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# EFEITOS DO AMBIENTE ÁCIDO E DO NITRATO EM JUVENIS DO BIJUPIRÁ *Rachycentron canadum*

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**FURG**

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**Programa de Pós-Graduação em Aquicultura**

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Tese apresentada como parte dos requisitos para a obtenção do grau de doutor em Aquicultura no programa de Pós-Graduação em Aquicultura da Universidade Federal do Rio Grande.

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**DEDICATÓRIA**

***Dedico este trabalho aos meus pais***

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**RESUMO GERAL**

O presente estudo avaliou o efeito agudo e crônico do ambiente ácido e do nitrato em juvenis do bijupirá *Rachycentron canadum*. Foi determinada a concentração letal mediana ($CL_{50}$-96h) e os efeitos histopatológicos do nitrato em juvenis do bijupirá. A $CL_{50}$-96 h foi estimada em 1829 mg $L^{-1}$ $NO_3^-$-N. A exposição aguda ao nitrato induziu alterações histopatológicas nas brânquias, esôfago e cérebro do bijupirá. Um segundo estudo foi realizado para avaliar o efeito agudo (24 h) do ambiente ácido (pH = 7,9 (controle), pH 6,5. 6,0 e 5,5) nos parâmetros fisiológicos e histológicos do bijupirá. O ambiente ácido induziu uma acidose sanguínea de acordo com a redução do pH, que culminou com uma redução das concentrações de bicarbonato e saturação do oxigênio no sangue dos peixes. Em resposta foi observado uma elevação do hematócrito e das concentrações de hemoglobina e glicose no sangue dos peixes comparando com o tratamento controle. O ambiente ácido afetou negativamente a histologia das brânquias e da pele, sendo que os dados foram mais severos de acordo com a redução do pH. Hiperplasia com fusão completa das lamelas secundárias foram observados para todos os tratamentos de redução do pH, enquanto telangiectasia e proliferação das células de cloreto foram observadas nos peixes expostos aos pHs 6,0 e 5,5. Na pele foram observados hiperplasia e hipertrofia das células mucosas com presença de necrose focal dessas nos peixes expostos aos pHs 6,0 e 5,5. O terceiro estudo avaliou a possibilidade de reduzir o pH para 6,5, o efeito de elevadas concentrações de nitrato e a interação entre ambos os parâmetros de qualidade de água durante 42 dias na sobrevivência, crescimento, parâmetros fisiológicos e histológicos do bijupirá. Os juvenis de bijupirá foram expostos as concentrações de nitrato de 0, 100 e 200 mg/L $NO_3^-$-N no pH 6,5 ou 8 e alimentados até a saciedade. Dois outros tratamentos avaliaram a relação do crescimento devido o consumo alimentar dos peixes em baixo pH ou elevado valor de

nitrato. Esses dois grupos foram mantidos em pH 8/0 mg/L $NO_3^-$-N. Um grupo foi alimentado com a mesma quantidade de alimento consumido pelos peixes mantidos com pH 8/ 200 mg/L $NO_3^-$-N. O outro grupo foi alimentado com a mesma quantidade de alimento consumido pelos peixes expostos ao pH 6,5/0 mg/L $NO_3^-$-N. A sobrevivência do bijupirá foi reduzida em 10% apenas nos peixes expostos ao pH 6.5/ 200 mg/L $NO_3^-$-N. O crescimento foi afetado pelo nitrato, mas não pelo pH. A exposição crônica ao nitrato reduziu significativamente o ganho de peso, a taxa de crescimento específico e o fator de condição, assim como elevou a conversão alimentar dos peixes. O consumo de alimento foi geralmente reduzido entre os peixes expostos ao nitrato. Os níveis de glicose plasmática foram reduzidas nos peixes expostos ao nitrato e ao pH 6,5. As concentrações de lactato e lisozima foram reduzidos nos peixes expostos ao nitrato em ambos os níveis de pH. Contudo, não houve influência do pH e do nitrato nas concentrações plasmáticas de cortisol e osmolaridade. Os bijupirás expostos ao nitrato apresentaram hiperplasia e telangiectasia nas lamelas secundárias. Desta forma podemos concluir que o bijupirá podem ser criados em pH 6,5 com reduzidos valores de nitrato. Porém, nesse pH deve-se ter um cuidado especial com elevadas concentrações de nitrato. Além disso, a concentração igual ou superior a 100 mg/L $NO_3^-$-N prejudicam o crescimento do bijupirá independente dos valores de pH.

**ABSTRACT**

The present study evaluated the acute and chronic effects of acid envinronment and nitrate on juvenile cobia *Rachycentron canadum* juvenile. The median lethal concentration ($LC_{50}$-96h) and the histopathological effects were determined for juvenile cobia. The $LC_{50}$-96h was estimated to be 1829 mg $L^{-1}$ $NO_3^-$-N. Cobia exposed to sub-lethal nitrate concentrations showed histopathogical alterations in the gills, esophagus and brain. The second study evaluates the acute effects (24 h) of acid water (pH = 7,9 (control), pH 6,5. 6,0 e 5,5) on physiological and histopathological effects of cobia. Acid water induced blood acidosis accordingly pH reduction. In response could be observed decrease in bicarbonate ($HCO_3^-$) and saturated $O_2$ ($sO_2$) in fish blood. However, hematocrit, hemoglobin and glucose concentration increased their values comparing to control treatment. Acid environment negatively affect gills and skin histology, and the damage increased within pH reduction. Hyperplasia with completely fusion of secondary lamella was observed in all pH treatments, while telangiectasia and proliferation of chloride cells was present for fish exposed to pH 6.0 and 5.5. In skin was observed hyperplasia and hypertrophy of mucous cells, with necrosis of these cells for fish exposed to pH 6.0 and 5.5. The third study evaluated the possibility of decreasing the pH to 6.5, the effects of high nitrate concentrations and the interactions of both water quality parameters during 42 days, on growth, survival, feeding performance, physiological parameters and histopathology on juvenile cobia. Juvenile cobia were exposed to 0, 100, or 200 mg $L^{-1}$ $NO_3^-$-N at a pH of 6.5 or 8.0 and fed to apparent satiation. Two other treatments evaluated the relationship of growth with feed intake at either low pH or high nitrate concentration. The two groups were kept at pH 8.0 with no nitrate. One group was offered the same amount of food consumed by fish reared at pH 8.0/ 200 mg/L $NO_3^-$-N. Another group of cobia, was fed on the same

amount of food voluntarily consumed by fish exposed to pH 6.5/0 mg/L $NO_3^-$-N. Survival of fish was in 10% only in fish exposed to pH 6.5/ 200 mg/L $NO_3^-$-N. Growth performance was affected by nitrate concentration but not pH. Chronic nitrate exposure yielded significantly reduced weight gain, standard growth rate, and conditional factor, and elevated food conversion rates values. Feed intake was generally reduced among fish exposed to nitrate. Plasma glucose was generally reduced among fish exposed to nitrate and those cultured at pH 6.5. Plasma lactate and lysozyme activity were reduced among fish exposed to nitrate at either water pH. However, there was no influence of pH and nitrate on cortisol and osmolality concentration. Cobia exposed to nitrate presented hyperplasia and telangectasia in the secondary lamella. We conclude that cobia could be reared in pH 6.5 with no nitrate. However, special attention must be given with nitrate in this pH. In addition, concentration of 100 mg/L $NO_3^-$-N or higher hampers growth of cobia even in pH 6.5 or 8.

## INTRODUÇÃO GERAL

### *Descrição da espécie*

O bijupirá, *Rachycentron canadum* (Linnaeus, 1766), originalmente descrito como *Gastorosteus canadus*, é o único representante da família Rachycentridae (Collette, 1981). No Brasil *R. canadum* também é conhecido popularmente como beijupirá e pirambijú (Figueiredo e Menezes, 1980). É uma espécie de grande porte podendo chegar a 2 m de comprimento e mais de 60 Kg (Figura 1). Na natureza, o bijupirá geralmente é encontrado sozinho ou formando pequenos cardumes, comumente associado a estruturas flutuantes como plataformas de petróleo ou mesmo dispersos na coluna d'água. O bijupirá é uma espécie carnívora oportunista, alimentando-se de várias espécies de peixes, crustáceos e moluscos (Shaffer e Nakamura, 1989), sendo que no litoral do nordeste brasileiro o bijupirá se alimenta principalmente de teleósteos (Cavalli et al., 2011).

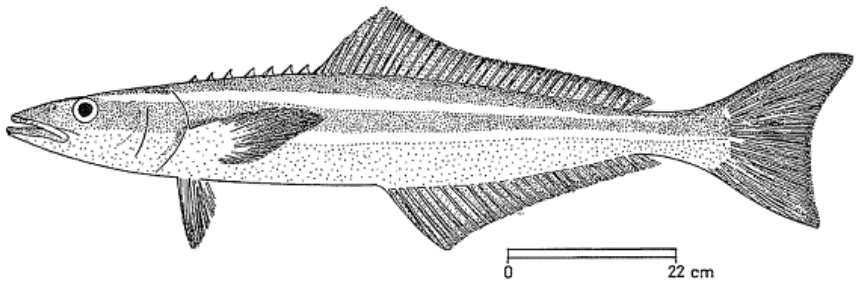


Figura 1. Ilustração de um exemplar adulto de *Rachycentron canadum* (Collette 1981).

Esta espécie habita a zona costeira, estuários rasos, assim como a plataforma continental até 1.200 m de profundidade. É um peixe migratório pelágico, não possui vesícula gasosa, apresentando hábito natatório ativo. A distribuição do bijupirá é fortemente influenciada pela temperatura com preferência por regiões quentes. Contudo, ocorre em regiões temperadas durante os meses quentes do ano (Shaffer e Nakamura, 1989). O bijupirá ocorre em todos os mares tropicais e subtropicais, com exceção da

parte central e oriental do Oceano Pacífico (Shaffer e Nakamura, 1989) (Figura 2), sendo que esta espécie foi introduzida nos mares Mediterâneo, Negro e Vermelho (Goren e Dor, 1994; Golani et al., 2002). Enquanto no Brasil, *R. canadum* está presente em todo o litoral, com maior abundância na região nordeste (Figueiredo e Menezes, 1980). Contudo, o bijupirá ainda é uma espécie pouco conhecida no Brasil. Devido ao hábito de não formar cardumes, não existe uma frota pesqueira direcionada para o bijupirá. A produção mundial da pesca de bijupirá atingiu 10.113 toneladas em 2009 (FAO, 2011).

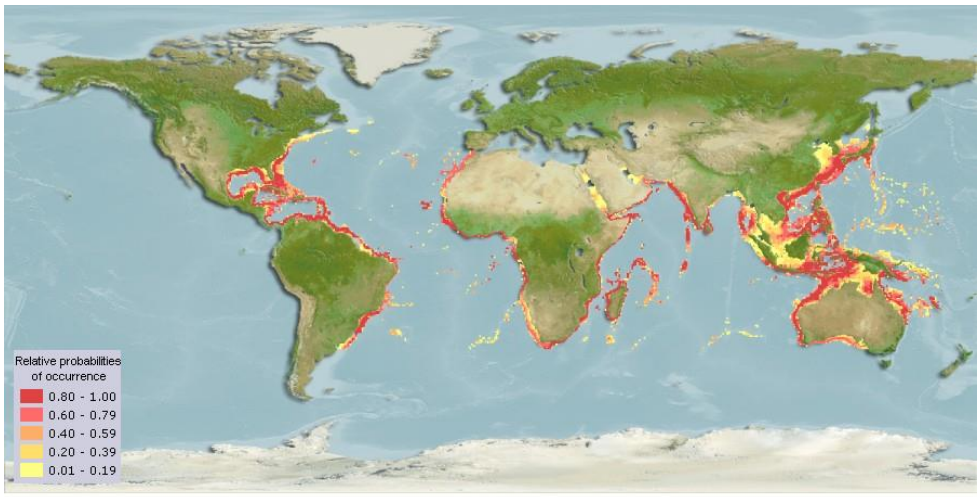


Figura 2. Distribuição geográfica e probabilidade relativa de ocorrência de *Rachycentron canadum* (Froese e Pauly, 2011).

***Produção do bijupirá***

O primeiro estudo sobre a produção de bijupirá é da década de 70 nos Estados Unidos (Hassler e Rainville, 1975). Foram coletados ovos do ambiente natural e as larvas foram criadas em laboratório, os juvenis foram mantidos até 131 dias de idade. Estes autores observaram um rápido crescimento, facilidade de manejo e tolerância a variações de qualidade de água, o que evidenciou o potencial dessa espécie para aquicultura. Porém, foi apenas nos anos 90, que o bijupirá começou a ser produzido

comercialmente em Taiwan, onde atualmente é a principal espécie de peixe marinho produzida (Liao et al., 2004). Contudo, ainda é considerada uma espécie emergente para a aqüicultura, com rápido potencial de expansão devido à suas excelentes características zootécnicas (Liao e Leaños, 2007). Entre os atributos que confirmam estas características estão sua elevada fecundidade, além da facilidade de obtenção de desovas naturais em cativeiro (Arnold et al., 2002). Outra importante característica relacionada à reprodução são as múltiplas desovas dentro do mesmo período reprodutivo (Lotz et al., 1996), o que permite que uma única fêmea produza milhões de larvas. O bijupirá é uma espécie eurialina e euritérmica, tolerando uma ampla faixa de salinidade (5 – 44) e temperatura (16,8 – 32,2 °C) (Shaffer e Nakamura, 1989; Resley et al., 2006). Possui uma elevada taxa de crescimento, alcançando de 4 a 6 Kg em 12 meses de criação (Liao et al., 2004; Benetti et al., 2008) e entre 8 a 10 Kg após 18 meses (Liao et al., 2004). O bijupirá aceita com facilidade dietas comerciais com elevadas taxas de sobrevivência após o desmame (Liao e Leaños, 2007; Maclean et al., 2009). Os juvenis utilizam com eficiência diferentes fontes de proteína e lipídios sem alterar suas características de produção (Craig et al., 2006). Essa espécie possui também uma elevada qualidade de carne e bom rendimento durante o processamento, assim como elevado valor comercial (Liao e Leaños, 2007).

O sucesso conseguido em Taiwan tem servido para estimular o desenvolvimento da criação dessa espécie ao redor do mundo. Além da Ásia, atualmente o bijupirá vem sendo criado na Europa, Austrália, Estados Unidos, América Central e Brasil (Liao et al., 2004; Benetti et al., 2010; Cavalli et al., 2011; Sampaio et al., 2011). A produção mundial de bijupirá em cativeiro em 2010 foi de 24.860 toneladas (FAO, 2010), produção superior a captura pela frota pesqueira mundial. Atualmente é considerada uma das espécies de peixes marinho mais promissoras do

mundo para a aquicultura (Liao et al., 2004; Benetti et al., 2010). Contudo, ainda existem limitações significativas para sua produção em larga escala, como produção inconsistente de formas jovens, ausência de laboratórios com esquema de biossegurança e diversidade genética, o mercado ainda em desenvolvimento e ausência de dietas específicas para a espécie (Holt et al., 2007; Cavalli et al., 2011).

***Sistemas de produção de R. canadum***

O bijupirá vem sendo produzido em diferentes sistemas de produção. Para a larvicultura são relatados o uso de viveiros e tanques em sistema de fluxo contínuo ou em sistemas de recirculação de água (Weirich et al., 2004; Holt et al., 2007; Benetti et al., 2008). Para engorda o sistema que está sendo amplamente utilizado ao redor do mundo são os tanques-rede, tanto "near-shore" como "off-shore" (Benetti et al., 2010; Sampaio et al., 2011). Contudo, mais recentemente essa espécie vem sendo introduzida em sistemas de recirculação de água (SRA) com excelentes resultados (Liao e Leaños, 2007), principalmente em países com restrições ao uso de regiões costeiras e/ou que possuam temperaturas inadequadas para a sua produção. Os sistemas de recirculação permitem um excelente controle da qualidade da água conjugada com uma reduzida utilização de água comparado aos sistemas tradicionais, assim como elevada produtividade por área utilizada (Liao e Leaños, 2009), excelente biosegurança e controle de doenças (Weirich et al., 2003), propicia a criação de organismos aquáticos marinhos longe de regiões costeiras e próximo a mercados consumidores, reduzindo custos com transporte (Liao e Leaños, 2007), além de facilitar a obtenção de licenças para cultivo (Resley et al., 2006). Contudo, são limitadas as informações referentes a produção de bijupirá nesse sistema de produção (Webb Jr. et al., 2007).

***Sistemas de recirculação e a qualidade da água***

***Efeitos do pH em peixes***

Pouco se conhece sobre os parâmetros de qualidade da água durante a criação do bijupirá em SRA. Esses sistemas geralmente operam com elevadas densidades de estocagem que podem gerar um acúmulo de metabólitos nos sistemas, como a amônia e o dióxido de carbono, que resultam em um efeito negativo sobre a produção (Person-Le Ruyet et al., 2003; Foss et al., 2004).

O processo de nitrificação ocorre através da oxidação do íon amônio ($NH_4^+$) para nitrito e posteriormente para nitrato. Este processo libera íons $H^+$, que consequentemente são acumulados na água e acidificam o meio (Timmons et al., 2007). Contudo os sistemas de recirculação são rotineiramente mantidos em pHs mais baixos que o da água marinha típica, especialmente para uma melhor performance dos biofiltros e menor custo para tamponamento dessa água (Abbink et al., 2011).

O pH interfere na eficiência do processo de nitrificação. Esse processo é menos eficiente em baixos pHs, o que acarreta em acúmulo de amônia ionizada nos sistemas, enquanto em pHs alcalinos o nitrato tende a acumular em sistemas de recirculação (Villaverde et al., 1997). O pH também é importante no equilíbrio da amônia ionizada ($NH_4^+$) e não ionizada ($NH_3^-$) na água, sendo que em baixo pH predomina a forma não ionizada, cuja toxicidade é menor para os peixes (Thurston et al., 1981). Desta forma, uma alternativa para uma produção mais intensiva de peixes em baixo pH, permitindo concentrações elevadas de amônia total, vem sendo avaliada e considerada eficiente por Eshchar et al. (2006). Esse autor determinou que essas condições, além de não prejudicarem o crescimento e a sobrevivência dos peixes, proporcionam uma redução de custos durante o ciclo de produção devido a redução do bombeamento de água, aumento das densidades de estocagem e utilização de filtros biológicos menores. Todos estes

aspectos caracterizam o pH como um importante parâmetro na criação de peixes em SRA.

O pH também é um importante parâmetro de qualidade de água durante o transporte de peixes em sistema fechado. Durante o transporte pode ocorrer uma rápida redução da qualidade da água devido ao acúmulo de dióxido de carbono na água. O dióxido de carbono pode acidificar a água abruptamente, comprometendo o bem estar dos peixes. Foi verificada uma redução de 7,5 para 6 nos valores de pH em apenas 6 horas de transporte do bacalhau *Gadus morhua* com uma densidade relativamente baixa (10 g/L) (Treasurer, 2012).

Os efeitos agudo e crônico do ambiente ácido têm sido relatado para várias espécies de peixe de água doce, especialmente em salmonídeos (Milligan et al., 1982; Dockray et al., 1998). Por outro lado, pouco se conhece sobre os efeitos do estresse ácido em peixes marinhos. A $CL_{50}$-96 h do ambiente ácido para juvenis do linguado *Paralichthys orbignyanus* foi estimada em 4,40 (Wasieleski et al., 1997). Enquanto que Abbink et al. (2011) observaram que juvenis do olhete *Seriola lalandi* mantidos em um pH igual a 6,60 resultou em aumento da mortalidade e inibição do crescimento e da conversão alimentar aparente. O efeito do pH sobre o crescimento de juvenis do bijupirá já foi previamente avaliado por Sampaio et al. (2008), sendo que pHs iguais ou inferiores a 6 possuem um efeito deletério sobre o seu crescimento, quando comparado aos pHs 7 e 8. Por outro lado não se sabe se o pH igual a 7 é o valor mínimo que esta espécie pode ser criada sem efeitos prejudiciais ao seu crescimento e sobrevivência. Também se desconhece o efeito de alterações abruptas do pH sobre o bijupirá.

***Efeitos do nitrato em peixes***

O efeito tóxico da amônia e do nitrito sobre os processos fisiológicos e alterações estruturais são relativamente bem conhecidos para os peixes (Thurston et al., 1981; Person-Le Ruyet et al., 2003; Kroupova et al., 2005). Estudos relacionados à toxicidade aguda da amônia e do nitrito já foram realizados para juvenis do bijupirá, sendo que essa espécie possui uma boa resistência a estes dois compostos nitrogenados quando comparado a outras espécies de peixe (Weirich et al., 2006; Rodrigues et al., 2007). Sendo que a tolerância à amônia do bijupirá decresce com a redução da salinidade (Barbieri e Doi, 2011). Contudo, anteriormente a essa tese, não havia informações sobre o efeito do nitrato para o bijupirá.

O nitrato é considerado o menos tóxico entre os compostos nitrogenados (Tomasso, 1994; Timmons et al., 2007), dessa forma poucos estudos existem com este produto nitrogenado em peixes. Devido ao processo de nitrificação, o nitrato tende a acumular nos sistemas de recirculação que não utilizam filtros denitrificantes (Timmons et al., 2007), chegando a concentrações superiores a 500 mg/L $NO_3^-$-N (Pierce at al., 1993), sendo incerto o efeito que estas concentrações podem causar na maioria dos organismos criados. Algumas variáveis podem alterar a toxicidade do nitrato. Por exemplo, a toxicidade do nitrato aumenta com o decréscimo da salinidade para juvenis do camarão marinho *Penaeus monodom* (Tsai e Chen, 2002). Hamlin (2006) demonstrou um aumento na susceptibilidade ao nitrato à medida que os juvenis do esturjão siberiano *Acipenser baeri* crescem. Para essa espécie, juvenis de 7 e 674 g apresentam $CL_{50}$-96 h de 1028 e 397 mg/L $NO_3^-$-N, respectivamente. A comparação entre a toxicidade do nitrato entre espécies também é difícil, devido a toxicidade aguda do nitrato ser extremamente variável, apresentando $CL_{50}$ inferior a 200 mg/L $NO_3^-$-N para "fathead minnow" *Pimephales promelas* e superior a 5000 mg/L $NO_3^-$-N para *Heteromycteris capensis* (Tabela 1).

Tabela 1. Toxicidade aguda do nitrato para diferentes espécies de peixe.

| Espécie | $CL_{50}$-96h mg/L $NO_3^-$-N | Referência |
|---|---|---|
| *Oncorhynchus mykiss* | 1364 | Westin, 1974 |
| *Ictalurus punctatus* | 1409 | Colt e Tchobanoglous, 1976 |
| *Poecilia reticulata* | 191 | Rubin e Elmaraghy, 1977 |
| *Lithognathus mormyrus* | 3450 | Brownell, 1980 |
| *Diplodus saegus* | 3560 | Brownell, 1980 |
| *Heteromycteris capensis* | 5050 | Brownell, 1980 |
| *Micropterus treculi* | 1261 | Tomasso e Carmichael, 1986 |
| *Monocanthus hispidus* | 573 | Pierce et al., 1993 |
| *Raja eglanteria* | >960 | Pierce et al., 1993 |
| *Trachinotus carolinus* | 1006 | Pierce et al., 1993 |
| *Centropristis striata* | 2415 | Pierce et al., 1993 |
| *Pomacentrus leucostritus* | >3000 | Pierce et al., 1993 |
| *Pimephales promelas* | 1341 | Scott e Crunkilton, 2000 |
| *Coregonus clupeaformis* | 1902-2185 | McGurk et al., 2006 |
| *Salvelinus namaycush* | 1121-2342 | McGurk et al., 2006 |
| *Mugil platanus* | 1522 | Poersch et al., 2007 |
| *Acipenser baeri* | 397-1028 | Hamlin, 2008 |

Os efeitos crônicos do nitrato ainda não foram adequadamente estudados em peixes criados com fins comerciais. Spotte (1979) relatou que níveis aceitáveis de nitrato para organismos criados em água marinha são geralmente considerados até 20 mg/L $NO_3^-$-N, enquanto Pierce et al. (1993) considera seguro níveis de até 500 mg/L $NO_3^-$-N. Contudo, níveis de segurança aplicados na aqüicultura para nitrato são

geralmente baseados em informações empíricas relacionados a valores de $CL_{50}$ ou baseado em práticas rotineiras de aquicultura (Van Bussel et al., 2012). Frakes e Hoff Jr. (1982) determinaram que concentrações de 100 mg/L $NO_3^-$-N prejudicam o crescimento e a coloração do peixe palhaço *Amphiprion ocellaris*. Enquanto que concentrações iguais ou superiores a 125 mg/L prejudicam o crescimento do "turbot" *Psetta máxima* (Van Bussel et al., 2012). O bagre africano *Clarias gariepinus* pode ser criado em concentrações de até 140 mg/L $NO_3^-$-N sem prejuízos ao seu crescimento e sobrevivência (Schram et al., 2013). Efeitos subletais do nitrato incluem alterações endócrinas que alteraram o metabolismo, a reprodução e o desenvolvimento de organismos aquáticos. Concentrações de 4 - 5 mg/L $NO_3^-$-N afetaram a reprodução do peixe mosquito *Gambusia holbrooki*, como a redução do gonopódio e da fecundidade (Edwards et al., 2006).

O mecanismo de toxicidade do nitrato em peixes ainda não foi bem determinado (Camargo et al., 2005; Hamlin, 2006). O principal mecanismo de toxidade do nitrato foi associado à formação de metahemoglobina no sangue dos peixes, diminuindo assim a capacidade de transporte de oxigênio do sangue, resultando na morte dos peixes por asfixia (Camargo et. al., 2005), similar ao mecanismo de toxicidade do nitrito (Kroupova et al., 2005). De acordo com Bodansky (1951) o nitrato é reduzido a nitrito antes da oxidação da hemoglobina para metahemoglobina. Contudo apenas um estudo com truta arco-íris *Oncorhynchus mykiss* relatou o aumento de metahemoglobina no sangue dos peixes expostos durante 11 semanas a concentrações de nitrato entre 26,2 e 30,6 mg/L $NO_3^-$-N (Grabda et al., 1979). Em contraste, a própria truta arco-íris e o "turbot" *Psetta maxima* não apresentaram elevações nas concentrações de metahemoglobina quando submetidos a elevadas concentrações de nitrato (Stormer et al., 1996; Van Bussel et al., 2012). Contudo, apenas recentemente a

toxicidade do nitrato tem recebido atenção pelo seu potencial de alterar a função endócrina (Guillette e Edwards, 2005). O mecanismo proposto por estes autores para os distúrbios esteroidogênicos induzidos pelo nitrato incluem a sua conversão ao nitrito e posteriormente ao óxido nítrico (NO). O NO possui a capacidade de se ligar a porção heme das enzimas do citocromo P450, que estão presentes em vários locais ao longo da via esteroidogênica e liberação dos hormônios esteróides.

***Efeitos dos estressores em peixes***

O pH, assim como os compostos nitrogenados possuem um importante papel na fisiologia dos peixes, tendo influência no balanço ácido-básico, regulação iônica, elevação das concentrações plasmáticas de glicose, lactato e cortisol, excreção de amônia, crescimento somático e reprodução dos peixes (Fromm et al., 1980; Iger et al., 1994; Dockray et al., 1998; Person-Le Ruyet et al., 2003). Portanto o ambiente ácido e os compostos nitrogenados são considerados estressores para os peixes (Dockray et al., 1998; Miron et al., 2008). A resposta fisiológica dos estressores é dividida em estresse primário, secundário e terciário. O estresse primário é caracterizado pela ativação do sistema nervoso simpático, que culmina com a elevação das concentrações plasmáticas das catecolaminas e do cortisol, enquanto o estresse secundário é a resposta da ação direta desse hormônio sobre os tecidos, estando entre eles a elevação das concentrações de glicose e lactato no sangue. O estresse terciário é a resposta dos organismos em termos de crescimento (Wendelaar Bonga, 1997), sendo de interesse direto da aqüicultura. Portanto, diversos estudos têm avaliado a elevação plasmática das concentrações de cortisol, glicose e lactato no sangue dos peixes para avaliação do efeito de estressores (Dockray et al., 1998; Person-Le Ruyet et al., 2003; Eshchar et al.,

2006). Esses parâmetros de estresse já foram previamente utilizados em teste de estresse agudo em juvenis de bijupirá (Cnaani e McLean, 2009; Trushenski et al., 2010).

A lisozima faz parte do sistema imunológico inespecífico dos peixes. A lizosima impede o assentamento e colonização de microorganismos nocivos aos peixes, ajudando a prevenir infecções e doenças (Alexander e Ingram, 1992). A redução das concentrações plasmáticas de lisozima indica uma condição de imunossupressão nos peixes. Sua atividade foi avaliada em juvenis do bijupirá submetidos a uma situação de estresse agudo (Cnaani e McLean, 2009).

Outro importante efeito deletério do pH e dos compostos nitrogenados são os efeitos histopatológicos que eles podem causar em diferentes órgãos dos peixes, sendo esses efeitos também considerado um estresse secundário. Ambientes ácidos geralmente induzem alterações histopatológicas nas brânquias e pele dos peixes, devido ao seu contato direto e permanente com a água. Entre os principais efeitos do ambiente ácido nesses órgãos estão à indução de hiperplasia de células mucosas com o intuito de proteger os peixes (Iger et al., 1994; Iger and Wendelaar Bonga, 1994). Enquanto a hiperplasia das células de cloreto pode ocorrer como mecanismo para reestabelecer o pH sanguíneo (Wendelaar Bonga et al., 1990). Lemly e Smith (1987) avaliaram o efeito crônico do pH sobre os efeitos histopatológicos dos órgãos sensoriais de *Pimephales promelas*. Miron et al. (2008) observaram diferentes alterações histopatológicas nas brânquias de *Rhamdia quelen* submetidos a diferentes pHs e concentrações de amônia em testes agudos. Por outro lado, poucos estudos existem na literatura sobre o efeito histopatológico do nitrato. Concentrações próximas a 100 mg/L de nitrato resultaram em efeitos histopatológicos no fígado, rim e brânquias do "medaka" *Oryzias latipes* (Shimura et al., 2004). Enquanto Kuhn et al. (2011) relataram que as histopatologias (no presente estudo potencialmente causados pelo ambiente ácido e pelo nitrato) podem

comprometer esses órgãos em longo prazo causando efeitos letais e subletais, que são muito importantes para a aqüicultura.

A diminuição do consumo de alimento pelos peixes é influenciado por agentes estressores, entretanto isso tem sido pouco estudado (Wendelar Bonga, 1997). Dockray et al. (1998) avaliaram o efeito da restrição de alimento pelo pH e pela temperatura sobre o crescimento e parâmetros fisiológicos de *Oncorhynchus mykiss*. Em outro estudo, Pichavant et al. (2001) avaliaram o efeito da restrição alimentar pela redução de oxigênio na água sobre juvenis de *Dicentrarchus labrax* e de *Psetta maxima*. Sampaio et al. (2008) observaram que o pH baixo (igual ou inferior a 6) reduz o consumo alimentar de juvenis de bijupirá em ambiente ácido, afetando diretamente o crescimento dos peixes. Estudos prévios relatam também que ingestão e assimilação do alimento é influenciado pelo nitrato (Van Bussel et al., 2011; Schram et al., 2013). Reconhecendo que o pH e o nitrato podem afetar a fisiologia dos peixes bem como o comportamento alimentar e por consequência causar efeitos subletais nos peixes, é importante caracterizar o efeito do pH e do nitrato em juvenis do bijupirá.

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## OBJETIVOS

### OBJETIVO GERAL

Determinar o efeito do ambiente ácido e do nitrato sobre juvenis do bijupirá *R. canadum*.

### OBJETIVOS ESPECÍFICOS

- Determinar a concentração letal mediana ($CL_{50}$-96 h) e as alterações histopatológicas causadas pela toxicidade aguda do nitrato em juvenis do bujupirá *R. canadum*.
- Avaliar o efeito agudo do ambiente ácido sobre parâmetros fisiológicos e histológicos de juvenis do bijupirá.
- Investigar o efeito crônico do ambiente ácido, elevadas concentrações de nitrato e a interação entre ambos os parâmetros no crescimento, alimentação, histopatologias e parâmetros bioquímicos sanguíneos de juvenis do bijupirá *R. canadum*.

# CAPÍTULO 1

## ACUTE EXPOSURE OF JUVENILE COBIA *Rachycentron canadum* TO NITRATE INDUCES GILL, ESOPHAGEAL AND BRAIN DAMAGE

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**Acute exposure of juvenile cobia *Rachycentron canadum* to nitrate induces gill, esophageal and brain damage**

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**Abstract**

Cobia *Rachycentron canadum* is a fast growing fish with world-wide potential for aquaculture, and has been considered for rearing in recirculating aquaculture systems (RAS). Nitrate is considered the least toxic nitrogenous product in the ammonia nitrification process, but as it may accumulate in RAS, toxic levels can be reached. The objective of this study was to evaluate the acute toxicity and the histopathological effects of nitrate on juvenile cobia. Juveniles (6.87 ± 0.36 g; 11.8 ± 0.19 cm) were acutely exposed to six concentrations of nitrate (500 – 3000 mg/L $NO_3^-$-N) plus a control during 96 h. At the end of this period of exposure, juvenile cobia were sampled for histopathological evaluation. The estimated LC50 of nitrate to juvenile cobia was equal to 2407 and 1829 mg/L $NO_3^-$-N at 24 and 96 h, respectively. Cobia exposed to sub-lethal nitrate concentrations showed histopathological alterations in the gills, esophagus and brain. The gills revealed epithelial hyperplasia with complete lamellar fusion, telangiectasia, and lamellar shorting induced by necrosis, and the esophagus presented hyperplasia of epithelium and mucus cells. In the brain, glial cells proliferation, satellitosis (microglial cells surrounding neurons with swollem and prenecrotic neurons), and Virchow-Robin spaces (enlarged perivascular spaces, EPVS) were observed. The results of the present study indicate that juvenile cobia have a high tolerance to acute exposure of nitrate. However, assorted histopathological responses were observed for cobia at sub-lethal nitrate concentrations. Therefore, further studies are needed to estimate safe chronic nitrate levels for juvenile cobia culture.

## 1. Introduction

Cobia *Rachycentron canadum* is a migratory pelagic species widely distributed in tropical and subtropical waters, except for the central and eastern Pacific Ocean (Shaffer and Nakamura, 1989). Over the last decade, cobia have received increased interest for aquaculture due to their impressive growth rates, reaching over 6 Kg in 1 year (Liao et al., 2001), ease of spawning in captivity (Arnold et al., 2002), high post-weaning survival, and good feed efficiency (McLean et al., 2009).

Cobia has been successfully reared in cages since the mid-1990s, and it is among the most economically important marine fish for aquaculture in Taiwan (Liao et al., 2004). However, due to regulatory constraints, minimal biosecurity and disease issues associated with cage culture, cobia production has begun in recirculating aquaculture systems (RAS) (Weirich et al., 2003). Nevertheless, little information is available regarding culture parameters of cobia in RAS.

Nitrate is considered the least toxic nitrogenous product in the ammonia nitrification process. However as the final product of nitrification, it may accumulate in RAS (Hamlin, 2006) and species-specific toxic levels may be reached (Timmons and Ebeling, 2007). Despite this issue, toxicity of nitrate has received little attention compared to ammonia and nitrite in RAS. Furthermore, no safe levels for nitrate have been established for aquatic animals (US Environmental Protection Agency, 1986).

Shimura et al. (2002) observed that nitrate can negatively affect growth and reproduction of medaka *Oryzias latipes* exposed to 75 mg/L of $NO_3^-$-N. Nitrate also has the potential to disrupt endocrine function and induces secondary stress responses in Siberian sturgeon *Acipenser baeri* (Hamlin et al., 2008). However, the mechanism of nitrate toxicity in fish is still unclear (Hamlin, 2006).

Different studies have documented the histopathlogical effects of ammonia and nitrite on fish (Benli et al., 2008; Frances et al., 1998). On the other hand, little is known about histopathological effects of nitrate on fish. Shimura et al. (2004) observed different histopathological effects of nitrate in medaka *Oryzias latipes*, but no further results were found in the literature concerning histological effects of nitrate in fish.

As there is no information in literature describing the effects of nitrate on cobia, the aim of the present study was to evaluate the acute toxicity and histopathological effects of nitrate to juvenile *R. canadum*.

## 2. Material and methods

Fertilized cobia eggs were air transported from Troutlodge Marine Farms (Vero Beach, Florida, USA) to Virginia Tech's Virginia Seafood Agricultural Research and Extension Center (VSAREC), located in Hampton, Virginia (USA). Juvenile cobia were produced at the VSAREC in specialized RAS according to standardized VSAREC larviculture protocols (McLean et al., 2009).

Prior to toxicity trials, the fish were acclimated to experimental conditions in a 300 L tank connected to a marine RAS for 1 week. During this acclimation period, the fish were fed a commercial diet (Zeigler, Finfish Starter, 55% protein: 15% lipids, 2 mm, Zeigler Bros. Inc., Gardners, Pennsylvania, USA) 4 times per day to satiation.

Juvenile cobia (6.87 ± 0.36 g; 11.8 ± 0.19 cm; n = 25) were acutely exposed during 96 h to 6 concentrations of nitrate $NO_3^-$-N (500, 1000, 1500, 2000, 2500 and 3000 mg/L), plus one control where no nitrate was added. The experimental concentrations and the control were conducted with three replicates each. The desired test concentrations were obtained using sodium nitrate ($NaNO_3$; Fischer Chemical, New

Jersey, USA), and dechlorinated freshwater was added to compensate for the resulting increase in $Na^+$ induced salinity.

A semi-static system was used to evaluate the acute toxicity of nitrate. Water was continuously aerated by air stones and fully replaced daily in the experimental concentrations and controls. The volume of each tank was 9 L, and the stocking density was 4 fish per tank. Fish were not fed 1 day prior to and throughout the 96 h experiment. Fish behavior and mortality were observed twice daily (9 AM: 9 PM). Fish were considered dead when they were motionless on the bottom of the tank, exhibited no opercular movement, and showed no response to mechanical stimuli.

Water quality parameters were analyzed once daily. Temperature, salinity and dissolved oxygen were measured with a digital YSI Model 55A oxygen meter (Yellow Springs Instruments, Yellow Springs, USA) and pH was measured with a YSI Model pH 100 meter (Yellow Springs Instruments, Yellow Springs, USA). Alkalinity was determined using the Hach digital titration method. TA-N, $NO_2^-$-N and $NO_3^-$-N were determined via colorimetric assays, methods 10031, 8153 and 8039 respectively, using a DR 2800 spectrophotometer (Hach, Loveland, Colorado, USA). $NH_3^-$-N levels were determined according to TA-N, temperature, salinity and pH values using the equations of Ostrensky et al. (1992) adapted from Whitfield (1974) and Bower and Bidwell (1978).

After 96 h of exposure to different concentrations of nitrate, cobia were euthanized with 100 mg/L of tricaine methanesulfonate (MS 222, Western Chemical Inc., Ferndale, Washington, USA). Fish were dissected and samples of gill, esophagus, liver, kidney, spleen, and brain were fixed in 10% buffered formalin for subsequent histopathological evaluation. The samples were dehydrated in a graded series of ethanol, embedded in paraplast, sectioned (5 μm) and the slides were stained with

hematoxylin and eosin. Slides from the brain were stained with silver staining technique. Slides were examined by light microscopy (Olympus BH-2, Center Valley, Pennsylvania, USA) and the images were registered with a digital camera.

Median lethal concentrations ($LC_{50}$) and their respective confidence intervals (95%) were calculated after 12, 24, 48, 72 and 96 h using the software Trimmed Spearman Karber Method (Hamilton et al., 1977). Comparisons among median toxic lethal concentrations for nitrate were made by one-way ANOVA followed by Tukey's test with significance level of 95%, using the software Statistic 7.0 according to Bhujel (2008).

**3. Results and discussion**

Aside from the treatment variable, water quality parameters presented no statistical differences ($P$>0.05) among treatments, and was determined to be: temperature 25.5 ± 0.2 °C; pH 7.89 ± 0.1; minimum dissolved oxygen 6.0 mg $L^{-1}$; maximum unionized ammonia 0.08 mg $L^{-1}$; maximum nitrite nitrogen ($NO_2^-$-N) 0.3 mg $L^{-1}$; salinity 25.4 ± 0.2‰; alkalinity 130 ± 2.4 mg $L^{-1}$ as $CaCO_3$. Water quality parameters were considered adequate for cobia, including the other nitrogenous compounds (ammonia and nitrite), according to the safe levels determined by Rodrigues et al. (2007) and did not affect nitrate toxicity.

After 12 h of acute exposure to concentrations equal to or higher than 1000 mg $L^{-1}$ $NO_3^-$-N, cobia presented clinical signs of toxicity (erratic swimming, mucus in the water and lethargy). In addition, moribund fish presented clinical signs of hyperventilation and all dead fish had their operculum flared wide open. However, fish in the control tanks, and those exposed to 500 mg/L $NO_3^-$-N showed no clinical signs of toxicity. Similar responses to nitrate toxicity, like hyperventilation and lethargy were

observed for juvenile Siberian sturgeon *Acipenser baeri* (Hamlin, 2006). Juvenile medaka *Oryzias latipes* exposed to nitrate presented loss of appetite and lethargic behavior (Shimura et al., 2004). Moribund juvenile cobia acutely exposed to ammonia and nitrite, also presented clinical signs of toxicity such as erratic swimming (Rodrigues et al., 2007). These clinical sings of toxicity are important and practical information to identify lethal and sub-lethal concentrations of nitrogenous compounds in routine aquaculture practices, but they are not specific to nitrogenous compounds.

Median lethal concentrations of nitrate to juvenile cobia were estimated after 12, 24, 48, 72 and 96 h (Table 1). The estimated $LC_{50}$ to nitrate for juvenile cobia increased with the exposure time. $LC_{50}$ was equal to 2407 and 1829 mg/L $NO_3^-$-N at 24 and 96 h , respectively, indicating that this species has a good resistance to nitrate. Nitrate toxicity can vary within species as it depends on variables such as fish size (Hamlin, 2006) and environment salinity for example (Tisai and Chen, 2002). Comparison among species is also difficult, as the acute toxicity of nitrate ($NO_3^-$-N) to fish is highly variable, presenting toxicity on the range of 200 mg/L $NO_3^-$-N for fathead minnow *Pimephales promelas* (Rubin and Elmaraghy, 1977) to over 3000 mg/L $NO_3^-$-N for beaugregory *Stegastes leucostictus* (Pierce et al., 1993). However, the results of the current study indicate that cobia response to toxicity of nitrate-N is similar to several other marine finfish, on the range of 1000 mg/L $NO_3^-$-N or more. For example, the $LC_{50}$-96 h to nitrate for juvenile Florida pompano was determined to be 1006 mg/L $NO_3^-$-N (Pierce et al., 1993), while the $LC_{50}$-96 h for juvenile mullet *Mugil platanus* was calculated to be 1522 mg/L $NO_3^-$-N (Poersch et al., 2007).

The histopathological evaluation of juvenile cobia acutely exposed to nitrate showed no effects on liver, kidney and spleen. However, several abnormalities on gills, esophagus and brain tissue were observed. These alterations were more conspicuous

with increasing nitrate concentrations. The major branchial abnormalities observed in the gills were hyperplasia of the epithelial cells that was observed in every nitrate concentrations, but at higher concentrations this effect was more severe as denoted by the complete lamellar fusion (Fig. 1B). At concentrations equal to or higher than 1500 mg/L $NO_3^-$-N, telangiectasia was also observed (Fig. 1C), as was lamellar shorting induced by necrosis (Fig. 1 B). Gill morphology is commonly used as a biomarker of aquatic toxicology because it is the first organ to respond to adverse environmental conditions (Bernet et al., 1999). Several authors have reported similar gill histopathologies for different fish species exposed to excessive nitrogenous compounds. Benli et al. (2008) observed epithelium hyperplasia of the secondary lamella and telangiectasia of Nile tilapia *Oreochromis niloticus* exposed to ammonia. Frances et al. (1998) related hypertrophy, hyperplasia, epithelial lifting and telangiectasia in silver perch *Bidyanus bidyanus* exposed to nitrite. Furthermore, Shimura et al. (2004) detected that short-term exposure of medaka to nitrate caused disruption of cell alignment, hyperplasia and necrosis in the gill.

The esophagus also presented hyperplasia of the epithelial and mucosal cells (Fig. 2B). This esophagus histopathology was observed in pejerrey *Odontesthes argentinensis* larvae exposed to toxic levels of hydrocarbons (Rodrigues et al., 2010). The branchial and esophageal histopathologies found in the present study are probably reactive, protecting the fish from nitrate toxicity. The protection is a result of the reduced surface area exposed to the toxicant, as proposed by Mallat (1985).

The mechanism of nitrate toxicity in fish is still unclear (Hamlin, 2006). However, Camargo et al. (2005) associated nitrate toxicity with the formation of methemoglobin in fish blood, thus decreasing the oxygen carrying capacity of blood resulting in fish death by suffocation. The hyperplasia with complete lamellar fusion

and telangiectasia observed in the present experiment is directly related to the ability of fish to capture oxygen from the water, probably inducing the fish to suffocation, as suggested by fish behavior.

Control fish showed normal histological brain architecture without any indication of pathology (Fig. 3 A), while fish exposed to concentrations equal to or higher than 1000 mg/L $NO_3^-$-N, showed glial cells proliferation, satellitosis (microglial cells surrounding neurons with swollen and prenecrotic neurons), Virchow-Robin spaces (enlarged perivascular spaces, EPVS), and cellular necrosis (Fig. 3B). These brain histopathologies were severe and probably contributed to fish death. Although this is the first report of brain pathology caused nitrate toxicity, fish exposed to hypoxic conditions showed the same histopathologies (Heal, 1995). Therefore, it is not possible to determine whether the histopathology found in the brain of juvenile cobia exposed to nitrate were caused by the direct action of nitrate or indirectly caused by the possible condition of hypoxia which fish were exposed due to sever branchial alteration observed.

Despite the variability in nitrate toxicity among species and across environmental conditions, acute toxicity testing can be a useful tool to examine nitrate toxicity of fish. In addition, it is a useful device to help to establish safe levels of nitrate in culture systems, especially for RAS, where nitrate tends to accumulate. However, according to this study, sub-lethal concentrations of nitrate induce histopathologies in the esophagus, and especially in gills and b rain of cobia. The histopathological results found in the present study contribute to understanding the nitrate toxicity mechanism in fish. However, more studies are needed to determine safe nitrate levels for cobia cultured in RAS.

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Table 1 – Median lethal concentrations ($LC_{50}$) of nitrate-N to juvenile cobia *Rachycentron canadum*. Data in brackets represent the 95% confidence interval of the $LC_{50}$.

| Time (h) | $NO_3^-$-N (mg/L) |
|---|---|
| 12 | 2661 (2561 – 2764)[a] |
| 24 | 2407 (2236 – 2592)[ab] |
| 48 | 2071 (1929 – 2222)[bc] |
| 72 | 2028 (1888 – 2179)[c] |
| 96 | 1829 (1758 – 1903)[c] |

Values followed by different letters represent significant difference ($P<0.05$) after one-way ANOVA followed by Tukey´s test.

Figure 1. Photomicrography of gills of cobia *Rachycentron canadum* juvenile stained with haematoxylin-eosin. (A) Control treatment showing normal structure of gills (200×); (B) juvenile exposed to 1500 mg/L $NO_3^-$-N for 96 h, showing hyperplasia in the secondary lamella with complete lamellar fusion (arrow), and lamellar shorting (arrow head; 200×); (C) juvenile exposed to 1500 mg/L $NO_3^-$-N for 96 h, showing telangiectasia of lamella (arrow) (400×).

Figure 2. Photomicrography of esophagus of cobia *Rachycentron canadum* juvenile stained with haematoxylin-eosin. (A) Control treatment showing normal structure of esophagus (400×); (B) juvenile exposed to 2000 mg/L $NO_3^-$-N for 96 h, showing hyperplasia of esophagus epithelium (long arrow) and the hyperplasia of mucous cells (short arrow) (400×).

Figure 3. Photomicrography of brain of cobia *Rachycentron canadum* juvenile stained with silver stain technique. (A) Control treatment showing normal structure of brain (400×); (B) juvenile exposed to 1500 mg/L $NO_3^-$-N for 96 h, showing proliferation of the glial cells (○), satellitosis (□), Virchow-Robin space indicating a severe perivascular edema (long arrow) and cellular necrosis (short arrow) (400×).

**Fig. 1**

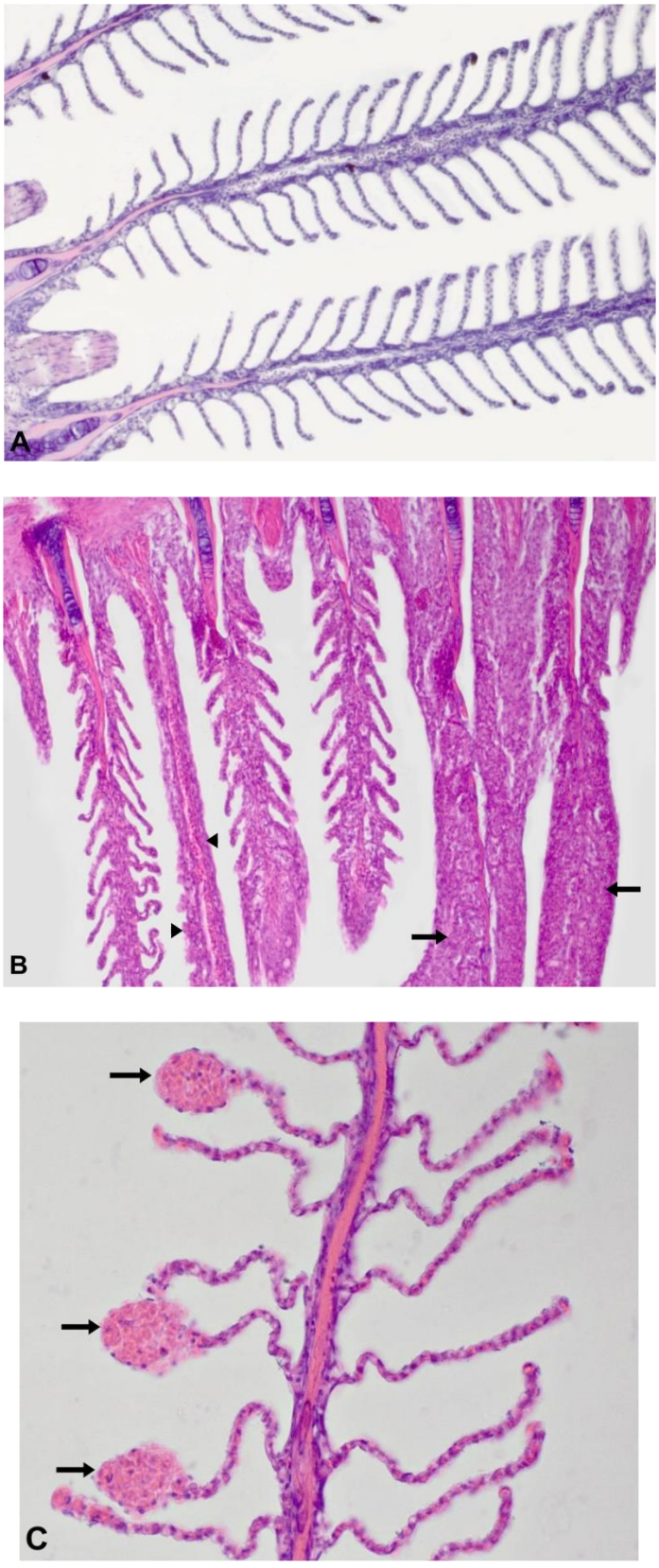


**Fig. 2**

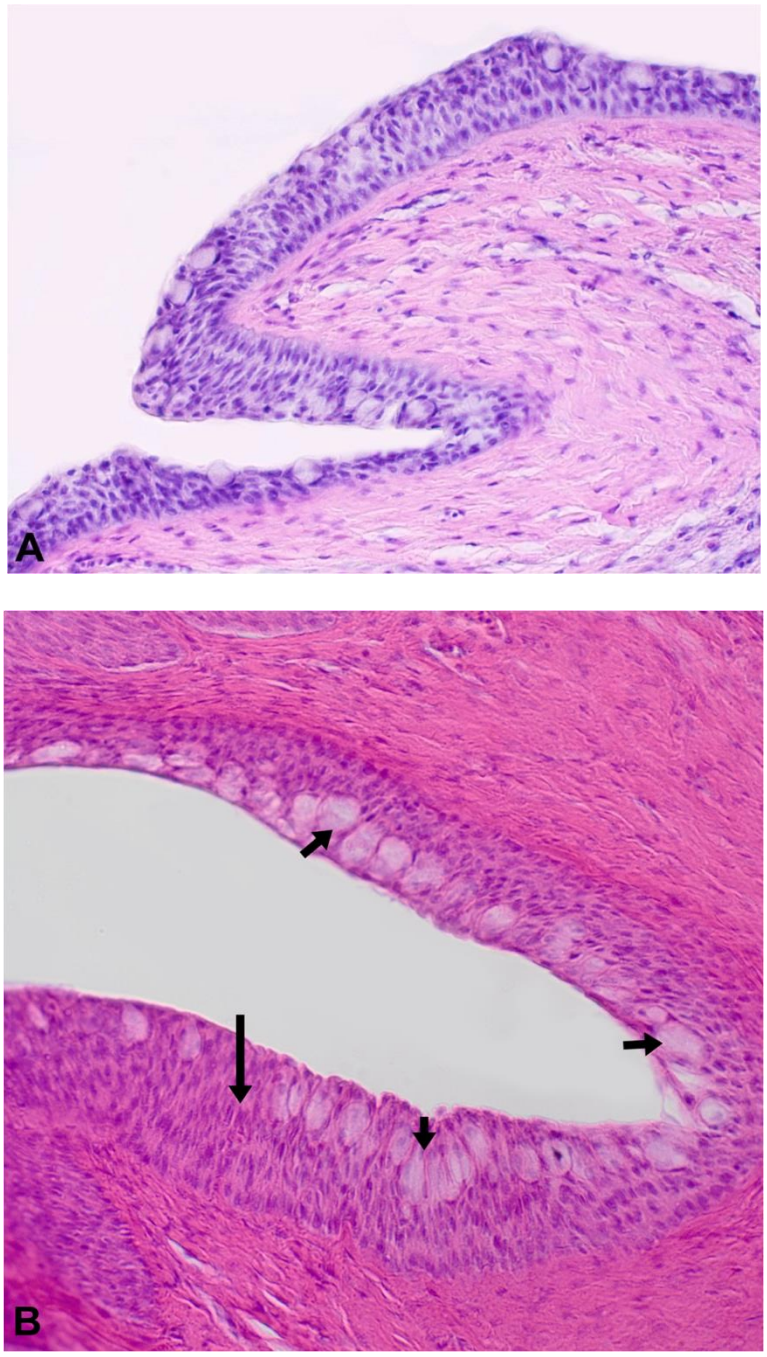
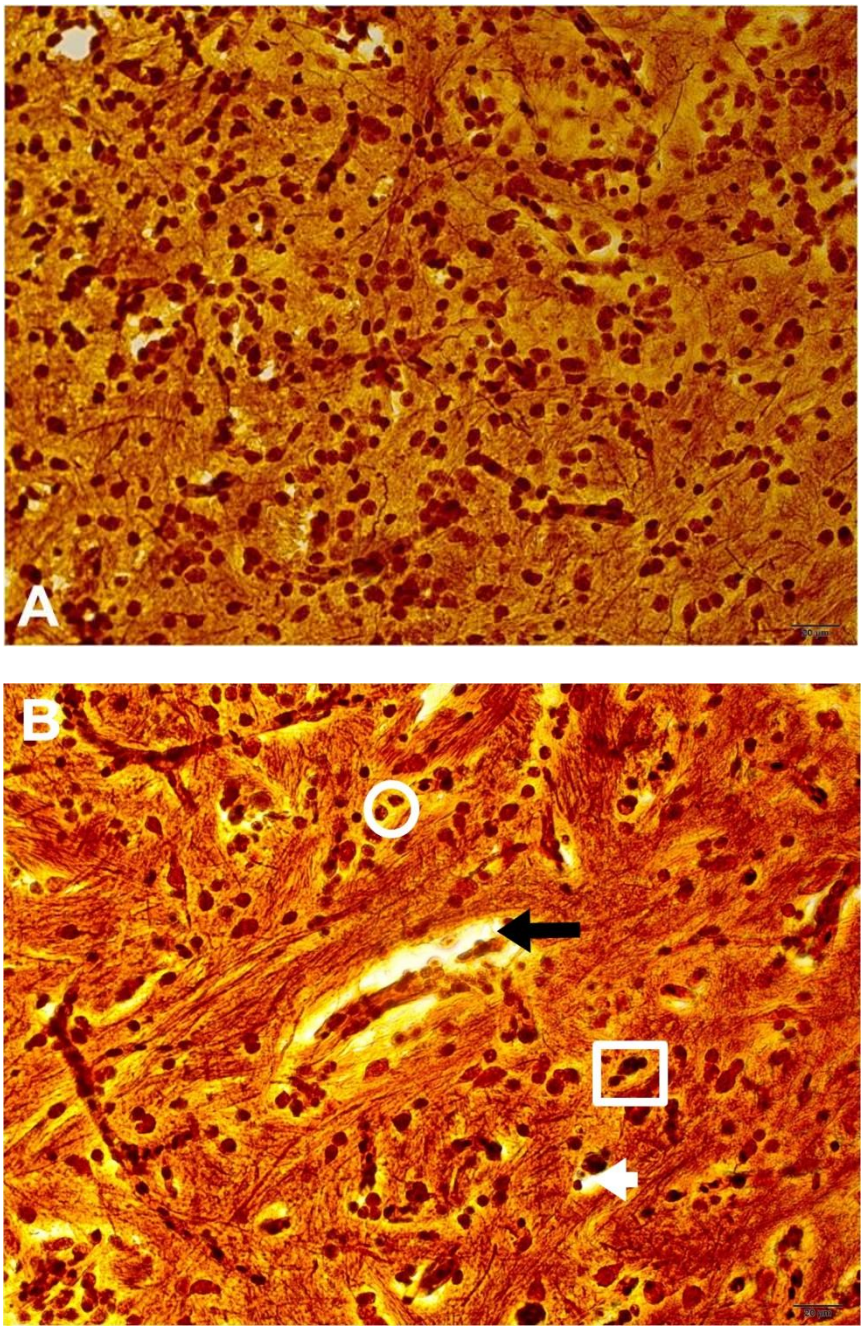


**Fig. 3**



# CAPÍTULO II

## ACUTE RESPONSES OF JUVENILE COBIA *Rachycentron canadum* TO ACID STRESS

Artigo submetido para a revista Aquaculture Research

**Acute responses of juvenile cobia *Rachycentron canadum* (Linnaeus 1766) to acid stress**

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**Abstract**

Fish are potentially submitted to water acidification when reared in recirculating aquaculture systems. The present study evaluated the responses of juvenile cobia *Rachycentron canadum* after acute exposure to acid water. Juvenile cobia (12.6 ± 0.5 g; 14.2 ± 0.2 cm) were acutely exposed to four pH levels (7.9 (control), 6.5, 6.0, and 5.5). After 24 h of exposure to different pH values, fish were sampled for physiological and histopathological evaluation. Acid water affected physiological parameters and induced morphological histopathologies on gill and skin of juvenile cobia, and these effects were more conspicuous with decreasing pH values. Acid stress induced blood acidosis in juvenile cobia, coupled to a decrease in bicarbonate ($HCO_3^-$) and saturated $O_2$ ($sO_2$) in fish blood. On the other side, hematocrit, hemoglobin, and glucose concentration increased their values ($P$<0.01) comparing to control level. Hyperplasia with completely fusion of secondary lamella was observed in all pH treatments (6.5. 6.0 and 5.5), while telangiectasia and proliferation of chloride cells was present for fish exposed to pH 6.0 and 5.5. In skin hyperplasia and hypertrophy of mucous cells, with necrosis of these cells for fish exposed to pH 6.0 and 5.5 was observed. The results of the present study demonstrate that acute acid water exposition affected physiology and histopathology in juvenile cobia, especially at pH values below 6.5. Accordingly, particular attention must be given to pH during cobia reared in recirculating aquaculture.

**Key-words:** pH, fish physiology, skin, gill, histopathology

## Introduction

The first report considering cobia, *Rachycentron canadum* (L), for aquaculture was in the early 1970s, when eggs were still collected from the wild (Hassler & Rainville 1975). However, mass production of cobia in cages began only during the 1990s in Taiwan (Liao, Huang, Tsai, Hsueh, Chang & Leaño 2004). Since then, cobia aquaculture has expanded around the world, mainly due to their rapid growth rates, excellent flesh quality and good adaptability to culture conditions (Liao *et al*. 2004). Nowadays, cobia is considered one of the species with the largest potential for marine fish for warm-water aquaculture expansion around the world (Benetti, O'Hanlon, Rivera, Welch, Maxey & Orhun 2010).

Cobia has mainly been produced in off-shore and near-shore cages (Liao *et al*. 2004; Benetti *et al*. 2010; Sampaio, Moreira, Miranda-Filho & Rombenso 2011). However, cobia has also been considered for intensive inland production, especially in recirculating aquaculture systems (RAS) (Holt, Faulk & Schwarz 2007). However, water quality deterioration can occur in intensive rearing conditions, in particular due to the accumulation of fish metabolites, such as ammonia and carbon dioxide (Timmons & Ebeling 2010). In RAS, the oxidation of ammonia during the nitrification process and the interaction of carbon dioxide with the water tend to acidify the environment by the liberation of hydrogen ions (Timmons & Ebeling 2010).

The main effect of acid water on fish is related to ion regulation that induces a subsequent variety of physiological responses (Fromm 1980; Milligan & Wood 1982; Dockray, Morgan, Reid & Wood 1998; Parra & Baldisserotto 2007). Additionally, fish exposed to acid water also present histopathological changes, especially in gills and skin of fish (Iger & Wendelaar Bonga 1994; Iger, Balm & Wendelaar Bonga 1994) caused by the direct and permanent contact of these organs with water. Therefore, due to its

characteristics, gills and skin are suitable biomarkers to assess aquatic pollution (Bernet, Schmidt, Meier, Burkhardt-Holm & Wahli 1999).

Acute and chronic effects of acid waters have been determined for a number of freshwater fish, with special attention to salmonids (Milligan & Wood 1982; Dockray et al. 1998). On the other hand, little is known related to acid tolerance for marine fish. The 96 h LC50 of acid water to Brazilian flounder *Paralichthys orbignyanus* (Valenciennes) was estimated to be 4.40 (Wasielesky, Bianchini, Santos & Poersch 1997). While, Abbink, Garcia, Roques, Partridge, Kloet & Schneider (2011) describe that pH must be maintained higher than 7.16 to prevent acidity related consequences in juvenile yellowtail kingfish *Seriola lalandi* (Valenciennes) reared in RAS. In a previous study, Sampaio, Santos, Delbos & Schwarz (2008) showed that juvenile cobia growth was reduced when this species was reared in pH 6 and 5 when compared to pH 7 or 8. However, there is no information in the literature related to the abrupt effects of acid environment, and their effects on cobia. Accordingly, the purpose of the present study was to evaluate the physiological and histopathological stress responses after acute acid exposure on juvenile cobia.

**Material and Methods**

Juvenile cobia were obtained from a commercial hatchery (Camanor Produtos Marinhos, Brazil) and air transported to the Federal University of Rio Grande, Rio Grande, Brazil, where the experiment was conducted. Prior to the experiment, juvenile cobia were acclimated to experimental conditions for 2 weeks in a 300 L tank coupled to a RAS. During the acclimation period fish were hand fed a commercial diet (NRD, INVE, Grantsville, UT, USA) 4 times per day, and the water quality was determined to be: temperature 26.5 ± 0.5 °C; pH 7.9 ± 0.1; minimum dissolved oxygen 5.8 mg/L;

maximum total ammonia nitrogen (TAN) 0.5 mg/L; maximum nitrite nitrogen ($NO_2^-$-N) 0.8 mg/L; nitrate above 5 mg/L $NO_3^-$-N, and salinity was maintained at 29 ± 0.5 ‰.

Juvenile cobia (12.6 ± 0.5 g; 14.2 ± 0.2 cm) were acutely exposed to four pH levels (7.9(control), 6.5, 6.0 and 5.5) during 24 h. The test pH levels and the controls were all conducted with three replicates each (3 fish/tank; 9 fish/treatment) in independent static systems comprised of 50 L tanks. The desired pH level was obtained from a stock solution made with hydrochloric acid 20%. During the exposure time, pH values and fish behavior were observed every 2 hours.

Temperature was maintained at 26 ºC and salinity at 29 ‰. Fish were not fed one day prior to and throughout the 24 h of experiment. After 24 h of exposure to different pH levels, all juvenile cobia were euthanized in a benzocaine bath (100 mg/L). Blood samples were collected from the caudal vein using heparinized 1 mL syringe. Immediately after sampled, the whole blood was analyzed using an i-STAT Portable Clinical Analyzer (Abbott Laboratories, Chicago, IL, USA). The i-STAT analyzer was used with an $CG8^+$ cartridge measuring sodium ($Na^+$), potassium ($K^+$), ionized calcium ($Ca^+$), pH level, hemoglobin, hematocrit, partial gas pressure of $CO_2$ ($PCO_2$), $O_2$ ($PO_2$), saturated $O_2$ ($sO_2$) and displaying calculated values of blood bicarbonate ($HCO_3^-$). Values for pH, $pCO_2$ and $HCO_3^-$ were temperature-corrected to experimental temperature according to the manufacture´s specifications. The efficacy of i-STAT measurements has been proved for several fish species (Cooke, Suski, Danylchuk, Danylchuk, Donaldson, Pullen, Bulte, O'Toole, Murchie, Koppelman, Shultz, Brooks & Goldberg 2008; Kristensen, Rosseland, Kiessling, Djordevic & Massabau 2010; Paust, Foss & Imsland 2011). Lactate was determined using a portable meter (Accutrend® Plus, Roche, Germany).

After blood samples were taken, fish were dissected and samples of gill (gills arches from the right side) and skin (from the dorsal part of the head) were fixed during 24 h in Bouin solution and preserved in 70% ethanol for subsequent histopathological evaluation. The samples were dehydrated in a graded series of ethanol, embedded in regular paraplast (Sigma-Aldrich, St. Louis, MO, USA), sectioned (5 μm) and the slides were stained with hematoxylin and eosin. Slides were examined under light microscopy (Olympus BX 45, Center Valley, PA, USA) and the images were registered with a digital camera (Olympus DP 72, Center Valley, PA, USA). Chloride cells densities were obtained from 3 randomly selected histological fields of branchial tissue per fish (n=9). Chloride cell density (cell number $mm^{-2}$) was analyzed using the software Image J 1.45e (National Institutes of Health, USA).

Temperature and dissolved oxygen concentration were measured with a YSI Model 550A meter (Yellow Springs Instruments, Yellow Springs, OH, USA), salinity with a hand refratometer (Atago, S/Mill, Japan), and the pH was measured with a YSI Model 60 meter (Yellow Springs Instruments, Yellow Springs, OH, USA). TAN and nitrite were determined according to Solorzano (1969) and Bendschneider & Robinson (1952) respectively. Alkalinity was measured according to APHA (1998).

Statistical analyses were conducted using STATISTICA 7.0. Normality was verified by the Kolmogorov-Smirnov test, and homogeneity of variances was verified using Levene´s test. The effects of acute exposure of pH on juvenile cobia were analyzed using one-way ANOVA, followed by Duncan´s multiple-range test. Significance level was taken as $P<0.05$. The percentage values were transformed (square root arcsine) prior to the analysis (Bhujel 2008), but only original data are presented. All results are shown as average ± standard error of means.

**Results**

It was observed that alkalinity was closely related to the tested pH values, and they were significantly different among treatments ($P<0.01$). The other water quality parameters presented no differences among treatments ($P>0.05$) (Table 1). No mortality was observed during the experimental time. The blood pH values decreased accordingly to the environmental pH and they were significantly different among treatments ($P<0.05$). Similar responses were also observed for blood bicarbonate ($HCO_3^-$), partial gas pressure $O_2$ ($PO_2$), and saturated $O_2$ ($sO_2$). However, opposite results were observed for hematocrit, hemoglobin, and glucose concentration, their values increased significantly ($P<0.01$) with pH decrease (Table 2).

Control treatment did not show any histopathological changes in gill and skin of cobia (Fig. 1A and 3A). The acid environment negatively affected gills and skin histology. The damage observed increased accordingly to the pH reduction. The gills of fish exposed to all pH treatments (except control) showed hyperplasia of interlamellar epithelium with fusion of secondary lamella (Fig. 1B). Telangiectasia was observed in fish acutely exposed to pH 6.0 and 5.5 (Fig. 1C). However, hyperplasia and hypertrophy of chloride cells were observed only in fish exposed to pH 5.5 (Fig. 1D). The significant increase ($P<0.05$) in chloride cells densities was observed accordingly to pH reduction. Control treatment presented ($16.6 \pm 0.8$ cell $mm^{-2}$), and cobia exposed to pH 5.5 ($26.1 \pm 1.6$ cell $mm^{-2}$) (Fig. 2).

Skin of fish exposed to acid water presented hyperplasia and hypertrophy of mucous cells, comparing to control (Fig. 3B), and these alterations were more conspicuous with pH decreasing. Necrosis of mucous cells was observed in pH values of 6.0 and 5.5 (Fig. 3C).

**Discussion**

The present results demonstrate that survival of juvenile cobia acutely exposed to acid water (down to pH 5.5) during 24 h was not affected. However, blood physiology was substantially affected. There were also several histopathological effects on gills and skin of cobia.

Acid water induced blood acidosis on juvenile cobia, followed by a reduction in bicarbonate concentration in the blood. The reduction in bicarbonate is a response aiming to neutralize the excess of hydrogen ion on blood. This process induces an increase in blood $CO_2$ concentration, which could have been eliminated in the present study through the gills (for review see Perry & Gilmour 2006), because a hypercapnia was not observed in fish exposed to acid water. In response the oxygen saturation decreases in fish blood. These results could be explained by Bohr effects (Withers 1992) where the affinity of hemoglobin for carrying oxygen capacity is affected in the present situation. In response to reduction of saturated oxygen concentrations in fish blood, an elevation in hemoglobin values was observed, in an attempt to increase the oxygen carrying capacity.

The higher hematocrit observed was also observed in juvenile brook trout *Salvelinus fontinalis* (Mitchill) (Dively, Mudge, Neff & Anthony 1977), rainbow trout *Oncorhynchus mykiss* (Walbaum) (Milligan & Wood 1982, and for three Indian major carps (*Catla catla* (Hamilton), *Labeo rohita* (Hamilton), and *Cirrhinus mrigala* (Hamilton) (Das, Ayyappan & Jena 2006) exposed to acid water. These results could be explained by reduction in plasma volume, probably associated to erythrocytes swelling, and increase in erythropoiesis in response to tissue hypoxia induced by acid stress (Fromm 1980). The hypoxia condition could be observed by reduction in oxygen

saturation on fish blood, whereas no increase of lactate concentrations in blood plasma was observed.

The elevation in plasma glucose level is the most frequently assessed indicator of the secondary effects of stress in fish (Wendelaar Bonga 1997). The glucose level observed was directly affected by acute acid exposure, which expressed a two-fold increase in glucose level after 24 h of exposition to pH 5.5. Increase in glucose level was also observed for *C. catla*, *L. rohita*, and *C. mrigala* exposed during 21 days to acid water (Das *et al*. 2006). Similar responses in elevations of blood glucose levels seen in the present study were also described for cobia submitted to acute stress challenges (Cnaani & MacLean 2009; Trushenski, Schwarz, Takeuchi, Delbos & Sampaio 2010).

Acid water can damage gills, resulting in ionic imbalance. A slight increase was observed only in $Na^+$ concentration in fish exposed to acid stress. The $Na^+$ increases could be attributed to the $Na^+/H^+$ exchanger and/or via a $Na^+$ channel linked to a V-type $H^+$-ATPase, which in salt water fish is obviously favorable in the apical membrane of chloride cells (Perry & Gilmours 2006).

Gills are considered the primary target for water contaminants (Fernandes & Mazon 2003), and structural changes in fish gills might be used as a criterion for acceptable water quality (Lease, Hansen, Bergman & Meyer 2003). Some reports have shown that exposure of fish to acid water induces gill histopathologies (Iger & Wendelaar Bonga 1994). The histopathologies found in the present study, like hyperplasia of the interlamellar epithelium with fusion of secondary lamella, increase its thickness, thus minimizing the potential contact between the environment and blood, protecting the fish against the stressor (Mallat 1985). Nevertheless, these histopathologies are not toxicant-specific and were firstly observed for juvenile cobia acutely exposed to nitrate (Rodrigues, Schwarz, Delbos, Carvalho, Romano & Sampaio

2011). Thus, the morphological alterations found in the present study can affect gas exchange capabilities of cobia, especially at the lowest pH tested (5.5). However, fish exposed to acid water could increase their ventilation rate, to compensate low oxygen uptake, $CO_2$ excretion or ionoregulation imbalance (Fernandes & Mazon 2003). This behavior was observed for brook trout *S. fontinalis* (Dively *et al*. 1977) and Brazilian flounder *Paralichthys orbignyanus* (Wasielesky *et al*. 1997) acutely exposed to acid water. In the present study, cobia probably increasing the excretion of $CO_2$ across gills; nevertheless it was not enough to maintain the saturated oxygen values. Acid water also induced proliferation of chloride cells, probably trying to increase $HCO_3^-$ uptake via $Cl^-/HCO_3^-$ carrier mechanism in the apical membranes of chloride cells (Perry & Gilmours 2006). The increase on chloride cells number may also be due to a mechanism to compensate inhibition of the $Cl^-/HCO_3^-$ pump. However, this mechanism was not enough to increase bicarbonate concentration in fish blood. Similar response in proliferation of chloride cells were observed for tilapia *Oreochromis mossambicus* acutely and gradually exposed to acidified water (Wendelaar Bonga, Flik, Balm & Van der Meij 1990). These authors observed a two-fold and five-fold increase in chloride cells after 2 and 42 days, respectively, after acid water exposition, coupled to a higher turn-over of these cells along the experimental period.

The skin of fish is a metabolic active tissue representing an important protective barrier between the organism and the water. It also reacts quickly against a wide variety of stressors, including acidified water (Iger *et al*. 1994). Hyperplasia and hypertrophy of mucous cells in the skin of cobia represent enhanced mucous production which was observed in fish kept in water with decreasing pH levels, representing a protective response of cobia against acid environment. Similar responses of stimulation of mucous secretion were also observed for juvenile *Salmo trutta trutta* (Linnaeus)

(Ledy et al. 2003), *O. mykiss* (Iger *et al*. 1994), and *Cyprinus carpio* (Linnaeus) (Iger &Wendelaar Bonga 1994) exposed to acid waters.

Acute exposure to acid water negatively influences blood physiology and histopathology of juvenile cobia, especially at pH values below 6.5. These effects were more evident as the pH was further reduced. Consequently, particular attention must be given to pH during cobia reared in recirculating aquaculture systems.

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Figure 1. Gill sections of juvenile cobia *Rachycentron canadum*. (A) Gill of cobia in the control treatment showing normal structure (400×); (B) gill of cobia exposed to pH 5.5 showing hyperplasia of interlamellar epithelium with fusion of secondary lamella (400×); (C) secondary lamella with telangiectasia (arrow) in a fish acutely exposed to pH 6.0 (400×); (D) hyperplasia and hypertrophy of chloride cells in juvenile cobia acutely exposed to pH 5.5 (400×).

Figure 2. Chloride cell density in gills of cobia *Rachycentron canadum* exposed for 24 h to different pH. Different letters indicate significant differences ($P<0.05$) among treatments.

Figure 3. Skin sections of juvenile cobia *Rachycentron canadum*. (A) Skin of cobia in the control treatment showing normal structure (400×); (B) skin of fish exposed to pH 5.5 during 24 h showing hyperplasia and hypertrophy of mucus cells (400×); (C) necrosis of mucus cells in fish exposed to pH 5.5 (arrow) (400×);

Table 1 – Water quality parameters (mean±SE) during the evaluation of acute exposure to low pH in juvenile cobia *Rachycentron canadum*. Different letters at the same line represent significant differences among pH treatments ($P<0.05$).

| Parameter | pH | | | |
|---|---|---|---|---|
| | 7.9 | 6.5 | 6.0 | 5.5 |
| pH | 7.9±0.02[a] | 6.50±0.04[b] | 6.02±0.01[c] | 5.49±0.03[d] |
| Dissolved oxygen (mg/L) | 6.35±0.04 | 6.24±0.04 | 6.24±0.04 | 6.27±0.05 |
| TAN (mg/L $NH_4^+$+$NH_3$-N) | 0.4±0.03 | 0.53±0.03 | 0.61±0.01 | 0.56±0.04 |
| $NH_3$-N mg/L | 0.017±0.003 | 0±0 | 0±0 | 0±0 |
| Temperature (ºC) | 25.9±0.1 | 25.7±0.1 | 25.7±0.1 | 25.7±0.1 |
| Salinity | 29±0 | 29±0 | 29±0 | 29±0 |
| Alkalinity (mg/L as $CaCO_3$) | 136.7±1.7[a] | 21.7±1.7[b] | 13.3±1.7[c] | 5±0[d] |

Table 2 – Blood parameters (mean±SE) of juveline cobia *Rachycentron canadum* exposed for 24 h to different pH. Different letters at the same line represent significant differences among pH treatments ($P<0.05$).

| Treatment | pH | | | |
|---|---|---|---|---|
| | 7.9 | 6.5 | 6.0 | 5.5 |
| pH | 7.28±0.02$^{a}$ | 7.07±0.03$^{b}$ | 7.00±0.04$^{bc}$ | 6.94±0.02$^{c}$ |
| $pCO_2$ (mm Hg) | 7.11±0.18$^{b}$ | 8.63±0.43$^{a}$ | 7.57±0.30$^{b}$ | 6.73±0.27$^{b}$ |
| $HCO_3^-$ (mmol/L) | 3.33±0.13$^{a}$ | 2.54±0.22$^{b}$ | 1.89±0.11$^{c}$ | 1.45±0.10$^{c}$ |
| $pO_2$ (mm Hg) | 45.2±7.4 | 40.4±7.8 | 33.5±7.7 | 27±4.4 |
| $sO_2$ (%) | 98.6±0.8$^{a}$ | 92.1±2.9$^{a}$ | 83.5±9.0$^{a}$ | 73.1±10.2$^{b}$ |
| Hemoglobin (mmol/L) | 6.52±0.59$^{b}$ | 7.6±0.42$^{ab}$ | 8.47±0.30$^{a}$ | 8.15±0.52$^{a}$ |
| Hematocrit (%) | 19.2±1.7$^{b}$ | 22.4±1.2$^{ab}$ | 25±0.9$^{a}$ | 24±1.5$^{a}$ |
| Glucose (mg/dL) | 61.2±3.3$^{b}$ | 71.8±7.4$^{b}$ | 82.9±7.8$^{ab}$ | 120.8±24.9$^{a}$ |
| Lactate (mmol/L) | 2.2±0.26 | 2.7±0.19 | 2.26±0.15 | 2.12±0.17 |
| $Na^+$ (mmol/L) | 165.6±0.9$^{b}$ | 173±1.6$^{a}$ | 169.7±0.9$^{ab}$ | 170.5±2.1$^{a}$ |
| $K^+$ (mmol/L) | 4.62±0.15 | 4.77±0.43 | 4.81±0.21 | 4.28±0.13 |
| $Ca^{+2}$ (mmol/L) | 0.99±0.14 | 1.23±0.13 | 1.22±0.07 | 1.16±0.11 |

Fig. 1

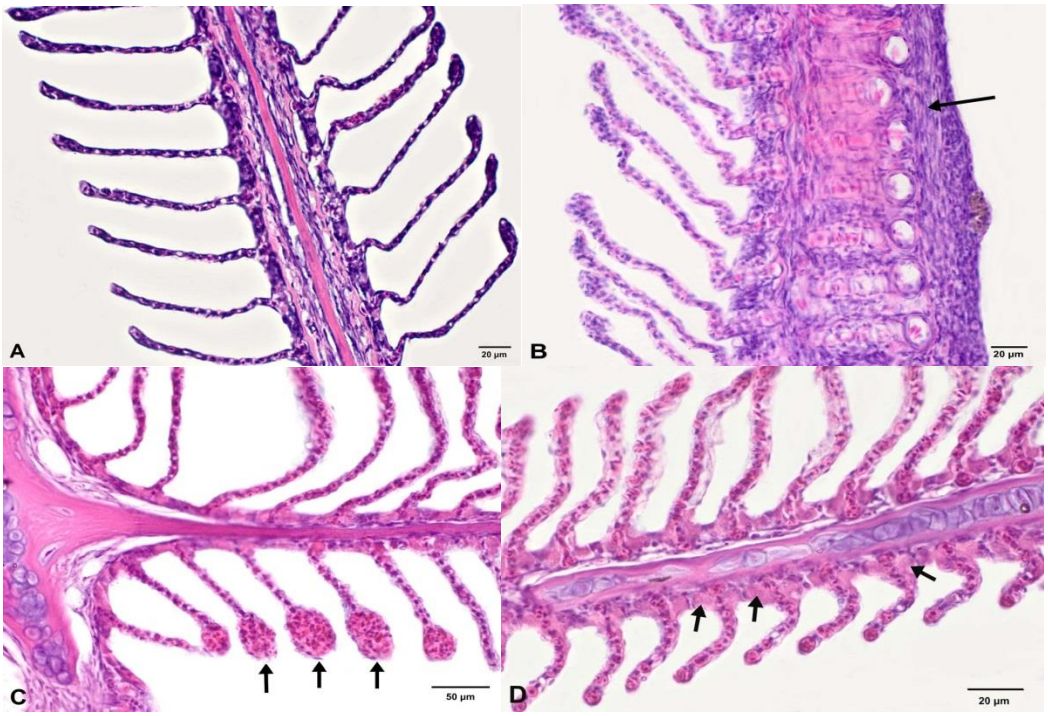


Fig. 2

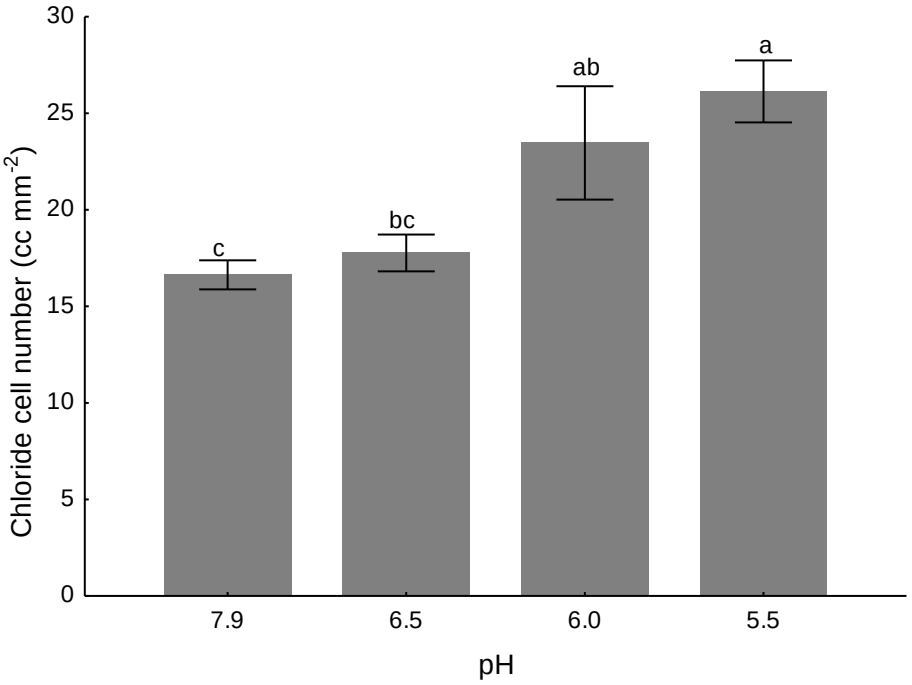
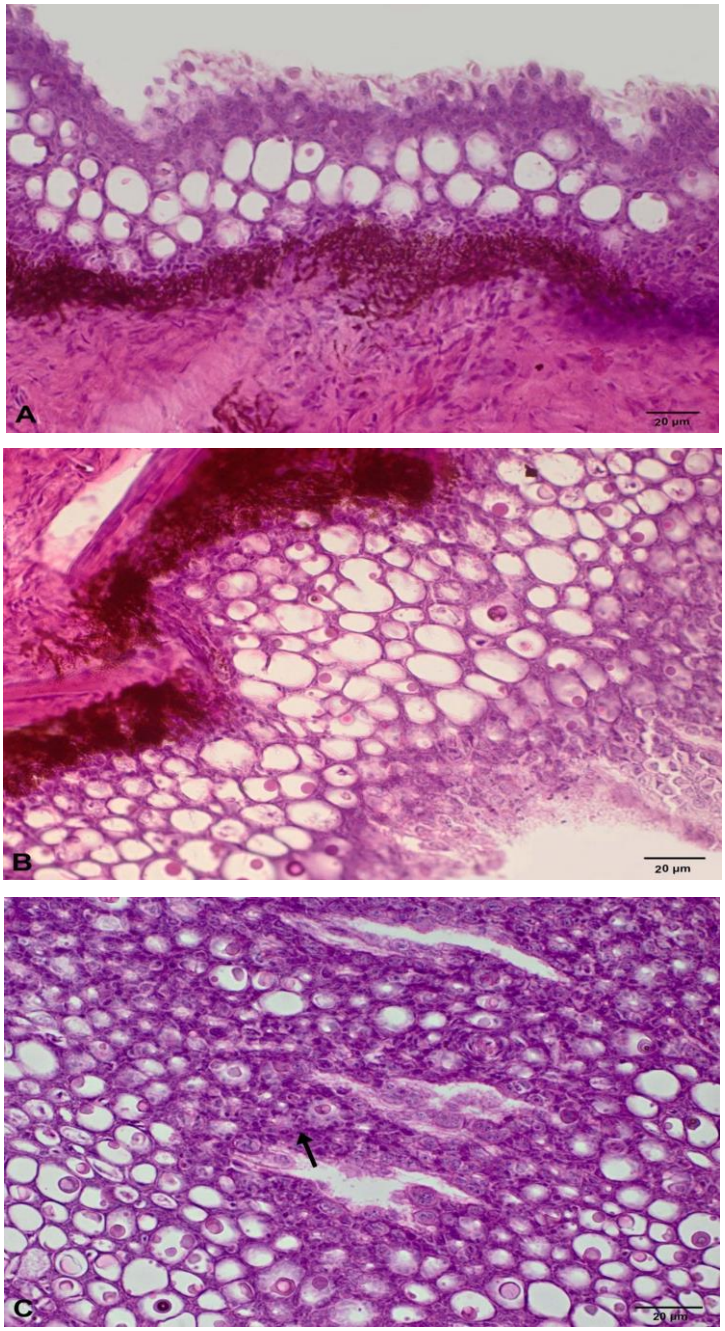

30
25
20
15
10
5
0
Chloride cell number (cc mm-2)
c
bc
ab
a
7.9
6.5
6.0
5.5
pH

Fig. 3



## CAPÍTULO III

# CHRONIC LOW PH AND HIGH NITRATE EXPOSURE ON COBIA *Rachycentron canadum* JUVENILE REARED IN RECIRCULATING AQUACULTURE SYSTEM

Artigo nas normas da revista Aquaculture

**Chronic low pH and high nitrate exposure on cobia *Rachycentron canadum* juvenile reared in recirculating aquaculture system**

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**Abstract**

The aim of the present study was evaluate the chronic effects of chronic low pH and high nitrate on juvenile cobia. Fish (~50 g, 20 cm) were stocked in independent RAS (10 fish/tank, 3 tanks/treatment) and reared under eight treatments: fish were exposed to 0, 100, or 200 mg $L^{-1}$ $NO_3^-$-N at a pH of 6.5 or 8.0 and fed to apparent satiation. Two other treatments evaluated the relationship of growth with feed intake at either low pH or high nitrate concentration. The two groups were kept at pH 8.0 with no nitrate. One group was offered the same amount of food consumed by fish reared at pH 8.0/ 200 mg/L $NO_3^-$-N. Another group of cobia, was fed on the same amount of food voluntarily consumed by fish exposed to pH 6.5 and no nitrate. Survival of fish was reduced in 10% only in fish exposed to pH 6.5/ 200 mg/L $NO_3^-$-N. After 42 days, growth performance was affected by nitrate concentration but not pH. Chronic nitrate exposure yielded significantly reduced weight gain, SGR, and K, and elevated FCR values. Feed intake was generally reduced among fish exposed to nitrate, though differences were not statistically significant in all cases. Plasma glucose was generally reduced among fish exposed to nitrate and those cultured at pH 6.5. Plasma lactate and lysozyme activity were reduced among fish exposed to nitrate at either low pH. However, there was no influence of pH and nitrate on cortisol and osmolality concentration. Cobia exposed to nitrate presented hyperplasia and telangectasia in the secondary lamella. We conclude that cobia could be reared in pH 6.5 with no nitrate. However, special attention must be given with nitrate in this pH. In addition, concentration of 100 mg/L $NO_3^-$-N or higher hampers growth of cobia even in pH 6.5 or 8.

**Key-words:** nitrate toxicity, physiology, histopathology

## 1. Introduction

Cobia, *Rachycentron canadum*, is a pelagic, carnivorous fish with a worldwide distribution in tropical and subtropical seas (Shaffer and Nakamura, 1989). It has been cultured in Asia since the 1990s (Liao et al., 2004), and in recent years, it has emerged as culture species in the Americas (Benetti et al., 2008; Sampaio et al., 2011). Cobia are noted for their exceptionally high growth rate, and can grow from larvae to 4-6 kg in one year (Chou et al., 2001). Beyond rapid growth rates, cobia exhibit many other characteristics that make them well-suited to intensive culture, such as volitional spawning and high fecundity in captivity (Arnold et al., 2002; Benetti et al., 2008), high survival rate after weaning (MacLean et al., 2009), high protein and lipid conversion efficiencies (Craig et al., 2006), excellent flesh quality, and high market value (Shiau, 2007).

Cobia have been typically reared in off-shore and near-shore cages (Liao et al., 2004; Benetti et al., 2010; Sampaio et al., 2011). However, increasing interest in land based aquaculture, has led researchers to focus on refining methods to rear cobia in recirculating aquaculture systems (RAS) (Resley et al., 2006; Holt et al., 2007). These systems are attractive in that they are more biosecure and use less water than other production systems, they can be located anywhere, and offer year-round rearing of fish under optimal conditions. Unfortunately, continuous water reuse results in the accumulation of metabolites that may be toxic to fish. Nitrogenous waste is excreted by fish in the form of ammonia, which must be processed or removed from the system. Commonly, this is accomplished by nitrifying bacteria which occupy biofiltration media and transform ammonia to less-toxic nitrite and the resultant nitrite to comparatively non-toxic nitrate. Although nitrate is generally considered to be non-toxic, in RAS, nitrate can accumulate to extremely high levels and may negatively affect fish

(Timmons and Ebeling, 2010 2007). Acute toxicity of nitrate has been studied for a few fish species (Pierce et al., 1993; Poersch et al., 2007; Hamlin et al., 2006), including cobia (Rodrigues et al., 2011). The median lethal concentration ($LC_{50}$-96 h) of nitrate-N was estimated in 1,829 mg/L $NO_3^-$-N for juvenile cobia (Rodrigues et al., 2011). Unfortunately, there is very little information available about the effects of chronic nitrate exposure in fish, though the information available suggests that growth suppression may be a consequence of long-term exposure (Van Bussel et al., 2012). The nitrification of ammonia and the interaction of carbon dioxide with water tend to acidify the environment by the liberation of hydrogen ions (Timmons and Ebeling, 2010). However, in order to reduce cost with buffers and the actual better performance of the bio filters, pH values in RAS are routinely maintained below typical seawater levels (Abbink et al., 2011). In a preliminary investigation, it has been shown that cobia growth is hampered at pH 6.0 when compare to pH 7 and 8 (Sampaio et al., 2008). Thus, it is important to determine the minimum pH at which cobia can be reared.

Although the acute toxicity of ammonia, nitrite and nitrate are known for juvenile cobia (Atwood et al., 2004; Rodrigues et al., 2007; Barbieri and Doi, 2011; Rodrigues et al., 2011) and many other aquatic species, in general, nitrate toxicity is relatively poorly understood (Camargo et al., 2005). The effects of long-term exposure and the influence of other environmental parameters such as pH, have not been well-characterized for cobia or other aquatic taxa (Camargo et al., 2005). Given the interest in culturing cobia in RAS and the associated likelihood of long-term exposure to elevated nitrate concentrations under varying environmental conditions, we evaluated the effects of chronic nitrate exposure under different pH conditions on growth and physiological condition of juvenile cobia.

## 2. Material and Methods

### *2.1 Fish and experimental system*

Newly hatched cobia larvae (Troutlodge Marine Farms, Vero Beach, Florida, USA), were shipped to the Virginia Seafood Agricultural Research and Extension Center (VSAREC; Hampton, VA, USA). The larvae were reared according to standard VSAREC larviculture protocols (McLean et al., 2009) until they reached the size desired for the experiment (~50 g, ~20 cm total length).

Eight individual RAS were used to create the environmental conditions desired for each experimental treatment. Each RAS comprised three culture tanks (300 L) and a reservoir (370 L) that received effluent water from the tanks and also served as a fluidized bed biofilter (Kaldnes® biological filtration media; R&B Aquatics, Waring, TX, USA). From the reservoir, water was pumped via a 1/3 HP pump (AmpMaster 5600/4700, Pensacola, USA) to a bubble-washed bead filter (BBF-XS4000, Aquaculture Systems Technologies, New Orleans, USA) for solids removal, continued through an UV sterilizer (025080, Emperor Aquatics, Pottstown, USA), and then proceeded to the distribution manifold. This distribution manifold was valved to each of the three culture tanks, as well as back to the reservoir. Bead filters were backwashed daily to remove solids and maintain a consistent 10% daily water exchange. On each reservoir, as a closed side-loop, a magnetic drive pump (4-MDQ-SC, Little Giant, Oklahoma City, USA) was connected to a double venture protein skimmer (TF300, Top Fathom, Hudsonville, USA) for removal of fine suspended solids and dissolved organics. pH control was achieved through high turnover rates between culture tanks and the RAS (1800 L/h), plus constant monitoring and control within the reservoir. To achieve this, systems (pH 6.5) were configured with a dosing pump with an integrated

pH controller (Hanna Instruments, BL 7916-1 model, Rubano, Italy), connected to an independent 50 L carboy containing muriatic acid (31.45% HCl, Transchem Inc., Port Allen, USA) diluted 10 fold with fresh water. The resultant 3.1% HCl solution was automatically injected based upon pre-determined pH digital set points (± 0.1) to maintain experimental parameters. Sodium bicarbonate was added daily to systems to keep pH 8. Experimental nitrate concentrations were carried out using sodium nitrate.

The makeup water for the RAS came from Chesapeake Bay water adjacent to the VSAREC facility. The seawater was sterilized with 20 mg/L chlorine and maintained under continuous circulation through a sand filter (L190, Jacuzzi Brothers, Little Rock, AR, USA) and a diatomaceous earth filter. Prior to use the seawater was dechlorinated with sodium thiosulfate.

Fish were stocked in the culture tanks (10 fish/tank) and acclimated to the experimental tanks for two weeks before weighing and measuring the fish (fish were sedated prior to handling in a bath of 100 mg/L tricaine methanesulfonate [MS 222]; Tricaine-S, Western Chemical Inc., Ferndale, Washington, USA) and formally initiating the trial. Throughough the acclimation and experimental periods, the photoperiod was maintained at 18 h of light per day. Fish were hand-fed a commercial diet (Zeigler Gold, 5mm pellet: 42% protein, 16% lipid, Zeigler Bros. Inc., Gardners, PA, USA) twice a day (0900 and 1800 h).

*2.2 Experimental design*

Groups of fish were exposed to one of six different water chemistry treatments for 42 days. Triplicate tanks of fish were exposed to 0, 100, or 200 mg/L $NO_3^-$-N at a pH of 6.5 or 8.0 and fed to apparent satiation. Recognizing that nitrate and pH could affect physiologically as well as by altering feeding behavior (and therefore growth

performance), two additional groups of fish were maintained as reference or "feeding control" groups: three tanks of fish were kept at pH 8.0 with no nitrate (0 mg/L $NO_3^-$-N) and were offered the same amount of food consumed by fish in the pH 8.0/200 mg/L $NO_3^-$-N treatment ($NO_3$ Feeding Control), and three tanks of fish were kept at pH 8.0 with no nitrate and were offered the same amount of food consumed by fish in the pH 6.5/ 0 mg $L^{-1}$ $NO_3$-N treatment (pH Feeding Control).

Every 2 weeks and at the end of the trial, all fish were anaesthetized using 100 mg/L MS-222 measured and weighed, and feed consumption data were recorded on a daily basis throughout the trial. Production performance metrics were calculated as follows:

$$\text{Weight Gain (\%)} \frac{\text{(average individual final weight - average individual initial weight)}}{\text{average individual initial weight}}$$

$$\text{Feed Conversion Ratio (FCR)} = \frac{\text{average individual feed intake}}{\text{average individual weight gain}}$$

$$\text{Specific Growth Rate (SGR, \% body weight/day)} = 100 \times \frac{\log_e \text{average individual final weight} - \log_e \text{average individual initial weight}}{\text{days of feeding}}$$

$$\text{Feed Intake (\% body weight/day)} = 100 \times \frac{\text{average individual feed intake}}{(\text{average individual initial weight} \times \text{average individual final weight})^{0.5} / \text{days of feeding}}$$

$$\text{Condition Factor (K)} = \frac{\text{average individual final weight (g)}}{\text{average individual final length (cm)}^3}$$

Temperature, salinity, and dissolved oxygen (DO) were measured daily using the YSI 550A meter (Yellow Springs Instruments, Yellow Springs, USA), whereas pH was measured twice daily with a YSI pH100 meter (Yellow Springs Instruments,

Yellow Springs, USA). Total ammonia nitrogen (TAN), nitrite nitrogen, and nitrate nitrogen were determined daily via spectrophotometric analysis (methods 10031, 8153 and 8039; DR 2010 spectrophotometer; Hach Company, Loveland, CO, USA). Unionized ammonia nitrogen ($NH_3$-N) levels were determined according TAN, temperature, salinity and pH values using the equations of Ostrensky et al. (1992) adapted from Whitfield (1974) and Bower and Bidwell (1978).

*2.3 Blood chemistry analyses*

At the end of the experiment (day 42), 3 fish per tank were randomly sampled and immediately anesthetized with MS-222 (100 mg/L). Blood samples were collected from the caudal vasculature using heparinized, evacuated blood collection assemblies (Vacutainer®; Becton Dickinson and Co., Franklin Lakes, New Jersey, USA) and stored on ice prior to analyses. Subsamples of whole blood were used for the determination of glucose and lactate using portable meters (Rite Aid, USA and Lactate Plus, Nova Biomedical, Waltham, MA, USA) according to Trushenski et al. (2010). Remaining blood was centrifuged (Model 5417 R; Eppendorf, Hamburg, Germany; 10,856 x *g*, 10 min, 4°C) and the resultant plasma was stored at -80˚C until shipment for further analysis.

Frozen plasma samples were packed in ice and shipped overnight to the Fisheries and Illinois Aquaculture Center (FIAC) (Carbondale, Illinois, USA) where the samples were stored at -80˚C prior to analysis. Plasma osmolality was determined using a vapor-pressure osmometer (Vapor 5520; Wescor Inc., Logan, UT, USA). Plasma cortisol was quantified using a diagnostic ELISA kit for human plasma/serum samples according to the manufacturer's instructions (EIA 1887; DRG International, Mountainside, New Jersey, USA). Plasma lysozyme was quantified following

procedures adapted from Welker et al. (2007). Briefly, 400 µL of a suspension of *Micrococcus lysodeiktus* was aliquoted into triplicate wells of a 96-well plate containing 20 µL of plasma, and absorbance (450 nm) was measured immediately and following a 10-min incubation period. The difference in absorbance was converted to lysozyme concentrations (µg/mL) using a standard curve created using hen egg lysozyme as an external standard.

*2.4 Histopathological evaluation*

At the end of experiment 3 fish per tank were euthanized with MS 222 (100 mg /L) and gills, liver, kidney and spleen were sampled and fixed in 10% buffered formalin for histopathological evaluation . Tissues were dehydrated in a graded series of ethanol, embedded in Paraplast, sectioned (5 µm) and the slides were stained with hematoxylin and eosin. Slides were examined by light microscopy (BH-2 microscope, Olympus, Melville, NY, USA) and images were captured using with a microscope-mounted digital camera.

*2.5 Statistical analysis*

All production performance and blood chemistry were analyzed by one-way analysis of variance (ANOVA) to determine whether significant differences existed among treatment groups. For parameters exhibiting significant treatment effects, Tukey's Honestly Significant Difference pairwise comparison tests were applied to allow for comparisions between the experimental groups. To determine the significance of nitrate concentration and pH as main effects and to test for an interaction between these treatment factors, these data (excluding the pH and $NO_3$ Feeding Control groups) were also analyzed by two-way ANOVA. For these analyses, tanks were used as

experimental units ($N$ = 3), effects were considered signficant at $P < 0.05$, and all tests were conducted using the SAS 9.3 (SAS Institute, Cary, North Carolina, USA).

**3. Results**

Temperature (29 °C), salinity (23 g/L) and dissolved oxygen higher than 6 mg/L were uniform among all treatments throughout the experiment. However, there were small, but differences on ammonia, pH, and alkalinity. The water quality parameters varied according nitrate concentrations increased as planned for the different treatments (Table 1). As expected significant differences ($P$<0.05) in nitrate concentration are observed among treatment. The others water quality parameters are also showed significant differences ($P$<0.05) among treatments.

Survival of juvenile cobia was not affected by exposure to nitrate or pH treatments; it was reduced only in fish exposed to pH 6.5 at 200 mg/L $NO_3^-$-N. Actually, the mortality results did not vary within treatments, thus variance could not be assessed to determine significance.

Growth performance was affected by nitrate concentration but not pH (Table 2). Unfortunately, variation in fish size was not strictly controlled during initial stocking, and thus significant differences in initial fish size were observed among treatment groups; however, the magnitude of these differences was relatively small, as average initial fish weight ranged from 52.0-59.5 g. Chronic nitrate exposure yielded significantly reduced weight gain, SGR, and K, and elevated FCR values. Feed intake was generally reduced among fish exposed to nitrate, though differences were not statistically significant in all cases.

Blood chemistry of cobia was also influenced by nitrate concentration, and, in some cases, pH (Table 3). Plasma glucose was generally reduced among fish exposed

to nitrate and those cultured at pH 6.5. Plasma lactate and lysozyme activity were reduced among fish exposed to nitrate at either water pH. There were no significant differences in plasma cortisol concentrations or osmolality among treatments.

No histopathological changes on liver, spleen and kidney were observed on juvenile cobia exposed to nitrate during 42 days. However, the gills were affected, and the severity of histopathologies was directly related to nitrate concentration. Fish maintained at 100 and 200 mg/L $NO_3^-$- N showed hyperplasia of primary lamella and hyperplasia of primary and secondary lamella with complete lamellar fusion (Fig. 2C). Fish exposed to 200 mg/L $NO_3^-$- N also presented telangiectasia (Fig. 2C). While, fish exposed to positive and negative control showed no histopathological alterations in the gills (Fig. 2A).

**4. Discussion**

Nitrate has been was considered for a long time with as a non- toxic compound to aquatic organisms, and few studies are related to chronic nitrate exposure effects to aquaculture conditions are available (Hamilin, 2006; Van Bussel et al., 2012). However, the intensification of the aquaculture in the recent decades, especially the use of RAS, which is known to accumulate nitrate, points led to the need to know evaluate the possible toxic effects of nitrate on aquatic organisms.

The results of the present study demonstrated that water quality parameters (other than those under direct investigation) presented small differences throughout the experiment. Ammonia concentration was higher in system with pH 6.5, shown by high TAN results. Higher TAN values could be explained by reduced nitrification efficiency at low pH values (Villaverde et al., 1997). However, the unionized ammonia was equal and practically zero for all treatments. Therefore, the water quality were kept within

appropriate levels for fish culture in RAS (Timmons and Ebeling, 2007), especially those parameters known for cobia, such as ammonia (Atwood et al., 2004; Rodrigues et al., 2007; Barbieri and Doi, 2011). Therefore, the results observed in present study are only directly affected by parameters under investigation.

Survival of cobia was 100% in all treatments with pH 8.0. These results are in accordance with previous studies related to the chronic effects of nitrate. Frakes and Hoff (1982) reported no mortality for juvenile false percula *Amphiprion ocellaris* reared at 100 mg/L $NO_3^-$-N. While, the mortality of turbot *Psetta maxima* and the Pacific white shrimp *Litopennaeus vannamei* were impacted only at concentrations higher than 200 mg $L^{-1}$ $NO_3^-$-N (Van Bussel et al., 2012; Khun et al., 2011). On the other hand, medaka *Oryzia latipes* is relatively more sensitive to nitrate, and its survival was affected at 75 mg/L $NO_3^-$-N (Shimura et al., 2002). Survival of cobia reared at pH 6.5/0 mg/L $NO_3^-$-N was not affected. Similar result was observed for Sampaio et al. (2008) which not related mortality in cobia reared in pH 5 during 42 days. Cobia survival was affected only in fish exposed to pH 6.5/200 mg/L $NO_3^-$-N, presenting 10% of mortality.

Growth of juvenile cobia was significantly affected by nitrate, as it has been shown to juvenile false percula, which presented reduced growth when exposed to concentrations of 100 mg/L $NO_3^-$-N (Frakes and Hoff, 1982). Juvenile turbot exposed to concentrations $\geq$ 125 mg $L^{-1}$ $NO_3^-$-N (Kuhn et al., 2011) and medaka exposed to 75 mg/L $NO_3^-$-N (Shimura et al., 2002) also presented reduced growth.

In a previous study (Sampaio et al., 2008) it was shown that juvenile cobia growth was reduced when these fish were reared in pH 6 when compare to pH 7 or 8. It was hypothesized that one of the mechanisms by which low pH reduced growth in juvenile cobia was a decrease in food intake. In the present study, feed intake was not affected at pH 6.5, while it was directly affected by nitrate. This result are in line with

results from Van Bussel et al. (2012) who related a significant reduction of FI for juvenile turbot exposed to nitrate concentrations equal to or higher than 125 mg/L $NO_3^-$. However, FI for the African catfish *Clarias gariepinus* was reduced only in concentrations above 140 mg/L $NO_3^-$-N (Schram et al., 2013). Gastro-intestinal nitrate exposure via water ingestion may become significant when fish start drinking or ingests water during feeding (Schram et al., 2013). Therefore the reduction of FI could be a mechanism reducing nitrate exposure via ingested water.

Growth of juvenile cobia fed a restricted ration to nitrate was intermediary between the control and fish exposed to nitrate. Hence, decrease in total feed intake is a direct mechanism with which cobia chronically exposed to nitrate reduces their growth, decreasing the energy available to growth. However, the feed efficiency was affected only in fish exposed to nitrate, and directly affects conditional factor and specific growth rates of cobia. So in the present study the reduced growth performance is also related to increased energetic costs to maintain the basal metabolism in fish exposed to nitrate, such as proposed by van Bussel et al. (2012) for turbot chronically exposed to nitrate.

Reduced total feed intake does not fully explain the reductions in performance we observed; other metabolic perturbations may contribute to the reduced growth observed among juvenile cobia cultured in high-nitrate conditions. The mechanism of increased energetic costs to maintain the basal metabolism could be supported by reduced values of glucose and lactate observed in the present study, indicating an metabolic depression (Wendelar Bonga, 1997).

The question whether cortisol is a suitable parameter to measure chronic stress has been brought up by Van Weerd and Komen (1998), as the relationship between cortisol levels and depressed growth is not consistent. In the present study, cortisol was

not altered in fish exposed to nitrate or in the controls. As such, despite cortisol being considered an adequate indicator of acute stress for juvenile cobia (Cnaani and McLean, 2009; Trushenski et al., 2010), it is important to evaluate the role of cortisol as an appropriate stress indicator for cobia chronically exposed to other toxicants. Therefore, nitrate exposure not affected osmolality of cobia, such as observed for *C. gariepinus* chronic exposed to nitrate (Schram et al., 2013).

Lysozyme is a part of the nonspecific immune system. It impedes the settlement and colonization of harmful microorganisms, helping to prevent infection and diseases (Alexander and Ingram, 1992), and its activity is affect by stress. In the present study, lysozyme concentration of juvenile cobia exposed to nitrate was reduced. The suppression of lysozyme activity was also observed on juvenile cobia acutely stressed by air exposition (Cnaani and McLean, 2009). The results suggest that chronic nitrate exposure can have an immunosuppressive action on juvenile cobia.

Gills are target organ to determine aquatic pollution in fish, for their directly and permanently contact with the water. Gills pathologies, such as hyperplasia, lamellar fusion and necrosis were observed in medaka *Oryzias latipes* acute exposed to 100 mg/L of nitrate (Shimura et al., 2004). In addition, histopathological alterations observed in the present study were also related for juvenile cobia acutely exposed to high concentrations of nitrate (Rodrigues et al., 2011). As these authors observed, the histopathological abnormalities are probably a reactive response to the organism against nitrate toxicity. Lamellar fusion of gills could be also a protective effect, in order to diminish the gill surface area exposed to the toxicant (Mallat, 1985). However, the loss of regular branchial structure can compromise fish respiration (Ip and Chew, 2010). The main toxic action of nitrate is probably the methemoglobin formation that is incapable

of carrying oxygen (Camargo et al., 2005). Therefore, the branquial histopathologies can aggravate the oxygen disponibility to maintain basal metabolism.

## 5. Conclusions

In conclusion, juvenile cobia could be reared at pH 6.5 with no deleterious effects on their growth and survival. However, special attention must be given with nitrate in this pH. Growth of juvenile cobia is hampered when they are exposed to nitrate at concentrations equal to or above 100 mg/L $NO_3^-$-N for six weeks. It is clear that feed utilization directly affects growth of cobia exposed to nitrate; however metabolic disturbances and gills histopathologies can also contribute to the reduced growth. Furthermore, nitrate also affects immunosuppressive action on juvenile cobia. This information of the present study will help to determine future chronic safe levels of nitrate to cobia. However, close attention must be given to this toxic in RAS.

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Figure 1. Gill of juvenile cobia *Rachycentron canadum*. (A) Control treatment showing the normal structure of gill (400 ×). (B) juvenile exposed to 200 mg/L $NO_3^-$-N for 42 days, showing telangiectasia in the secondary lamella (arrows) (400 ×). (C) juvenile exposed to 200 mg/L $NO_3^-$-N for 42 days, showing hyperplasia in the primary lamella (short arrow), and hyperplasia of primary and secondary lamella with complete lamellar fusion (long arrow) (400 ×).

Table 1. Water quality parameters during chronic exposition of juvenile cobia exposed to different environmental pH and nitrate levels. Least-square means ± SE are shown for each treatment factor combination in plain text. Means with common letter labels are not significantly different based on 1-way ANOVA and post-hoc Tukey's HSD pairwise comparison tests ($P \geq 0.05$).

| | NO3 | pH | | pH = 6.5 | | | pH = 8.0 | | | P values from 1-Way ANOVA |
|---|---|---|---|---|---|---|---|---|---|---|
| | Feeding | Feeding | 0 | 100 | 200 | 0 | 100 | 200 | | |
| Parameter | Control | Control | | | | | | | PSE | |
| Temperature (ºC) | 29.0 | 29.2 | 29.1 | 29.0 | 29.1 | 28.8 | 28.9 | 29.0 | 0.08 | >0.05 |
| Salinity (‰) | 23.2 | 23.3 | 23.1 | 23.6 | 24 | 23.2 | 23.6 | 23.8 | 0.07 | >0.05 |
| Dissolved oxygen (mg/L) | 6.38 | 6.22 | 6.29 | 6.37 | 6.36 | 6.19 | 6.36 | 6.31 | 0.04 | >0.05 |
| TAN (mg/L) | 0.17 | 0.19 | 1.26 | 2.13 | 0.67 | 0.24 | 0.20 | 0.21 | 0.03 | >0.05 |
| $NH_3$-N (mg/L) | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0 | >0.05 |
| $NO_2^-$-N (mg/L) | 0.08 | 0.06 | 0.06 | 0.13 | 0.19 | 0.11 | 0.16 | 0.23 | 0.02 | >0.05 |
| $NO_3^-$-N (mg/L) | $11.3^c$ | $12.2^c$ | $9.2^c$ | $110.5^b$ | $199.4^a$ | $12.6^c$ | $102.8^b$ | $191.3^a$ | --- | <0.01 |
| pH | $7.94^a$ | $7.88^a$ | $6.49^b$ | $6.48^b$ | $6.47^b$ | $7.88^a$ | $7.92^a$ | $7.93^a$ | --- | <0.01 |
| Alkanity (mg/L as $CaCO_3$) | $169^a$ | $169.3^a$ | $52.5^b$ | $49.8^b$ | $47.8^b$ | $171^a$ | $174.1^a$ | $176.1^a$ | --- | <0.01 |

2049 Table 2. Production performance of juvenile cobia exposed to different environmental pH and nitrate levels. Least-square means and pooled SE
2050 (PSE) are shown for each treatment factor combination. Means with common letter labels are not significantly different based on 1-way
2051 ANOVA and post-hoc Tukey's HSD pairwise comparison tests ($P \geq 0.05$). *P*-values resulting from the 2-way ANOVA test (excluding both
2052 control groups) are also provided.

| Parameter | NO3 Feeding Control | pH Feeding Control | pH = 6.5 | | | pH = 8.0 | | | PSE | P values from 2-Way ANOVA | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 100 | 200 | 0 | 100 | 200 | | pH | $NO_3$ | pH×$NO_3$ |
| Initial Weight (g) | 59.5$^{a}$ | 58.9$^{a}$ | 56.7$^{ab}$ | 54.3$^{ab}$ | 52.1$^{b}$ | 56.4$^{ab}$ | 52.0$^{b}$ | 54.8$^{ab}$ | 2.0 | 0.962 | 0.020 | 0.120 |
| Final Weight (g) | 110.2$^{b}$ | 162.1$^{a}$ | 159.7$^{a}$ | 78.5$^{c}$ | 75.7$^{c}$ | 166.7$^{a}$ | 75.8$^{c}$ | 81.9$^{c}$ | 6.5 | 0.406 | <0.001 | 0.569 |
| Weight Gain (%) | 86$^{b}$ | 176$^{a}$ | 182$^{a}$ | 44$^{c}$ | 45$^{c}$ | 195$^{a}$ | 46$^{c}$ | 50$^{c}$ | 11 | 0.302 | <0.001 | 0.689 |
| SGR (% body weight/day) | 1.47$^{b}$ | 2.41$^{a}$ | 2.47$^{a}$ | 0.87$^{c}$ | 0.89$^{c}$ | 2.57$^{a}$ | 0.89$^{c}$ | 0.96$^{c}$ | 0.11 | 0.309 | <0.001 | 0.863 |
| Feed Intake (% body weight/day) | 2.63$^{c}$ | 4.06$^{a}$ | 4.17$^{a}$ | 3.20$^{bc}$ | 3.80$^{ab}$ | 4.34$^{a}$ | 3.12$^{bc}$ | 3.16$^{bc}$ | 0.20 | 0.177 | <0.001 | 0.066 |
| FCR | 1.76$^{b}$ | 1.62$^{b}$ | 1.62$^{b}$ | 3.76$^{a}$ | 4.23$^{a}$ | 1.61$^{b}$ | 3.53$^{a}$ | 3.29$^{a}$ | 0.32 | 0.090 | <0.001 | 0.223 |
| Conditional Factor (K) | 0.62$^{b}$ | 0.73$^{a}$ | 0.73$^{a}$ | 0.58$^{c}$ | 0.57$^{c}$ | 0.72$^{a}$ | 0.58$^{c}$ | 0.59$^{c}$ | 0.01 | 0.541 | <0.001 | 0.240 |
| Survival | 100 | 100 | 100 | 100 | 90 | 100 | 100 | 100 | 0[1] | --- | --- | --- |

2053 [1]Observations did not vary within treatments, thus variance could not be assessed to determine significance.

Table 3. Plasma chemistry of juvenile cobia exposed to different environmental pH and nitrate levels. Least-square means and pooled SE (PSE) are shown for each treatment factor combination. Means with common letter labels are not significantly different based on 1-way ANOVA and post-hoc Tukey's HSD pairwise comparison tests ($P \geq 0.05$). *P*-values resulting from the 2-way ANOVA test (excluding both control groups) are also provided.

| Parameter | NO3 Feeding Control | pH Feeding Control | pH = 6.5 | | | pH = 8.0 | | | PSE | P Values from 2-way ANOVA | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 100 | 200 | 0 | 100 | 200 | | pH | $NO_3$ | pH×$NO_3$ |
| Glucose (mg/dL) | $61^{ab}$ | $52^{ab}$ | $51^{b}$ | $37^{b}$ | $32^{b}$ | $87^{a}$ | $31^{b}$ | $55^{ab}$ | 11 | 0.036 | 0.03 | 0.043 |
| Lactate (mmol/L) | $1.7^{abcd}$ | $1.8^{ab}$ | $1.7^{abc}$ | $1.3^{bcd}$ | $1.0^{d}$ | $2.0^{a}$ | $1.0^{d}$ | $1.1^{cd}$ | 0.2 | 0.850 | 0.001 | 0.180 |
| Cortisol (ng/mL) | 69 | 52 | 71 | 56 | 42 | 47 | 38 | 81 | 26 | 0.977 | 0.846 | 0.465 |
| Lysozyme (µg/mL) | $24^{abc}$ | $29^{a}$ | $28^{ab}$ | $21^{bc}$ | $19^{c}$ | $25^{abc}$ | $19^{c}$ | $22^{abc}$ | 2 | 0.624 | 0.002 | 0.087 |
| Osmolality (mOsm/kg) | 336 | 345 | 351 | 346 | 345 | 349 | 342 | 347 | 7 | 0.897 | 0.548 | 0.855 |

2059 Fig. 1

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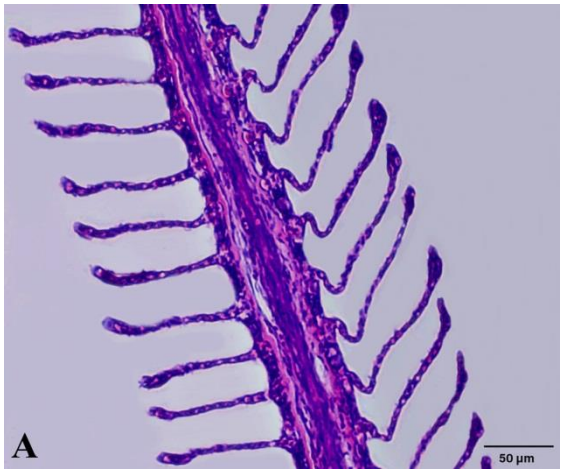


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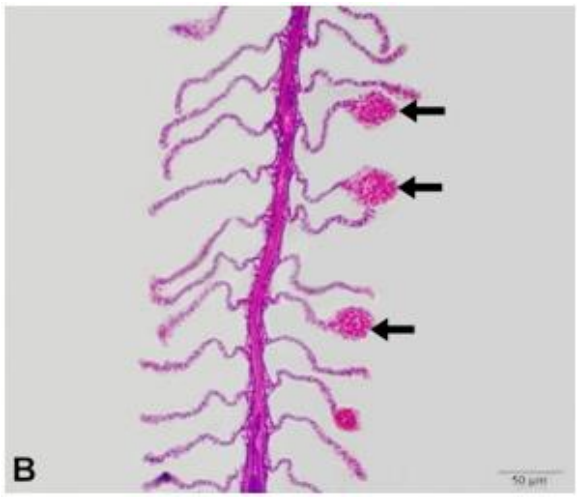


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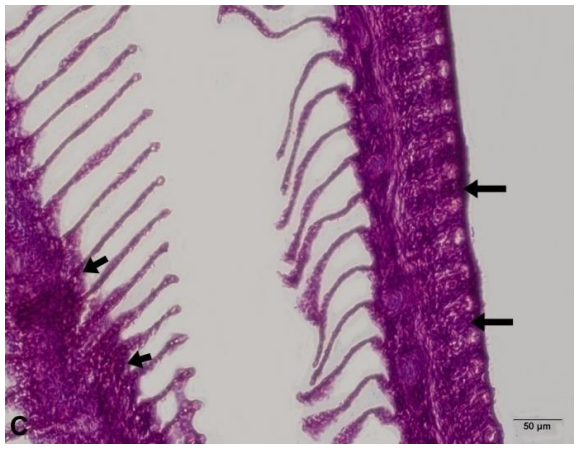


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## DISCUSSÃO GERAL

O efeito do ambiente ácido e do nitrato em peixes marinhos foi por muito tempo negligenciado na aquicultura. Contudo, nos últimos anos devido à utilização cada vez maior dos sistemas de recirculação de água o estudo desses dois parâmetros de qualidade de água para aquicultura se faz cada vez mais importante.

O presente estudo estimou a concentração letal mediana ($CL_{50}$-96 h) do nitrato para juvenis do bijupirá em 1829 mg/L $NO_3^-$-N, indicando que esta espécie possui uma boa resistência a exposição aguda ao nitrato. Os resultados do presente estudo indicam respostas similares de $CL_{50}$ ao nitrato para várias espécies de peixe marinho, com valores próximos a 1000 mg/L $NO_3^-$-N ou mais. Por exemplo, (Pierce et al., 1993) determinaram a $CL_{50}$-96 h do nitrato para juvenis do pampo *Trachinotus carolinus* em 1006 mg/L $NO_3^-$-N, enquanto a $CL_{50}$-96 h para juvenis da tainha *Mugil platanus* foi calculada em 1522 mg/L $NO_3^-$-N (Poersch et al., 2007).

A avaliação histopatológica dos juvenis evidenciou que a exposição aguda ao nitrato induziu várias anormalidades nas brânquias, esôfago e cérebro do bijupirá. A exposição aguda e crônica ao nitrato induziram os mesmos tipos de histopatologias branquiais. Um dos principais mecanismos de toxidade do nitrato é a formação de metahemoglobina no sangue que é incapaz de transportar oxigênio (Camargo et al., 2005). Desta forma as histopatologias nas brânquias podem agravar a captação e disponibilização do oxigênio para manutenção do metabolismo basal dos peixes. Histopatologias branquiais foram previamente observadas para juvenis do "medaka" *Oryzias latipes* expostos ao nitrato (Shimura et al., 2004). Porém, este é primeiro estudo que reporta observações histopatológicas no cérebro de peixes que foram expostos ao nitrato. Estas histopatologias são severas e provavelmente contribuíram para a morte dos peixes. Desta forma os valores de $CL_{50}$, assim como as respostas histológicas

demonstraram ser uma ferramentas efetivas para a toxicidade aguda e importantes para o estabelecimento de níveis de segurança para o nitrato em sistemas aquícolas.

O efeito agudo e crônico do ambiente ácido tem sido determinado para várias espécies de peixe de água doce, com especial atenção para os salmonídeos (Milligan et al., 1982; Dockray et al., 1998). Contudo pouco se sabe sobre o efeito do ambiente ácido em peixes marinhos. Em um estudo prévio, Sampaio et al. (2008) observaram que o crescimento de juvenis do bijupirá é comprometido quando submetidos a pH igual ou inferior a 6, quando comparado aos pHs 7 e 8. Contudo, os resultados do presente estudo indicam que o bijupirá pode ser criado em pH 6,5 com reduzidos valores de nitrato sem prejuízos a sua sobrevivência, crescimento e parâmetros fisiológicos e histopatológicos, sendo este o pH mínimo que esta espécie pode ser mantido.

Quanto a exposição aguda ao ambiente ácido, a sobrevivência de juvenis do bijupirá não foi afetada com a exposição aguda ao ambiente ácido (pH 5,5). Contudo, o baixo pH alterou parâmetros fisiológicos e induziu histopatologias nas brânquias e na pele de *R. canadum*. Essas alterações foram mais severas de acordo com a redução do pH.

Entre as alterações mais significativas, o ambiente ácido resultou em uma redução do pH sanguíneo (acidose), acompanhado de decréscimo dos valores de bicarbonato no sangue dos peixes. Esse processo provavelmente induziu uma elevação nos valores de dióxido de carbono, que no presente estudo provavelmente foram eliminados pelas brânquias (Perry e Gilmours, 2006). Em resposta a saturação do oxigênio decaiu no sangue dos peixes e pode ser explicada pelo Efeito Bohr (Whithers, 1992). Uma resposta na tentativa de aumentar a capacidade de transporte de oxigênio é o aumento nos valores de hematócrito e da hemoglobina no sangue, que foram observados no presente estudo.

Foi observada uma elevação nos níveis plasmáticos de glicose nos peixes expostos ao estresse agudo, sendo que seus valores dobraram nos peixes expostos ao pH 5,5. A elevação das concentrações plasmáticas de glicose é o indicador mais utilizado de estresse secundário em peixes (Wendelaar Bonga, 1997), e foi previamente utilizado como indicador de estresse para juvenis de bijupirá submetidos a desafios de estresse agudo (Cnaani and MacLean, 2009; Trushenski et al., 2010).

A morfologia das brânquias e pele dos peixes são bons biomarcadores em peixes, pelo direto e permanente contato com o meio (Bernet et al., 1999). A avaliação histopatológica revelou que o ambiente ácido induziu hiperplasia no epitélio lamelar com fusão das lamelas secundárias e telangiectasia. Entretanto, essas histopatologias não são específicas da toxicidade do ambiente ácido e foram também observadas em juvenis do bijupirá expostos a elevadas concentrações de nitrato no experimento agudo e crônico. Essas alterações morfológicas são provavelmente reativas aos tóxicos na tentativa de proteger os organismos contra suas toxicidades (Mallat, 1985). Também foi observado hiperplasia e hipertrofia das células de cloreto nos peixes expostos ao ambiente ácido, provavelmente na tentativa de aumentar a absorção de bicarbonato através dos transportadores $Cl^-/HCO3^-$ presentes nas membranas apicais das células de cloreto (Perry e Gilmours, 2006).

No estudo crônico, a sobrevivência dos peixes foi igual a 100% em todos os tratamentos com pH 8. Estes resultados são similares a estudos prévios com nitrato, onde a sobrevivência de *Psetta maxima* e *Lipopenaeus vannamei* foi impactada apenas em concentrações superiores a 200 mg/L $NO_3^-$-N (Khun et al., 2011; Van Bussel et al., 2012). No presente estudo apenas os peixes expostos ao tratamento pH 6,5/200 mg/L $NO_3^-$-N apresentaram 10% de mortalidade.

No estudo crônico o crescimento foi afetado pelo nitrato, mas não pelo pH. O nitrato também influenciou negativamente a ingestão de alimento. O nitrato pode ser assimilado pelo trato digestório pela ingestão de água durante a alimentação (Van Bussel et al., 2012). Portanto, a redução da ingestão de alimento pelo nitrato pode ser um mecanismo pelo qual os peixes reduzem a exposição ao nitrato pela ingestão de água. A redução do crescimento nos peixes expostos ao nitrato pode também em parte ser explicado pelo aumento do custo energético para manutenção do metabolismo basal, sendo suportado pelas reduções de glicose e lactato observadas.

Os resultados do presente estudo indicam que o bijupirá pode ser criado em pH 6,5 com reduzidos valores de nitrato sem prejuízos a sua sobrevivência e crescimento, sendo esse o pH mínimo que esta espécie pode ser mantido. Contudo uma atenção especial deve-se ter com elevadas concentrações de nitrato nesse pH. Além disso, a concentração igual ou superior a 100 mg/L $NO_3^-$-N prejudicam o crescimento do bijupirá independente dos valores de pH.

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## CONCLUSÕES

- A concentração letal mediana $CL_{50}$ – 96 h do nitrato foi estimada em 1829 mg/L $NO_3^-$-N para juvenis do bijupirá, sendo que a exposição aguda ao nitrato induziu histopatologias nas brânquias, esôfago e cérebro do bijupirá.
- A exposição de juvenis do bijupirá ao ambiente ácido durante 24 h resultou em uma série de respostas fisiológicas e alterações histopatológicas nos peixes. Dentro deste período de exposição deve-se evitar a redução do pH abaixo de 6,5.
- Os juvenis de bijupirá podem ser mantidos no pH de 6,5 com reduzido níveis de nitrato sem prejuízos a seu crescimento e sobrevivência. Contudo, concentrações iguais ou maiores a 100 mg/L $NO_3^-$-N afetam negativamente o crescimento, a sobrevivência e a alimentação de juvenis do bijupirá.