of exposure, fish exposed to this lower ammonia concentration presented a recovery of basal levels of some parameters (gill GST activity, liver ACAP), which were transiently changed only on day 1, indicating an adaptation to this level of the environmental ammonia

The ammonia production, excretion and toxicity in fish involve several physiological and biochemical mechanisms in different organs such as brain, liver, and gill (Ip and Chew 2010), and exposure to undesirable levels of environmental ammonia can also result in histopathogical alterations to these tissues (Rodrigues et al. 2014). However, no negative effect was evidenced in histological sections of brain, liver and gills of juvenile Brazilian flounder during ammonia exposure or subsequent recovery in ammonia free-water. No hispathological alterations were also demonstrated in gill of silver perch *Bidyanus bidyanus* exposed to 0.14 mg NH_3-N L^{-1} for 39 days (Frances et al. 2000). Medeiros et al. (2016) showed enhanced gill lesion in false clownfish *Amphiprion ocellaris* only at 0.57 mg NH_3-N L^{-1}, while concentrations up to 0.23 mg NH_3-N L^{-1} did not differ from the control, in acute toxicity test (96 h). The absence of histopathological effects while the parameters of oxidative stress presented several alterations indicating the toxic effects of ammonia, is related to the fact that these biochemical responses can be early indicators of biological damage (Bernet et al. 1999). Similar results were also demonstrated

in fish exposed to other environmental contaminants (Velisek et al. 2011; Zivna et al. 2013; Sevcikova et al. 2016). This result demonstrates a good tolerance of the species to this nitrogenous compound, since its effects were limited to biochemical level without leading to a more severe impairment of assessed organs, which can affect its vital functions.

It is important to point out that present results also demonstrate the relevance of using multiple biomarkers of different organization levels for assessment of the effects related to environmental stressors on aquatic organisms. Furthermore, oxidative stress parameters can be used as a more sensitive tool to determine water quality criteria for aquaculture, aiming a more appropriate management, especially in intensive systems, such as RAS.

The present study shows that ammonia exposure to concentrations ranging from 0.12 to 0.57 mg NH_3-N L^{-1} throughout 10 days induces different changes in oxidative stress parameters in a time- and concentration-dependent way, but does not cause histological changes in juvenile Brazilian flounders. The activation of antioxidant defenses in an adaptive response to prevent organs from oxidative damage, and a reduction in the total antioxidant competence were observed in all tested concentrations, indicating that ammonia exposure can lead to a disruption of homeostasis even at the lowest concentration. However, only in fish exposed to 0.28 and 0.57 mg NH_3-N L^{-1} a mild oxidative stress was evidenced. In addition, the recovery period (10 days) was not sufficient to complete re-establishment of fish homeostasis and induced further changes in antioxidant defenses.

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Conflict of interest

The authors declare that they have no conflict of interest.

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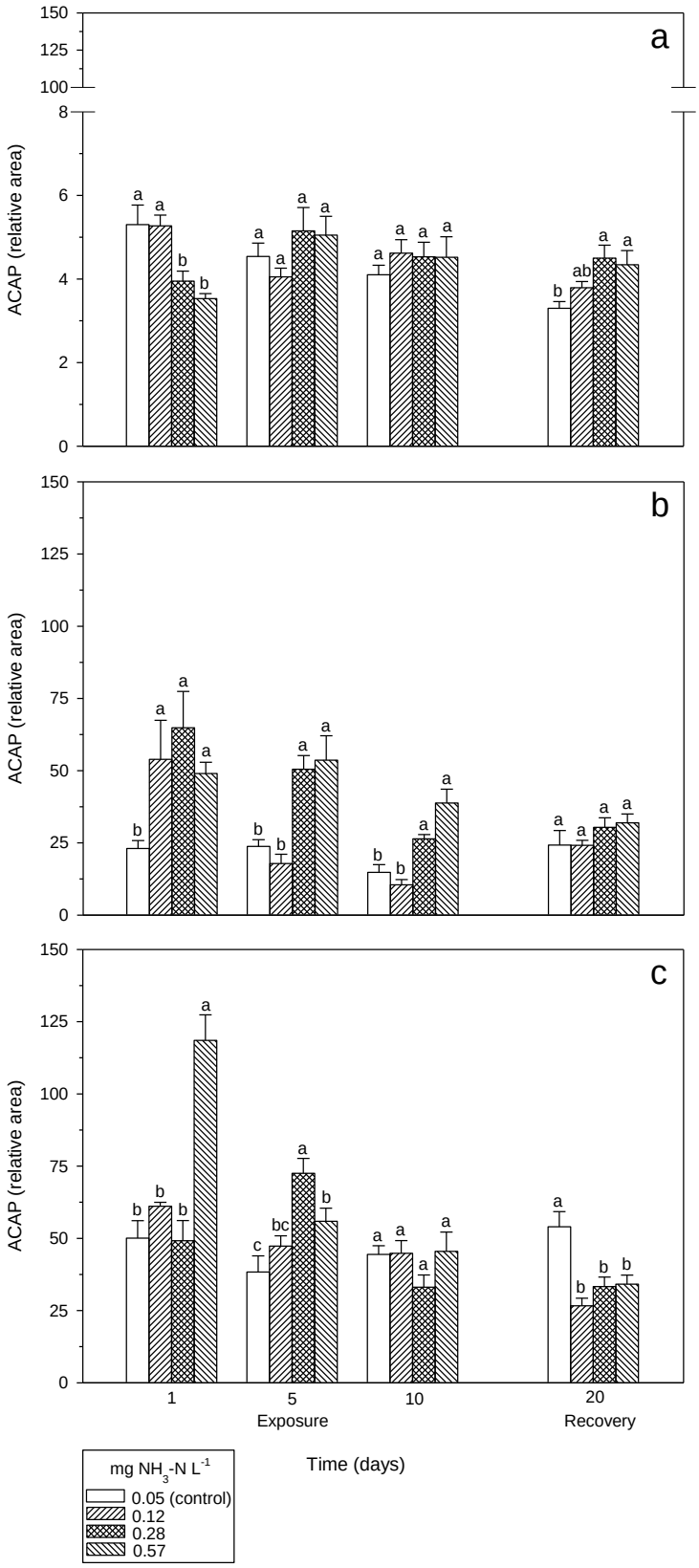
Figure Captions

Fig. 1 Total antioxidant capacity against peroxyl radicals (ACAP) (relative area) in gill (a), liver (b) and muscle (c) of juvenile Brazilian flounder *Paralichthys orbignyanus* exposed to ammonia and after recovery in ammonia-free water. Data were expressed as means ± SEM (n=9). Different lowercase letters indicate significant differences among treatments within the same sampling day (Tukey's test, $p < 0.05$).

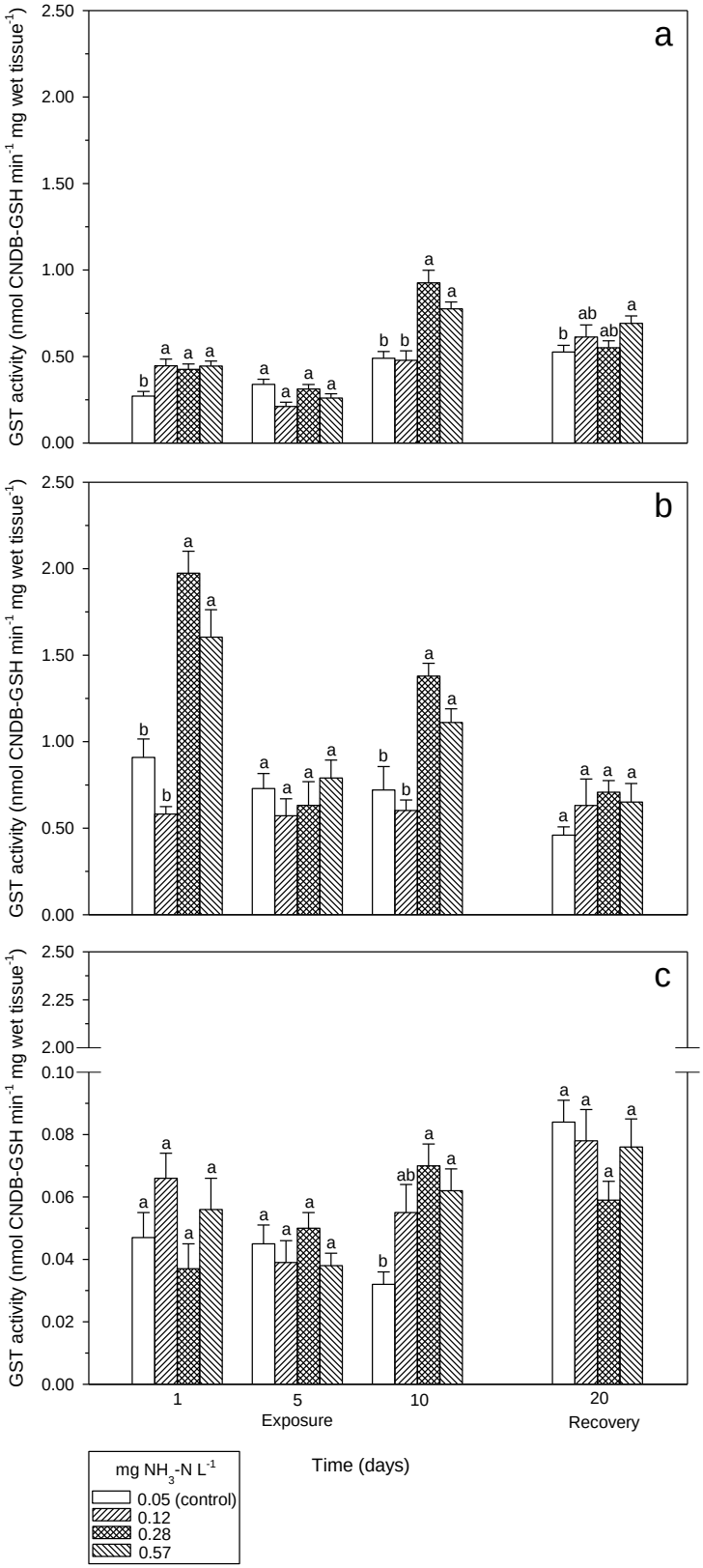
Fig. 2 Glutathione-S-transferase (GST) activity (nmol CNDB-GSH min^{-1}mg wet $tissue^{-1}$) in gill (a), liver (b) and muscle (c) of juvenile Brazilian flounder *Paralichthys orbignyanus* exposed to ammonia and after recovery in ammonia-free water. Data were expressed as means ± SEM (n=9). Different lowercase letters indicate significant differences among treatments within the same sampling day (Tukey's test, $p < 0.05$).

Fig. 3 Thiobarbituric acid reactive substances (TBARS) content (nmol TMP mg wet $tissue^{-1}$) in gill (a), liver (b) and muscle (c) of juvenile Brazilian flounder *Paralichthys orbignyanus* exposed to ammonia and after recovery in ammonia-free water. Data were expressed as means ± SEM (n=9). Different lowercase letters indicate significant differences among treatments within the same sampling day (Tukey's test, $p < 0.05$).

Fig.1

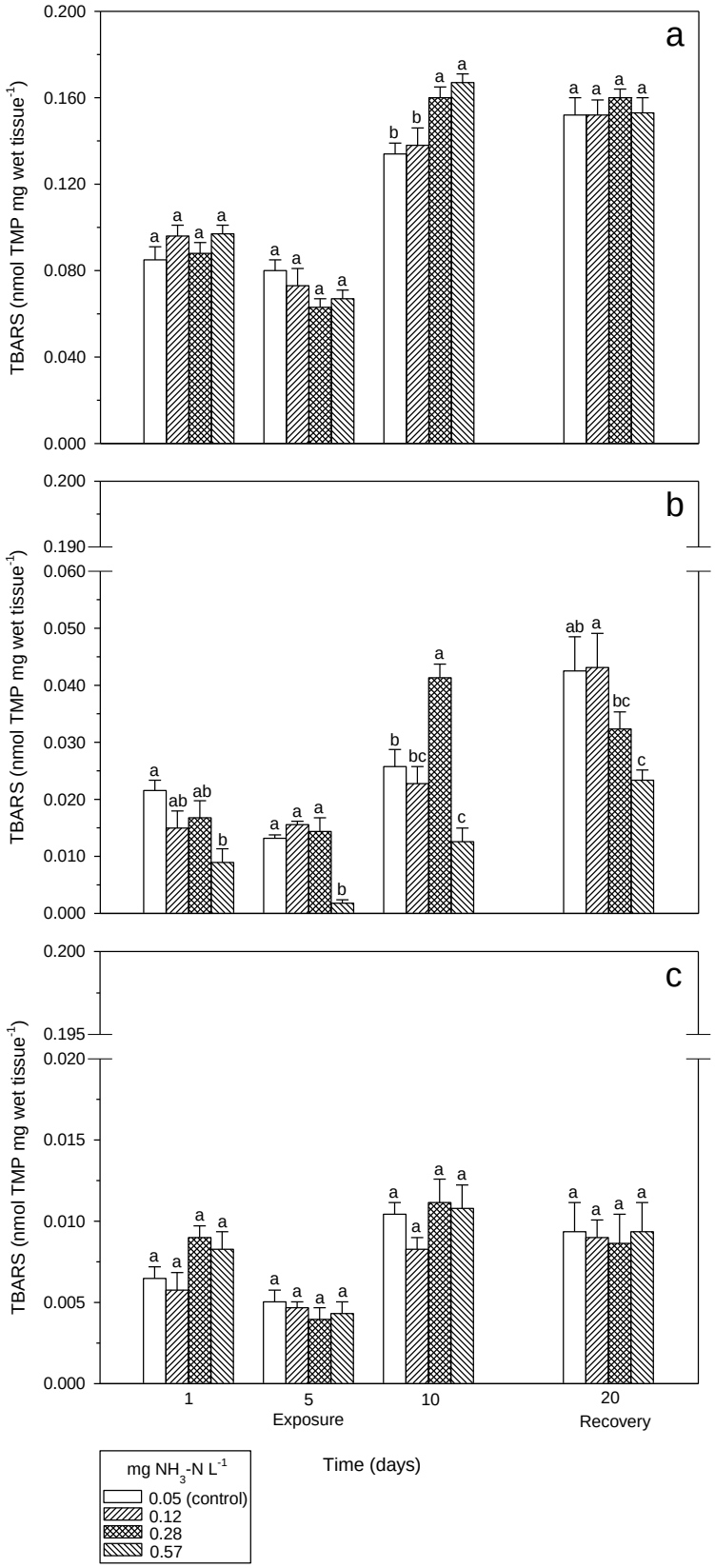

a
b
c
ACAP (relative area)
Exposure
Recovery
Time (days)
mg NH3-N L-1
0.05 (control)
0.12
0.28
0.57

1 **Fig. 2**


a
b
c
GST activity (nmol CNDB-GSH min-1 mg wet tissue-1)
2.50
2.00
1.50
1.00
0.50
0.00
2.25
0.10
0.08
0.06
0.04
0.02
1
5
10
20
Exposure
Recovery
Time (days)
mg NH3-N L-1
0.05 (control)
0.12
0.28
0.57

2

1 **Fig. 3**


a
b
c
TBARS (nmol TMP mg wet tissue^{-1})
0.200
0.160
0.120
0.080
0.040
0.000
0.190
0.060
0.050
0.040
0.030
0.020
0.010
0.195
0.015
0.005
1
5
10
20
Exposure
Recovery
Time (days)
mg NH_3-N L^{-1}
0.05 (control)
0.12
0.28
0.57

2

CAPÍTULO 3

Oxidative stress and antioxidant responses in juvenile Brazilian flounder *Paralichthys orbignyanus* exposed to sub-lethal levels of nitrite

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Running title: Nitrite-induced oxidative stress in *Paralichthys orbignyanus*

ms. has 29 pages, 6 figures

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Abstract

This study evaluated the effects of short-term exposure to sub-lethal levels of nitrite on total protein content, oxidative stress parameters and histology of juvenile Brazilian flounder *Paralichthys orbignyanus*. An assessment of fish recovery was also performed. Fish were exposed to 0.08 (control), 5.72, 10.43 and 15.27 mg NO_2-N L^{-1} for 10 days followed by the same recovery time. Gill, liver and muscle samples were collected after 1, 5 and 10 days of exposure and after recovery for the measurement of total protein content, antioxidant capacity against peroxyl radicals (ACAP), glutathione-S-transferase (GST) activity, content of non-protein (NPSH) and protein thiols (PSH), and lipid peroxidation levels by thiobarbituric acid reactive substances (TBARS) content. For the histopathological evaluation, samples of gill, liver and brain tissues were collected. Nitrite exposure induced alterations pertained to reduced total protein content, compromising of the overall antioxidant system (reduced ACAP and GST activity) and enhanced oxidative damage in lipids and proteins. Increases in GST activity, NPSH and PSH contents were also demonstrated. No nitrite-induced histopathological effects were found. The recovery period allowed for resumption of basal levels for all (treatment 5.72 mg NO_2-N.L^{-1}) or some of the evaluated parameters (other treatments). In conclusion, exposure to nitrite concentrations from 5.72 to 15.27 mg NO_2-N.L^{-1} caused changes in protein content and induced oxidative stress and antioxidant responses of juvenile Brazilian flounder. The recovery period led to complete or partial resumption of homeostasis depending on the nitrite concentrations at which the fish were previously exposed.

Key words: fish, lipoperoxidation, nitrogen compound, recovery, thiol groups, toxicity

1. Introduction

Nitrite (NO_2^-) is an intermediate product in bacterial nitrification and denitrification processes and therefore constitutes a component of the nitrogen cycle in aquatic environments. In intensive fish culture systems, especially in recirculation aquaculture systems (RAS), imbalances of nitrifying bacteria activity present in biofilter, may lead to a build-up of nitrite. This nitrogen compound causes a potential risk to cultured species due to its high toxicity (Jensen, 2003; Kroupova et al., 2005). Besides, the deleterious effects of

nitrite exposure can adversely affect growth (Ciji et al., 2014), immune system (Jia et al., 2016) and survival of fish (Wuertz et al., 2013) resulting in production losses.

The mechanisms of nitrite uptake and toxicity are widely discussed for freshwater organisms but poorly studied on estuarine and marine fish (Tomasso, 2012; Jia et al., 2015). Nitrite is generally less toxic in saltwater due to the high chloride concentration and to the opposite osmotic gradients, which reduce nitrite influx via competition with chloride active uptake in gill (Jensen, 2003). However, potential routes for nitrite uptake have been demonstrated in intestinal (Grosell and Jensen, 2000) and gill (inward diffusion) epithelium of saltwater species (Jensen, 2003), and can lead to a build-up of this compound in blood plasma (Park et al., 2013) and other organs, such as muscle, gills and liver (Huertas et al., 2002). The main physiological disorder related to nitrite accumulation is the oxidation of functional hemoglobin (Hb) to methemoglobin (metHb), which cannot transport oxygen. In addition to inducing functional hypoxia due to methemoglobinemia, exposure to nitrite can affect multiple physiological functions in fish (Jensen, 2003).

The imbalance between pro-oxidants and antioxidant defenses which leads to an oxidative stress condition (Halliwell and Gutteridge, 2015) is one of these nitrite-induced disturbances at the biochemical level. Exposure of fish to nitrite can enhance the intracellular generation of reactive oxygen species (ROS) (Sun et al., 2014a) and reactive nitrogen species (RNS) (Jensen and Hansen, 2011; Jensen et al., 2015), and/or reduce antioxidant defenses (Jia et al., 2015). Therefore, increased levels of oxidative damage in different macromolecules such as lipids and proteins have already been demonstrated (Sun et al., 2014a; Jia et al., 2015). Furthermore, the non-enzymatic antioxidant defenses such as reduced glutathione (GSH) plays an important role in preventing Hb oxidation by nitrite (Smith and Nunn, 1984; Doblander and Lackner, 1996) and elevated activities of several antioxidant enzymes provide protection against oxidative damage during nitrite exposure (Ciji et al., 2012).

At a higher biological organization level, exposure to environmental stressors and related biochemical responses can also induce histopathological changes in specific target organs resulting in impairment of their vital functions (Bernet et al., 1999). These kinds of changes were already demonstrated in fish exposed to nitrite by some authors (Saoud et al., 2014; Medeiros et al., 2016). Differently, other studies did not find a relationship between histological lesions and nitrite toxicity (Wuertz et al., 2013; Roques et al., 2015). In fact,

the application of multiple biomarkers provides a more reliable and integrative assessment of toxic effects related to contaminants (Saleh and Marie, 2016).

Despite the different toxic effects, undesirable nitrite concentrations are often transient in aquatic ecosystems and aquaculture systems (Jensen, 2003). The return of fish to nitrite-free water after an exposure episode is generally sufficient to induce nitrite detoxification from the organism and to reverse physiological disturbances within a few days as demonstrated by previous studies (Jensen et al., 1987; Knudsen & Jensen, 1997; Gisbert et al., 2004). Fish are capable of detoxifying nitrite by oxidising it to low-toxic nitrate through different biochemical pathways involving the enzymes catalase and cytochrome oxidase, and mainly via haemoglobin oxidation (Doblander and Lackner, 1996).

Since this mechanism of nitrite detoxification is the same involved on metHb formation, the metHb reductase systems play an important role not only for the ressumptions of normal Hb levels but also indirectly assist the nitrite detoxification process (Jensen, 2003). Excretion of nitrite to the environment across the gills and via the urine are also mechanisms involved in the elimination thereof (Zachariasen, 2001). However, the recovery responses of fish after nitrite exposure are still understudied and there are no works that perform this evaluation using oxidative stress parameters. For aquaculture purposes, the provision of a suitable recovery period for nitrite-exposed fish is critical to minimize the chances of cumulative effects if fish are subject to additional stressors (Wedemeyer, 2012).

The Brazilian flounder *Paralichthys orbignyanus* (Valenciennes, 1839) is a native species to South America, inhabiting estuarine and coastal waters from Brazil to Argentina (Figueiredo and Menezes, 2000; Millner et al., 2005). This is a promising species for intensive aquaculture due its commercial importance, easy adaptation to captivity conditions and wide tolerance to distinct water quality conditions (Bianchini et al., 2010). However, the knowledge about nitrite toxicity, a potential problem in intensive systems, is quite limited regarding this species (Bianchini et al., 1996) and the sub-lethal effects of exposure to this compound and recovery responses are still unknown.

Therefore, the aim of this study was to investigate the effects of exposure to sublethal concentrations of nitrite on oxidative stress parameters and histopatology in different tissues of juvenile Brazilian flounder *P. orbignyanus*. Moreover, an assessment of fish recovery after a period in water with minimum nitrite concentrations was also performed.

2. Materials and methods

2.1 Ethical procedures

The experimental protocol was approved by the Committee on Animal Experimentation of FURG (process number 23116.001030/2016-15).

2.2 Fish and experimental conditions

The juveniles Brazilian flounder (310.0 ± 5.6 g and 29.4 ± 0.2 cm) were obtained from the Aquaculture Marine Station of the Universidade Federal do Rio Grande - FURG in Rio Grande do Sul, south Brazil. Prior to the experiment, 180 fish were randomly distributed in twelve 300 L circular tanks (15 fish per tank) organized in four recirculation systems which were connected to their respective biological and mechanical filters. Fish were acclimated for 20 days, fed twice daily (3% of total biomass) with commercial diet (46% crude protein) throughout the acclimation and experimental periods. Photoperiod was fixed at 12 h Light :12 h Dark.

The fish were exposed to 0.08 ± 0.01(control), 5.72 ± 0.02, 10.43 ± 0.05 and 15.27 ± 0.04 mg NO_2-N.L^{-1} in triplicate for 10 days in a semi-static system. Test concentrations were chosen above the safety level (3.06 mg NH_3-N.L^{-1}) for the species in seawater (Bianchini et al., 1996) and reached through the addition of aliquots from a sodium nitrite ($NaNO_2$; Synth, Brazil) solution to the experimental units. The analytical verification of the desired nitrite concentrations was performed daily according to Bendschneider and Robinson (1952). After the exposure period, fish remained for 10 days in a recirculation system to recover under minimum nitrite concentrations (0.05 ± 0.01 mg NO_2-N.L^{-1}).

The water quality parameters temperature (23.91 ± 0.05 ºC) and dissolved oxygen (7.34 ± 0.02 mg.L^{-1}) (oximeter YSI 55), salinity (23.91 ± 0.05) (refractometer), pH (8.17 ± 0.01) (pH meter, WTW 315i), total (0.27 ± 0.02 mg.L^{-1}) (UNESCO, 1983) and un-ionized ammonia (< 0.01 mg.L^{-1}) (Colt, 2002), and total alkalinity (232.19 ± 1.50 mg $CaCO_3$.L^{-1}) (Eaton et al., 2005) were measured daily during the acclimation and experimental periods, and always before the first feeding.

2.3 Organ sampling

After 1, 5 and 10 days of exposure and after a recovery period (day 20), nine fish were randomly netted from each treatment and immediately euthanized with a lethal concentration of hydrochloride benzocaine (500 ppm). Samples of gills, liver, brain and muscle tissues were collected. Feeding was ceased 24 hours prior to each sampling day.

2.4 Organ homogenization and total protein content

Samples of gill, liver and muscle tissues were stored at -80 ºC and then homogenized (1:5,w/v) in a Tris–HCl (100 mM, pH 7.75) buffer with EDTA (2 mM) and Mg^{2+} (5 mM). The supernatants resulting from the centrifugation of the homogenates (10000 x *g*, 20 minutes, at 4ºC) were used for all analyses (da Rocha et al., 2009). The total protein content of homogenized samples was determined in a microplate reader (BioTek LX 800) using a commercial kit (Doles®) based on the Biuret assay (550 nm).

2.5 Total antioxidant capacity against peroxyl radicals

All samples were previously diluted with homogenization buffer to 2.0 mg protein mL^{-1} and the total antioxidant capacity against peroxyl radicals (ACAP) was determined according to the method described by Amado et al. (2009). Fluorometry (excitation: 485 nm; emission: 520 nm) was measured through a spectrofluorimeter (Victor 2, Perkin Elmer, MA, USA) with readings at every 5 minutes, during 30 minutes (gill and liver samples) or at every 20 minutes, during 120 minutes (muscle samples). The ACAP values (expressed as a relative area) were calculated using the expression proposed by Monserrat et al. (2014). For interpretation purposes, a higher relative area means a lower antioxidant capacity.

2.6 Glutathione-S-transferase activity

Glutathione-S-transferase (GST) activity was determined following the conjugation of 1 mM glutathione (GSH) and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) according to Habig et al. (1974) and Habig and Jakoby (1981). The absorbance (340 nm) was read in 96-wells transparent microplates using a microplate reader (Biotek Elx 800).

2.7 Content of non-protein and protein thiols

For the measurement of non-proteic (NPSH) and proteic (PSH) thiols, the employed method using DTNB (5,5-dithiobis-(2-nitrobenzoic acid; Sigma) was based on Sedlak and

Lindsay (1968). Determination of NPSH content in the samples was measured after deproteinization with trichloro acetic acid (TCA 50%). The pellet formed by the precipitated protein was re-suspended with homogenization buffer for determination of PSH content. The absorbance readings (405 nm) were performed using a microplate reader (Biotek Elx 800).

2.8 Lipid Peroxidation

The lipid peroxidation levels were measured according Oakes and Van Der Kraak (2003). This method quantifies malondialdehyde (MDA) levels, a by-product of lipid peroxidation, by measuring thiobarbituric acid-reactive substances (TBARS). Fluorometric measurements (excitation: 520 nm, emission: 580 nm) was performed in spectrofluorimeter (Victor 2, Perkin Elmer, MA, USA) and the results were expressed as nmol TMP mg wet tissue^{-1}, where TMP stands for tetramethoxypropane (ACROS Organics), employed as a standard.

2.9 Histopathological evaluation

Gill samples were fixed in Bouin's solution, while brain and liver samples were fixed in 10% buffered formalin. Samples were dehydrated using sequentially increasing concentrations of ethanol, cleared in xylene and embedded in paraplast with an automatic tissue processor (LUPETEC, PT 05, Brazil). The sections (5 µm) were prepared using a microtome (LUPETEC, MRP03, Brazil), stained with hematoxylin-eosin and slides were analyzed using an optical microscope (ZEISS, PRIMO STAR).

2.10 Statistical analysis

All data were expressed as means ± standard error (S.E.M). One-way ANOVA and Tukey's test were performed to check differences among treatments on the same day. The data of non-protein thiols content in gills did not meet the assumptions for ANOVA and therefore the non-parametric Kruskall-Wallis'test followed by a multiple comparisons' test. The significance level was set at 5% in all cases ($p<0.05$).

3. Results

No mortality occurred for any of the experimental treatments throughout the exposure and recovery periods.

3.1 Total protein content

The total protein content in gill was significantly lower in juvenile Brazilian flounder exposed to 5.72 mg NO_2-N.L^{-1} compared to other experimental groups at day 1, and compared to treatment 10.43 mg NO_2-N.L^{-1} at the 10th day of exposure. After recovery, the values were higher in the gill of fish subjected to 5.72 mg NO_2-N.L^{-1} compared to those exposed to 10.43 and 15.27 mg NO_2-N.L^{-1}. However, treatments did not differ significantly from controls at day 10 and after recovery (Figure 1A). The total protein content was significantly lower in liver of fish exposed to 10.43 and 15.27 mg NO_2-N.L^{-1} in relation to control and 5.72 mg NO_2-N.L^{-1} at day 10 (Figure 1B). The total protein content of muscle was also significantly lower in fish exposed to 15.27 mg NO_2-N.L^{-1} in relation to other treatments, and in fish exposed to 10.43 mg NO_2-N.L^{-1} compared to control fish and to those exposed to 5.72 mg NO_2-N.L^{-1} at day 5 (Figure 1C). No differences were found among treatments after recovery in liver and muscle (Figure 1B-C).

3.2 Total antioxidant capacity against peroxyl radicals

Reduced antioxidant competence in relation to control were observed in gills of fish exposed to all NO_2-N.L^{-1} concentrations on day 1 and a similar condition was observed in gills of fish exposed to 15.27 mg NO_2-N.L^{-1} on day 5 (Figure 2A). The antioxidant competence in the liver of fish exposed to treatment 15.27 mg NO_2-N.L^{-1} was significantly lower than that of their respective control during the entire period of exposure (days 1, 5 and 10). Moreover, treatments 5.72 and 10.43 mg NO_2-N.L^{-1} showed lower antioxidant competence compared to controls at days 5 and 10 (Figure 2B). Exposure to 15.27 and 5.72 mg NO_2-N.L^{-1} reduced antioxidant competence in muscle as compared to controls on day 1 and at the end of exposure period (day 10), respectively (Figure 2C). Regardless of organ, no differences were found among treatments after recovery (Figure 2A-B-C).

3.3 Glutathione-S-transferase activity

The GST activity in gills was lower in treatment of 15.27 mg NO_2-N.L^{-1} compared to control group and to concentration of 5.72 mg NO_2-N.L^{-1}, and in treatments of 10.43 mg

NO_2-N.L^{-1} compared to control at day 5. No differences were found among treatments after recovery (Figure 3A). Liver of fish exposed to 10.43 and 15.27 mg NO_2-N.L^{-1} showed enhanced GST activity compared to other experimental groups at day 10. Differently, fish exposed to 15.27 mg NO_2-N.L^{-1} had a lower GST activity in relation to control and treatment 5.72 mg NO_2-N.L^{-1} after recovery (Figure 3B). During the exposure period, muscle GST activity showed higher values in fish exposed to 15.27 mg NO_2-N.L^{-1} compared to all groups at day 5, and lower values in treatment 5.72 mg NO_2-N.L^{-1} at day 10, in comparison to all other treatments. Reduced GST activity was observed in treatment 15.27 mg NO_2-N.L^{-1} compared to control after recovery (Figure 3C).

3.4 Content of non-protein thiols

The NPSH content were higher in the gill of fish exposed to the treatment 15.27 mg NO_2-N.L^{-1} in relation to control and to treatment 5.72 mg NO_2-N.L^{-1}, and in treatment 10.43 mg NO_2-N.L^{-1} in relation to control at day 10. After recovery, higher contents of NPSH were found in treatments 10.43 and 15.27 mg NO_2-N.L^{-1} compared to control and to treatment 5.72 mg NO_2-N.L^{-1} (Figure 4A). On day 1, hepatic NPSH content were higher in fish exposed to the treatment 15.27 mg NO_2-N.L^{-1} in relation to control fish and to those exposed to 5.72 mg NO_2-N.L^{-1}. On day 5, higher values of this parameter were observed in treatments 10.43 and 15.27 mg NO_2-N.L^{-1} compared to control and to treatment 5.72 mg NO_2-N.L^{-1}. NPSH contents were also higher in liver of fish exposed to 15.27 mg NO_2-N.L^{-1} compared to control and to treatment 5.72 mg NO_2-N.L^{-1}, and in fish exposed to 10.43 mg NO_2-N.L^{-1} compared to control fish, at day 10. NPSH content was higher in liver of fish exposed to 15.27 mg NO_2-N.L^{-1} compared to control after the recovery (Figure 4B). In muscle, NPSH content was higher in fish exposed to 15.27 mg NO_2-N.L^{-1} compared to other groups on the 10th day of exposure. Elevations in NPSH content were also found in treatments 10.43 and 15.27 mg NO_2-N.L^{-1} compared to control and to treatment 5.72 mg NO_2-N.L^{-1} after recovery (Figure 4C).

3.5 Content of protein thiols

Exposure of fish to 15.27 mg NO_2-N.L^{-1} and all NO_2-N treatments led to a reduced content of PSH in gill in comparison with controls at day 1 and 5, respectively. No differences were found among treatments after recovery (Figure 5A). In liver, enhanced PSH content was

observed in treatments 10.43 and 15.27 mg NO_2-N.L^{-1} compared to control and to treatment 5.72 NO_2-N.L^{-1} at the end of exposure and after recovery (Figure 5B). Nitrite exposure induced a progressive enhanced of muscle PSH content over increasing concentrations on days 5 and 10. At these days, PSH contents were higher in fish exposed to 15.27 mg NO_2-N.L^{-1} compared to all groups, and higher in fish exposed to 10.43 mg NO_2-N.L^{-1} compared to control fish. After recovery, treatment 5.72 mg NO_2-N.L^{-1} presented higher PSH content than that of the 10.43 mg NO_2-N.L^{-1} – exposed fish, however, both treatments did not differ from control (Figure 5-C).

3.6 Lipid Peroxidation – TBARS

The TBARS levels in gill of Brazilian flounder exposed to all NO_2-N treatments were higher than those of the control group at day 1. On the 5th day of exposure, the treatment 5.72 mg NO_2-N.L^{-1} showed higher values compared to control, whereas treatment 15.27 mg NO_2-N.L^{-1} had lower TBARS content in relation to any of the treatments. No differences were found among treatments after recovery (Figure 6A). The liver of fish had lower TBARS levels in treatments 10.43 and 15.27 mg NO_2-N.L^{-1} compared to control and to treatment 5.72 mg NO_2-N.L^{-1} at day 10. A reduced TBARS level was also observed in fish subjected to treatment 15.27 mg NO_2-N.L^{-1} in comparison to control fish after recovery (Figure 6B). Significant differences in muscle TBARS levels were detected only on day 10, when the values were higher in fish exposed to 15.27 mg NO_2-N.L^{-1} compared to control and treatment 5.72 mg NO_2-N.L^{-1}. After recovery, no differences were found among treatments (Figure 6C).

3.7 Histopathological investigation

The histological investigation did not reveal any morphological alterations in gills, liver and brain of flounder juveniles during nitrite exposure or after recovery period.

4. Discussion

In natural environments, nitrite typically occurs at very low concentrations, however, toxic levels of this compound can be found in polluted waters or aquaculture production systems, particularly in RAS (Kroupová et al., 2016). Understanding how nitrite affects animals is

essential to establish appropriate management strategies, to avoid deleterious effects (Tomasso, 2012). In the present study, exposure to nitrite concentrations ranging from 5.72 to 15.27 mg NO_2-N.L^{-1} for 10 days led to changes in protein content and caused oxidative stress, without the occurrence of histopathological alterations in the assessed organs of juvenile Brazilian flounder. The observed nitrite-induced effects were organ-, time- and concentration-dependent.

Metabolic alterations, which are triggered during stress condition (Bonga, 1997), can lead to reduced protein content in tissues of fish exposed to environmental contaminants (Liu et al., 2010; Feng et al., 2014), as seen with nitrite toxicity reported in this study. The reduction in total protein could indicate either an inhibition of synthesis or increased degradation or breakdown (Firat et al., 2011). The protein catabolism is a general stress response to cope with the high energy demand, when glycogen reserves are depleted (Oruc, 2011). While proteins are the major organic constituents of fish tissues and closely involved with biological processes (Halver and Hardy, 2002), changes in its normal metabolism can be linked to further physiological alterations.

The oxidative stress induced by environmental contaminants in fish is a result of enhanced ROS production and/or weakening of the antioxidant system (Lushchak, 2016), which is comprised of low-molecular-weight antioxidants and several enzymes (Martínez-Álvarez et al., 2005). Nitrite exposure led to reduced total antioxidant competence in gill, liver and muscle of Brazilian flounder, making these organs more susceptible to further ROS attack. The impairment of a specific enzymatic defence was also demonstrated by some inhibition of GST activity in muscle and gill, thus corroborating the nitrite-induced deleterious effects in antioxidant and detoxifying system. Similarly, reduced enzymatic and non-enzymatic antioxidant defences were demonstrated in gills of juvenile turbot (*Scophthalmus maximus*) exposed for 96 h to similar concentrations (5.60 and 11.20 mg NO_2-N.L^{-1}) of nitrite used herein (Jia et al., 2015). These results suggest an enhanced production of free-radicals, as ROS and RNS, resulting in expenditure or inhibition of antioxidant components due to oxidative damage (Sun et al., 2012; Jia et al., 2015). Furthermore, coping with stress involves several physiological adjustments to supply increased energy demand (Barton, 2002), which can lead to a deficiency in antioxidants production in the organism. The lowering of protein content seems support this idea.

Otherwise, in certain conditions of ROS overproduction, the activation of redox-sensitive transcription factors and ROS-mediated covalent modification of antioxidant proteins can trigger antioxidant responses (Hermes-Lima et al., 2015). Enhanced gene expression and activity of antioxidant enzymes have already been described in fish as an adaptive mechanism to prevent oxidative stress induced by environmental nitrite (Ciji et al., 2012; Sun et al., 2014a). In the present study, fish exposed to 10.43 and 15.27 mg NO_2-N.L^{-1} triggered similar protective response mechanisms within the antioxidant system, characterized by increases in GST activity and more evidently by elevations in the thiol contents.

GST represent the major group of xenobiotic detoxification enzymes and are often used as biomarkers of aquatic environmental contamination. The main role of these enzymes is the catalysis of nucleophilic attack of the sulfur atom of GSH to electrophilic substrates providing cellular defence against chemically induced toxicity (Blanchette et al., 2007). Enhanced GST activity mediated by a prooxidant condition promotes the detoxification of some toxic carbonyl-, peroxide-, and epoxide-containing metabolites produced by oxidative damage (Hayes and Pulford, 1995).

Sinhorin et al. (2014) observed that when enzymatic defences are impaired or insufficient to counteract oxidative radicals, non-enzymatic antioxidants including thiols levels are enhanced so as to prevent the occurrence of auto-oxidation reactions. Increased levels of NPSH (all tissues) and PSH (liver and muscle) in nitrite-exposed Brazilian flounder could result either from enhanced synthesis, or decreased consumption and/or degradation (Husak et al., 2017). It is recognized that nitrite accumulation in fish organism can cause excessive production of nitric oxide (NO) (Jensen and Hansen, 2011) and the GSH biosynthetic pathway can be stimulated in response to enhanced NO levels to protect cells against oxidative or nitrosative stress (Moellering et al., 1998). These mechanisms can explain the enhanced NPSH contents in the present study, since GSH is the predominant non-protein thiol in cells (Dickinson and Forman, 2002). GSH is synthesized mainly in the liver and released to the bloodstream before being distributed to other tissues (Peña et al., 2000). This could explain the early increment in hepatic NPSH content on the first day of exposure, whereas increases in this parameter were observed only on day 10 for gill and muscle tissues. Besides the important role as an antioxidant, GSH can covalently modify cysteine residues of protein thiols in a reversible process called S-glutathionylation,

avoiding irreversible oxidative damage and regulate protein function (Jakob and Reichmann, 2013; Halliwell and Guteridge, 2015). Thus, enhanced PSH content is an indication of reduced oxidative damage in proteins, possibly mediated by higher GSH availability.

Considering the metahemoglobinemia as the main effect of nitrite toxicity (Jensen, 2003), the up regulation of certain antioxidants, including GSH, can improve the tolerance of fish to this compound, by shielding oxidation of haemoglobin (Smith and Nunn, 1984). Zhou et al. (2013) reported an improved antioxidant capability and a higher tolerance to nitrite in GIFT *Oreochromis niloticus* previously fed with dietary GSH. However, future studies evaluating the relation between oxidative stress responses in blood and metahemoglobin content can shed light on the role of the antioxidant system of Brazilian flounder against nitrite-induced haemoglobin oxidation.

When antioxidant system is unable to efficiently counteract ROS and RNS, enhanced levels of oxidative damage occurs. The higher TBARS values showed in gill on days 1 and 5, and in muscle of fish exposed to 15.27 mg NO_2-N.L^{-1} at the end of exposure, indicate enhanced levels of lipid peroxidation end products (Halliwell and Gutteridge, 2015). Concomitantly, the reduction in PSH content in gill could be also interpreted as a signal of a more oxidative environment in cells leading to protein oxidation (Mitton et al., 2016). Our results are in agreement with previous works that demonstrated nitrite-induced oxidative damage in lipids and proteins of fish tissues (Sun et al., 2014a; Jia et al., 2015). The gill was the most affected organ and the enhanced levels of oxidative damage in the first 5 days of exposure are consistent with the impairment of antioxidant system at these days. Oxidative stress responses can be influenced both by metabolism and basal redox condition of each organ (da Rocha et al., 2009). Among the analyzed organs, the gill presented higher basal levels of TBARS and lower content of thiol groups which is an indication of a pre-existing and more intense pro-oxidant environment, making it more susceptible to oxidative damage during a stress condition.

After enhanced TBARS values on day 1, the gills of fish exposed to 15.27 mg NO_2-N.L^{-1} had lower levels of this parameter on day 5. This result may indicate more severe effects, possibly related to a decrease in polyunsaturated fatty acids levels due to early oxidative degradation (Sun et al., 2014b). Similarly, this is a possible explanation for the reduced TBARS levels in the liver of fish exposed to 10.43 and 15.27 mg NO_2-N.L^{-1} on day 10.

However, the reduced hepatic TBARS could also be related to a higher detoxification of LPO end products as the MDA, due to the concurrently increase in GST activity.

Generally, it is important to highlight that some of nitrite-induced effects showed in this study were transitory, occurring on the first and/or fifth day of exposure with subsequent recovery of homeostasis, even during the exposure period. This pattern suggests that Brazilian flounder presents biochemical compensatory mechanisms which confer to the species a certain degree of adaptation to high levels of environmental nitrite.

Adverse biochemical and physiological changes, including enhanced oxidative damage of macromolecules as lipids and proteins result in pathological alterations in tissues of fish exposed to nitrite (Sun et al., 2014a). Although nitrite exposure induced oxidative stress in juvenile Brazilian flounder, no histological abnormalities were found, indicating that toxic effects of this compound were limited to the biochemical level. These results are in agreement with other studies that evaluated the effects of contaminants on fish (Velisek et al., 2011; Zivna et al., 2013; Sevcikova et al., 2016; Simonato et al., 2016), in which time and/or nitrite concentrations were insufficient to cause further severe tissue damage (Wuertz et al., 2013; Roques et al., 2015). Thus, the absence of histological alterations in fish exposed to concentrations above the safety level (Bianchinni et al., 1996) suggest a higher tolerance to nitrite than it would have been expected for this species.

The recovery period of 10 days was sufficient to restore basal levels of all analysed parameters in fish exposed to the lowest nitrite concentration indicating a complete reestablishment of homeostasis in this case. Differently, fish exposed to the other two highest nitrite concentrations presented partial recovery, with return to basal levels only for some parameters, and then a longer time is possibly required for complete recovery. NPSH content in all tissues (except in liver of fish exposed to 10.43 mg NO_2-N.L^{-1}) and hepatic PSH levels were elevated in both treatments, suggesting a remaining effect of exposure period and/or a role of thiols in nitrite detoxification and recovery of related physiological disturbances. The recovery of basal TBARS content was demonstrated in fish exposed to 10.43 mg NO_2-N.L^{-1}, while remained altered in liver of fish subjected to 15.27 mg NO_2-N.L^{-1} as well as at the end of the exposure period. This is explained by a more severe effect at higher concentrations. Reduced GST activity in liver and muscle was also observed in treatment 15.27 mg NO_2-N.L^{-1} after recovery. There is no evidence of a pro-oxidant scenario triggered during the recovery period that can result in an inhibition of GST activity

by oxidative damage. On the contrary, antioxidant enzymes activity as GST is modulated by ROS action and concentrations of oxidative damage products in tissues (Hermes-Lima et al., 2015). Thus, the down-regulation of GST activity also can be expected due to a less oxidant condition and/or reduced levels of oxidative damage products, as showed for liver TBARS.

For Brazilian flounder aquaculture, negative implications can result from nitrite-induced oxidative stress. This condition is known to be responsible for a variety of adverse health effects and diseases in living organism (Livingstone, 2003; Valavanidis et al., 2006), and also can lead to an impairment of final product due to higher oxidative degradation (Zhang et al., 2016). Additionally, the increased energy cost related to stress, can result in tertiary stress responses as reduced growth and reproductive performances (Barton, 2002). Hence, concentrations of nitrite at 5.72 mg NO_2-N.L^{-1} or above should be avoided in production systems, even in the case of short-term exposure. Therefore, the constant monitoring of nitrite levels becomes critical to ensure an adequate management of water quality, especially in intensive production systems. As further research endeavours, we also suggest that investigations on the use of strategies to improve the antioxidant system, such as the inclusion of dietary antioxidants, should be conducted for it can potentially minimize nitrite toxicity in this species.

In conclusion, the results of the present study revealed that short-term (10 days) exposure to nitrite concentrations from 5.72 mg NO_2-N.L^{-1} compromises the overall antioxidant system and induced oxidative stress, without inducing histopathological alterations in juvenile Brazilian flounder. Enhanced non-protein and protein thiols were also demonstrated as an adaptive antioxidant response against nitrite toxicity. In addition, the recovery period was sufficient for a complete (treatment 5.72 mg NO_2-N.L^{-1}) or partial resumption (treatment 10.43 and 15.27 mg NO_2-N.L^{-1}) of physiological condition depending on the nitrite concentrations at which the fish were exposed.

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Legends

Figure 1. Total protein content in gill, liver and muscle of juvenile Brazilian flounder. Total protein content (mg g wet tissue^{-1}) in gill (A), liver (B) and muscle (C) of juvenile Brazilian flounder *Paralichthys orbignyanus* exposed to nitrite for 1, 5 and 10 days and after recovery (day 20). Data were expressed as means ± S.E.M (n=9). Different lowercase letters indicate significant differences among treatments at the same day ($p < 0.05$).

Figure 2. Total antioxidant capacity against peroxyl radicals in gill, liver and muscle of juvenile Brazilian flounder. Total antioxidant capacity against peroxyl radicals (ACAP) (relative area) in gill (A), liver (B) and muscle (C) of juvenile Brazilian flounder *Paralichthys orbignyanus* exposed to nitrite for 1, 5 and 10 days and after recovery (day 20). Data were expressed as means ± S.E.M (n=9). Different lowercase letters indicate significant differences among treatments at the same day ($p < 0.05$).

Figure 3. Glutathione-S-transferase activity in gill, liver and muscle of juvenile Brazilian flounder. Glutathione-S-transferase (GST) activity (nmol CNDB-GSH min^{-1} mg wet tissue^{-1}) in gill (A), liver (B) and muscle (C) of juvenile Brazilian flounder *Paralichthys orbignyanus* exposed to nitrite for 1, 5 and 10 days and after recovery (day 20). Data were expressed as means ± S.E.M (n=9). Different lowercase letters indicate significant differences among treatments at the same day ($p < 0.05$).

Figure 4. Content of non-protein thiols in gill, liver and muscle of juvenile Brazilian flounder. Content of non-protein thiols (NPSH) (nmol SH mg wet tissue^{-1}) in gill (A), liver (B) and muscle (C) of juvenile Brazilian flounder *Paralichthys orbignyanus* exposed to nitrite for 1, 5 and 10 days and after recovery (day 20). Data were expressed as means ± S.E.M (n=9). Different lowercase letters indicate significant differences among treatments at the same day ($p < 0.05$).

Figure 5. Content of protein thiols in gill, liver and muscle of juvenile Brazilian flounder. Content of protein thiols (PSH) (nmol SH mg protein^{-1}) in gill (A), liver (B) and muscle (C) of juvenile Brazilian flounder *Paralichthys orbignyanus* exposed to nitrite for

1, 5 and 10 days and after recovery (day 20). Data were expressed as means ± S.E.M (n=9). Different lowercase letters indicate significant differences among treatments at the same day ($p < 0.05$).

Figure 6. Content of thiobarbituric acid reactive substances in gill, liver and muscle of juvenile Brazilian flounder. Thiobarbituric acid reactive substances (TBARS) content (nmol TMP mg wet tissue^{-1}) in gill (A), liver (B) and muscle (C) of juvenile Brazilian flounder *Paralichthys orbignyanus* exposed to nitrite for 1, 5 and 10 days and after recovery (day 20). Data were expressed as means ± S.E.M (n=9). Different lowercase letters indicate significant differences among treatments at the same day ($p < 0.05$).

Figure 1

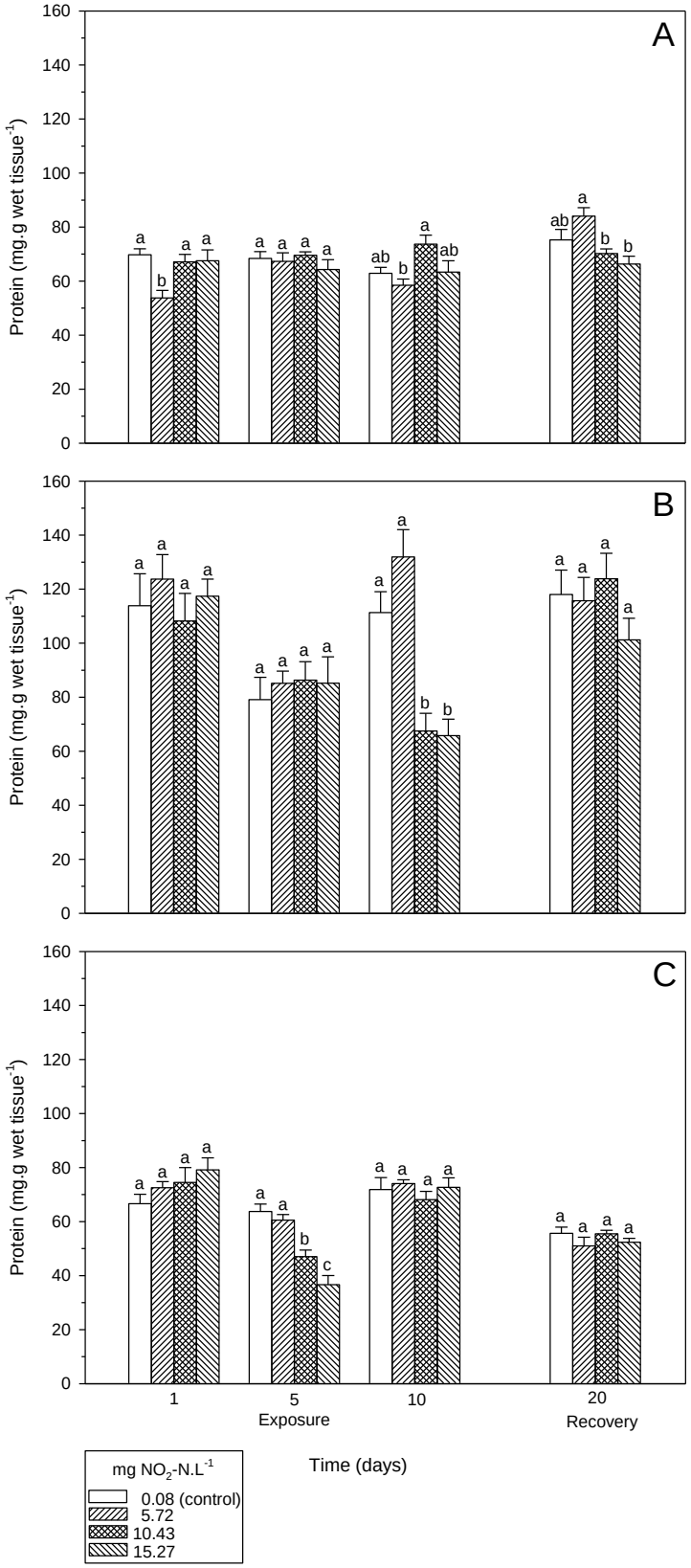

A
B
C
Protein (mg.g wet tissue^{-1})
Exposure
Recovery
Time (days)
mg NO_2-N.L^{-1}
0.08 (control)
5.72
10.43
15.27

Figure 2

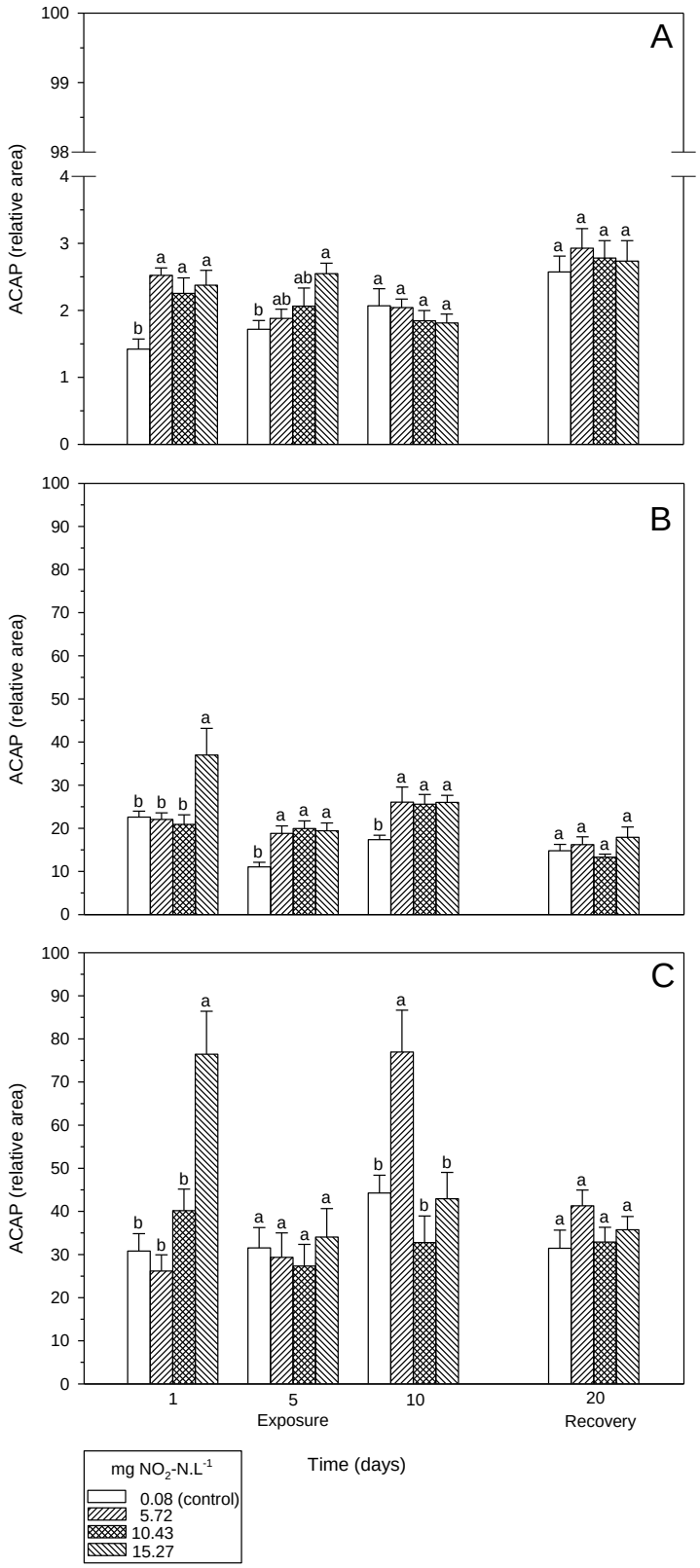

A
B
C
ACAP (relative area)
Exposure
Recovery
Time (days)
mg NO2-N.L-1
0.08 (control)
5.72
10.43
15.27

Figure 3

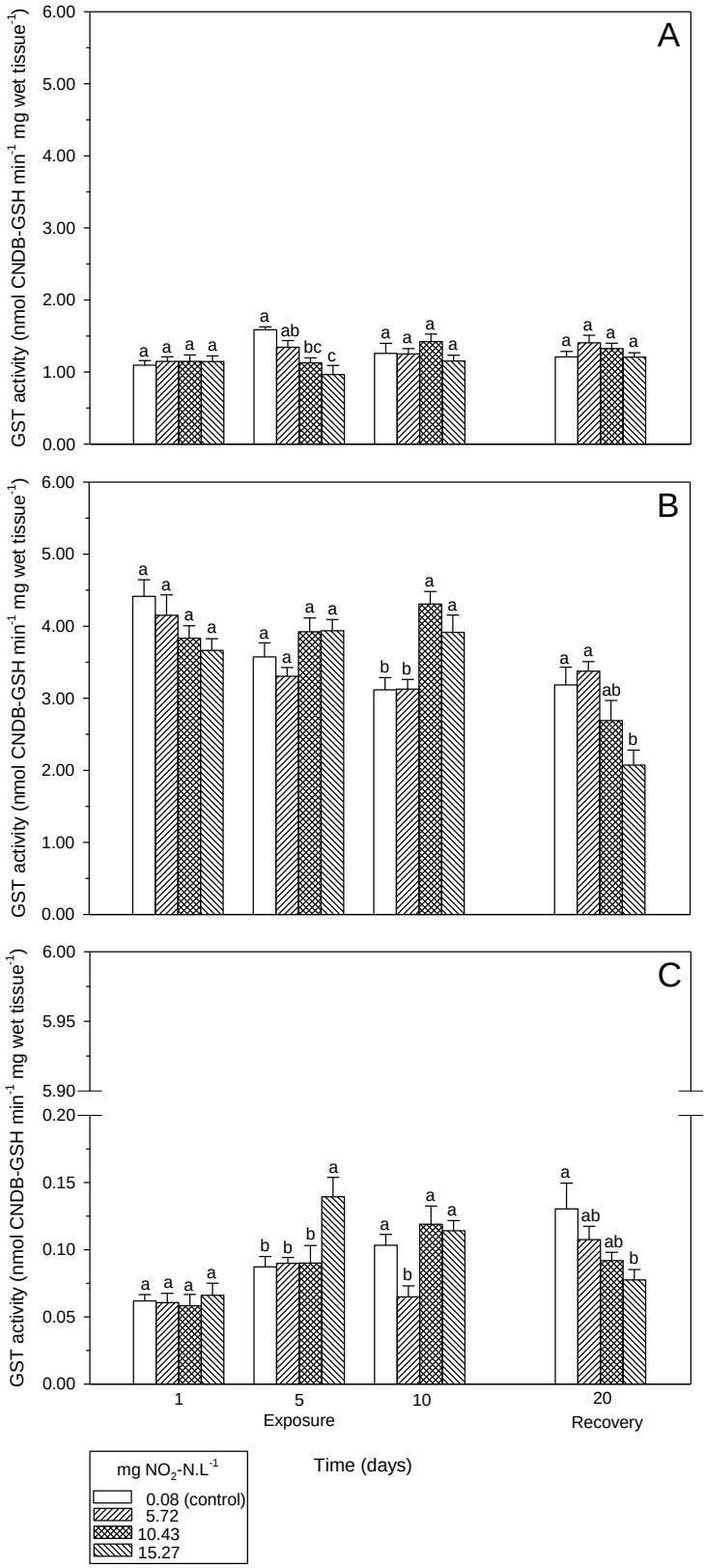

A
B
C
GST activity (nmol CNDB-GSH min-1 mg wet tissue-1)
Exposure
Recovery
Time (days)
mg NO2-N.L-1
0.08 (control)
5.72
10.43
15.27

Figure 4

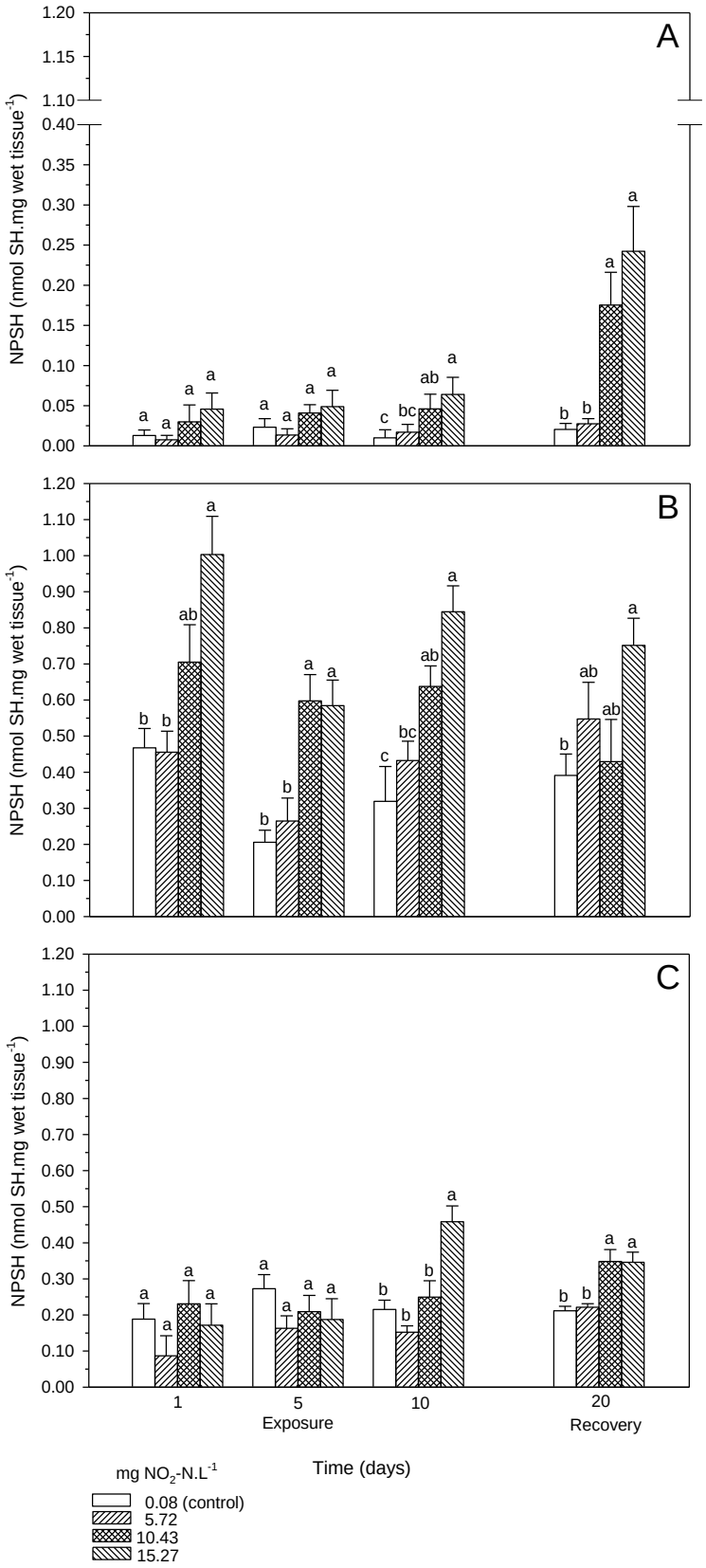

A
B
C
NPSH (nmol SH.mg wet tissue^{-1})
1.20
1.15
1.10
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
1.00
0.90
0.80
0.70
0.60
0.50
1
5
10
20
Exposure
Recovery
Time (days)
mg NO_2-N.L^{-1}
0.08 (control)
5.72
10.43
15.27

Figure 5

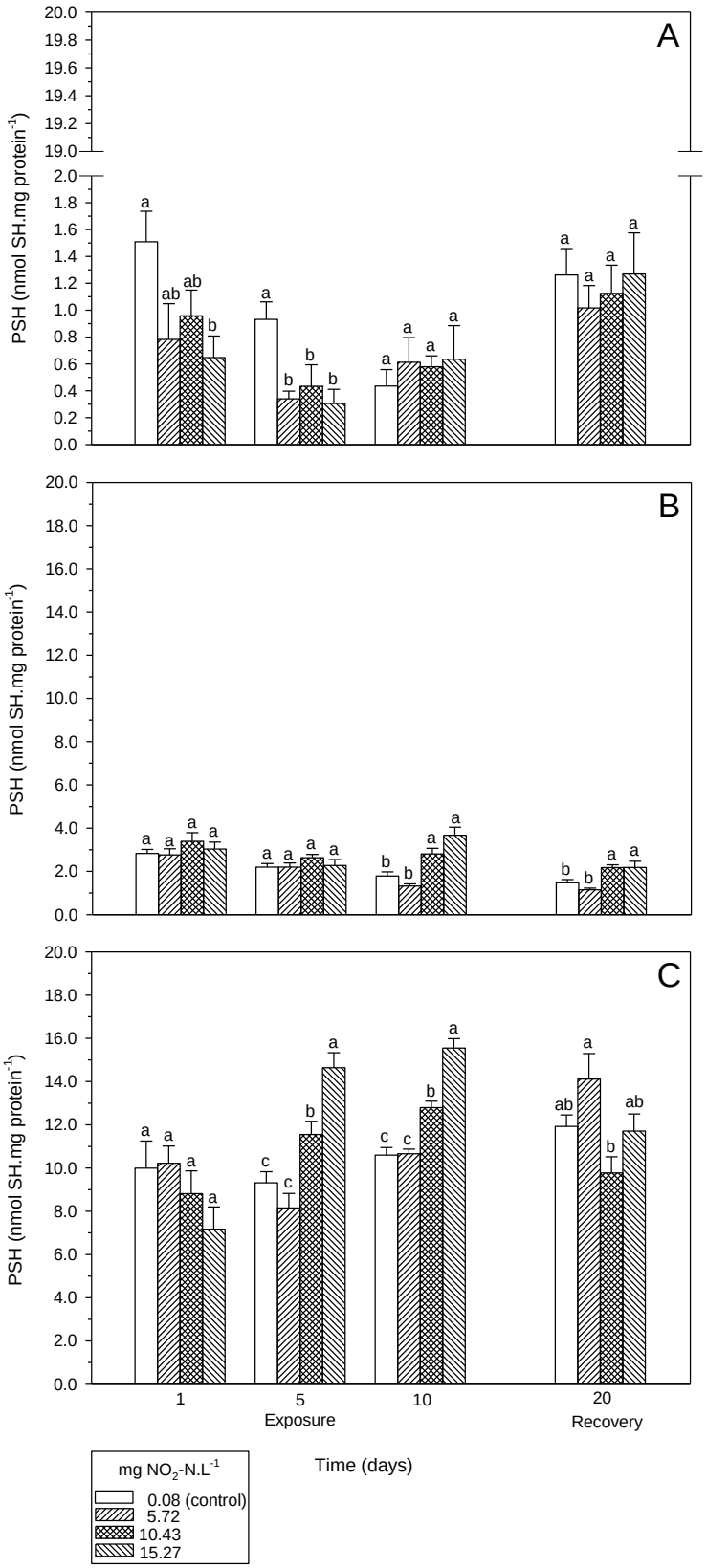
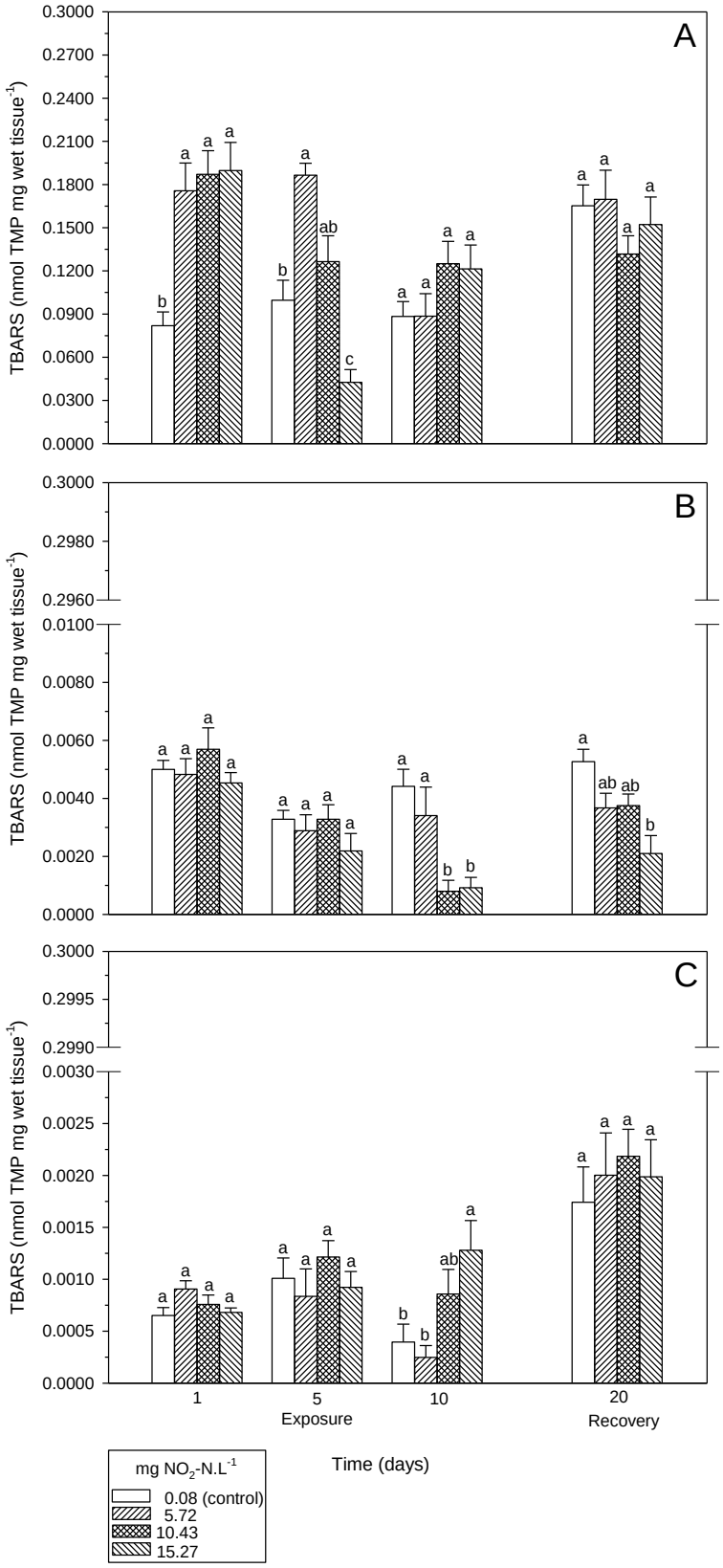

A
B
C
PSH (nmol SH.mg protein-1)
Exposure
Recovery
Time (days)
mg NO2-N.L-1
0.08 (control)
5.72
10.43
15.27

Figure 6


A
B
C
TBARS (nmol TMP mg wet tissue^{-1})
Exposure
Recovery
Time (days)
mg NO_2-N.L^{-1}
0.08 (control)
5.72
10.43
15.27

5. CONCLUSÕES

- A exposição a concentrações partir de 0,12 mg NH_3-N.L^{-1} e 5,72 mg NO_2-N.L^{-1} por um período de 10 dias induzem diversas alterações fisiológicas e bioquímicas, de modo tempo e concentração-dependentes, em juvenis de *P. orbignyanus*.

- Os efeitos decorrentes da exposição à amônia e nitrito em *P. orbignyanus* incluem distúrbios metabólicos, osmorregulatórios, ácido-base, e na resposta imune, além da indução ao estresse oxidativo e a ativação de respostas antioxidantes.

- A exposição a concentrações de até 0,57 mg NH_3-N.L^{-1} e 15,27 mg NO_2-N.L^{-1} por um período de 10 dias não foi suficiente para ocasionar alterações histopatológicas no cérebro, brânquia e fígado de *P. orbignyanus*.

- O período de recuperação de 10 dias foi capaz de reverter todas ou parte das alterações nos parâmetros sanguíneos e de estresse oxidativo ocasionados nos juvenis de *P. orbignyanus*, dependendo do composto e concentração que foram expostos. A recuperação completa foi demonstrada apenas nos peixes expostos a 5,72 mg NO_2-N.L^{-1}.

6. CONSIDERAÇÕES FINAIS E PERSPECTIVAS

- Com base nos resultados obtidos, concentrações de amônia e nitrito a partir de 0,12 mg NH_3-N.L^{-1} e 5,72 mg NO_2-N.L^{-1}, respectivamente, devem ser evitadas nos sistemas de produção do linguado *P. orbignyanus*, uma vez que foram capazes de causar estresse nos peixes, mesmo em curto prazo (1 a 10 dias). Vale ressaltar que esta é uma recomendação para condições de cultivo semelhantes as do presente estudo e não deve ser generalizada, uma vez que a toxicidade destes compostos pode ser influenciada por diversos fatores bióticos e abióticos. No presente estudo, mesmo as concentrações mais baixas testadas de ambos os compostos induziram alterações na homeostase dos peixes. Portanto, recomenda-se que futuros trabalhos sejam realizados avaliando os efeitos da exposição a concentrações inferiores a 0,12 mg NH_3-N.L^{-1} e 5,72 mg NO_2-N.L^{-1}, bem como a interação com outras

variáveis, o que poderá permitir o estabelecimento mais preciso de quais os níveis seguros de amônia e nitrito na água para espécie em diferentes condições.

- O monitoramento não só da qualidade de água, mas também do estado fisiológico dos peixes deve ser uma prática rotineira nos sistemas de produção aquícola, permitindo a detecção de condições estressantes para os animais e a tomada de medidas necessárias de maneira precoce.

- Os parâmetros sanguíneos e de estresse oxidativo aplicados nesta tese demonstraram ser excelentes biomarcadores para detectar os efeitos deletérios ocasionados pela exposição de juvenis de linguado a níveis subletais de amônia e nitrito.

- Uma vez que o estresse oxidativo foi um dos efeitos tóxicos da amônia e nitrito para os juvenis de linguado, a busca por alternativas que melhorem o sistema antioxidante, como a inclusão de antioxidantes na dieta, pode potencialmente minimizar ou evitar este efeito. Portanto, recomenda-se que esta hipótese seja testada em futuros estudos com a espécie.

- Um período de 10 dias de recuperação em água livre ou com concentrações mínimas de nitrito é recomendado para restabelecer as condições fisiológicas normais de juvenis de linguado que tenham sido expostos a concentrações até 5,72 mg NO_2-N.L^{-1}. Para peixes expostos a concentrações de amônia e nitrito a partir de 0,12 mg NH_3-N.L^{-1} e 10,43 mg NO_2-N.L^{-1}, respectivamente, sugere-se que um período de recuperação mais longo seja necessário, o qual deverá ser estabelecido a partir de novos estudos.