UNIVERSIDADE FEDERAL DO PARANÁ
DEPARTAMENTO DE CIÊNCIAS BIOLÓGICAS
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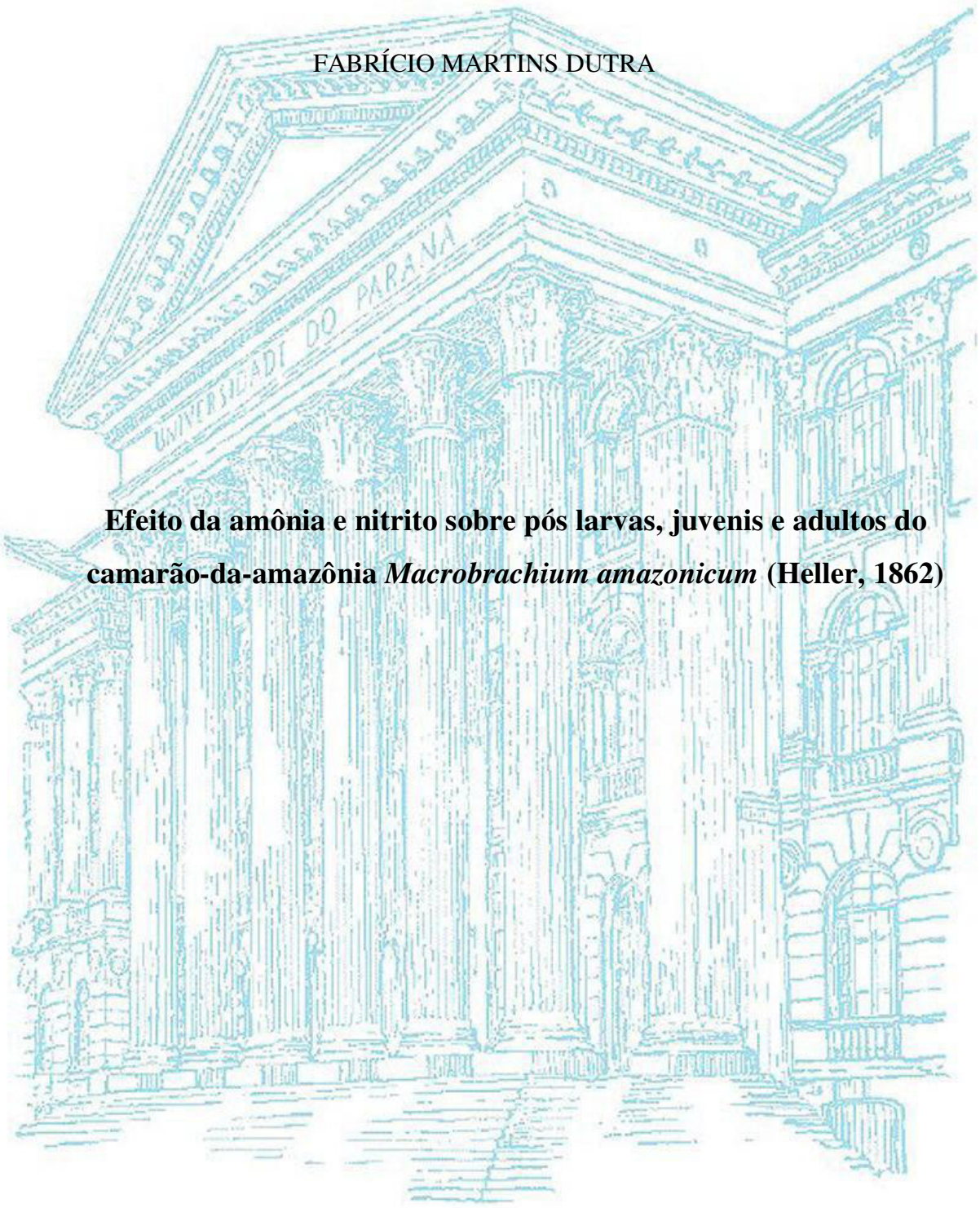
FABRÍCIO MARTINS DUTRA



Efeito da amônia e nitrito sobre pós larvas, juvenis e adultos do camarão-da-amazônia *Macrobrachium amazonicum* (Heller, 1862)

PALOTINA - PR
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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas - Zoologia, Setor de Ciências Biológicas da Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Ciências Biológicas. Área de concentração: Zoologia.

Orientador: Prof. Dr. Eduardo Luis Cupertino Ballester

Coorientadora: Prof.ª Dr.ª Carolina Arruda de Oliveira Freire

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ZOOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **FABRICIO MARTINS DUTRA** intitulada: **Efeito da amônia e nitrito sobre pós-larvas, juvenis e adultos do camarão-da-amazônia *Macrobrachium amazonicum* (Heller, 1862)**, após terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela sua Aprovação.

Curitiba, 02 de Fevereiro de 2017.

EDUARDO LUIS CUPERTINO BALLESTER
Presidente da Banca Examinadora (UFPR)

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KLEBER CAMPOS MIRANDA FILHO
Avaliador Externo (UFMG)

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“Negar nossa posição única e especial no mundo natural pode parecer uma atitude convenientemente modesta aos olhos da eternidade. Mas essa mesma negativa poderia ser usada como uma desculpa para fugir às nossas responsabilidades. A verdade é que nenhuma outra espécie, em tempo algum, teve um controle tão completo e absoluto sobre tudo o que existe na Terra, vivo ou morto, como nós temos hoje. Esse poder nos lega, independentemente da nossa vontade, uma responsabilidade terrível. Em nossas mãos se encontram não apenas nosso próprio futuro, mas o de todos os outros seres vivos com os quais compartilhamos a Terra.”

David Attenborough

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

Martin Luther King

RESUMO

O objetivo do presente trabalho foi determinar o efeito de diferentes concentrações de amônia e nitrito sobre pós-larvas, juvenis e adultos do camarão-da-amazônia *Macrobrachium amazonicum*, durante 96 horas de exposição. Cada estágio de vida foram submetido a diferentes concentrações de amônia total (0, 5, 10, 20, 40 e 80 $mg.L^{-1}$) e nitrito (0, 1, 2, 4, 8 e 16 $mg.L^{-1}$). Brânquias de juvenis foram retiradas para realização da avaliação histopatológica. A concentração letal mediana (CL_{50}-96h) de amônia (*capítulo 1*) foi calculada pelo modelo matemático Probit. A CL_{50}-96h para pós-larvas, juvenis e adultos de *M. amazonicum* foi de 21,14; 21,65 e 36,59 $mg.L^{-1}$ de amônia total, ou 0,67, 0,75 e 1,08 $mg.L^{-1}$ de amônia não ionizada, respectivamente. Os níveis de segurança para a produção deste camarão foi de 0,06; 0,07 e 0,1 $mg.L^{-1}$ para pós-larvas, juvenis e adultos, respectivamente. No teste de concentração letal mediana (CL_{50}-96h) de nitrito (*Capítulo 2*), realizado pelo modelo matemático Weibull I, observou-se que a CL_{50}-96h para pós larvas, juvenis e adultos de *M. amazonicum* foi de 1,49; 2,36 e 2,34 $mg.L^{-1}$ de nitrito, respectivamente. Os níveis de segurança para produção foram de 0,1 mg L^{-1} a pós-larvas e 0,2 $mg.L^{-1}$ nitrito para juvenis e adultos. Quando avaliamos as alterações em brânquias de juvenis de *M. amazonicum* submetidos a diferentes concentrações de amônia total e nitrito (*Capítulo 3*), observou-se que os danos às brânquias nos tratamentos com mortalidade de 100%, corresponderam à alta ocorrência de danos progressivos, regressivos, circulatórios e inflamação. Os demais tratamentos apresentaram, principalmente, inflamação e danos regressivos, aumentando sua ocorrência de acordo com o aumento da concentração. A análise histológica confirmou que quanto maiores as concentrações de amônia total e nitrito, maiores foram os danos causados à estrutura da brânquia. Na avaliação do processo osmótico (*Capítulo 4*), ao comparar as fases de vida para cada concentração de amônia total, observou-se que a atividade da anidrase carbônica (AAC) foi maior para juvenis, estatisticamente diferente ($p<0,05$) de pós larvas. Para nitrito, a fase adulta apresentou maior atividade na concentração de 8 $mg.L^{-1}$, sendo diferente ($p<0,05$) de pós larvas e juvenis. Os valores de ACC nas demais concentrações são semelhantes entre as fases de vida ($p>0,05$). Ao comparar as concentrações dos compostos em cada fase de vida, observou-se que camarões na fase adulta apresentaram maior AAC quando expostos a 5 $mg.L^{-1}$ de amônia total, diferindo estatisticamente ($p<0,05$) da AAC em 20 e 40 $mg.L^{-1}$ de amônia total. Quando submetidos ao nitrito, camarões adultos apresentaram maior AAC na concentração de 8 $mg.L^{-1}$ e menor na concentração de 16 $mg.L^{-1}$, sendo diferente estatisticamente ($p<0,05$) entre si. Nas demais fases a AAC foi semelhante ($p>0,05$) entre todas as concentrações, tanto para amônia total quanto para nitrito. Avaliando a osmolalidade da hemolinfa, observamos que nas concentrações de 0 e 5 $mg.L^{-1}$ de amônia total, pós larvas apresentaram os maiores valores, sendo diferente estatisticamente das demais fases ($p<0,05$). Já para nitrito, pós larvas apresentaram os menores valores de osmolalidade, diferindo estatisticamente ($p<0,05$) de juvenis e adultos. Ao comparar as concentrações dos compostos em cada fase de vida, observamos que os maiores valores de osmolalidade são encontrados em pós larvas e juvenis nas concentrações de 5 e 10 $mg.L^{-1}$ de amônia total, que apresentaram diferença estatística ($p<0,05$) em relação às menores concentrações osmóticas, observadas nas concentrações de 40 e 0 $mg.L^{-1}$ de amônia total, respectivamente. Para nitrito, foi observada diferença ($p<0,05$) na fase de pós larvas, que apresentou maior valor de osmolalidade em 0 $mg.L^{-1}$ e o menor em 2 $mg.L^{-1}$ de nitrito. Portanto, Pós larvas de *M. amazonicum* tem menor capacidade de manter sua AAC quando submetida ao aumento da concentração de amônia total, bem como, menor capacidade de manter a concentração osmótica na hemolinfa quando submetido ao aumento na concentração de nitrito.

Palavras chave: Camarão de água doce, Compostos nitrogenados, Toxicologia, Alteração branquial, Atividade enzimática

ABSTRACT

The objective of the presente study was to determine the effect of different concentrations of ammonia and nitrite on post larvae, juveniles and adults of the Amazon river prawn *Macrobrachium amazonicum*, during 96 hours of exposure. Each life stage were submitted to different concentrations of total ammonia (0, 5, 10, 20, 40 and 80 $mg.L^{-1}$) and nitrite (0, 1, 2, 4, 8 and 16 $mg.L^{-1}$). Juvenile gills were removed for the histopathological evaluation. The median lethal concentration (LC_{50}-96h) of ammonia (Chapter 1) was calculated by the mathematical model Probit. LC_{50}-96h for post larvae, juveniles, and adults of *M. amazonicum* was of 21.14, 21.65 and 36.59 $mg.L^{-1}$ of total ammonia or 0.67, 0.75 and 1.08 $mg.L^{-1}$ of non-ionized ammonia. Thus, from these results, safe levels for the production of this prawn are of 0.06, 0.07 and 0.1 $mg.L^{-1}$ from post larvae, juveniles and adults, respectively. In the test of median lethal concentration (LC_{50}-96h) of nitrite (Chapter 2), performed with the mathematical model Weibull I, it was observed that the LC_{50}-96h for post larvae, juveniles and adults of *M. amazonicum* were of 1.49, 2.36 and 2.34 $mg.L^{-1}$ of nitrite, respectively. Safe levels for the production were of 0.1 $mg.L^{-1}$ to post larvae and 0.2 $mg.L^{-1}$ of nitrite to juvenile and adults. When we evaluated the alterations in gills of juveniles of *M. amazonicum* submitted to different concentrations of total ammonia and nitrite (Chapter 3), it was observed that damage to gills in treatments with 100% mortality, corresponded to the high occurrence of progressive, regressive, circulatory, and inflammation damages. The other treatments had mainly inflammation and regressive damages, whose occurrence increased according to the increase in concentration. The histological analysis confirmed that the higher the total ammonia and nitrite concentrations, the larger the damages caused to the gill structure. In the evaluation of the osmotic process (Chapter 4), when comparing the life stages for each concentration of total ammonia, it was observed that the Activity of carbonic anhydrase (CAA) was lower for post larvae and higher for juveniles exposed to total ammonia, differing statistically ($p<0.05$) between them. For nitrite, the adult stage presented higher activity in the concentration of 8 $mg.L^{-1}$, differing ($p<0.05$) from post larvae and juveniles. In the other concentrations, similar activity values were observed among the life stages ($p>0.05$). Comparing the concentrations of the compounds in each life stage, it is observed that adult prawns presented higher CAA when exposed to 5 $mg.L^{-1}$ of total ammonia, differing statistically ($p<0.05$) from CAA in 20 and 40 $mg.L^{-1}$ of total ammonia, which presented the lowest values. When submitted to nitrite, adult prawn presented higher AAC at the concentration of 8 $mg.L^{-1}$ and lower at the concentration of 16 $mg.L^{-1}$, differing ($p<0.05$) from each other. In the other stages the AAC was similar ($p>0.05$) among all concentrations, to both total ammonia and nitrite. Evaluating the hemolymph osmolality, we observed that in the concentrations of 0 and 5 $mg.L^{-1}$ of total ammonia, post larvae had the highest values. To nitrite, post larvae present the lowest values of osmolality, differing statistically from the other stages ($p<0.05$). Comparing the concentrations of the compounds at each life stage, it is observed that the highest values for post larvae and juveniles are observed in the concentrations of 5 and 10 $mg.L^{-1}$ of total ammonia, which presented statistical difference ($p<0.05$) in relation to the lower concentrations, observed in the concentrations of 40 and 0 $mg.L^{-1}$ of total ammonia, respectively. To nitrite, was observed difference ($p<0.05$) in the post larval stage, which has a higher osmolality value in 0 $mg.L^{-1}$ and the lowest at 2 $mg.L^{-1}$ nitrite. Therefore, post larvae of *M. amazonicum* have a lower capacity to maintain their CAA when submitted to an increase in total ammonia concentration. Pos larvae also has a lower ability to maintain the osmotic concentration in the hemolymph when submitted to an increase in nitrite concentration.

Keywords: Freshwater prawn, Nitrogen compounds, Toxicology, Gill alteration, Enzymatic activity

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CAPÍTULO I: Acute toxicity of ammonia to various life stages of the Amazon river prawn *Macrobrachium amazonicum*, Heller, 1862

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SUMÁRIO

1. INTRODUÇÃO GERAL

Os crustáceos compreendem um dos maiores e mais diversos grupos de invertebrados, aos quais tem sido atribuída grande importância ecológica, devido ao seu papel na cadeia trófica e ciclagem de energia no ambiente em que se encontram (MCLAUGHLIN, 1980; MARTIN et al., 2009). Dentre os crustáceos, a ordem Decapoda compreende o grupo com maior diversidade, com representantes marinhos, dulcícolas e terrestres (MCLAUGHLIN, 1980). Além da diversidade e importância ecológica, alguns camarões de águas continentais têm despertado interesse econômico por possuírem potencial para produção em cativeiro, bem como, por apresentarem uma forma sustentável de produção (VALENTI, 2002). Das 665 espécies dulcícolas pertencentes à infraordem Caridea (GRAVE et al., 2008), 18 pertencem ao gênero *Macrobrachium*, sendo encontradas em águas continentais brasileiras (BARROS; SILVA, 1997; MELO, 2003).

O camarão-da-amazônia *Macrobrachium amazonicum* pertence ao grupo de espécies continentais de desenvolvimento larval completo (ODINETZ-COLLART, 1993). Sua ocorrência foi revisada por Maciel e Valenti (2009) e bem descrita para rios, lagos e planícies aluviais de regiões tropicais e subtropicais da América do Sul, inclusive com ocorrência na planície de inundação do alto rio Paraná (BIALETZKI et al., 1997; MELO, 2003). A espécie apresenta importância ecológica como componente da cadeia trófica, bem como, apresenta importância econômica, sendo um dos principais recursos explorados na região Norte e Nordeste do Brasil (ODINETZ-COLLART, 1993; VIEIRA, 2003). Entretanto, também está a mercê das alterações causadas por poluições: urbana, industrial e agrícola (BECKER et al., 2009), que são responsáveis pela redução na qualidade de água (PEREIRA; MERCANTE, 2005). Dentre os fatores que causam depleção na qualidade de água, os compostos nitrogenados estão entre os que mais apresentam danos à biota aquática (SIPAÚBA-TAVARES et al., 1995; DAMATO; BARBIERI, 2011). A exploração pela pesca e a poluição ambiental também podem contribuir para a diminuição desta espécie, como já ocorre com a espécie nativa *Macrobrachium carcinus* (PORTZ et al., 2006; BECKER et al., 2009; SARAIVA, 2009), que se encontra na lista nacional das espécies, de invertebrados aquáticos e peixes, ameaçadas de extinção (IBAMA, 2004). Portanto, estudos que gerem informações para compreender a biologia da espécie e que proporcionem a manutenção da espécie no ambiente natural e em sistema de produção são necessários, minimizando-se os riscos do seu esgotamento.

Os compostos nitrogenados apresentam grande importância no ambiente aquático natural e artificial. Sua principal atribuição está relacionada aos processos metabólicos e na

formação de proteínas, por serem componentes básicos da biomassa. Quando em quantidades diminutas nos ecossistemas aquáticos, atuam como fator limitante para a produção primária (ESTEVES, 1998). O nitrogênio está presente como nitrato (NO_3^-), nitrito (NO_2^-), amônia (NH_3), amônio (NH_4^+), óxido nitroso (N_2O), nitrogênio molecular (N_2), nitrogênio orgânico dissolvido (ureia, peptídeos, purinas, aminas, aminoácidos, etc.) e nitrogênio orgânico particulado (bactérias, fitoplâncton, zooplâncton e detritos) (VIEIRA et al., 2006). As principais fontes destes compostos são: a chuva, material orgânico e inorgânico de origem alóctone e autóctone e a fixação de nitrogênio molecular dentro do próprio ambiente (EPA, 1975) (Figura 1).

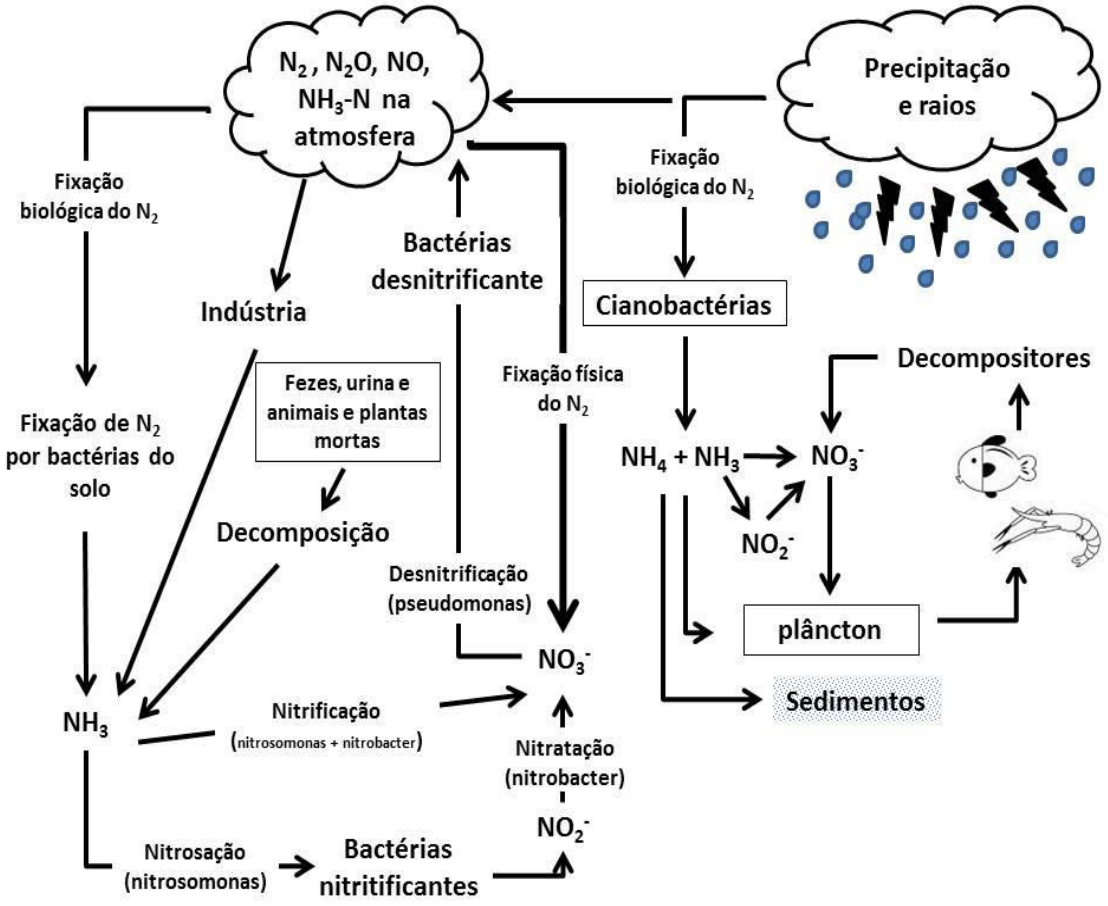

Figura 1. Diagrama do ciclo e fonte de nitrogênio.
FONTE: Dutra (2017)

No ambiente aquático, os compostos nitrogenados aparecem sob três formas. O nitrato é a principal forma de nitrogênio encontrada nas águas e, quando em elevadas concentrações, pode conduzir a um processo de produção primária exagerada, denominada de eutrofização; pode também apresentar toxicidade em sistemas fechados de produção por ser bioacumulativo.

O nitrogênio amoniacal (amônia total), é uma substância tóxica não persistente e não acumulativa e que, em concentrações baixas, não causa nenhum dano fisiológico aos animais. O nitrito, que é uma forma química do nitrogênio, normalmente encontrada em quantidades diminutas nas águas superficiais, é instável na presença do oxigênio. Sua presença indica processos biológicos ativos, influenciados por poluição orgânica (GORSEL; JENSEN, 1999). As concentrações desses compostos nitrogenados no solo e em águas superficiais contribuem para a degradação dos ecossistemas aquáticos. Consequentemente, os organismos aquáticos sofrem os efeitos tóxicos desse processo de eutrofização (SMITH et al., 1999; HOWARTH et al., 2000).

A amônia é um composto nitrogenado que ocorre naturalmente no ambiente (MARTÍN; FREDERICO, 2001), sendo também induzida pela poluição industrial, doméstica, agrícola e por mudanças ambientais (REBELO et al., 2000). É também o principal produto gerado pelo catabolismo proteico da maioria dos organismos aquáticos. Bem como, é originado pela decomposição de alimentos não digeridos e outros resíduos orgânicos (NOGA, 1996). Portanto, o composto é comprovadamente tóxico, influenciando no crescimento, na alimentação, na sobrevivência e na susceptividade a parasitos e doenças em camarões e outros organismos aquáticos (ARMSTRONG et al., 1978; WICKINS, 1976; DANIELS et al., 1992; KIR et al., 2004; MUGNIER; JUSTONS, 2004). No meio aquático, a amônia se encontra sob duas formas: forma ionizada (NH_4^+) e não ionizada (NH_3); a soma das duas constitui a amônia total ($NH_4^+ + NH_3$) e seu equilíbrio no meio vai depender do pH, temperatura, salinidade, pressão parcial, etc (WHITFIELD, 1974; EMERSON et al., 1975; THURSTON, 1980; ARANA, 1997). A forma da amônia não-ionizada é quimicamente mais tóxica devido a sua capacidade de difusão pelas membranas celulares, causando danos ao epitélio branquial e, como consequência, desestabilizando o sistema de osmorregulação (BALL, 1967; FROMM; GILLETE, 1968; SMART, 1976; THURSTON, 1980; EIFAC, 1983; YU; HIRAYAMA, 1986).

O nitrito é o composto intermediário na nitrificação bacteriana da amônia a nitrato, podendo apresentar alta toxidez, dependendo de sua concentração no meio e do estágio de desenvolvimento do organismo (MIRANDA-FILHO et al., 1995). Em altas concentrações provoca a oxidação do átomo de ferro da molécula da hemoglobina do sangue, convertendo-a em meta-hemoglobina, molécula incapaz de transportar oxigênio, estabelecendo-se um quadro de hipóxia e cianose em peixes (DUBOROW et al., 1997). Conforme Chen e Cheng (1996), nos crustáceos, o nitrito provoca um aumento da pressão parcial de oxigênio, sugerindo uma elevação do O_2 livre e um decréscimo de O_2 ligado a hemocianina (oxihemocianina). O mesmo

efeito foi observado em *Penaeus japonicus* (CHEN; CHENG, 1995), *Penaeus monodon* (Cheng e Chen, 1999) e em *Macrobrachium rosenbergii* (CHEN; LEE, 1997). Segundo Needhan (1961) e Armstrong et al. (1976), a hemocianina é menos afetada pelo nitrito que a hemoglobina.

Segundo Thurston et al. (1986), o nitrato é considerado uma substância com baixa toxicidade, mas, por ser o produto final da nitrificação, pode acumular-se em grandes quantidades, portanto, pode causar efeitos letais ou subletais em diferentes organismos, ou ainda, atuar sinergicamente com outras formas nitrogenadas (OSTRENSKY, 1997). Por ser considerado um composto nitrogenado de baixa toxicidade, poucos são os estudos sobre o nitrato (KAISER; WHEATON, 1983; RUSSO, 1991; STORMER et al., 1996).

Camarões de água doce, em sua história de vida, adaptaram-se a ambientes dulcícolas. Esta característica, por meio de pressão seletiva, solicitou a estes animais um ajuste nos seus processos fisiológicos, destacando-se o aumento na absorção de íons, diminuição da permeabilidade de íons pelo exoesqueleto, aumento da produção de metabólitos como urina e diminuição nos níveis de pequenas moléculas orgânicas que atuam como osmólitos no controle de volume celular (PÉQUEUX, 1995).

Animais de água doce são osmorreguladores e, em crustáceos, as enzimas Na^+/K^+-ATPase e anidrase carbônica (AC) têm papel fundamental na regulação do equilíbrio ácido/base e influxo iônico pelas brânquias, resultando na manutenção da concentração osmótica e de Na^+/Cl^- na hemolinfa sempre mais alta do que na água (PÉQUEUX, 1995; ROMANO; ZENG, 2013). Neste processo, a enzima anidrase carbônica catalisa e converte o CO_2 e H_2O em H^+ e HCO_3^-. Estes, por sua vez, são utilizados como substratos na absorção ativa de Na^+/Cl^- através da brânquia por meio dos trocadores Na^+/H^+ e Cl^-/HCO_3^-. Assim, o desafio de animais de água doce é manterem-se hiperosmóticos em relação ao meio, utilizando-se desse mecanismo para a absorção de sal (WHEATLY; HENRY, 1987; HENRY, 1988; MITCHELL; HENRY, 2014).

Devido aos mecanismos de regulação iônica e osmótica dos crustáceos de água doce incluírem a absorção ativa de Na^+ através do epitélio branquial (AUGUSTO et al., 2009), a amônia, por estar intimamente ligada ao processo de osmorregulação, pode difunde-se facilmente através das brânquias para a hemolinfa, onde transfere prótons para tornar-se NH_4^+. O NH_4^+, em seguida, substitui os íons de K^+ na brânquia por meio da via basolateral, localizada na Na^+/K^+-ATPase. O NH_4^+ é finalmente excretado para o meio através da via apical, localizado na Na^+/NH_4^+ permutador e/ou através da liberação de exocitóticos (ROMANO; ZENG, 2013). O nitrito, por sua vez, é captado pelo sistema de captação do Cl^- pelas brânquias, que é

responsável por regular o transporte de Cl^- na hemolinfa, bem como, pelo equilíbrio ácido-base, através da troca Cl^- influxo e HCO_3^- efluxo (Cl^-/HCO_3^-) (JENSEN, 1995) (Figura 2).

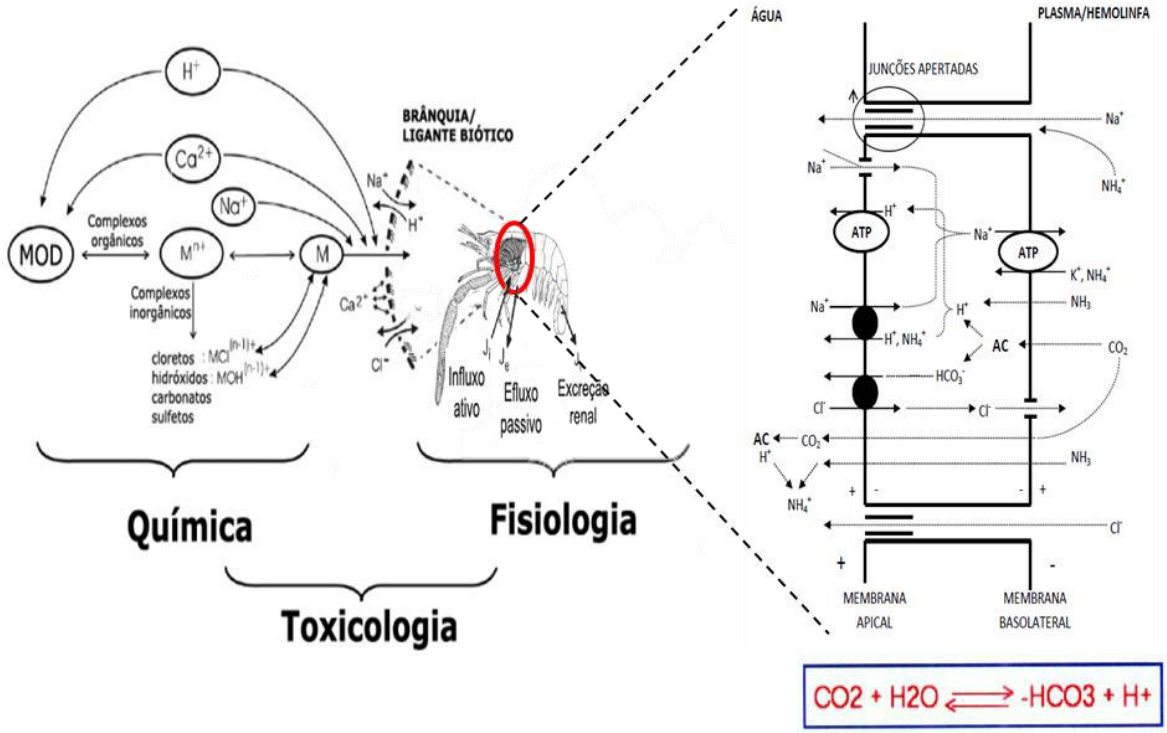

Figura 2. Representação esquemática dos aspectos toxicológicos no processo de osmorregulação.

FONTE: Adaptado de Paquin et al. (2002) e Grosell et al. (2002).

A inibição da AC pode responder satisfatoriamente a alterações ambientais, sendo considerada um bom marcador para detectar efeitos da exposição aos compostos tóxicos em organismos aquáticos (ARASHISAR et al., 2004). Trabalhos avaliando a anidrase carbônica já foram realizados para camarões marinhos e dulcícolas. Abdel-Mohsen (2009) observaram, em estudo com *Penaeus japonicus*, que a poluição ambiental não afetou a atividade da enzima AC. Maraschi et al. (2015) não encontraram alteração na atividade da enzima AC em *Macrobrachium acanthurus* expostos à variação de salinidade. Por outro lado, Roy et al. (2007) observaram variação na atividade da AC em brânquias de *Litopennaeus vannamei* expostos à aumento da salinidade. Entretanto, estudos sobre os efeitos da amônia e do nitrito sobre a atividade da AC em camarões são escassos.

O equilíbrio ácido-base pode ser alterado devido as trocas realizadas entre organismo e ambiente (TRUCHOT, 1983). Assim, a diminuição na osmolalidade devido a um efeito tóxico, provocaria um aumento do catabolismo dos aminoácidos (LANGE, 1972; LARSEN et al., 2014) e ou aumento do pH na hemolinfa sem a mudança na pressão parcial de gás carbônico

(PCO_2) (TRUCHOT, 1983). Portanto, a interpretação da osmolalidade da hemolinfa com foco em sua medição poderia ser utilizado como um indicador de stress em animais submetidos a agente tóxico (LIGNOT et al., 2000). Reduções na osmolalidade já foram observados em crustáceos quando submetidos a amônia (YOUNG-LAI et al., 1991; CHEN; CHENG, 1993).

Estudos toxicológicos por meio de testes de toxicidade aguda (CL_{50}) são recomendados para o fornecimento de informação básica a respeito da biologia da espécie (tolerância toxicológica) e para o conhecimento de possíveis danos causados a biota aquática (IBAMA, 1990). De acordo com o método escrito no APHA (1989), o valor de CL_{50} pode ser uma medida útil para testes de toxicidade, mas não representa concentrações seguras ou inofensivas em ambientes aquáticos sujeitos a poluição. Concentração de poluentes que não demonstrem toxicidade em 96 horas podem ser fatais sob condições de exposições contínuas. Assim, o valor de 96h CL_{50} pode representar apenas uma pequena fração do potencial tóxico do composto testado. Por outro lado, o valor de CL_{50} obtido em testes de toxicidade de curta duração, normalmente de 24 a 96 horas, geram resultados seguros da concentração tóxica em questão, podendo nortear outros testes de médio e longo prazo (APHA, 1992). Devido ao grau de toxidez dos compostos nitrogenados apresentarem variações específicas relevantes, o estudo dos efeitos tóxicos desta substância para os diferentes organismos é de extrema importância (WASIELESKY et al., 1994).

Por outro lado, mesmo que o estudo toxicológico apresente resultados seguros sobre a concentração tóxica de um determinando composto, este não fornece informações sobre o dano provocado ao organismo decorrente da intoxicação. Assim, a histopatologia tem sido uma ferramenta comumente utilizada na identificação dos danos provocados a organismos submetidos a um agente tóxico (MIRON et al., 2008). Embora alguns estudos com análises histológicas tenham avaliado o efeito de pesticidas (LIGNOT et al., 1997; SARAVANA BHAVAN; GERALDINE, 2000), metais pesados (ASIH et al., 2014; BEN-KHEDHER et al., 2014), vírus (PAZIR et al., 2011; YUN et al., 2014) e irradiação gama cobalto-60 (STALIN et al., 2013a; STALIN et al., 2013b) nas brânquias de camarões, nenhum estudo avaliou os efeitos histológicos de compostos nitrogenados sobre as brânquiais de camarões de água doce.

Dentro deste contexto, podemos enfatizar que a presente tese apresenta fundamentais contribuições ao conhecimento da biologia do camarão *M. amazonicum*, por meio da determinação dos níveis letais dos compostos nitrogenados amônia e nitrito; das análises histológicas dos efeitos causados à estrutura branquial por diferentes concentrações destes

compostos; da avaliação da atividade da enzima anidrase carbônica e; da avaliação da osmolalidade em camarões submetidos à amônia e ao nitrito.

1.1. OBJETIVOS

1.1.1. Objetivo geral

Determinar o efeito de diferentes concentrações de amônia e nitrito sobre pós-larvas, juvenis e adultos do camarão-da-amazônia *Macrobrachium amazonicum* (Heller, 1862), durante 96 horas de exposição.

1.1.2. Objetivos específicos

- ✓ Analisar a tolerância de pós-larvas, juvenis e adultos de *M. amazonicum* à amônia e ao nitrito;
- ✓ Determinar a Concentração Letal Mediana (CL_{50}) durante 96 horas e o Nível de Segurança de amônia e nitrito para pós-larvas, juvenis e adultos de *M. amazonicum*;
- ✓ Avaliar ocorrência de alterações nas brânquias de juvenis de *M. amazonicum* submetidos à exposição de amônia e nitrito por meio de análises histológicas.
- ✓ Verificar a influência da amônia e nitrito no processo de osmorregulação do *M. amazonicum*;

CAPÍTULO I:

Acute toxicity of ammonia to various life stages of the Amazon river prawn *Macrobrachium amazonicum*, Heller, 1862 [*]

(Formatado conforme "Instruções aos Autores" em anexo I)

[*] Capitulo publicado na revista Aquaculture, 453 (2016) 104-109, doi:10.1016/j.aquaculture.2015.11.038. Autores: Fabrício Martins Dutra, Sandra Carla Forneck, Claudia Caramelo Brazão, Carolina Arruda Freire, Eduardo Luis Cupertino Ballester.

Abstract

The buildup of nitrogenous wastes in the water reflects biological processes potentially stimulated by organic pollution. These wastes may cause physiological harm and even mortality in produced aquatic species. This study aimed at determining the effects of ammonia on different life stages of the Amazon river prawn *Macrobrachium amazonicum*. Experimental set up used 240 prawns to each life stage (post larvae, juveniles and adults), stocked in 24 experimental units, under a totally casualized arrangement, for each life stage. Individuals were exposed to six different concentrations of total ammonia (0, 5, 10, 20, 40 and 80 $mg.L^{-1}$), and the median lethal concentration after 96 hours was determined (LC_{50}-96 h). Water oxygen, pH, and temperature were monitored daily; at the start and end of the experimental periods, hardness, alkalinity, ammonia, nitrite and nitrate were also measured. LC_{50}-96 h was calculated through the probit method, later submitted to a linear regression ($p<0.05$). LC_{50}-96 h for post larvae, juveniles, and adults of *M. amazonicum* was of 21.14, 21.65 and 36.59 $mg.L^{-1}$ of total ammonia, or 0.67, 0.75 and 1.08 $mg.L^{-1}$of non-ionized ammonia. Thus, from these results, safe levels for the production of this prawn are of 0.06, 0.07 and 0.1 $mg.L^{-1}$, from post larvae, juveniles and adults, respectively.

Keywords: Freshwater prawn; Toxicology; Nitrogen compound; Ontogeny.

1. Introduction

Brazil has many native species with great potential for aquaculture exploitation. However, most of them are rather largely unexplored scientifically and technologically (Martino et al., 2002; Pérez et al., 2000). The Amazon river prawn *Macrobrachium amazonicum* is the native prawn species with the greatest potential for production, being widely consumed by Amazonian populations and regions of the semi-arid region of northeastern Brazil (Moraes-Valenti et al., 2010). Due to its wide distribution, fast growth and ease of maintenance in captivity (Silva et al., 2004), *M. amazonicum* has been the most exploited species of freshwater prawn by artisanal fisheries in Brazil (Maciel and Valenti, 2009). It has high economic and gastronomic importance (Marques and Moraes-Valenti, 2012). An example of the production potential of native species of palaemonid prawn is that of *Macrobrachium nipponense*, which has great economic importance in some Asian countries (Hongtuo et al., 2012).

Macrobrachium amazonicum has been studied in its ecology (Collart, 1990; Moreira and Collart, 1993; Dutra et al., 2014), biology (Moraes-Riodades and Valenti, 2004; Boudour-Boucheker, et al., 2013; Meireles et al., 2013) and production (Anger and Haydn, 2010; Moraes-Valenti et al., 2010; Freire et al., 2012). However, studies on the effect of the ammonia on the species are still needed, in order to assess its sensitivity to this relevant contaminant in production conditions.

Ammonia is a nitrogen compound that occurs naturally in the environment (Martín and Frederick, 2001), but it is also the result of industrial pollution, domestic, and agricultural runoffs (Rebelo et al., 2000). It is the main product generated by protein catabolism in most aquatic organisms, and likewise originates from the decomposition of undigested food and other organic wastes (Noga, 1996). The ammonia non-ionized form is demonstrably toxic (Armstrong et al., 1978) and negatively influences growth, feeding, survival and susceptibility to diseases and parasites in prawn and other aquatic organisms (Daniels et al., 1992; Mugnier and Justou, 2004). In the water, ammonia can be found in its ionized (NH_4^+) or unionized form (NH_3); the sum of the two is the total ammonia. Their proportion in the water depends on pH, temperature, salinity, partial pressure, among others factors (Bower and Bidwell, 1978). Many studies confirm that non-ionized ammonia is chemically more toxic due to its ability to diffuse through cell membranes, causing damage to the gill epithelium and, consequently, disturbing osmoregulatory mechanisms (Ball, 1967; Fromm and Gillette, 1968; Smart, 1976; Thurston, 1980; Yu and Hirayama, 1986). The first toxicological evaluation of a certain combination "organism + toxicant" in general involves the determination of median lethal concentration levels, in other words, that causes mortality of 50% of individuals (LC_{50}). LC_{50} values obtained from toxicity tests of short duration, typically 24 to 96 hours, are believed to generate reliable results (APHA, 1992). Within this context, the aim of the present study was to assess the effect of ammonia on different life stages of the prawn-Amazon - *M. amazonicum*, through the determination of LC_{50}-96 h concentrations of ammonia.

2. Material and Methods

The experimental work was conducted at the Prawn Culture Laboratory, located at the Federal University of Paraná - Sector Palotina, with animals provided by the Prawn Aquaculture of CAUNESP Center (Universidade Estadual Paulista "Julio de Mesquita Filho").

2.1. Experimental design

The methodology used here in assessing the median lethal concentration (LC_{50}-96 h) was based on the manual of the Environmental Protection Agency USA (Peltier and Weber, 1985). Experiments used 240 prawns of each life stage (post-larvae, juveniles and adults), randomly divided into 24 experimental units. The units consisted of a beaker with a volume of 1 L to post-larvae (0.08 ± 0.02 g, 2.25 ± 0.20 cm (total length)), or glass aquaria containing 10 L of useful volume to juvenile (2.07 ± 0.62 g, 6.29 ± 0.71 cm) and to adult (6.16 ± 1.41 g, 9.26± 0.70 cm). Experimental units were equipped with aeration systems and a photoperiod of 12/12 h (light/dark). The design was completely randomized, with six different total ammonia concentrations (0, 5, 10, 20, 40 and 80 $mg \cdot L^{-1}$ Ammonium Chloride PA - Synth®; São Paulo; Brazil) and four replicates per ammonia concentration. The ammonia levels resulted from the addition of appropriate volumes of the stock solution of NH_4Cl (stock solution of 1000 $mg \cdot L^{-1}$ NH_4Cl) to produce the desired final total ammonia concentrations. The concentrations used were indicated by preliminary tests.

The criterion used to determine the lethal concentration (LC) was: total absence of any kind of movement or reaction to mechanical stimuli using a glass rod. Prawns were observed every 1 h for the first 8 h. Between 8 h and 96 h, observations were performed every 12 h (Armstrong et al., 1976).

2.2. Analyses of water quality

The following variables of water quality were evaluated daily: dissolved oxygen (Oximeters, Hanna HI 9146), temperature (digital thermometer Incoterm) and pH (pHmeter, Tekna T-100). At the beginning and again at the end of the experiment, nitrite, nitrate, total ammonia, alkalinity, and hardness have also been determined (APHA, 2005).

The unionized ammonia fraction was calculated according to Emerson et al. (1975):

$$NH_3\text{-}N = \frac{[\ N\text{-}NH_3 + N\text{-}NH_4^+]}{1+10^{(pKa-pH)}}$$

Where:

NH_3-N = unionized ammonia;

$N\text{-}NH_3 + N\text{-}NH_4^+$ = total ammonia;

pKa - log Ka, calculated as pKa = 0.09018 + 2729.92 / *T;*

T = temperature in ° Kelvin;

The security level of the median lethal concentration of ammonia is determined by multiplying the value obtained in the test of toxicity by an application factor of 0.1, as recommended by Sprague (1971). Water quality variables were determined independently for each treatment in order to establish whether they were maintained at appropriate levels for the biology of the species (Moraes-Valenti and Valenti, 2010).

2.3. Statistical analysis

The median lethal concentrations (LC_{50}-96 h) of ammonia were calculated by the probit method (Finney, 1971), using the EPA Probit Analysis program, version 1.5 EPA (2012). This method establishes the relationship between the "probits" of observed cumulative mortality and the logarithm of the concentrations of ammonia. The relationship between the concentration of ammonia and mortality was then subjected to linear regression analysis.

3. Results

During the experimental period, water temperature ranged from 24.9 to 27.2 °C, dissolved oxygen from 5.2 to 7.6 $mg \cdot L^{-1}$, pH from 7.6 to 7.9, alkalinity from 16.7 to 27.7 $mg \cdot L^{-1}$ $CaCO_3$ and hardness from 23.6 to 53.7 $mg \cdot L^{-1}$. The higher mean nitrite values were 0.150 $mg \cdot L^{-1}$ for post-larvae, 0.060 $mg \cdot L^{-1}$ for juveniles and 0.448 $mg \cdot L^{-1}$ for adults. Nitrate values were lower than 0.105 $mg \cdot L^{-1}$ for all life stages (Table 1).

Mortality observed for the different life stages of control *M. amazonicum* (not exposed to increased ammonia) during the trial period may be related to the agonistic behavior of animals in competition for area (territorialism). Post-larvae of *M. amazonicum* exposed to ammonia showed 100% mortality after 48 h at concentrations of 40 $mg \cdot L^{-1}$ or 1.268 ± 0.073 $mg \cdot L^{-1}$ of NH_3-N. At a lower concentration, 20 $mg \cdot L^{-1}$ of total ammonia or 0.722 ± 0.115 $mg \cdot L^{-1}$ NH_3-N, mean mortality was of 40 ± 14% after 96 h. However, when comparing the relationship between mortality and total ammonia concentration by the probit method and regression analysis ($p < 0.05$), the lethal concentration for 50% of the prawns after 96 h can be predicted by the equation: $LC_{50} = - 0.092 + 0.0258X$ ($mg \cdot L^{-1}$ $N\text{-}NH_3 + N\text{-}NH_4^+$), $r^2 = 0.97$, resulting in a calculated LC_{50} of 21.143 $mg \cdot L^{-1}$ of total ammonia or 0.670 $mg \cdot L^{-1}$ of NH_3-N (Fig. 1). Thus, safe level of exposure for the post-larvae of *M. amazonicum* is of 2.114 $mg \cdot L^{-1}$ of total ammonia or 0.067 $mg \cdot L^{-1}$ of NH_3-N. The graph was adjusted to better represent the effect of mortality once 40 and 80 $mg \cdot L^{-1}$ of total ammonia showed 100% mortality

Table 1. Water quality parameters of LC_{50}-96 h tests, carried out for the different ontogenetic stages of *M. amazonicum*

Variables	0 mg.L^{-1}	5 mg.L^{-1}	10 mg.L^{-1}	20 mg.L^{-1}	40 mg.L^{-1}	80 mg.L^{-1}
Total ammonia in the experiments with the post-larvae						
Temperature H_2O (°C)	26.3±0.9	26.3±0.9	26.3±0.9	26.3±0.9	25.5±0.6	25.0±0.2
Dissolved Oxygen (mg.L^{-1})	6.9±0.3	6.7±0.4	6.8±0.4	7.0±0.2	6.7±0.4	6.7±0.5
pH	7.8±0.1	7.7±0.1	7.8±0.1	7.7±0.1	7.7±0.1	7.7±0.1
Alkalinity (mg.L^{-1} $CaCO_3$)	21.4±1.5	20.8±1.1	19.5±0.8	20.7±1.1	21.4±0.8	20.6±2.1
Hardness (mg.L^{-1})	30.8±3.2	29.6±4.4	29.1±2.3	28.2±1.5	28.1±2.1	31.9±6.5
Total ammonia (mg.L^{-1})	0.240±0.343	5.276±0.276	10.398±0.667	21.797±2.533	40.906±1.337	80.745±1.738
Un-ionized ammonia (mg.L^{-1})	0.008±0.012	0.176±0.025	0.366±0.033	0.722±0.115	1.268±0.073	2.428±0.225
Nitrite (mg.L^{-1})	0.016±0.009	0.061±0.097	0.015±0.004	0.150±0.270	0.030±0.014	0.012±0.009
Nitrate (mg.L^{-1})	0.040±0.026	0.105±0.127	0.024±0.017	0.022±0.020	0.008±0.009	0.059±0.029
Total ammonia in the experiments with the juvenile						
Temperature H_2O (°C)	25.0±0.1	25.1±0.3	25.2±0.3	25.2±0.2	25.0±0.1	25.1±0.1
Dissolved Oxygen (mg.L^{-1})	6.6±0.4	6.5±0.4	6.6±0.4	6.5±0.4	6.2±0.6	6.6±0.3
pH	7.8±0.1	7.8±0.1	7.8±0.2	7.8±0.1	7.8±0.1	7.8±0.1
Alkalinity (mg.L^{-1} $CaCO_3$)	24.5±1.1	25.8±1.9	24.7±1.2	22.8±2.1	21.7±1.0	20.5±1.3
Hardness (mg.L^{-1})	37.6±7.0	45.5±5.0	44.1±9.6	35.3±11.7	37.9±6.4	43.3±9.8
Total ammonia (mg.L^{-1})	0.404±0.117	5.377±0.239	10.039±0.114	20.402±0.586	40.870±0.434	80.254±0.845
Un-ionized ammonia (mg.L^{-1})	0.015±0.004	0.206±0.024	0.331±0.078	0.722±0.060	1.424±0.138	2.869±0.207
Nitrite (mg.L^{-1})	0.034±0.001	0.040±0.006	0.054±0.015	0.060±0.022	0.035±0.001	0.045±0.011
Nitrate (mg.L^{-1})	5.10^{-6}±4.10^{-6}	6.10^{-6}±5.10^{-6}	1.10^{-6}±4.10^{-6}	2.10^{-6}±9.10^{-7}	5.10^{-7}±1.10^{-7}	1.10^{-6}±4.10^{-7}
Total ammonia in the experiments with the adult						
Temperature H_2O (°C)	25.8±0.7	26.0±0.5	26.2±0.5	25.8±0.6	26.0±0.4	25.9±0.4
Dissolved Oxygen (mg.L^{-1})	6.1±0.9	5.9±0.7	6.0±0.7	6.0±0.8	6.1±0.6	6.7±0.9
pH	7.7±0.1	7.7±0.1	7.7±0.1	7.7±0.1	7.7±0.1	7.7±0.1
Alkalinity (mg.L^{-1} $CaCO_3$)	21.8±1.8	18.4±0.8	20.4±1.3	19.9±0.7	19.4±1.1	19.0±2.3
Hardness (mg.L^{-1})	29.4±5.8	27.8±4.2	30.2±4.2	29.3±1.8	30.0±7.4	24.9±5.1
Total ammonia (mg.L^{-1})	0.158±0.137	5.380±0.447	10.776±0.755	20.746±1.625	41.422±0.510	80.231±0.754
Un-ionized ammonia (mg.L^{-1})	0.003±0.008	0.159±0.016	0.336±0.027	0.590±0.037	1.155±0.070	2.327±0.550
Nitrite (mg.L^{-1})	0.448±0.017	0.360±0.037	0.336±0.142	0.411±0.086	0.445±0.054	0.391±0.181
Nitrate (mg.L^{-1})	3.10^{-5}±4.10^{-5}	8.10^{-6}±8.10^{-6}	4.10^{-6}±7.10^{-6}	4.10^{-6}±5.10^{-7}	5.10^{-5}±9.10^{-5}	5.10^{-5}±1.10^{-4}

FONTE: Dutra (2017)

Juvenile *M. amazonicum* exposed to ammonia concentrations of 40 mg·L^{-1} of total ammonia or 1.424 ± 0.138mg·L^{-1} of NH_3-N exhibited 100% mortality after 72 h. Mortality of juveniles, as related to total ammonia concentration, is represented by the equation: LC_{50} = -0.024 + 0.0242X (mg·L^{-1} N-NH_3 + N-NH_4^+), r^2 = 0.98. Calculated LC_{50} is of 21.653 mg·L^{-1} for total ammonia and 0.755 mg·L^{-1} for NH_3-N (Fig. 2). Therefore, safe exposure level for *M. amazonicum* juveniles is of 2.165 mg·L^{-1} of total ammonia or 0.075 mg·L^{-1} of NH_3-N. The graph was adjusted to better represent the effect of mortality once 40 and 80mg·L^{-1} of total ammonia showed 100% mortality.

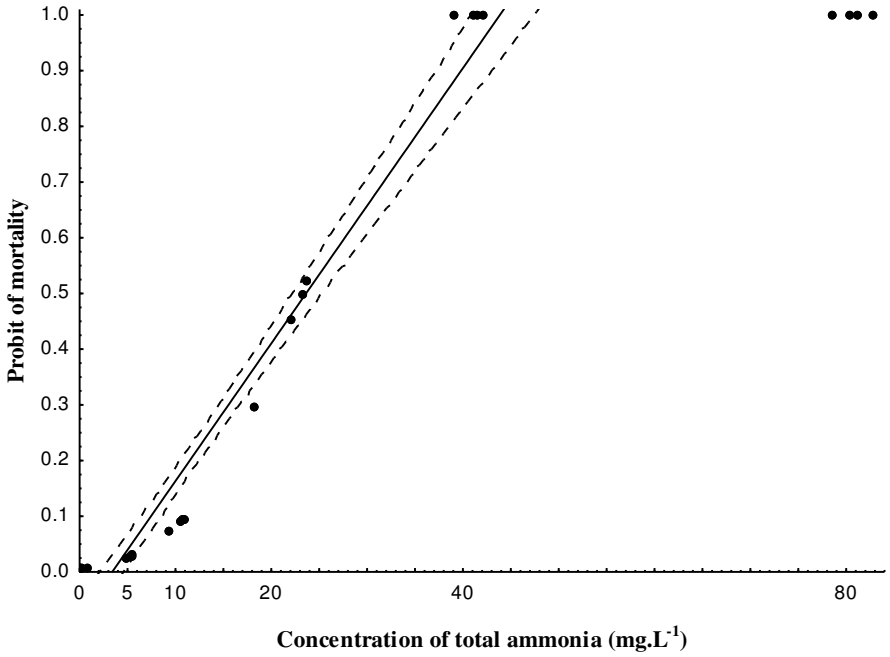

Figure 1. Linear regression of the LC_{50} for post-larvae of *M. amazonicum* after 96 h of exposure to ammonia. Confidence intervals (95%) are represented by the dashed lines. Graph adjusted to 40 mg.L^{-1} to better represent the effect of mortality.

FONTE: Dutra (2017)

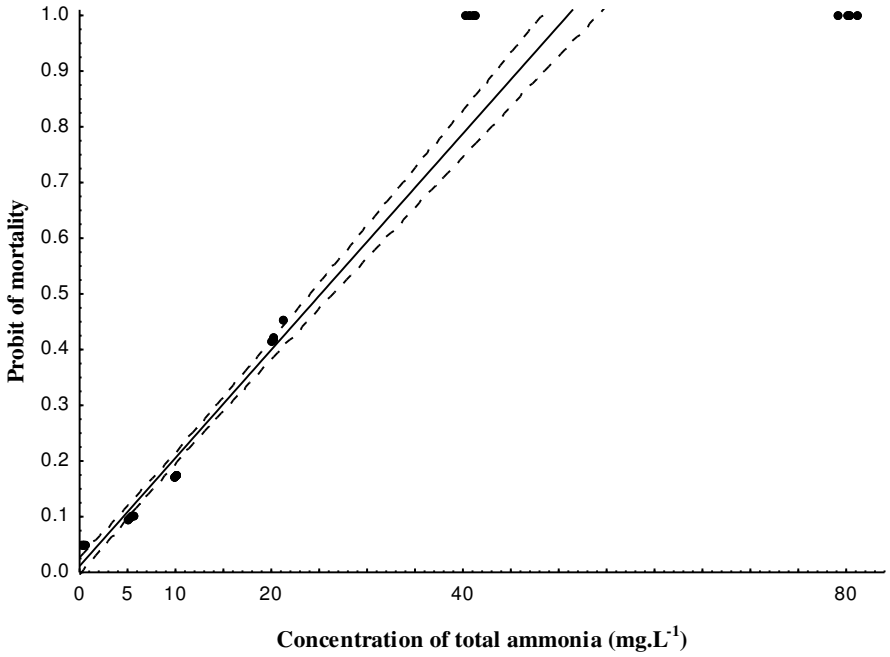

Figure 2. Linear regression of the LC_{50} for juveniles of *M. amazonicum* after 96 h of exposure to ammonia. Confidence intervals (95%) are represented by dashed lines. Graph adjusted to 40 $mg.L^{-1}$ to better represent the effect of mortality.

FONTE: Dutra (2017)

For adult *M. amazonicum*, 100% mortality was observed at a concentration of 80 $mg \cdot L^{-1}$ total ammonia, or 2.327 ± 0.550 $mg \cdot L^{-1}$ of NH_3-N after 48 h. The concentration of 40 $mg \cdot L^{-1}$ of total ammonia or 1.155 ± 0.070 $mg \cdot L^{-1}$ of NH_3-N produced an average mortality of 77 ± 21% after 96 h. median mortality for adults was predicted by the equation: $LC_{50} = 0.0137 + 0.0133X$ ($mg \cdot L^{-1}$ $N\text{-}NH_3 + N\text{-}NH_4^+$), $r^2 = 0.95$. Using this equation, we obtain a LC_{50} of 36.594 $mg \cdot L^{-1}$ for total ammonia or 1.078 $mg \cdot L^{-1}$ for NH_3-N (Fig. 3). Safe levels of exposure to ammonia for adult *M. amazonicum* would thus be 3.659 $mg \cdot L^{-1}$ for total ammonia and 0.108 $mg \cdot L^{-1}$ for NH_3-N.

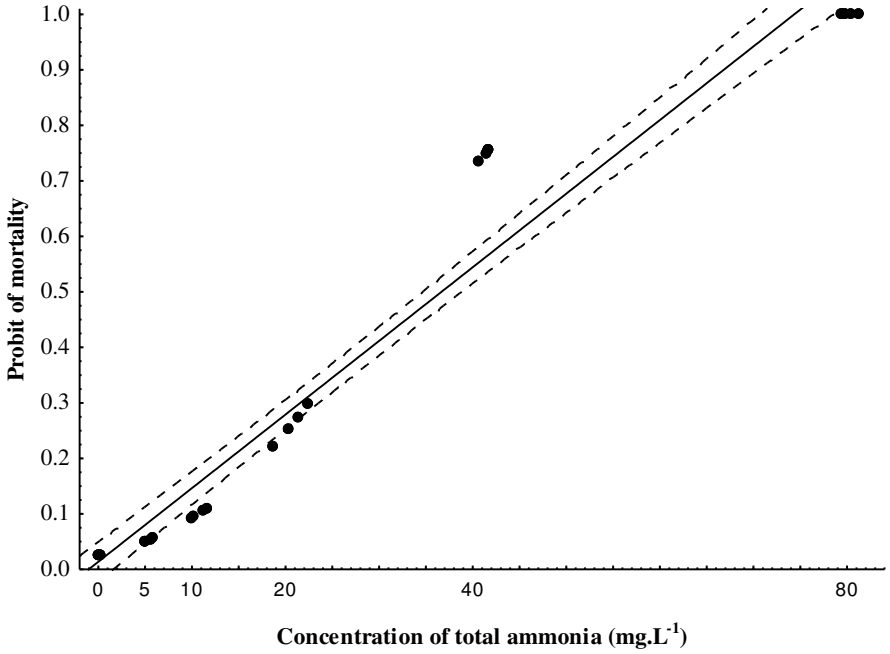

Figure 3. Linear regression of the LC_{50} for adults of *M. amazonicum* after 96 h of exposure to ammonia. Confidence intervals (95%) are represented by dashed lines.
FONTE: Dutra (2017)

Mortality rates of prawns exposed to ammonia concentrations of 5 and 10 $mg \cdot L^{-1}$ of total ammonia, for all life stages examined, were all below10%. The control (0$mg \cdot L^{-1}$ of total ammonia) for all ontogenetic stage presented mortality below 6%. This mortality rate is already expected in experimental conditions as demonstrated in previous research (Armstrong et al., 1976; Straus et al., 1991; Ostrensky and Wasielesky, 1995).

4. Discussion

Water quality parameters remained within the range reported by other authors for the production of *M. amazonicum* or other prawns of the same genus. Temperature ranges from 22–31 °C, dissolved oxygen ranges from 4.6 to 6.1 $mg \cdot L^{-1}$, pH ranges from 7.4–8.4 (Sampaio et al., 2007), hardness (for *Macrobrachium rosenbergii*) ranges from 20–200 $mg \cdot L^{-1}$ (Vasquez et al., 2007) and total alkalinity ranges from 20–60 $mg \cdot L^{-1}$ of $CaCO_3$ (New, 2002). Furthermore Coler et al. (1999) report that alkalinity values lower than 15 $mg \cdot L^{-1}$ $CaCO_3$ do not harm *M. amazonicum*. Nitrite and nitrate concentrations measured here were lower than those levels respectively suggested by Timmons et al. (2002), who recommended a threshold value for nitrite of 1 $mg \cdot L^{-1}$ in warm water aquaculture. Kozák et al. (2011) stated that the nitrite toxicity is strictly influenced by the presence of chlorides, and the threshold value is therefore highly

variable. Moraes-Valenti and Valenti (2010) suggested that nitrate values should not reach levels above 80 $mg \cdot L^{-1}$. Therefore, none of the water quality parameters measured in this study was limiting or stressful for the prawns. Thus, it can be argued that the lethal effects observed were due to the added ammonia.

The physiological processes of aquatic organisms, decomposition of organic matter and the supply of exogenous food in excess are the main sources of nitrogen compounds in aquaculture production systems (Moriarty, 1997). Colt and Armstrong (1981), in their review, reported toxic ammonia values (NH_3-N) for crustaceans in the range of 0.40 to 2.31 $mg \cdot L^{-1}$. The values determined in this study for the three life stages of *M. amazonicum* were within the range of LC_{50}-96 h reported for other prawns, palaemonids and peneids (Table 2).

Comparing the toxicity of NH_3-N after 96 h for the different species of prawns and shrimps (Table 2), one can see that the larvae of *M. rosenbergii* are more sensitive than later life stages of the same specie or other species (Armstrong et al., 1978; Mallasen and Valenti, 2005; Figueroa-Lucero et al., 2012). It should be noted that sensitivity to ammonia is related to other factors such as pH and temperature (Straus et al., 1991). In this last study, Straus et al. (1991) observed LC_{50} values for *M. rosenbergii* juveniles as 0.54 $mg \cdot L^{-1}$ NH_3-N at pH 9.5 and 29 °C. Under less alkaline pH levels, the LC_{50} increased to 2.18 $mg \cdot L^{-1}$ and 1.45, respectively, at pH 8.5 and 9.0 for post-larvae. For juveniles of *M. rosenbergii*, in that same study, the LC_{50} was 2.02 $mg \cdot L^{-1}$ at pH 9.0, during 72 h. Therefore, the sensitivity to ammonia depends on other factors such as temperature and pH, and is very close between these closely related species, *M. rosenbergii* and *M. amazonicum*.

When lethal ammonia levels determined for freshwater and marine species of shrimps are confronted (Table 2), we observe that marine species have higher LC_{50} values than those found for the freshwater species. Studies show that at lower salinity levels, higher levels of ammonia nitrogen are measured in the hemolymph (Chen et al., 1990a; Chen and Lin, 1991; Ostrensky and Wasielesky, 1995), when compared to levels found in the same species at higher salinities (Allan et al., 1990; Chen et al., 1990b; Lin and Chen, 2001; Romano and Zeng, 2013). The reason for these differences should be at least partially, the solubility of ammonia, lower in water containing salt (Bower and Bidwell, 1978). Consistently, sea water (32–40‰) displays ~20% less NH_3 than in fresh water with pH 7.5–8.5 and at 25 °C (Bower and Bidwell, 1978).

Table 2. Ammonia toxicity for freshwater prawns (*Macrobrachium*, Palaemonidae) and marine shrimps (Penaeidae) in different life stages.

Species	Life Stage	LC_{50}-96h -Total ammonia ($mg.L^{-1}$)	LC_{50}-96h - NH_3-N ammonia ($mg.L^{-1}$)	pH	Temperature (ºC)	Salinity ‰	Authors
Macrobrachium rosenbergii	Larvae	79.74-12.65	0.26 - 1.35(144 h)	6.8-8.3	28	12	Armstrong et al. (1978)
Macrobrachium rosenbergii		1 - 8	0.43 - 3.41	9,0	30	12	Mallasen and Valenti (2005)
Macrobrachium tenellum		12.66	0.41 (72 h)	7,7	28	20	Figueroa-Lucero et al. (2012)
Penaeus paulensis		9.39-21.98	0.73-0.85	8.2-7.9	25	28	Ostrensky e Wasielesky Jr (1995)
Macrobrachium amazonicum	Post-larva	***21,14***	***0,67***	***7,7***	***25,9***	-	Present study
Macrobrachium rosenbergii		-	2.18 - 1.45(72 h)	8.5-9.0	29	3	Straus et al. (1991)
Macrobrachium nipponense		36.6	1.97*	8,0	24	-	Wang et al. (2003)
Metapenaeus macleayi		26.3	1.39	8	25,1	34,5	Allan et al. (1990)
Penaeus monodon		37,4	1,69	8	26	34	Allan et al. (1990)
Penaeus paulensis		5,49	0,32	8,1	25	28	Ostrensky e Wasielesky Jr (1995)
Macrobrachium amazonicum	Juvenile	***21,65***	***0,75***	***7,8***	***25,1***	-	Present study
Macrobrachium rosenbergii		-	2.02 - 0.54(72 h)	9.0-9.5	29	3	Straus et al. (1991)
Litopenaeus vannamei		24.39-35.4-39.54	1.2 - 1.57 - 1.6	8,0	23	15 - 25 - 35	Lin e Chen (2001)
Penaeus chinensis		37.71	1.53	7,9	26	33	Chen et al. (1990b)
Penaeus monodon		53.4	0.96	7,6	24,5	20	Chen et al. (1990a)
Penaeus paulensis		38.72	1,10	7,8	25	28	Ostrensky e Wasielesky Jr (1995)
Penaeus penicillatus		24.88 - 29.77	0.99 - 1.11	8,1	21	25-34	Chen e Lin (1991)
Macrobrachium amazonicum	Adult	***36.59***	***1.08***	***7,7***	***25,9***	-	Present study
Penaeus paulensis		42.49	1.11	7,7	25	28	Ostrensky e Wasielesky Jr (1995)

* Estimated value of toxic ammonia

FONTE: Dutra (2017)

Beyond the question of solubility and higher volatilization of ammonia in the presence of salt, the reason is, most certainly, salt transport and acid–base regulation, which are different in marine and freshwater species. Penaeid marine shrimps are good hyper-regulators in diluted seawater and hypo-regulators in seawater (Lemaire et al., 2002; Charmantier et al., 2009). Freshwater hyper-regulators absorb Na^+ and Cl^- from the water, and ammonia (especially at lower pH values, typical of freshwater) would be in a greater extent found in the protonated form, NH_4^+, which can be absorbed by the absorption mechanisms of monovalent cations.

In fish, the higher the salinity, the greater the ability to excrete ammonia, and the lower the salinity, the higher the ammonia concentration found in the internal medium, such as found for crustaceans (Wright and Wood, 2012). Despite the differences between fish and crustaceans, there is convergence in the transport mechanisms that allow these comparisons (Kirschner, 2004; Freire et al., 2008; McNamara and Faria, 2012). Hyporegulating peneids would therefore have lower input of ammonia (Chen and Lin, 1991; Lin et al., 1993). In general, for osmoregulators such as palaemonid and penaeid shrimps, of intense interest for production, it is clear that ammonia, in any salinity affects osmoregulation, but more so in dilute seawater or freshwater (Chen and Lin, 1991; Lin et al., 1993).

The toxic effects of ammonia on post-larvaes, juveniles and adults are observed in various species of prawn (Table 2), but there are few studies reporting the effects on *M.*

amazonicum. Previous research with other species has shown reduction in development (Armstrong et al., 1978; Cavalli et al., 2000; Mallasen and Valenti, 2005), increase in mortality rate (Allan et al., 1990; Ostrensky and Wasielesky, 1995) and changes in physiological processes such as increased oxygen consumption (Wang et al., 2003; Barbieri, 2010) and alterations in nitrogen excretion (Barbieri, 2010; Romano and Zeng, 2013). Therefore, the LC_{50} and the safe levels determined in the present study are very important for the rearing of *M. amazonicum.* Comparisons among the life stages showed greater resistance of adults to ammonia indicating that early life stages require more care during the production cycle.

5. Conclusions

According to the results obtained, it was possible to determine the safe level of the total ammonia to each life stage of *M. amazonicum* allowing more reliability for the production of this species. The information obtained here have important implications for the production of this prawn, and other freshwater prawns of commercial relevance, the indication of precise limit levels of exposure to ammonia, enable the handling and management of these animals in production systems.

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CAPÍTULO II:

Acute Toxicity of Nitrite to Various Life Stages of the Amazon River Prawn *Macrobrachium amazonicum*, Heller, 1862[*]

(Formatado conforme "Instruções aos Autores" em anexo II)

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Abstract

This study determined the effects of nitrite on different life stages of the Amazon river prawn *Macrobrachium amazonicum*. Prawns of each life stage (postlarvae, juveniles and adults) were stocked in 24 experimental units (n = 10 prawns), under a complete randomized design. Individuals were exposed to nitrite (0, 1, 2, 4, 8 and 16 mg L−1). The median lethal concentration after 96 h (96 h LC_{50}) was calculated through the Weibull I. The mortality results showed that *M. amazonicum* is slightly less tolerant to nitrite than other species of *Macrobrachium*. The 96 h LC_{50} for postlarvae, juveniles and adults of *M. amazonicum* were of 1.49, 2.36 and 2.34 mg nitrite/L, respectively. Nitrite intoxication risk quotient suggest moderated risk to low risk to the species. Usually in production systems nitrite values are lower than safe levels suggested in this study (0.1 mg L^{-1} to postlarvae and 0.2 mg L^{-1} nitrite to juvenile and adults), which makes our results appropriate for the production of this species.

Keyword: Freshwater prawn. Nitrogen compound. Macrobrachium. Toxicity

1. Introduction

Freshwater prawn farming has shown great increase worldwide in the last two decades (FAO 2014; Hayd et al. 2014). Although the main reared prawn in Brazil is still the exotic species *Macrobrachium rosenbergii*, the indigenous species *M. amazonicum* has demonstrated similar production potential (Moraes-Valenti et al. 2010). The production of native species is essential for the conservational aspect of good aquacultural practices. Production of native species of prawns has become a trend, as the production of *Macrobrachium nipponense* in China (Kutty and Weimin 2010) and *Macrobrachium malcolmsonii* in India (Kutty 2005; Kutty and Valenti 2010). This practice prevents possible problems caused by the introduction of exotic species in the environment (Bridger and Garber 2002), besides reducing the uncontrolled exploitation caused by fishing (Silva et al. 2007).

Physiological processes of aquatic organisms, decomposition of organic matter, and food leftovers are the main sources of nitrogen compounds in aquaculture production systems (Campos et al. 2012). Nitrite is the intermediate compound in the bacterial nitrification of ammonia to nitrate. It may present high toxicity, depending on its concentration in the environment and organism developmental stage (larvae to adult) (Miranda-Filho et al. 1995). Several studies with *M. amazonicum* were already carried out to better understand its biology (Boudour-Boucheker et al. 2013; Meireles et al. 2013), ecology (Moreira and Collart 1993;

Dutra et al. 2014), and production features (Anger and Hayd 2010; Moraes-Valenti et al. 2010). However, studies on nitrite effects available in the literature are limited to larvae (Hayd et al. 2014), since the studies to cultured shrimps are mainly focused on penaeids (e.g., Cheng and Chen 2002; Chen and Cheng 2000). Mallasen and Valenti (2006) recommend nitrite values below 2.0 mg L^{-1} in the production of freshwater prawns.

Therefore, based on the hypothesis that the early stages of development of *M. amazonicum* are more sensitive to nitrite than later stages, the aim of the study was to assess the effect of nitrite on different life stages of the Amazon river prawn - *Macrobrachium amazonicum*, Heller, 1862, through the determination of 96 h LC_{50} concentrations of nitrite. To determine LC_{50} values, the prawns were submitted to six different nitrite concentrations (0, 1, 2, 4, 8 and 16 mg nitrite/L) in four replicates, during 96 h. Afterwards, the mortalities were assessed for each life stage (postlarvae, juveniles and adults).

2. Material and Methods

The experimental work described here was conducted at the Prawn Culture Laboratory, located at the Federal University of Paraná - Sector Palotina, with animals provided by the Laboratory of Prawn Culture of the CA-UNESP (Universidade Estadual Paulista "Julio de Mesquita Filho"). The experiments used 240 prawns of each life stage (postlarvae, juveniles and adults). The prawns reach the post-larvae stage after they go through all larval development (nine different stages from zoea I to zoea IX), the juvenile stage is reached approximately 30 days after they reach the post-larvae stage and the prawns are considered adults when they reach the reproductive stage and can be identified through the gonadal development (Vega-Villasante and Carrillo 2006; New et al. 2010). The prawns were randomly divided into 24 experimental units, with 10 prawns in each experimental unit. The wet weight and the total length (rostrum to telson) were measured in postlarvae (0.088 ± 0.022 g; 2.250 ± 0.170 cm, mean ± SD), juveniles (2.023 ± 0.271 g; 6.729 ± 0.296 cm) and adults (6.240 ± 1.827 g; 9.084 ± 0.793 cm); n = 50 out of the 240 used, randomly chosen. As experimental units we chose beakers with a volume of 1 liter to post larvae, or glass aquaria containing 10 liters of useful volume to juveniles and adults. Experimental units were equipped with aeration systems and light sources for a light:dark rhythm of 12:12 h, in a climate-controlled room (25 to 27ºC). The design was completely randomized, with six different nitrite concentrations (0, 1, 2, 4, 8, and 16 mg nitrite/L), and four replicates per nitrite concentration. The nitrite levels resulted from the addition of appropriate volumes of the stock solution of $NaNO_2$ PA - Synth® (stock solution of

250 mg nitrite/L) to produce the desired final nitrite concentrations. The concentrations used were based on work done with *M. rosenbergii* (Mallasen and Valenti 2006). Mortality was assessed by total absence of movement or reaction to mechanical stimuli using a glass rod. Prawns were observed every 1 hour for the first 8 hours. Between 8 hours and 96 hours, observations were performed every 12 hours (Armstrong et al. 1976).

The following variables of water quality were daily evaluated: dissolved oxygen (Oxymeter, Hanna HI 9146), temperature (digital thermometer Incoterm), and pH (Phmeter, Tekna T-100). At the beginning and at the end of the experiment, alkalinity and hardness of water samples were assayed by titration. Total ammonia levels were determined according to Koroleff (1976), where the sample containing ammonia reacts with phenol and sodium hypochlorite in alkaline solution to form a blue color solution which is catalyzed by sodium nitroprusside. The resulting absorbance was measured in a spectrophotometer (BEL photonics 2000UV, Brazil) at 630 nm. Nitrite and nitrate levels were determined according to Mackereth et al. (1978). The nitrite concentration was determined through the formation of a purple-red solution formed by diazotization of sulfanilic acid with N-(1-naphthyl)-ethylenediamine dihydrochloride. The resulting absorbance was measured at 540 nm. Nitrate concentration was determined through the reduction of nitrate to nitrite by amalgamated cadmium. The resulting concentration (nitrite originally present in the sample plus reduced nitrate) was measured according to the methodology described above for nitrite. The concentration of nitrite originally present was subtracted from the total concentration of nitrite obtained. The nitrogenous compounds were detected and measured in a spectrophotometer, at detection limit of 0.02 mg L^{-1} (lower limit), 0.005 mg L^{-1}, and 0.05 mg L^{-1} to ammonia, nitrite and nitrate, respectively. Afterwards, absorbance data were read against a standard curve to calculate the concentrations of the different nitrogenous compounds in the samples using the software "win-spec" (version 2.3.1). As standard compounds to determine the standard curve of ammonia, nitrite and nitrate were used ammonium chloride, sodium nitrite and potassium nitrate, respectively. Water quality variables were determined independently for each treatment in order to establish whether they remained at appropriate levels for the species (Moraes-Valenti and Valenti 2009).

The determination of the median lethal concentration (96 h LC_{50}) was based on the manual of the Environmental Protection Agency USA (Peltier and Weber 1985). The safe level (SL) was determined by multiplying the value obtained in the test of toxicity by an application factor of 0.1, as recommended by Sprague (1971).

Scatter plots were built between the nitrite concentration (mg L^{-1}) and the cumulative mortality for post larvae, juveniles and adults separately. Various dose-response models were

tested and the best fit was achieved with the Weibull I function (Ritz et al. 2015). The choice of the "best fit" was based on the values of standard error (S) and of the coefficient of determination (r^2), on the residual analysis (Sokal and Rholf, 1995) and of the biological adequacy.

The intoxication risk classification by nitrite was performed by quotient method, adapted of Urban & Cook (1986). The quotient was calculated by ratio between the recommended concentration in literature to the genus *Macrobrachium* and the 96h LC_{50} calculated value to species in study. We used the following intoxication risk classes: without risk ($Q \leq 0.1$); low risk ($0.1 < Q \leq 1$); moderated risk ($1 < Q \leq 10$) and high risk ($Q > 10$).

3. Results and discussion

During the experiment, temperature, pH, water hardness and alkalinity remained within suitable ranges for freshwater prawns culture (Coler et al. 1999; Sampaio et al. 2007; Vasquez et al. 2007). Ammonia concentrations measured here were lower than those levels suggested by Timmons et al. (2002), who recommended a threshold value for ammonia of 2 mg L^{-1} in warm water aquaculture. In the same way, the nitrate concentrations were lower than those levels recommended by Moraes-Valenti and Valenti (2010), who suggested that nitrate values should not reach levels above 80 mg L^{-1} (Table 1). Therefore, none of the water quality parameters measured in this study, except nitrite, were limiting or stressful for the prawns. Thus, it can be argued that the lethal effects observed were due to the nitrite concentrations evaluated.

Mortality observed for the different life stages at the control treatment (not exposed to nitrite) during the trial period was below 10%, which may be related to the behavior of animals in competition for area (territorialism), as demonstrated in previous research (Armstrong et al. 1976; Ostrensky and Wasielesky Jr. 1995).

Independently of the life stage of prawns, it is possible to verify that mortality increased with increasing nitrite concentration. Postlarvae of *M. amazonicum* exposed to nitrite showed 48 ± 2% (mean ± SD) mortality after 96 hours of exposure to 1 mg nitrite/L. The concentration of 2 mg L^{-1} yields mortality of 90 ± 2% in 96 hours. At the concentrations of 4-16 mg nitrite/L, mortality was of 100% in 24 hours. When evaluating mortality caused by nitrite by the Weibull I function, the lethal concentration for 50% of the prawns (LC_{50}) after 96 hours is 1.49 mg nitrite/L (Table 2; Figure 1A).

Table 1. Water quality parameters (mean ± SD; n = 4) for experiments carried out for the different life stages of *M. amazonicum.*

Variables	Postlarvae in experiment with nitrite						Recommended values for prawn culture
	0 mg L^{-1}	1 mg L^{-1}	2 mg L^{-1}	4 mg L^{-1}	8 mg L^{-1}	16 mg L^{-1}	
Dissolved Oxygen (mg L^{-1})	6.60±0.41	6.67±0.35	6.50±0.30	6.60±0.39	6.50±0.29	6.34±0.33	3 - 7
Temperature H_2O (ºC)	25.01±0.28	25.12±0.33	24.99±0.22	24.83±0.05	24.90±0.14	24.85±0.06	24 - 30
pH	8.32±0.10	8.26±0.09	8.24±0.05	8.24±0.05	8.15±0.05	8.16±0.06	7- 8.5
Alkalinity (mg L^{-1} $CaCO_2$)	26.35±0.73	28.50±0.89	24.08±0.97	23.13±0.66	23.88±1.07	24.03±1.12	20 - 60
Hardness (mg L^{-1})	37.08±2.10	34.78±1.50	29.38±2.98	28.70±2.92	22.00±1.27	24.25±6.18	20 - 150
Total ammonia (mg L^{-1})	0.623±0.009	0.568±0.059	0.619±0.042	0.625±0.02	0.494±0.034	0.435±0.123	< 1
Nitrate (mg L^{-1})	0.159±0.027	4.10^{-5}±2.10^{-5}	9.10^{-5}±4.10^{-5}	2.10^{-5}±3.10^{-6}	4.10^{-4}±1.10^{-4}	7.10^{-4}±1.10^{-4}	80
Nitrite (mg L^{-1})	0.019±0.002	1.087±0.036	2.244±0.1	4.108±0.675	8.337±0.239	16.547±0.325	< 2
	Juveniles in experiment with nitrite						
Dissolved Oxygen (mg L^{-1})	6.84±0.49	6.71±0.56	6.62±0.46	7.16±0.31	7.02±0.10	7.02±0.26	3 - 7
Temperature H_2O (ºC)	25.04±0.16	25.06±0.15	25.11±0.18	25.27±0.09	25.37±0.05	25.02±0.05	24 - 30
pH	8.02±0.11	8.01±0.12	8.02±0.12	8.00±0.05	7.69±0.02	8.05±0.03	7- 8.5
Alkalinity (mg L^{-1} $CaCO_2$)	46.40±5.75	51.98±4.70	50.20±2.51	43.07±0.40	41.05±0.83	37.87±1.42	20 - 60
Hardness (mg L^{-1})	31.85±8.74	42.20±9.63	37.12±6.32	25.27±0.99	24.80±0.59	22.42±0.66	20 - 150
Total ammonia (mg L^{-1})	3.10^{-4}±4.10^{-6}	2.10^{-4}±2.10^{-5}	3.10^{-4}±4.10^{-6}	3.10^{-4}±5.10^{-6}	3.10^{-4}±1.10^{-6}	3.10^{-4}±1.10^{-6}	< 1
Nitrate (mg L^{-1})	1.10^{-4}±4.10^{-5}	4.10^{-5}±2.10^{-5}	9.10^{-5}±5.10^{-6}	1.10^{-4}±3.10^{-5}	3.10^{-4}±1.10^{-4}	7.10^{-4}±1.10^{-4}	80
Nitrite (mg L^{-1})	0.040±0.006	0.955±0.041	2.170±0.11	4.026±0.085	8.176±0.31	16.088±0.307	< 2
	Adults in experiment with nitrite						
Dissolved Oxygen (mg L^{-1})	6.43±0.27	6.40±0.33	6.51±0.29	6.44±0.19	6.67±0.17	6.21±0.45	3 - 7
Temperature H_2O (ºC)	25.98±0.48	25.88±0.38	26.14±0.43	25.63±0.41	25.78±0.37	25.60±0.36	24 - 30
pH	7.73±0.09	7.70±0.08	7.75±0.12	7.69±0.14	7.64±0.03	7.71±0.06	7- 8.5
Alkalinity (mg L^{-1} $CaCO_2$)	20.65±1.66	19.25±2.73	23.83±1.12	23.03±1.16	19.60±1.51	20.53±1.75	20 - 60
Hardness (mg L^{-1})	29.43±4.88	24.25±1.35	34.68±5.68	34.28±3.44	23.48±1.03	30.50±2.43	20 - 150
Total ammonia (mg L^{-1})	0.042±0.083	0.033±0.066	0.028±0.057	0.447±0.253	1.10^{-4}±3.10^{-5}	3.10^{-4}±2.10^{-5}	< 1
Nitrate (mg L^{-1})	1.10^{-5}±3.10^{-6}	4.10^{-4}±1.10^{-5}	1.10^{-5}±2.10^{-6}	1.10^{-4}±1.10^{-5}	3.10^{-4}±1.10^{-5}	7.10^{-4}±6.10^{-5}	80
Nitrite (mg L^{-1})	0.048±0.02	1.103±0.009	2.385±0.444	4.117±0.255	8.012±0.036	16.074±0.139	< 2

FONTE: Dutra (2017)

Juvenile *M. amazonicum* exposed to concentrations of 2 mg L^{-1} and 4 mg L^{-1} showed average mortality of 55 ± 3% and of 95 ± 1% after 96 hours, respectively. Concentrations of 8-16 mg nitrite/L exhibited 100% mortality after 24 hours. Lethal concentration for 50% of the juveniles in 96 hours was 2.36 mg nitrite/L (Table 2; Figure 1B).

For adult *M. amazonicum*, the concentrations of 1 mg L^{-1} and 2 mg L^{-1} produced average mortality of 27 ± 8% and 50 ± 8% in 96 hours, respectively. Concentration of 4 mg L^{-1} caused average mortality of 80 ± 3% in 96 hours. 100% mortality was also observed at 8-16 mg nitrite/L in 24 hours. Average mortality for 50% of the adults after 96 h is obtained with 2.34 mg nitrite/L (Table 2; Figure 1C). Sahoo and Chand (2006), evaluating the effect of nitrite on the immune response of adult *Macrobrachium malcolmsonii*, measured a LC_{50} of 3.14 mg L^{-1} after 96 hours. Thus, it seems that *M. amazonicum* adults are slightly less tolerant to nitrite than *M. malcolmsonii* adults as it shows lower LC_{50} values: 2.34 mg L^{-1}. Mallasen and Valenti (2006) recommend nitrite values during the production of freshwater prawns below 2.0 mg L^{-1}. Hayd et al. (2014) claim that safe levels below 0.8 mg nitrite/L may be used as a general reference for production system. However, in the present study results pointed out that the safe levels for the production of *M. amazonicum* are much lower (0.14 to 0.23 mg L^{-1}) (Table 2).

Regarding the classification of intoxication risk by nitrite to *M. amazonicum*, the values were of 1.34 to postlarvae, 0.85 to juveniles and 0.85 to adults, suggesting moderated risk to postlarvae and low risk to juvenile and adult (Urban & Cook 1986).

Table 2. LC_{50} calculated and their 95% confidence intervals of nitrite to 96h, safe level of nitrite, standard error (S) and r^2 for the different life stages of *M. amazonicum.*

Life stages	96h LC_{50} calculated of nitrite (mg L^{-1})	Confidence interval (95%)	Safe level to nitrite (mg L^{-1})	S	r^2
Post larvae	1.49	1.30 – 1.72	0.14	0.67	0.98
Juvenile	2.36	2.11 – 2,63	0.23	0.78	0.94
Adult	2.34	2.00 – 2,67	0.23	0.73	0.96

FONTE: Dutra (2017)

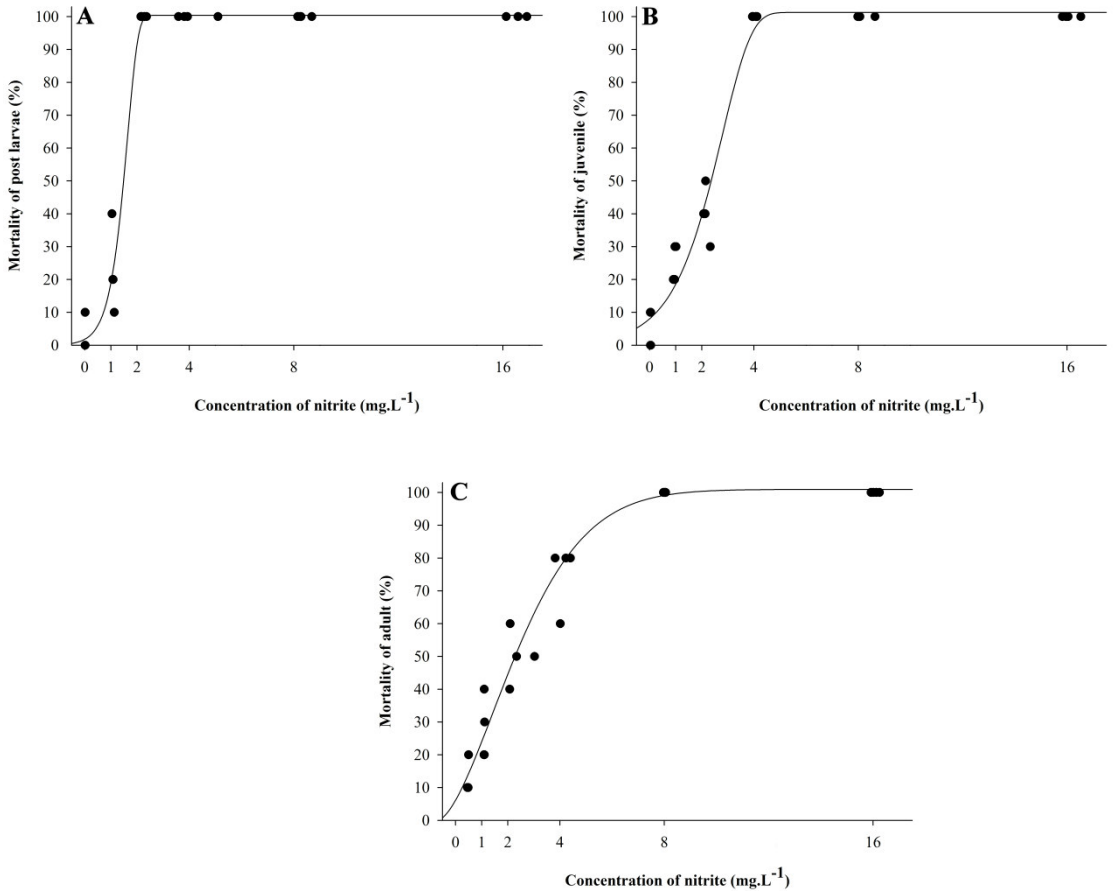

Figure 1 Mortality curves for each life stages of *M. amazonicum* after 96 hours of exposure to nitrite. **A**) Post larvae. **B**) Juveniles. **C**) Adults.

FONTE: Dutra (2017)

Comparing sensitivity to nitrite in the different life stages of *M. amazonicum*, it was found that the sensitivity to this compound is related to the animal's stage of development, where the later stages showed higher resistance. The sensitivity of an organism to a toxic agent can vary depending on its size, age and stage of development (Wajsbrot et al. 1993), because several enzymes may have differential activities along development or aging (Barbieri et al., 2002). In the early stages of development, the organisms are more sensitive generally due to increased mitotic activity (Barbieri 2008), and also possibly their higher surface to volume ratios, as they are smaller organisms. This variation in sensitivity of different ontogenetic stages has been observed previously in *Macrobrachium*, in studies with other compounds such as pesticides (Dai et al. 2014), heavy metals (Asih et al. 2013) and nitrogen compounds (ammonia and nitrite) (Lin et al. 1993; Mallasen and Valenti 2005, 2006).

In production systems, the nitrite values generally are below the safe limit determined in this study. Marques et al. (2012) observed in production systems in net pens nitrite values

ranging from 0.004 to 0.011 mg L^{-1}; Kimpara et al. (2011) found in ponds values ranging from 0.026 to 0.028 mg nitrite/L, while the recommended nitrite limits here are of 0.1 mg L^{-1} for post larvae and 0.2 mg L^{-1} for juveniles and adults. Thus, the results obtained have important implications for the production of this prawn and allow the indication of precise limit levels for the exposure of the different life stages of this species to nitrite, enabling the handling and management of these animals in production systems. Thus, monitoring this variable is important to avoid losses, mainly in larviculture and recirculation systems.

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CAPÍTULO III:

Histological alterations in gills of *Macrobrachium amazonicum* juveniles exposed to ammonia and nitrite [*]

(Formatado conforme "Instruções aos Autores" em anexo III)

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Abstract

This study aimed to assess histological changes in the gills of *Macrobrachium amazonicum* juveniles subjected to different concentrations of total ammonia and nitrite. The prawns were subjected to different concentrations of those compounds and their gills were removed and preserved for histological analysis. The gills were assessed for changes according to the Organ Index (Iorg) and, for each change, an importance factor (w) was attributed according to the degree of reversibility and applied according to the degree of extension or frequency of the damage. The damage to the gills in the treatments with mortality of 100%, both for ammonia and nitrite, corresponded to the high occurrence of progressive, regressive, circulatory, and inflammation damages. The other treatments had mainly inflammation and regressive damages, whose occurrence increased according to the increase in ammonia and nitrite concentration. The histological analysis confirmed that the higher the total ammonia and nitrite concentrations, the larger the damages caused to the gill structure and that lower nitrite concentrations caused similar damages to those caused by higher total ammonia concentrations, which reflects the lower capacity *M. amazonicum* has to tolerate this compound.

Keywords: Toxicology; Nitrogen compounds; Prawn production; Freshwater prawn.

1. Introduction

Crustaceans are invertebrates that depend on water quality for survival and are increasingly subjected to contaminated or low-quality waters in the different habitats across which they are distributed (Silva and Martinez, 2007). The easy exposure to contaminants or the alteration in physical and chemical water parameters causes stress responses in these organisms (Tomasso, 1994; Camargo and Alonso, 2006), such as growth inhibition, reproductive alterations, and changes in immune and behavioral responses (Kumar et al., 2015).

Pollutant discharges and the intensification of the pollution process in aquatic environments due to the accumulation of household, agricultural, and industrial effluents have been promoting changes in the physical, chemical, and biological characteristics of those environments (Paul and Meyer, 2001; Begun, 2004; Becker et al., 2009). The contamination of those environments by anthropic activities has caused from biochemical alterations in cells to the reduction in biological diversity (Paul and Meyer, 2001; Silva and Martinez, 2007; Clausen and York, 2008). This rampant influx of contaminants may cause poisoning and even death of the organisms (Boyd, 1986), leading to environmental and economic losses (Bennett et al.,

2001). Among contaminants, ammonia and nitrite are the compounds that most affect the health of aquatic organisms since they quickly reach toxic concentrations (Romano and Zeng, 2013).

Ammonia is a nitrogen compound that occurs naturally in the environment (Martín and Frederico, 2001), but that is also generated by industrial, household, and agricultural pollution and by environmental changes (Rebelo et al., 2000). It is also the main product generated by the protein catabolism of most aquatic organisms and by the decomposition of non-digested food and other organic residues (Noga, 1996). The compound has been proven to be toxic (Armstrong et al., 1978; Barbieri, 2009) and influences growth, feeding, survival, and the susceptibility to parasites and diseases in prawns and other aquatic organisms (Kir et al., 2004; Mugnier and Justons, 2004). In aquatic environments, ammonia is found in ionized (NH_4^+) and non-ionized (NH_3) forms, the sum of which constituted total ammonia ($NH_4^+ + NH_3$). Its toxic effect in the environment will depend on pH and temperature (Emerson et al., 1975). In fresh water, prawns tend to be hyper-regulators, absorbing Na^+ and Cl^- from the water and, consequently, ammonia (Freire et al., 2003; Jensen, 2003). Many researchers states that the non-ionized form of ammonia is chemically more toxic due to its capacity of diffusion through the lipid bilayers of the gill membranes, harming the gill epithelium and, consequently, destabilizing the osmoregulation system (Wang, 2003; Romano and Zeng, 2012; Romano and Zeng, 2013; Zhang et al., 2015).

Nitrite is a natural compound in aquatic and land ecosystems and its presence in the environment is a potential problem, with well-documented toxicity (Armstrong et al., 1976; Lewis and Morris, 1986; Chen and Lee, 1997a,b; Mallasen and Valenti, 2006; Hayd et al., 2014). Since it is an intermediate compound in the bacterial nitrification of ammonia into nitrate, it may be highly toxic depending on its concentration in the medium and of the development stage of the organism (Miranda-Filho et al., 1995; Romano and Zeng, 2013). Moreover, it plays an important role in the metabolism of many organisms since it is involved in the immune response of vertebrates and invertebrates (Wang et al., 2004; Kroupova et al., 2005; Lundberg et al., 2009). Nitrite is also known to diffuse in the hemolymph of crustaceans (Cheng and Chen, 2002) and to be transported instead of Cl^-. This nitrogen compound accumulates more in the extracellular liquid of freshwater crustaceans than in marine organisms (Cheng and Chen, 1998; Jensen, 2003), thus causing an increase in free O_2 and a decrease in O_2 bound to hemocyanin (oxy-hemocyanin). That reduces the hemolymph's capacity of transporting oxygen (Chen and Cheng, 1996), as well as induces the formation of metahemocyanin and harms the respiratory metabolism, thus leading to hypoxia of the respiratory tissue.

The Amazon river prawn (*Macrobrachium amazonicum*) belongs to a group of species with coastal and continental populations (Moraes-Valenti and Valenti, 2009). Its occurrence is well described for estuaries, rivers, lakes, and alluvial plains of tropical and subtropical regions of South America (Maciel and Valenti, 2009; Moraes-Valenti and Valenti, 2009). In Brazil, *M. amazonicum* is one of the native freshwater prawn species with the highest potential for aquaculture (New, 2005; Maciel and Valenti, 2009) due to its tasty flesh, its broad regional distribution, and its rusticity, which favors its rearing in captivity (Moraes-Riodades and Valenti, 2001; Maciel and Valenti, 2009).

Toxicology studies using lethality assays (CL50) are recommended to provide basic information on the toxic effects of a substance for a living organism (IBAMA, 1990), however, they do not provide information on the damage caused to the organism due to poisoning. Thus, histopathology has been a commonly employed tool in the identification of damage caused to organisms subjected to a toxic agent (Miron et al., 2008). Although some studies with histological analyses have assessed the effect of pesticides (Lignot et al., 1997; Saravana Bhavan and Geraldine, 2000), heavy metals (Asih et al., 2014; Ben-Khedher et al., 2014), viruses (Pazir et al., 2011; Yun et al., 2014), and cobalt-60 gamma radiation (Stalin et al., 2013a; Stalin et al., 2013b) on the gills of prawns, none assessed the effects of nitrogen compounds on the gills of freshwater prawns. Therefore, the present study aimed to assess histological alterations in the gills of *M. amazonicum* juveniles subjected to different total ammonia and nitrite concentrations and to compare them using the organ index.

2. Material and Methods

The experimental work was conducted at the Prawn Culture Laboratory and the histological analyses were performed at the Histopathology Laboratory, both located at the Federal University of Paraná - Sector Palotina, Brazil.

2.1. Experimental Design

Each toxicity test (total ammonia and nitrite) used 240 *M. amazonicum* juveniles divided into 24 experimental units with 10 prawns each. The prawns were weighed in an analytical balance (Marte® - AY220) and measured from the tip of the rostrum to the tip of the telson using digital calipers (Kingtools -502.300BL). The initial wet weight mean and total length mean were 2.07±0.62 g and 6.29±0.71 cm, respectively, in the experiment with total ammonia and 2.02±0.27 g and 6.72±0.29 cm for the experiment with nitrite. The design was completely

randomized, comprising six treatments corresponding to the concentrations of total ammonia (0, 5, 10, 20, 40, and 80 mg.L^{-1} total ammonia) and nitrite (0, 1, 2, 4, 8, and 16 mg.L^{-1} nitrite) with four replicates per treatment. Mortality was assessed by the lack of movement or reaction to mechanical stimuli.

2.2. Histological Analyses of Gills

The last five recently dead *M. amazonicum* juveniles and the survivors of the experiments were collected for each treatment and replicate and their cephalothorax was sectioned and fixated in ALFAC (Lightner and Bell, 1998) for 48 h. Next, the gills were removed from the cephalothorax and fixated in 70% alcohol.

For the preparation of the histological slides, the gills were placed in cassettes with filter paper and dipped in a graded alcohol series (70%, 80%, 90%, and absolute), diaphonized in xylol (three baths), and impregnated in histological paraffin at 56 °C for 40 min in each procedure. After the pieces were embedded and modeled in paraffin blocks, the tissues were cut into 5 µm-thick slices in a semi-automated rotary microtome (Leica® RM2245) and then stained with hematoxylin-eosin (H&E) (Bancroft and Cook, 1994; Tolosa et al., 2003). The images of the gills were captured using a microscope (Leica® DM1000) equipped with a 3-megapixel camera (Leica® DFC295) and visualized in the software LAS v 3.8 (Leica® Application Suite).

The alterations in the gills were assessed according to the organ index (I_{org}) (Bernet et al., 1999) adapted for invertebrates according to Costa et al. (2013). The alterations were classified according to the type of standard reaction (1 to 4), with an importance factor (w) being attributed to each alteration according to the degree of reversibility, where: 1 = easily reversible alterations; 2 = moderate alterations, reversible with the end of exposure, and 3 = irreversible alterations with partial or total loss of organ function (Table 1).

The score value (a) was according to the degree of distribution and intensity of organ damage, where: “0” = Unchanged; “1-2” = Mild occurrence; “3-4” = Moderate occurrence, and “5-6” = Accentuated occurrence.

From the above ratings were calculated the Index of the Organ (*Iorg*):

$$\boldsymbol{I_{org}} = \Sigma_{\mathbf{alt}} (\boldsymbol{a} \,.\, \boldsymbol{w})$$

Where: "I_{org}" = Organ Index; "alt" = alteration; "a" = Score value; and "w" = importance factors. The index represents the degree of organ injury. Therefore, an index with high value represents a high degree of organ injury.

Table 1. Histopathological assessment tools for gills of *M. amazonicum*. Importance factor (w) ranging from 1 to 3 for every alteration in its respective reaction pattern (rp) and alteration (alt).

Reaction pattern	Characteristic of the reaction	Description	Alteration	Importance factors (w)
1	Circulatory disturbance	Pathological condition of the flow of hemolymph and tissue fluids.	Clavate-globate "clubbing" *	1
2	Regressive changes	Pathological condition that shows functional reduction or loss in organ and/or structure.	Cellular tumefaction (lamellar swelling)†	1
			Thickening of the lamellar epithelium	1
			Edema	2
			Fused lamellae	2
			Necrosis	3
			Lifting of the lamellar cuticle	3
3	Progressive changes	Pathological condition causing increased cellular and/or tissue activity.	Hyperplasia ‡	2
4	Inflammation	Interstitial fluid containing high concentration of proteins and cellular debris that goes beyond the hemolymph vessels.	Hemocytic infiltration	2

FONTE: Dutra (2017)

2.3. Statistical Analyses

The results of mortality (CL_{50}) and I_{org} were subjected to the assumptions of normality and homogenicity of the variances (Sokal and Rohlf, 2012). After those assumptions were

*Clavate-globate "clubbing" is a specific tissue reaction to toxic substances in water or food that causes an agglomerate or congestion of the tissue with blood fluid.

† Cellular tumefaction is the intracellular accumulation of water due to failure of active transport, hypoxia, or damage caused by toxic substances.

‡ Hyperplasia is the increase in the number of cells of an organ or tissue promoted by the abnormal multiplication of cells after damage.

confirmed, they underwent one-way analysis of variance (ANOVA) and, when significant differences were found, Tukey's *a posteriori* test (α=0.05) was applied.

3. Results

Over the experimental period, the *M. amazonicum* juveniles exposed to total ammonia concentrations of 80 mg.L^{-1} had mortality of 100% after 48 h of exposure, which was significant compared to the other treatments ($p<0.05$), as seen in Figure 1. In 72 h, the treatment with concentrations of 40 mg.L^{-1} total ammonia reached 100% mortality, statistically differing from the treatments with 0, 5, 10, and 20 mg.L^{-1} total ammonia ($p<0.05$). By the end of the experimental period (96 h), mortalities of 8±5%, 10±8%, 20±8%, and 48±10% were observed in the treatments with 0, 5, 10, and 20 mg.L^{-1} total ammonia, respectively. The treatment with 20 mg.L^{-1} total ammonia statistically differed from the others ($p<0.05$; Figure 1).

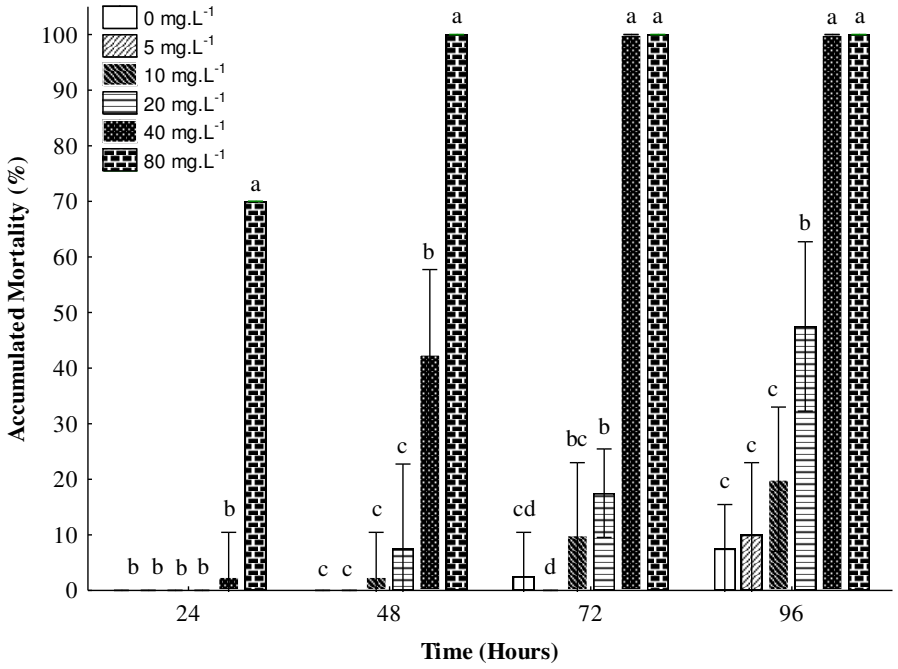


Figure 1. Mean±SD of mortality (%) of *M. amazonicum* juveniles exposed to total ammonia over 96 h.

FONTE: Dutra (2017)

At nitrite concentrations of 4 to 16 mg.L^{-1}, 100% mortality was observed in 24 h, with no significance among the treatments ($p>0.05$). At the concentration of 2 mg.L^{-1} nitrite, mean mortality after 96 h of exposure to the compound was 40±8%, significantly different from the other treatments ($p<0.05$). The same was observed at 1 mg.L^{-1} nitrite, with mean mortality of

27±6% over 96 h. The concentration of 0 mg.L^{-1}, with no nitrite or with negligible concentration values, had mean mortality of 5±6% over 96 h of exposure, significantly different from the other concentration ($p<0.05$; Figure 2).

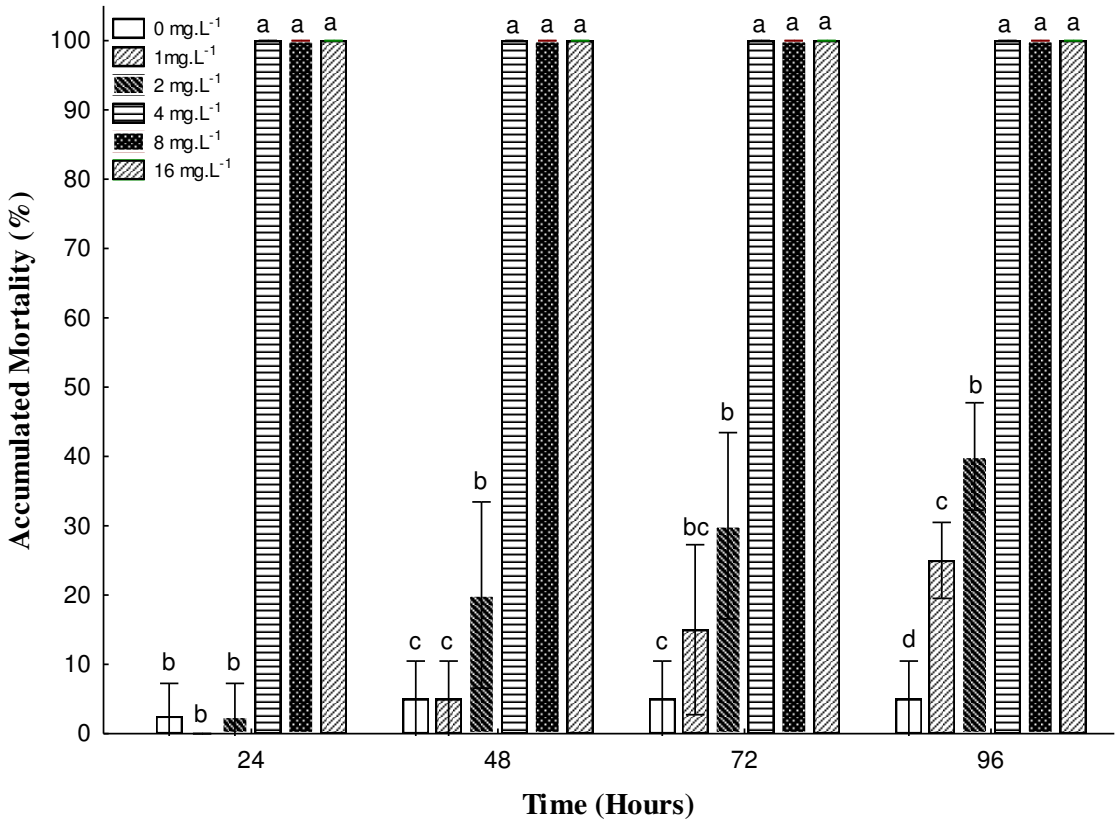


Figure 2. Mean±SD of mortality (%) of *M. amazonicum* juveniles exposed to nitrite over 96 h.
FONTE: Dutra (2017)

Gill structures are formed mainly by pillar cells, lamellar epithelium, and hemocytes. The pillar cells have wide base and apical extremities shaped as an elongated "I." Those cells play the role of supporting the organ, as well as forming spaces through which hemolymph runs. The lamellar epithelium has a generally thin shape, surrounded by a chitinous cuticle, thickening the basal and distal portions of the gill filament. Hemocytes are rounded and play the roles of gas transport and immunologic defense. The histopathological observations of *M. amazonicum* gills were carried out by comparing the control treatment with the other treatments for ammonia and nitrite (Figures 5 and 6). The degenerative effects on the gill structure are observed as concentration increases.

The analyses of the gills of *M. amazonicum* subjected to 80 mg.L^{-1} total ammonia showed that the high I_{org} value (50.5±6.1) for this treatment was statistically different from the

other treatments ($p<0.05$). This result was caused by the presence of several reaction patterns (progressive, regressive, and inflammation damages). The I_{org} for the concentrations of 40 and 20 mg.L^{-1} total ammonia were statistically similar ($p>0.05$) at 34.0±6.2 and 33.3±7.8, respectively. The treatment with concentration of 5 mg.L^{-1} did not significantly differ from the control treatment ($p>0.05$) and was statistically equal to the treatment with concentration of 10 mg.L^{-1}. The I_{org} values for those treatments were, respectively, 10.7±2.1 and 17.3±2.9, while the value for the control treatment was 3.7±1.9 (Figure 3).

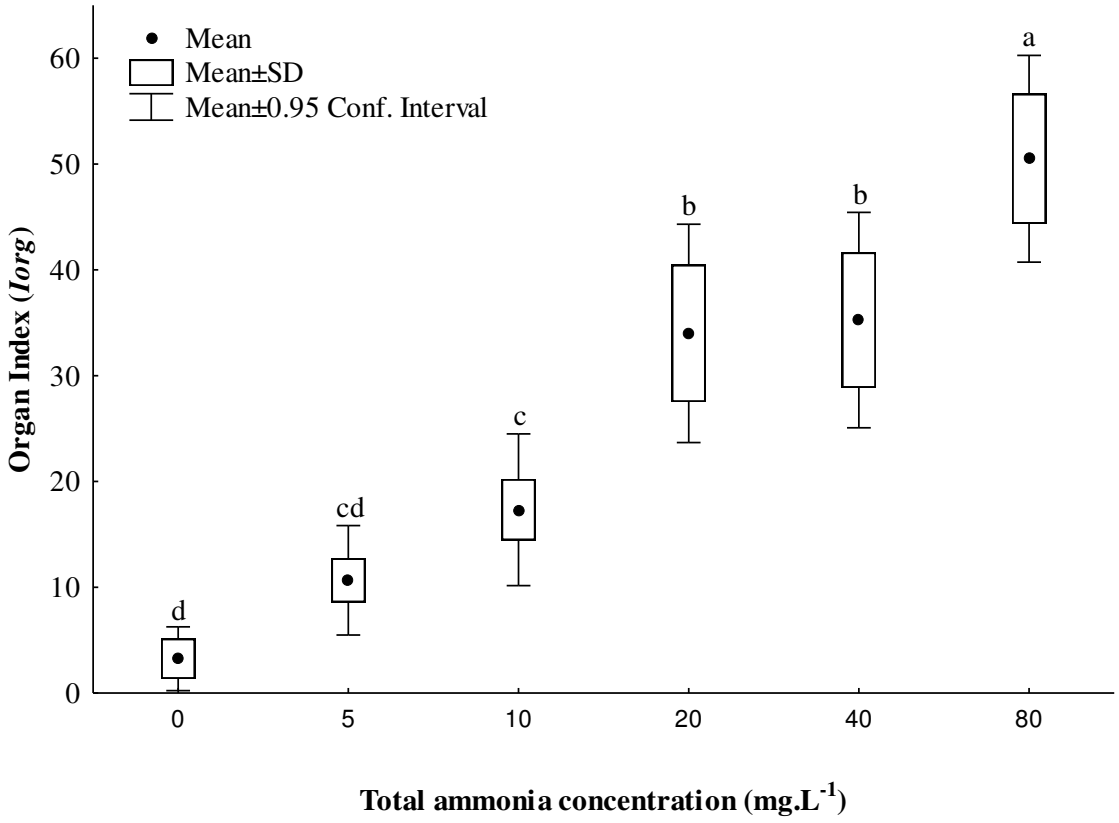


Figure 3. Organ index of *M. amazonicum* juveniles exposed to ammonia for 96 h (n=5).
FONTE: Dutra (2017)

The results of the I_{org} analysis of *M. amazonicum* juveniles subjected to different concentrations of nitrite (Figure 4) associated with the image of the histological cuts of the gills (Figure 6) support the results found in the mortality test, which shows that higher indexes are found in the treatments with 4, 8, and 16 mg.L^{-1} nitrite, with mean I_{org} values of 27.66±1.15, 28.33±2.51, and 30.33±2.51, respectively (Figure 4). Those treatments had extensive injury and/or degeneration of the gills caused mainly by progressive and regressive damage (Figure 6). The treatments with 1 and 2 mg.L^{-1} nitrite had intermediate I_{org} values of 12±2 and 12±1,

respectively. Those indexes are represented mainly by regressive and inflammatory damage. In the treatment with 0 mg.L^{-1} nitrite, with mean I_{org} of 4±1.73, little to no damage was seen in the gill structure. It can also be seen that the treatment with 0 mg.L^{-1} nitrite is significantly different from the other treatments ($p<0.05$), and that the treatments with 1 and 2 mg.L^{-1} nitrite did not differ from each other ($p>0.05$), nor did the treatments with 4, 8, and 16 mg.L^{-1} nitrite.

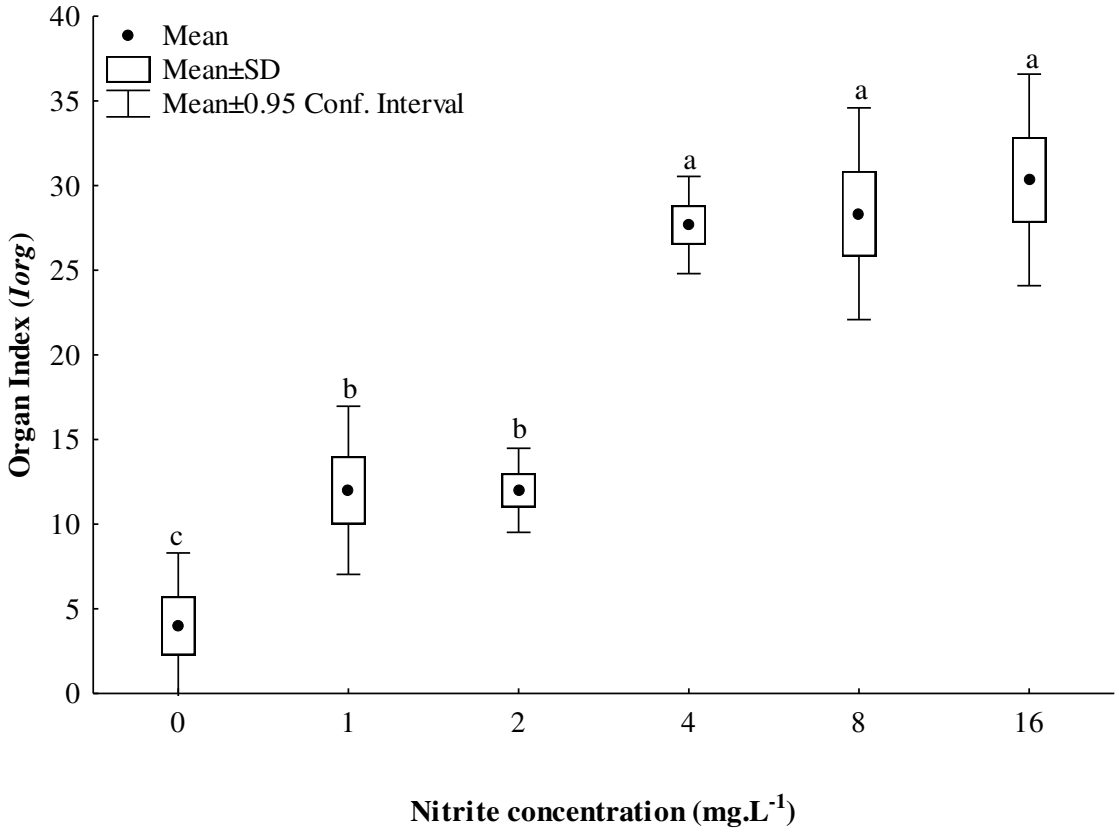
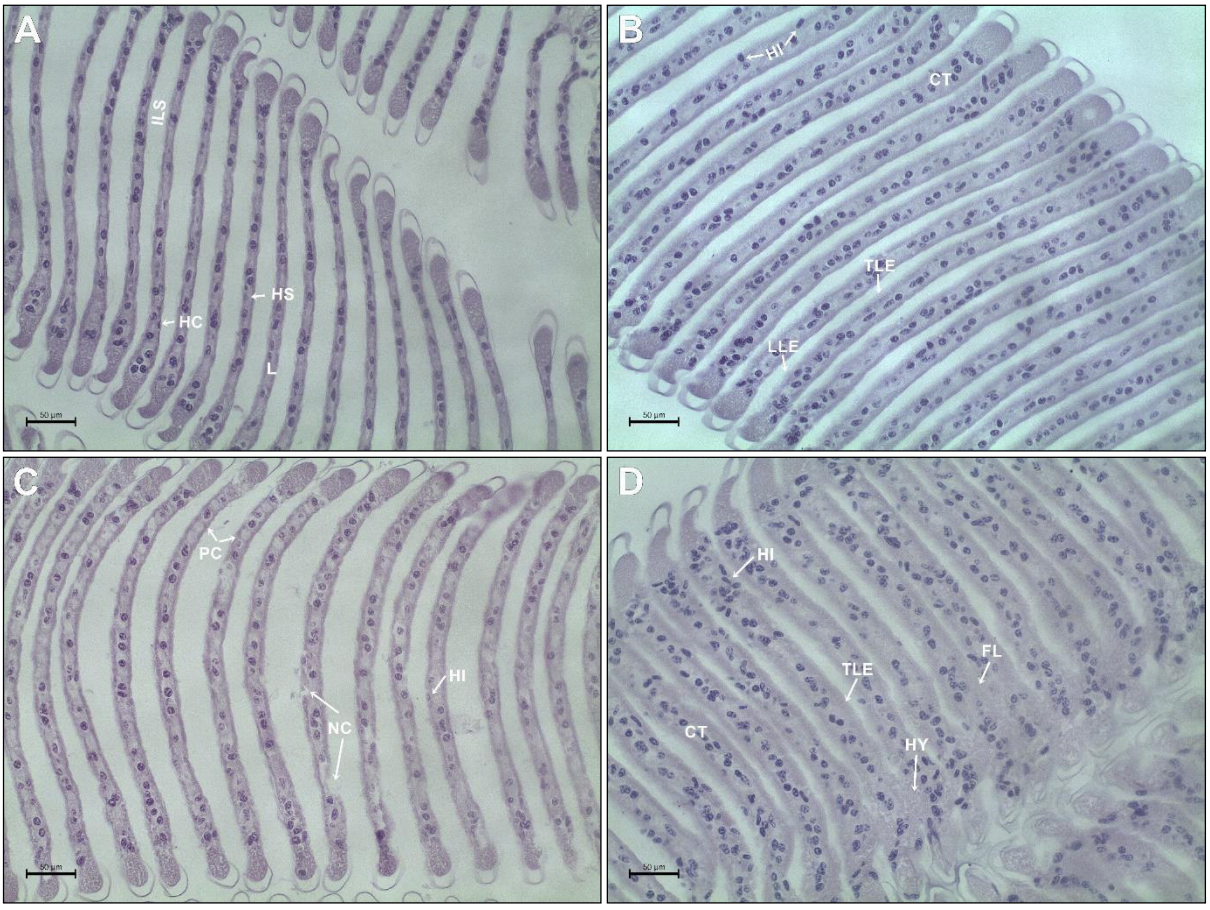


Figure 4. Organ index of *M. amazonicum* juveniles exposed to nitrite for 96 h (n=5).
FONTE: Dutra (2017)

The histological analysis of the gills of *M. amazonicum* subjected to different concentrations of total ammonia (Figure 5-A and B) showed that, comparing the control treatment (0 mg.L^{-1}) with the other treatments (5, 10, 20, 40, and 80 mg.L^{-1}) over 96 h of exposure, the gill structure suffered harmful effects. The treatment with 0 mg.L^{-1} total ammonia (control) had few alterations and, when they do occur, they are represented by negligible amounts of hemocytic infiltration. In the treatment with 5 mg.L^{-1} total ammonia, some regressive alterations and inflammations such as cellular tumefaction, thickening of the lamellar epithelium, hemocytic infiltration, and epithelium scalling are seen. The latter, despite having a high score for importance factor, did not impact the results due to its very low presence in the

gill structure (Figure 5-B). Inflammations and regressive damage were the most commonly found alterations in the treatment with 10 mg.L^{-1} total ammonia, found in small amounts (Figure 5-C). The treatment with 20 mg.L^{-1} total ammonia had higher prevalence of regressive damage, with low to moderate *w*, besides inflammations. However, the presence of progressive damage (hyperplasia) is also observed (Figure 5-D). At 40 mg.L^{-1} total ammonia, the alterations are well represented by the presence of regressive damage with low (cellular tumefaction and thickening of the lamellar epithelium) to high (necrosis) *w*. Inflammations (hemocytic infiltration) and progressive damage (hyperplasia) are also seen with moderate presence (Figure 5-E). At 80 mg.L^{-1} total ammonia, there is a prevalence of regressive damage with high *w* scores with the presence of many necroses and scalling of the epithelium, besides lamellar fusion and thickening of the lamellar epithelium. However, what calls a lot of attention is the edemas found in the gill structure, as shown in Figure 5-F.



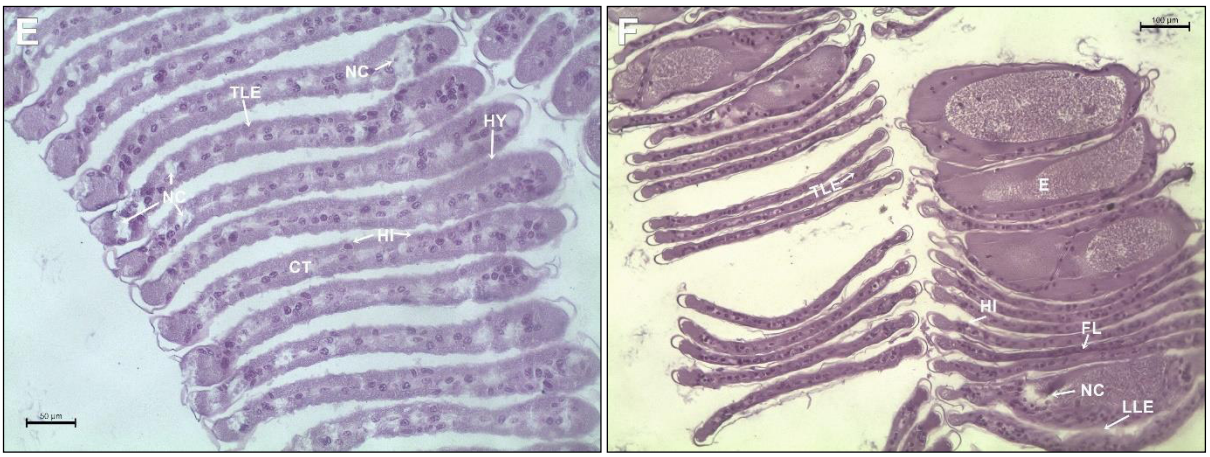


Figure 5. A) Gill of *M. amazonicum* exposed to 0 mg.L^{-1} of ammonia (control). Hemocoelic space (HS); Lamella (L); Hemocytes (HC); and Interlamellar space (ILS) (20x, 10x H&E). B) Gills exposed to 5 mg.L^{-1} of total ammonia. Cellular tumefaction (CT); Hemocytic infiltration (HI); Thickening of the lamellar epithelium (TLE); and Lifting of the lamellar epithelium (LLE) (20x, 10x H&E). C) Gills exposed to 10 mg.L^{-1} of total ammonia. Necrosis (NC); Pillar cells (PC); and Hemocytic infiltration (HI) (20x, 10x H&E). D) Gills exposed to 20 mg.L^{-1} of total ammonia. Cellular tumefaction (CT); Hemocytic infiltration (HI); Fused lamellae (FL); Hyperplasia (HY); and Thickening of the lamellar epithelium (TLE) (20x, 10x H&E). E) Gills exposed to 40 mg.L^{-1} of total ammonia. Necrosis (NC); Cellular tumefaction (CT); Hemocytic infiltration (HI); Hyperplasia (HY); and Thickening of the lamellar epithelium (TLE) (20x, 10x H&E). F) Gills exposed to 80 mg.L^{-1} of total ammonia. Necrosis (NC); Hemocytic infiltration (HI); Edema (E); Fused lamellae (FL); Lifting of the lamellar epithelium (LLE); and Thickening of the lamellar epithelium (TLE) (10x, 10x H&E).

FONTE: Dutra (2017)

When the gills of *M. amazonicum* exposed to nitrite concentrations are observed, it is seen that, in the treatment with 0 mg.L^{-1}, the gills are evenly distributed, with no degeneration of the lamellar structures observed (Figure 6-A) and, when it does occur, it is in negligible amounts with the presence of inflammations (hemocytic infiltration). At 1 and 2 mg.L^{-1} nitrite, regressive damages were observed such as cellular tumefaction and thickening of the lamellar epithelium, as well as the occurrence of lamellar fusion and necrosis at low frequency, besides inflammations (hemocytic infiltration) (Figure 6-B and C). At 4, 8, and 16 mg.L^{-1} nitrite, high degree of degeneration of the gill structures, caused mainly by circulatory disorders (clavate-globate “clubbing”), regressive damage (cellular tumefaction, lamellar fusion, and necrosis), progressive damage (hyperplasia) and inflammation (hemocytic infiltration) were seen (Figure 6-D, E, and F).

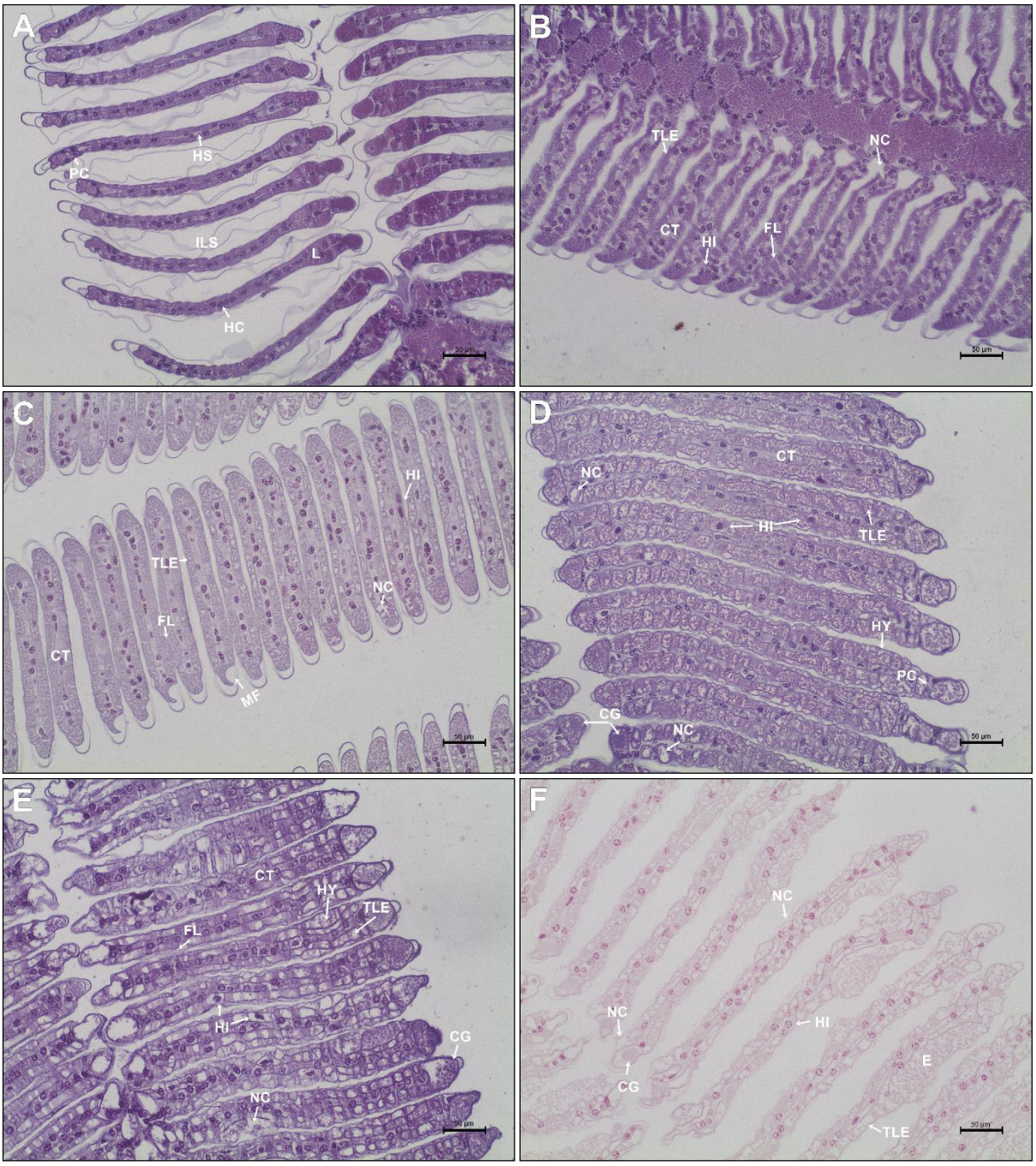


Figure 6 A) Gill of *M. amazonicum* exposed to 0 mg.L^{-1} of nitrite (control). Hemocoelic space (HS); Lamella (L); Hemocytes (HC); Pillar cells (PC) and Interlamellar space (ILS) (20x, 10x H&E). B) Gills exposed to 1 mg.L^{-1} of nitrite. Necrosis (NC); Cellular tumefaction (CT); Hemocytic infiltration (HI); Fused lamellae (FL), and Thickening of the lamellar epithelium (TLE) (20x, 10x H&E). C) Gills exposed to 2 mg.L^{-1} of nitrite. Necrosis (NC); Cellular tumefaction (CT); Hemocytic infiltration (HI); Fused lamellae (FL); Malformation (MF); and Thickening of the lamellar epithelium (TLE) (20x, 10x H&E). D) Gills exposed to 4 mg.L^{-1} of nitrite. Necrosis (NC); Cellular tumefaction (CT); Hemocytic infiltration (HI); Pillar cells (PC);

Clavate-globate “clubbing” (CG); Hyperplasia (HY); and Thickening of the lamellar epithelium (TLE) (20x, 10x H&E). E) Gills exposed to 8 $mg.L^{-1}$ of nitrite. Necrosis (NC); Cellular tumefaction (CT); Hemocytic infiltration (HI); Hyperplasia (HY); Fused lamellae (FL); Clavate-globate “clubbing” (CG); and Thickening of the lamellar epithelium (TLE) (20x, 10x H&E). F) Gills exposed to 16 $mg.L^{-1}$ of nitrite. Necrosis (NC); Hemocytic infiltration (HI); Clavate-globate “clubbing” (CG); Edema (E) and Thickening of the lamellar epithelium (TLE) (20x, 10x H&E).

FONTE: Dutra (2017)

4. Discussion

The gills are a vital organ for crustaceans, playing several important roles such as gas exchange, acid-base equilibrium, and excretion of nitrogen compounds. Since they are in close contact with water and subject to the action of toxic agents (Mantel and Farmer, 1983; Henry et al., 2012), their tissues may be damaged (Saravana Bhavan and Geraldine, 2000). Therefore, when the gills are exposed to certain concentrations of a toxic compound within a certain time frame (hours or days), lesions such as inflammatory responses may characterize a basic defense reaction (Werner, 2010; Vegad, 2015) by decreasing the gills’ vulnerable surface area and/or increasing the diffusion barrier against the contaminant (Erkmen and Kolankaya, 2000). In the present study, those inflammatory responses were seen mainly in the treatments with low concentrations of total ammonia and nitrite, represented particularly by hemocytic infiltration and cellular tumefaction. However, the tumefaction of the cell structure may lead to the worsening of the lesion, which may become a lamellar fusion. Trophon et al. (2003), in a study with sub-chronic exposure to cadmium for *Lates calcarifer*, found that the increase in lamellar epithelium often led to the partial or total fusion of the gill lamellas.

When the results of mortality are compared with the organ index (I_{org}) analyses and the alterations present in the histological analyses of the gills, it is evident that the more severe alterations in the gills are caused by the increase in total ammonia and nitrite concentrations. Rebelo et al. (2000) observed histopathological damages in the gills of *Chasmagnathus granulata* after acute exposure to ammonia. Damage to the gill structure was also observed by Romano and Zeng (2007) in a study that assessed the acute toxicity of ammonia for *Portunus pelagicus*. For nitrite, several studies found similar alterations, such as hyperplasia, lamellar fusion, thickening of the epithelium, and necrosis in the gills of fish (Michael et al., 1987; Gisbert et al., 2004; Saoud et al., 2014), but nothing has been reported for crustaceans. Romano and Zeng (2009), in a study with sub-chronic exposure to nitrite, potassium, and their

combinations, had already mentioned the lack of studies assessing the effect of nitrite on the gills of crustaceans. Many alterations found by those authors were also seen in the present study, such as hemocytic infiltration, hyperplasia, thickening of the epithelium, and necrosis.

Necrosis, rupture, and hyperplasia of the lamellar epithelium are lesions that reflect the direct effect of the contaminants (Temmink et al., 1983) and that occur under high concentrations of toxic compounds (Lease et al., 2003; Karasu and Köksal 2005). The increase in the presence of those alterations as a function of the increase in total ammonia and nitrite concentrations is also seen in this study.

This research showed alterations in the gill structure as total ammonia and nitrite concentrations increased, including cellular tumefaction, hemocytic infiltration, lamellar fusion, thickening of the lamellar epithelium, necrosis, clavate-globate "clubbing," and hyperplasia. Studies with exposure to heavy metals reported those same alterations, such as Ghate and Mulherkar (1979) for *Macrobrachium kistensis* subjected to concentrations of 0, 100, 200, 300, 400, and 500 $\mu g.L^{-1}$ copper sulfate, which reported the increase in necrosis and hemocytic infiltration as the compound concentrations increased. Victor et al. (1990) reported damage in the gill structures of *Macrobrachium idea* such as hyperplasia, lamellar, swelling, and hemocytic infiltration when those prawns were exposed for 30 days to 1 $\mu g.L^{-1}$ mercury. Li et al. (2007) observed that *Macrobrachium rosenbergii* exposed to concentrations of 0 to 0.4 $mg.L^{-1}$ copper for seven days had structural alterations such as lamellar swelling and lamellar fusion, besides clavate-globate "clubbing," scalling of the lamellar epithelium, and necrosis. Increases in structural alterations of the gills were observed as copper concentration increased. In a study on *M. rosenbergii* exposed for 96 h to different concentrations of mercury (0, 10, 50, 100, 200, 300, 400, and 500 μgL^{-1}), Kaoud et al. (2011) observed worsening of gill structure damage as the concentration increased. At low mercury doses, the prawns showed lamellar swelling and edema, whereas the animals exposed to higher doses had severe edema and hyperplasia, hemocyte accumulation in the hemocytic space, alterations in the gill extremities, necrosis, and clavate-globate "clubbing." Kaoud and Rezk (2011), in a study on *M. rosenbergii* subjected to concentrations of 0 to 100 $\mu g.L^{-1}$ cadmium, reported alterations such as swelling and edema at lower doses, however, severe edemas and hyperplasia were observed at high doses. Soegianto et al. (2013) also evaluated the exposure of *Macrobrachium sintangese* to concentrations of 0 to 10,000 $\mu g.L^{-1}$ cadmium over 96 h. The gills of the prawns had severe hyperplasia, clavate-globate "clubbing," and multiple necrosis, which resulted in lamellar swelling. The results show that the tolerance of *M. sintangese* to cadmium decreases as

exposure time increases. After the prawns exposed to cadmium were transferred to the control medium, the histopathological effects decreased.

Studies on pesticides also reported damage to prawn gills. Lignot et al. (1997) assessed the effect of fenitrothion (organophosphate insecticide) on *Penaeus japonicus* exposed to concentrations of 0 to 500 $\mu g.L^{-1}$ of the compound for 96 h and observed an increase in gill damage as concentration and duration of exposure increased. The main alterations found were hemocytic congestion (thrombosis, equivalent to clavate-globate "clubbing") and necrosis. Saravana Bhavan and Geraldine (2000), in a study on *Macrobrachium malcomsonii* exposed to concentrations of 0, 10.6, 16, and 32 $ng.L^{-1}$ endosulfan (organochlorine pesticide) over 21 days, observed an increase in gill lesions such as hemocytic infiltration, thickening of the basal lamellas, clavate-globate "clubbing," lamellar swelling and fusion, hyperplasia, and necrosis as the compound concentration increased. A study that assessed the exposure of *M. rosenbergii* to different concentrations (0, 0.1, 0.2, 0.4, 0.6, and 0.8 $mg.L^{-1}$) of Triclorfom (organophosphate insecticide) over 96 h observed that the greatest damages to gill structures are caused by the increase in concentration, which causes damages such as hemocytic infiltration, lamellar swelling and fusion, necrosis, hyperplasia, and clavate-globate "clubbing" (Chang et al., 2006).

Alterations in gill structures such as scalling of the lamellar epithelium, swollen lamellas, lamellar fusion, hyperplasia, necrosis, clavate-globate "clubbing," and complete lamellar disorganization were also observed in a study on *M. rosenbergii* subjected to ^{60}Co radiation at 0, 3, 30, 300, and 3,000 mGy/min over 96 h. More severe alterations were observed at the highest radiation dose (Stalin et al.,2013a; Stalin et al., 2013b).

All those authors stated that, since the main absorption pathway of those organisms is the gill, it becomes the main target of toxic compounds. Alterations such as swelling, elevation of the lamellar epithelium, hyperplasia, and hemocyte accumulation may reflect an adaptation to the stress caused by the exposure to the toxic substance, leading to thickening of the epithelium and, thus, increasing the distance between the internal and external media (Mallatt, 1985; Negro et al., 2011). Hence, severe and numerous alterations in the gill structure may compromise gill function as well as cause hypoxia and death in *M. amazonicum* (Rebelo et al., 2000).

A comparative analysis among the total ammonia and nitrite concentrations and their respective damages caused to the gills of *M. amazonicum* indicate that, in order to reach the same level of damage to the gill structure, with mortality of 50% of the individuals, total ammonia concentrations around 10-fold higher than nitrite concentrations would be required. In order to explain this fact, it is worth mentioning that ammonia production during protein

catabolism is a normal function through which the animal naturally produces and excretes ammonia (Noga, 1996), thus being familiarized with dealing with this compound throughout its life cycle. Nitrite, in turn, is an intermediate compound in the ammonia nitrification process (Romano and Zeng, 2013) that will normally only reach concentrations that cause damage to unbalanced systems since this compound does not prevail among the nitrogen substances in natural waters (Kroupova et al., 2005; Kroupova et al., 2008). Therefore, it seems natural that the prawns are not able to deal with this compound and are more drastically affected by it.

The histological assessment of the gills using the organ index developed for fish by Bernet et al. (1999) and applied to invertebrates by Costa et al. (1999) proved to be an efficient tool in explaining the prawns' health status, using a qualitative-quantitative analysis to present the effects caused to the gill structure after the exposure to different concentrations of total ammonia and nitrite.

5. Conclusion

The results of the histological analysis confirm that the higher the concentrations of total ammonia and nitrite, the greater the damages caused to the gill structure, matching the previously reported mortality data.

Regarding the comparative action of the two compounds, much lower nitrite concentrations cause similar damage as higher total ammonia concentrations, which reflects the lower capacity the prawns have to tolerate this compound.

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CAPÍTULO IV:

Influência de amônia e nitrito na osmorregulação de *Macrobrachium amazonicum* em diferentes estágios de vida [*]

(Formatado conforme "Instruções aos Autores" em anexo IV)

[*] Capítulo elaborado para submissão na Freshwater Biology. Autores: Fabrício Martins Dutra, Sandra Carla Forneck, Giovanna Carstens Castellano; Eduardo Luis Cupertino Ballester, Carolina Arruda Freire.

Resumo

O objetivo deste estudo foi avaliar como a amônia e o nitrito interferem na osmorregulação dos diferentes estágios de vida (pós larvas, juvenis e adultos) de *M. amazonicum*. Os camarões foram submetidos a diferentes concentrações de amônia total (0, 5, 10, 20, 40 e 80 $mg.L^{-1}$) e nitrito (0, 1, 2, 4, 8 e 16 $mg.L^{-1}$) durante 96 horas. Foi avaliada a atividade da Anidrase Carbônica (AAC) branquial e da osmolalidade da hemolinfa. Quando se compara as fases de vida para cada concentração dos compostos, a AAC foi menor para pós larvas e maior para juvenis expostos à amônia total, diferindo estatisticamente ($p<0,05$) entre si. Para nitrito, a fase adulta apresentou maior ACC na concentração de 8 $mg.L^{-1}$, sendo diferente ($p<0,05$) de pós larvas e juvenis. Nas demais concentrações foram observados valores de atividade semelhantes entre as fases de vida ($p>0,05$). Ao comparar as concentrações dos compostos em cada fase de vida, observa-se que camarões na fase adulta apresentaram maior AAC quando expostos a 5 $mg.L^{-1}$ de amônia total, sendo estatisticamente diferente ($p<0,05$) da AAC em 20 e 40 $mg.L^{-1}$ de amônia total, que apresentaram os menores valores. Quando submetidos ao nitrito, camarões adultos apresentaram maior AAC na concentração de 8 $mg.L^{-1}$ e menor na concentração de 16 $mg.L^{-1}$, sendo diferente estatisticamente ($p<0,05$) entre si. Nas demais fases a AAC foi semelhante ($p>0,05$) entre todas as concentrações, tanto para amônia total quanto para nitrito. Avaliando a osmolalidade da hemolinfa, observamos que nas concentrações de 0 e 5 $mg.L^{-1}$ de amônia total, pós larvas apresentaram os maiores valores, sendo diferente estatisticamente ($p<0,05$) das demais fases. Já para nitrito, as pós larvas apresentaram os menores valores de osmolalidade, diferindo estatitisticamente ($p<0,05$) de juvenis e adultos. Ao comparar as concentrações dos compostos em cada fase de vida, observa-se que os maiores valores para pós larvas e juvenis são observados nas concentrações de 5 e 10 $mg.L^{-1}$ de amônia total, que apresentaram diferença estatística ($p<0,05$) em relação as menores concentrações, observadas nas concentrações de 40 e 0 $mg.L^{-1}$ de amônia total, respectivamente. Para nitrito, foi observado diferença significativa ($p<0,05$) na fase de pós larvas, que apresentaram maior valor de osmolalidade em 0 $mg.L^{-1}$ e o menor em 2 $mg.L^{-1}$ de nitrito. Portanto, Pós larvas de *M. amazonicum* tem menor capacidade de manter sua AAC quando submetida ao aumento da concentração de amônia total, bem como, menor capacidade de manter a concentração osmótica na hemolinfa quando submetido ao aumento na concentração de nitrito.

Palavras chave: Camarão de água doce; carcinicultura; fisiologia, crustáceos.

Abstract

The objective of this study was to evaluate how ammonia and nitrite interfere in the osmoregulation of the different life stages (post larvae, juveniles and adults) of *M. amazonicum.* The prawn were submitted to different concentrations of total ammonia (0, 5, 10, 20, 40 and 80 $mg.L^{-1}$) and nitrite (0, 1, 2, 4, 8 and 16 $mg.L^{-1}$) for 96 Hours. Carbonic Anhydrase Activity (CAA) branchial and hemolymph osmolality were analyzed. When comparing the life stages for each concentration of the nitrogenous compounds, CAA was lower for post larvae and higher for juveniles exposed to total ammonia, differing statistically ($p<0.05$) between them. To nitrite, the adult stage presented higher CAA in the concentration of 8 $mg.L^{-1}$, differing ($p<0.05$) from post larvae and juveniles. In the other concentrations, similar activity values were observed among the life stages ($p>0.05$). Comparing the concentrations of the compounds in each life stage, it is observed that adult prawns presented higher CAA when exposed to 5 $mg.L^{-1}$ of total ammonia, differing statistically ($p<0.05$) from CAA in 20 and 40 $mg.L^{-1}$ of total ammonia, which presented the lowest values. When submitted to nitrite, adult prawn presented higher AAC at the concentration of 8 $mg.L^{-1}$ and lower at the concentration of 16 $mg.L^{-1}$, differing ($p<0.05$) from each other. In the other stages the AAC was similar ($p>0.05$) among all concentrations, to both total ammonia and nitrite. Evaluating the hemolymph osmolality, we observed that in the concentrations of 0 and 5 $mg.L^{-1}$ of total ammonia, post larvae had the highest values, differing statistically from the other stages ($p<0.05$). For nitrite, post larvae present the lowest values of osmolality, differing statistically ($p<0.05$) of juveniles and adults. Comparing the concentrations of the compounds at each life stage, it is observed that the highest values for post larvae and juveniles are observed in the concentrations of 5 and 10 $mg.L^{-1}$ of total ammonia, which presented statistical difference ($p<0.05$) in relation to the lower concentrations, observed in the concentrations of 40 and 0 $mg.L^{-1}$ of total ammonia, respectively. For nitrite, it was observed statistical difference ($p<0.05$) in the post larval stage, which has a higher osmolality value in 0 $mg.L^{-1}$ and the lowest at 2 $mg.L^{-1}$ nitrite. Therefore, post larvae of *M. amazonicum* have a lower capacity to maintain their CAA when submitted to an increase in total ammonia concentration. Pos larvae also has a lower ability to maintain the osmotic concentration in the hemolymph when submitted to an increase in nitrite concentration.

Keywords: Freshwater prawn; prawn farming; physiology; crustaceans.

1. Introdução

Na história de vida dos crustáceos, repetidas transições entre diferentes ambientes aquáticos fizeram com que estes adaptassem seu processo osmótico, permitindo sua sobrevivência em ambientes de água doce, onde há menor concentração de íons no ambiente externo em relação aos fluidos corporais (Lee e Bell, 1999). Os Palemonídeos, em especial, adaptaram-se aos mais diversos ambientes, estas adaptações ocorreram após saírem da água salgada para colonizar a água doce, demandando a estes animais um ajuste nos processos fisiológicos, como: o aumento na absorção de íons, a diminuição da permeabilidade a íons pelo exoesqueleto, o aumento da produção de metabólitos como urina e a diminuição nos níveis de pequenas moléculas orgânicas que atuam como osmólitos no controle de volume celular (Péqueux, 1995; Lucu et al, 2000; McNamara & Faria, 2012;). Entretanto, algumas espécies diádromas de água doce, como *Macrobrachium amazonicum*, *Macrobrachium olfersii, Macrobrachium acanthurus, Macrobrachium heterochirus e Macrobrachium potiuna*, ainda são dependentes de ambiente estuarino ou marinho para completar seu ciclo de vida, e, com isso, determinada fase de seu ciclo de vida apresenta variação no processo osmorregulatório (Moreira et al., 1983; Augusto et al., 2007).

A entrada de nutrientes por deposição de efluentes domésticos, agrícolas e/ou industriais (Becker et al., 2009) e o acúmulo de matéria orgânica, junto a uma variedade de compostos químicos são responsáveis pela redução na qualidade da água (Pereira e Mercante, 2005). Isto vem provocando a redução e até mesmo a inibição da capacidade dos crustáceos de realizar seu processo osmótico, promovendo efeitos subletais (redução de crescimento e capacidade reprodutiva) e até mesmo mortalidade (Lin et al., 1993; Tarazona et al., 2008). Compostos nitrogenados estão entre os fatores de qualidade de água que mais apresentam efeitos subletais e letais aos organismos aquáticos, dependendo da sua concentração no meio (Sipaúba-Tavares et al., 1995; Damato e Barbieri, 2011).

Compostos nitrogenados ocorrem naturalmente no ambiente aquático (Martín e Frederico, 2001), sendo também originados pela ação antrópica e por mudanças ambientais (Rebelo et al., 2000). Dentre estes compostos, a amônia é comprovadamente tóxica (Armstrong et al., 1978; Kir et al., 2004; Mugnier e Justons, 2004), devido a sua capacidade de difusão pelas brânquias, causando danos ao epitélio branquial e, como consequência a desestabilização do sistema osmorregulatório (Zhang et al., 2015). O nitrito por sua vez, pode apresentar alta toxidez (Miranda-Filho et al., 1995) devido ao aumento da pressão parcial de oxigênio,

sugerindo uma elevação do O_2 livre e um decréscimo de O_2 ligado a hemocianina (oxihemocianina) (Chen e Cheng, 1995; Chen e Lee, 1997).

O *Macrobrachium amazonicum* é um palemonídeo com ocorrência nas regiões tropicais e subtropicais da América do sul (Maciel e Valenti, 2009) que depende diretamente da qualidade da água para sua sobrevivência (Silva e Martinez, 2007). Devido às facilidades de exposição a contaminantes e a alterações nas propriedades físicas e químicas da água, respostas de stress podem ser causadas nesse organismo (Tomasso, 1994; Camargo e Alonso, 2006), estas respostas de stress apresentam-se em três níveis basicamente (primário, secundário e terciário). Respostas primárias envolvem a ativação cerebral, a qual resulta na liberação de hormônios; as secundárias são decorrentes da ação desses hormônios sobre o organismo e envolve ativação metabólica, aumento do consumo de oxigênio e perturbação do equilíbrio hidromineral; e as respostas terciárias, por sua vez, envolvem não somente o organismo, mas também podem ser extrapoladas às suas populações e comunidades, tendo como principais manifestações a inibição do crescimento, alterações reprodutivas e nas respostas imunes e comportamentais (Magalhães e Ferrão-Filho, 2008).

Animais de água doce são sempre osmorreguladores e, em crustáceos, as enzimas Na^+/K^+-ATPase e anidrase carbônica (AC) têm papel fundamental na regulação do equilíbrio ácido/base e influxo iônico pelas brânquias, resultando na manutenção de concentração osmótica e de NaCl na hemolinfa sempre mais alta do que na água (Péqueux, 1995; Romano e Zeng, 2013). Neste processo, a enzima anidrase carbônica catalisa e converte o CO_2 e H_2O em H^+ e HCO_3^-. Estes, por sua vez, são utilizados como substratos na absorção ativa de Na^+/Cl^- através da brânquia por meio dos trocadores Na^+/H^+ e $Cl^-/HCO3^-$. Assim, o desafio de animais de água doce é manterem-se hiperosmóticos em relação ao meio, utilizando-se desse mecanismo para a absorção de sal (Wheatly e Henry, 1987; Henry, 1988; Mitchell e Henry, 2014). Devido aos mecanismos de regulação iônica e osmótica dos crustáceos de água doce incluírem a absorção ativa de Na^+ através do epitélio branquial (Augusto et al., 2009), a amônia, por estar intimamente ligada ao processo de osmorregulação, difunde-se facilmente através das brânquias para a hemolinfa, onde transfere prótons para tornar-se NH_4^+. O NH_4^+, em seguida, substitui os íons de K^+ na brânquia por meio da via basolateral, localizada na Na^+/K^+-ATPase. O NH_4^+ é finalmente excretado para o meio através da via apical, localizado na Na^+/NH_4^+ permutador e/ou através da liberação de exocitóticos (Romano e Zeng, 2013). O nitrito, por sua vez, é captado pelo sistema de captação do Cl^- pelas brânquias, que é responsável por regular o transporte de Cl^- na hemolinfa, bem como, pelo equilíbrio ácido-base, através da troca Cl^- influxo e HCO_3^- efluxo (Cl^-/HCO_3^-) (Jensen, 1995). A inibição da AC pode responder

satisfatoriamente a alterações ambientais, sendo considerada um bom marcador para detectar efeitos da exposição aos compostos tóxicos em organismos aquáticos (Arashisar et al., 2004). Trabalhos avaliando a anidrase carbônica já foram realizados para camarões marinhos e dulcícolas. Abdel-Mohsen (2009) observaram, em estudo com *Penaeus japonicus*, que a poluição ambiental não afetou a atividade da enzima AC. Maraschi et al. (2015) não encontraram alteração na atividade da enzima AC em *M. acanthurus* expostos à variação de salinidade. Por outro lado, Roy et al. (2007) observaram variação na atividade da AC em brânquias de *L. vannamei* expostos à aumento da salinidade. Entretanto, estudos sobre os efeitos da amônia e do nitrito sobre a atividade da AC em camarões são escassos.

O equilíbrio ácido-base pode ser alterado devido as trocas realizadas entre organismo e ambiente (Truchot, 1983). Assim, a diminuição na osmolalidade devido a um efeito tóxico, provocaria um aumento do catabolismo dos aminoácidos (Lange, 1972; Larsen et al., 2014) e ou aumento do pH na hemolinfa sem a mudança na pressão parcial de gás carbônico (PCO_2) (Truchot, 1983). Portanto, a interpretação da osmolalidade da hemolinfa com foco em sua medição poderia ser utilizado como um indicador de stress em animais submetidos a agente tóxico (Lignot et al., 2000). Redução de osmolalidade já foi observada em crustáceos quando submetidos a amônia (Young-Lai et al., 1991; Chen e Cheng, 1993). Portanto, o objetivo deste estudo foi avaliar como a amônia e o nitrito interferem na osmorregulação dos diferentes estágios de vida de *M. amazonicum* durante 96 horas de exposição.

2. Material e Métodos

O trabalho foi conduzido no Laboratório de Carcinicultura da Universidade Federal do Paraná-Setor Palotina e as análises de anidrase carbônica e de osmolalidade foram realizadas no Laboratório de Fisiologia Comparativa da Osmorregulação da Universidade Federal do Paraná.

2.1. Delineamento experimental

Foram utilizados 240 camarões da espécie *M. amazonicum* para cada fase de vida (pós-larva, juvenil e adulto) e para cada teste de toxicidade (amônia total e nitrito), divididos aleatoriamente em 24 unidades experimentais (n=10 para cada unidade). O delineamento foi inteiramente casualizado, composto por seis tratamentos (concentrações), expostos por 96 horas à amônia total (0, 5, 10, 20, 40 e 80 $mg.L^{-1}$) e nitrito (0, 1, 2, 4, 8 e 16 $mg.L^{-1}$), com quatro repetições por tratamento. O peso médio úmido para o experimento de amônia total foi 0.08 ±

0.02 g; 2,07±0,62 g; 6.16 ± 1.41 g e comprimento médio total de 2.25 ± 0.20 cm; 6,29±0,71 cm; 9.26± 0.70 cm. O experimento de nitrito appresentou peso médio úmido de 0.08 ± 0.02 g; 2.02 ± 0.27 g; 6.24 ± 1.82 g e comprimento médio total de 2.25 ± 0.17 cm; 6.729 ± 0.296 cm; 9.084 ± 0.793 cm, respectivamente para pós larvas, juvenis e adultos. A mortalidade foi avaliada pela ausência de movimento ou reação a estímulos mecânicos.

2.2. Dosagem da Anidrase Carbônica (AC)

A análise da atividade da anidrase carbônica (AAC) branquial foi determinada conforme descrito por Vitale et al. (1999), baseado em Henry (1991). As brânquias foram seccionadas da porção do cefalotórax para cada fase (pós-larva, juvenil e adulto; n=8), pesadas (~150 µg de massa total, balança de 0,0001 de precisão Bioprecisa FA2104N®) para determinação do volume do tampão fosfato (manitol, 225 mM; sacarose, 75 mM; tris-fosfato, 10 mM; pH 7,4, 7 ml) e em seguida sonicadas a 10% PV (Peso de brânquia/Volume de tampão de homogenização) em sonicador (Fisher Scientifific® -Modelo FB 120). Após, o homogeneizado foi submetido à centrifugação (Hettich® MIKRO 200R centrifuge) a 13500 rpm por cinco minutos em temperatura de 4 ºC. Uma alíquota do sobrenadante foi utilizada para o ensaio da quantificação da AAC, enquanto a outra foi devidamente diluída em água deionizada em 1:9 (amostra: água deionizada) para dosagem de proteínas totais segundo método de Bradford (1976).

A quantificação da atividade da enzima foi obtida através da adição da alíquota do sobrenadante (50 µl) e de água destilada saturada com CO_2 (1 ml) em um meio contendo o mesmo tampão utilizado para a homogeneização das amostras. Imediatamente após a adição do sobrenadante, foi monitorada durante 20 segundos a queda do pH com o auxiliado de um pHmetro de bancada (inoLAB® E163694), com leitura do valor de pH a cada 4 segundos em temperatura de 2,5 a 4ºC. Os valores obtidos foram submetidos a uma regressão linear (pH x Tempo) para determinar a taxa de reação catalisada (TC). A taxa de reação não catalisada (TNC) foi realizada através de teste branco (sem adição de amostra), sendo o branco realizado no início e fim do procedimento e a cada cinco amostras (n=55).

O cálculo da AAC foi realizada com base em Burnett et al. (1981) e Vitale et al. (1999):

$$AAC = \frac{[\frac{TC}{TNC-1}]}{mg\ de\ proteína\ total}$$

Onde:

AAC = Atividade da Anidrase Carbônica;

TC = Taxa de reação catalisada;

TNC = Taxa de reação não catalisada.

2.3. Dosagem da osmolalidade na hemolinfa

Para a realização das análises de osmolalidade na hemolinfa de pós larvas, estas foram maceradas com auxílio de pistilo plástico e centrifugadas para retirada da alíquota do líquido extracelular. Para juvenis e adultos, a hemolinfa foi retirada com auxílio de uma micropipeta digital tipo monocanal de 200 µl (LabMate®) e colocada em eppendorf de 0,5 ml. Ambas as amostras foram coletadas e identificadas quanto à sua repetição para seus respectivos tratamentos e preservadas em *freezer* a -4º C até a realização das análises. As amostras foram submetidas à leitura de osmolalidade em micro-osmômetro de Pressão de Vapor Wescor, modelo VAPRO® 5520, em amostras não diluídas.

2.4. Análise estatística

Os resultados da AAC branquial e da dosagem de osmolalidade na hemolinfa foram submetidos a verificação da normalidade e homogeneidade das variâncias (Sokal e Rohlf, 2012). Após confirmados estes pressupostos, foram submetidos a análise de variância de duas vias (Two-Way ANOVA) (concentração de amônia total ou nitrito *vs* fase de vida), quando foram encontradas diferenças significativas foi aplicado o teste *a posteriori* de Tukey ($\alpha = 0,05$).

3. Resultados

Por meio da análise dos resultados da AAC branquial dos diferentes estágios ontogenéticos para cada concentração amônia total, foi demonstrado que na concentração de 0 $mg.L^{-1}$ de amônia total, as pós larvas apresentaram atividade estatisticamente inferior ($p<0,05$) aos valores encontrados para juvenis e adultos, estes últimos foram semelhantes entre si ($p>0,05$). Na concentração de 5 $mg.L^{-1}$ de amônia total a AAC foi maior para adultos, estaticamente diferente ($p<0,05$) de pós larvas. Juvenis apresentaram resultados semelhantes às demais fases de vida ($p>0,05$). Nas concentrações com 10, 20 e 40 $mg.L^{-1}$ de amônia total, os juvenis apresentaram valores maiores de AAC, diferindo ($p<0,05$) de pós larvas e adultos, enquanto entre pós larvas e adultos a AAC foi semelhante ($p>0,05$). Foi observada diferença entre as fases de vida ($p<0,05$) na concentração com 80 $mg.L^{-1}$ de amônia total, com o maior valor de atividade observado para juvenis, seguido por adultos e pós larvas (Figura 1).

Ao comparar às concentrações de amônia total em cada estágio de vida, observamos que pós larvas mantiveram sua atividade constante (p>0,05), independentemente da concentração à que estavam submetidas. Juvenis também não apresentaram variação significativa (p>0,05) na AAC entre as concentrações de amônia total. Na fase adulta a concentração de 5 $mg.L^{-1}$ de amônia total apresentou maior valor de AAC, sendo diferente (p<0,05) das concentrações de 20 e 40 $mg.L^{-1}$ de amônia total. As demais concentrações foram semelhantes entre si (p>0,05; Figura 1).

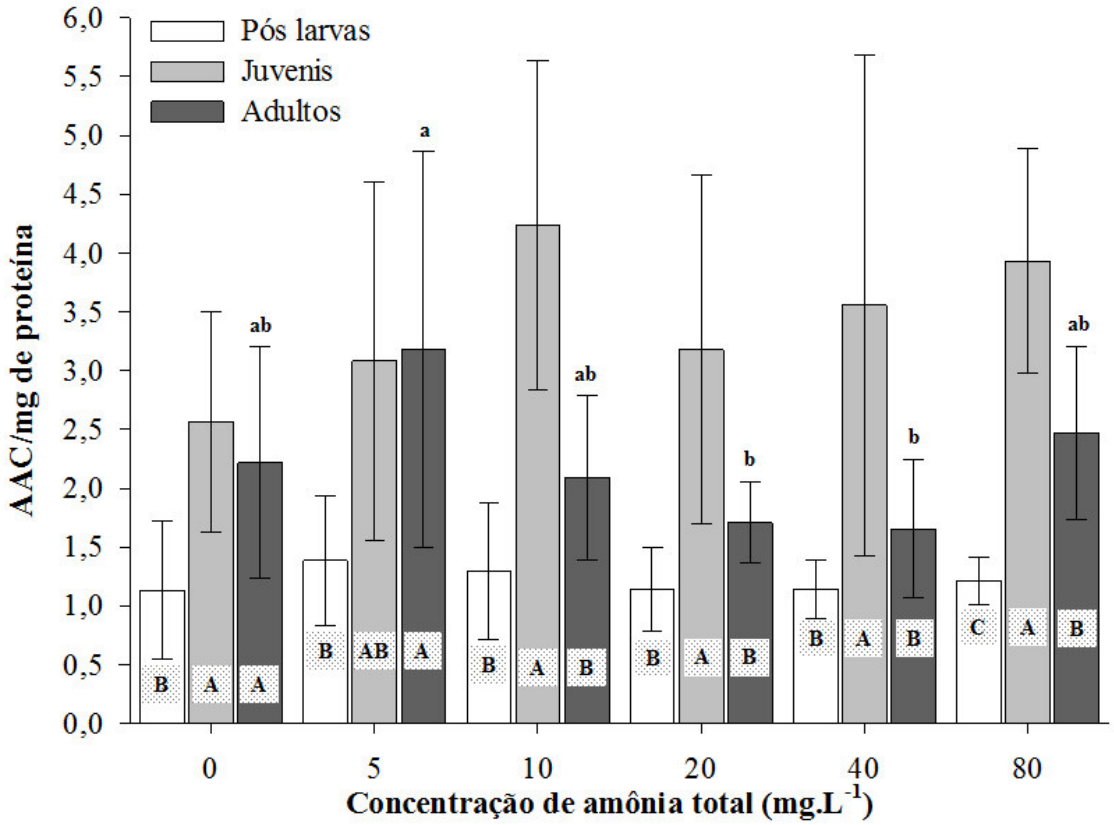

Figura 1. Valores médios (± Desvio Padrão) da atividade da anidrase carbônica branquial (AAC/mg de proteína) de *M amazonicum* submetido à diferentes concentrações de amônia total, durante 96 horas. Letras maiúsculas indicam diferença estatística (p<0,05) entre as fases de vida para cada concentração de amônia total. Letras minúsculas indicam diferença estatística (p<0,05) entre as concentrações de amônia total para cada estágio de vida. A ausência de letras nas indica que não houve diferença (p>0,05).

FONTE: Dutra (2017)

Ao observar a AAC branquial entre as fases de *M. amazonicum* de vida para cada concentrações de nitrito, visualizamos que na concentração de 8 $mg.L^{-1}$ de nitrito a AAC foi

maior em adultos, sendo diferente ($p<0,05$) de pós larvas e juvenis. Nas demais concentrações, a AAC foi semelhante entre as fases de vida ($p>0,05$; Figura 2).

Ao comparar às concentrações de nitrito para cada estágio de vida, observamos que pós larvas e juvenis apresentaram AAC semelhante entre as concentrações ($p>0,05$). Para a fase adulta, a maior AAC é observada na concentração de 8 $mg.L^{-1}$ de nitrito, diferindo estaticamente da concentração de 16 $mg.L^{-1}$ de nitrito ($p<0,05$). As demais concentrações foram semelhantes entre si ($p>0,05$; Figura 2).

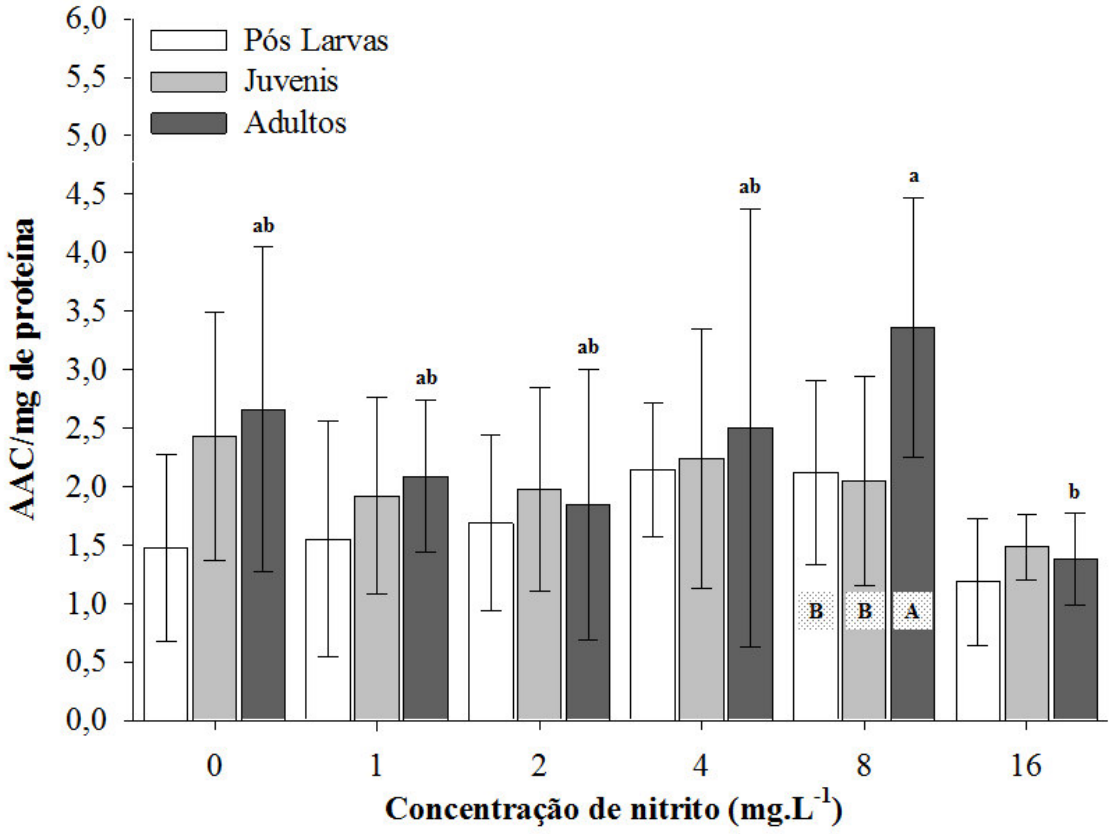

Figura 2. Valores médios (± Desvio Padrão) da atividade da anidrase carbônica branquial (AAC/mg de proteína) de *M amazonicum* submetido à diferentes concentrações de nitrito, durante 96 horas. Letras maiúsculas indicam diferença estatística ($p<0,05$) entre as fases de vida para cada concentração de nitrito. Letras minúsculas indicam diferença estatística ($p<0,05$) entre as concentrações de nitrito para cada estágio de vida. A ausência de letras nas indica que não houve diferença ($p>0,05$).

FONTE: Dutra (2017)

Ao analisar os resultados da osmolalidade da hemolinfa de *M amazonicum* observamos que, na ausência de amônia total (0 $mg.L^{-1}$), pós larvas apresentaram maior concentração osmótica, sendo diferente estaticamente ($p<0,05$) de juvenis e adultos, que mostraram ser

semelhantes entre si ($p>0,05$). A concentração de 5 $mg.L^{-1}$ de amônia total também apresentou maior concentração osmótica para pós larvas, sendo estatisticamente diferente ($p<0,05$) de adultos. Nesta mesma concentração observou-se que juvenis não diferiram estaticamente ($p>0,05$) das demais fases de vida. Nas concentrações de 10, 20, 40 e 80 $mg.L^{-1}$ de amônia total não foi observado diferença estatística ($p>0,05$) na osmolalidade entre os estágio de vida (Figura 3).

Quando observamos a osmolalidade da hemolinfa de *M. amazonicum* entre as concentrações de amônia total para cada estágio de vida (Figura 3), notamos que, para pós larvas, as maiores concentrações osmóticas são encontradas nas concentrações de 5 e 10 $mg.L^{-1}$ de amônia total, sendo estas estaticamente semelhantes ($p>0,05$) entre si. A menor concentração osmótica é visualizada em 40 $mg.L^{-1}$ de amônia total, que difere ($p<0,05$) das concentrações anteriomente mencionadas. As outras concentrações de amônia total são semelhantes às demais ($p>0,05$). Em juvenis, os maiores valores de osmolalidade também foram observados nas concentrações de 5 e 10 $mg.L^{-1}$ de amônia total, que diferiram estatisticamente ($p<0,05$) do controle (0 $mg.L^{-1}$ de amônia total), que apresentou o menor valor. As concentrações de 20 a 80 $mg.L^{-1}$ de amônia total foram selhantes entre si e às outras concentrações ($p>0,05$). Adultos apresentaram menor e maior valor de osmolalidade nas concentrações de 0 e 80 $mg.L^{-1}$ de amônia total, respectivamente. Entretanto, todas as concentrações foram estatisticamente semelhantes entre si ($p>0,05$).

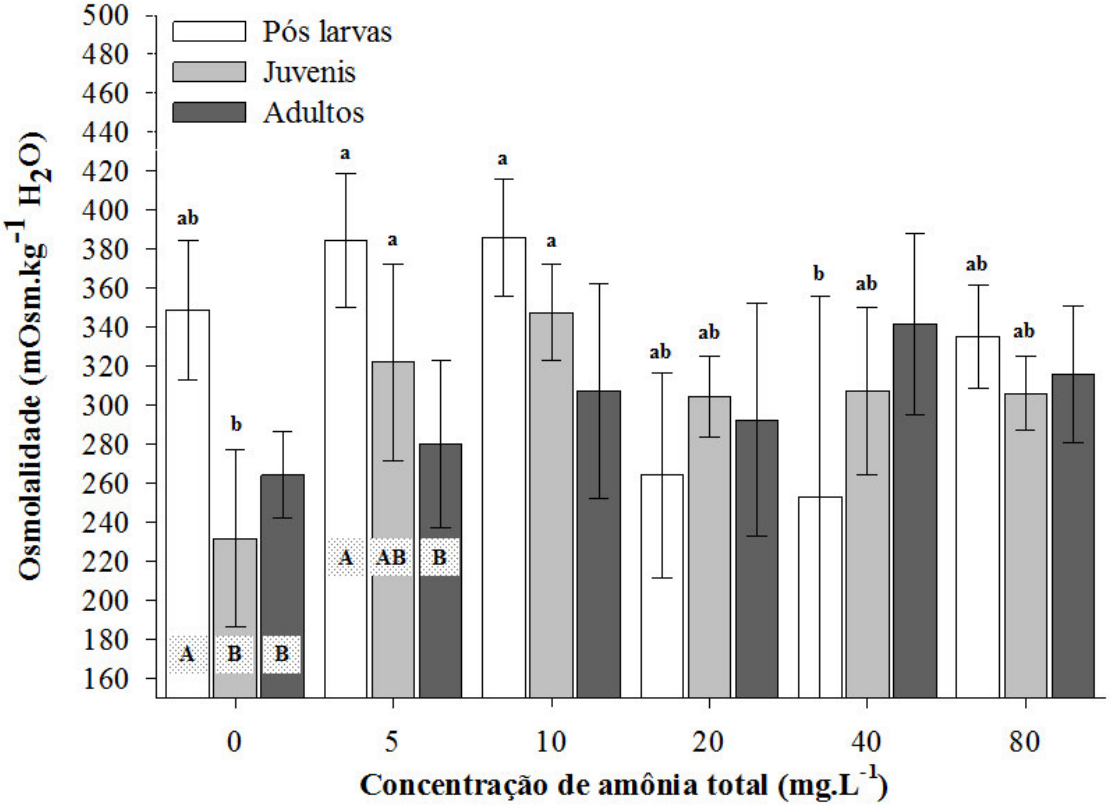

Figura 3. Valores médios (± Desvio Padrão) da osmolalidade da hemolinfa (mOsm.kg^{-1} H_2O) de *M amazonicum* submetido à diferentes concentrações de amônia total, durante 96 horas. Letras maiúsculas indicam diferença estatística ($p<0,05$) entre as fases de vida para cada concentração de nitrito. Letras minúsculas indicam diferença estatística ($p<0,05$) entre as concentrações de nitrito para cada estágio de vida. A ausência de letras nas indica que não houve diferença ($p>0,05$).

FONTE: Dutra (2017)

A osmolalidade na concentração de 0 mg.L^{-1} de nitrito foi estatisticamente semelhante ($p>0,05$) entre as fases de vida de *M. amazonicum*. Entretanto, ao comparar os estágios de vida concentrações de 1, 2 e 4 mg.L^{-1} de nitrito para seus respectivos, observamos que pós larvas apresentam valores de concentração osmótica estatisticamente inferiores ($p<0,05$) aos valores de juvenis e adultos, que são semelhantes entre si ($p>0,05$). Nas concentrações de 8 e 16 mg.L^{-1} de nitrito, pós larvas apresentaram valores de osmolalidade estatisticamente menores do que adultos ($p<0,05$), enquanto, juvenis apresentaram valores semelhantes aos outros estágios ($p>0,05$; Figura 4).

Quando comparamos as concentrações de nitrito para cada estágio de vida, percebemos que pós larvas de *M. amazonicum* apresentam maior concentração osmótica para 0 mg.L^{-1} de nitrito, diferindo ($p<0,05$) das concentrações de 1, 2, 4 e 8 mg.L^{-1} de nitrito. A concentração de

16 mg.L^{-1} de nitrito foi semelhante estatisticamente (p>0,05) à todas as concentrações. Juvenis e adultos não apresentaram diferença na osmolalidade entre as concentrações (p>0,05; Figura 4).

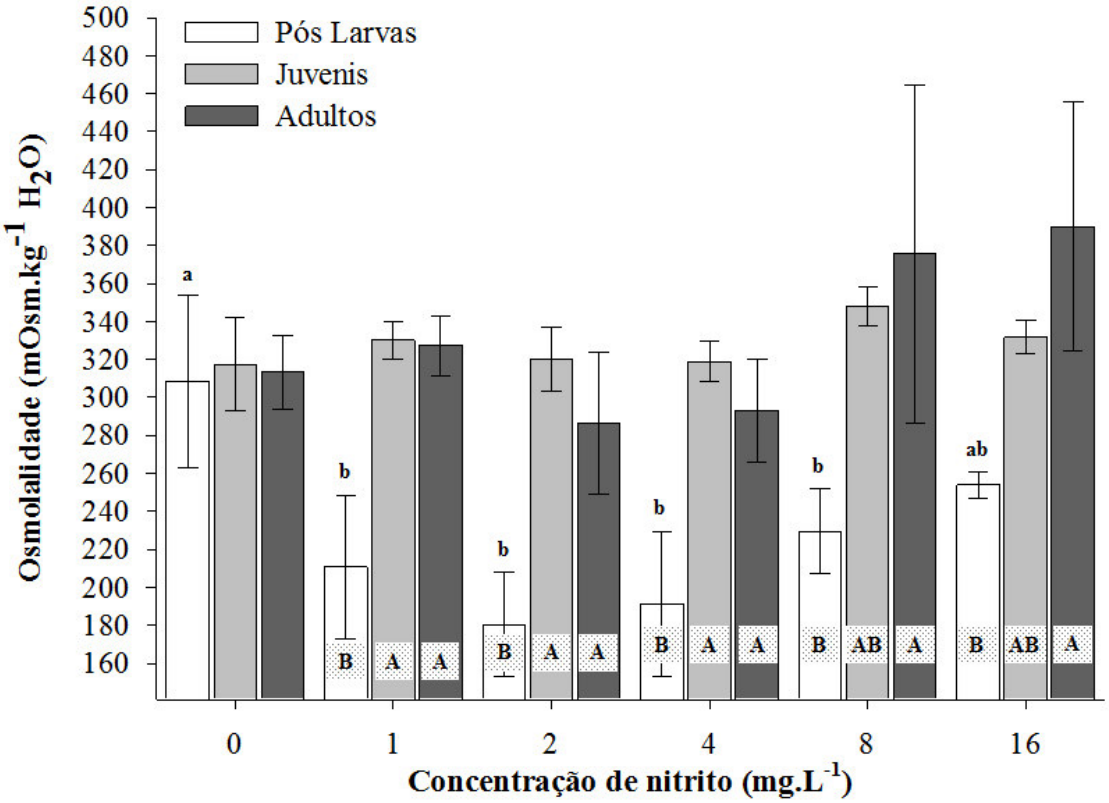

Figura 4. Valores médios (± Desvio Padrão) da osmolalidade da hemolinfa (mOsm.kg^{-1} H_2O) de *M amazonicum* submetido à diferentes concentrações de nitrito, durante 96 horas. Letras maiúsculas indicam diferença estatística (p<0,05) entre as fases de vida para cada concentração de nitrito. Letras minúsculas indicam diferença estatística (p<0,05) entre as concentrações de nitrito para cada estágio de vida. A ausência de letras nas indica que não houve diferença (p>0,05).

FONTE: Dutra (2017)

4. Discussão

A atividade da enzima AC para *M. amazonicum* apresentou pouca variação para adultos, não apresentando variação para pós larvas e juvenis quando observado cada fase de vida em comparação ao aumento da concentração de amônia total e nitrito. No entanto, quando observamos a atividade da enzima entre as fases de vida, notamos que a menor atividade é visualizada para pós larvas e a maior para juvenis submetidos a amônia total. Quando submetido a nitrito, observamos um leve crescimento da atividade da enzima AC em pós larvas até adulto.

Pós larvas, em geral, apresentam mecanismos regulatórios menos desenvolvidos do que juvenis e adultos, uma vez que pós larvas habitam áreas estuarinas, enquanto juvenis e adultos já vivem em água doce (Augusto et al., 2007). Assim, pela AC tratar-se de um mecanismo regulatório, presume-se que este também seja menos desenvolvido nas fases de vida iniciais. Valores de atividade da enzima AC reportada para adultos de *M. acanthurus* foram de aproximadamente 2 por mg de proteína, mesmo com aumento de salinidade de 0 para 25 ‰ em 24 horas (Maraschi et al., 2015), resultado próximo ao encontrado neste estudo para a maioria das fases de vida de *M. amazonicum*. Valores próximos também são visualizados em *Macrobrachium rosenbergii* de 10 a 20 g, onde foi observada variação na atividade da enzima AC entre 3,33 a 6,38 mg de proteína, este último valor é reportado para animais em pré-ecdise, decaindo para 3,98 em pós-ecdise (Jasmani et al., 2008). Em peixes expostos à amônia é observada redução na atividade da enzima AC com o aumento na concentração da amônia (ArasHisar et al., 2004; Souza-Bastos, 2015). Excesso de amônia na água pode reduzir a taxa de excreção da amônia, prejudicando o sistema de tamponamento dos tecidos, devido a retenção de CO_2, o que reduz o transporte de O_2 (Tomasso et al., 1980; Wilkie, 2002; ArasHisar et al., 2004). Isso impede o processo de hidratação do CO_2, prejudicando o equilíbrio ácido-básico e a respiração (Henry, 1996).

No presente estudo não foi observada redução na atividade da enzima com o aumento da concentração de amônia total para pós larvas e juvenis. Crustáceos podem apresentar mecanismos de combate à presença de amônia, como, capacidade de reduzir a difusão de amônia por meio do fechamento de canais iônicos presentes nas brânquias, ou por meio de mecanismo de desintoxicação, que é uma importante adaptação, uma vez que o acúmulo de amônia na hemolinfa é inevitável durante exposição prolongada ou elevada (Durand et al., 1999; Wang et al., 2004; Martin et al., 2011; Romano e Zeng, 2013). Esse mecanismo seria realizado por meio do aumento de aminoácidos não essenciais internos e aminoácidos livres, através da utilização do amoníaco (Durand et al., 1999; Wang et al., 2004). Tais mecanismos podem ter atuado nos animais aqui testados, uma vez que não foi visualizado declínio na atividade da AC. Já em peixes expostos a nitrito, a proliferação das células de cloreto pode ser vista como uma resposta compensatória que neutraliza a depleção do cloreto, mas também pode ser inativada pelo aumento na captação de nitrito (Willians e Eddy, 1988; Jensen, 2003). Entretanto, redução na atividade da enzima AC em função das concentrações de nitrito de *M. amazonicum* não foi observada para pós larvas e juvenis, o que pode indicar proliferação de células de Cl^- para compensar o transporte de íons pela brânquia. A fase adulta foi a única fase de vida que apresentou variação na AC entre as concentrações de amônia total e nitrito. Leone

et al. (2014) afirmam que a sensibilidade diminui durante a ontogenia de *M. amazonicum*, e que juvenis são menos adaptados a NH_4^+ do que adultos, uma vez que em juvenis ocorre ligação de K^+ e NH_4^+ a dois sítios distintos, mas equivalentes, na molécula da enzima Na^+/K^+-ATPase. Adultos tem a capacidade de ligar cada íon ao seu próprio sítio específico, proporcionando estimulação sinérgica considerável (≈ 50%) da atividade Na^+, K^+-ATPase. Esdtudos avaliando o nitrito são escassos para crustaceos na literatura, mas acreditamos que adultos de *M. amazonicum* podem apresentar mecanismos de enfrentamento semelhantes para este composto e que são mais adaptados a situações de estresse ambiental, ocasionado por mudanças na qualidade de água.

A capacidade de manter alta concentração osmótica na hemolinfa foi a característica fundamental para que *M. amazonicum* colonizasse com sucesso ambientes de água doce (McNamara, 1987; Ordiano et al., 2005). Assim, podemos observar no presente estudo, que a dosagem da osmolalidade da hemolinfa em água doce para os diferentes estágios de *M. amazonicum* submetidos a diferentes concentrações de amônia total e nitrito mostrou que a espécie manteve sua concentração osmótica. Bezerra (2010) encontrou para *M. amazonicum* osmolalidade de 326,71±20,73 mOsm/Kg H_2O, valor próximo ao encontrado neste estudo. Valores de osmolalidade próximos também foram reportados por Lima et al. (1997) para *M. olfersii* (340 mOsm/Kg H_2O) ao estudarem a regulação durante a aclimatação a meios de salinidade (0, 21 e 28‰) e por Freire et al. (2003) para a mesma espécie, onde a osmolalidade foi de 336 mOsm/Kg H_2O) ao analisar padrões adaptativos de regulação osmótica e iônica. Wang et al. (2004) encontraram para *Macrobrachium nipponense* osmolalidade de 330 mOsm/Kg H_2O, ao avaliarem as alterações de proteínas ligadas e aminoácidos livres na musculatura em diferentes salinidades. Entretanto, estes estudos observaram aumento na concentração osmótica na hemolinfa quando houve aumento na salinidade. Valores superiores de osmolalidade são descritos por diversos autores para espécies do gênero *Macrobrachium* (Tabela 1).

Tabela 1. Comparação da concentração osmótica da hemolinfa em camarões expostos a água doce (mOsm/Kg H_2O); ponto isosmótico (mOsm/Kg H_2O - Salinidade ‰) e temperatura (◦C) para diferentes espécies de *Macrobrachium*.Comparação da concentração osmótica da hemolinfa em camarões expostos a água doce (mOsm/Kg H_2O); ponto isosmótico (mOsm/Kg H_2O - Salinidade ‰) e temperatura (◦C) para diferentes espécies de *Macrobrachium*.

Espécie	Fase de vida	Osmolalidade em água doce (mOsm/Kg H_2O)	Ponto isosmótico (mOsm/Kg H_2O)- Salinidade (‰)	Temperatura (◦C)	Referência

M. amazonicum	Larvas	416	522-17	29	Chamantier e Anger (2011)
M. amazonicum		392	502-17	29	Chamantier e Anger (2011)
M. amazonicum	Pós larvas	416	522-17	29	Chamantier e Anger (2011)
M. rosenbergii		479	-	28	Sandifer et al. (1975)
M. rosenbergii		442	422-20	30	Herrera et al. (1993)
M. amazonicum	Juvenis	416	522-17	29	Chamantier e Anger (2011)
M. amazonicum		392	502-17	29	Chamantier e Anger (2011)
M. nipponense		330	450-15	24	Wang et al. (2004) [a]
M. rosenbergii		450	693-24	27	Armstrong et al. (1981)
M. rosenbergii		450	-	25	Castille e Lawrence (1981)
M. rosenbergii		743	515	28	Sandifer et al. (1975)
M. rosenbergii		450	566-20	30	Herrera et al. (1993)
M. tenellum		485	533-17	28	Aguilar et al. (1998)
M. tuxtlaense		406	700-25	24	Ordiano et al. (2005)
M. acanthurus	Adultos	440	640-22,4	20	Moreira et al. (1983)
M. acanthurus		421	632-23	20	Signoret e Brailovsky (2004)
M. amazonicum		403	642-22,1	-	Augusto et al. (2007)
M. amazonicum		380	480-24	24	Zanders e Rodríguez (1992) [a]
M. amazonicum		416	522-17	29	Chamantier e Anger (2011)
M. amazonicum		392	502-17	29	Chamantier e Anger (2011)
M. amazonicum		326	-	-	Bezerra (2010)
M. brasiliense		412	521-17	20 – 25	Freire et al. (2003)
M. australiense		520	475-20	22	Denne (1968) [b]
M. carcinus		441	492-17	-	Moreira et al. (1988)
M. carcinus		417	490-17	20	Signoret e Brailovsky (2004)
M. equidens		525	529-21	22	Denne (1968) [b]
M. heterochirus		425	647-22,6	20	Moreira et al. (1983)
M. ohione		462	643	25	Castille e Lawrence (1981)
M. olfersii		423	620-21,7	-	Augusto et al. (2007)
M. olfersii		340	737-21	-	Lima et al. (197)
M. olfersii		520	620-21,7	20	Moreira et al. (1983)
M. olfersii		336	428-14	20 – 25	Freire et al. (2003) [a]
M. olfersii		363	746-27	20	McNamara (1987) [a]
M. petersi		475	480-16	24	Read (1984)
M. potiuna		380	456-16	23	Souza e Moreira (1994) [a]
M. potiuna		493	552-19,3	20	Moreira et al. (1983)
M. potiuna		418	562-19	20 – 25	Freire et al. (2003)
M. rosenbergii		361	450-17	25 – 27	Singh (1980) [a]
M. rosenbergii		402-500	-	28	Wilder et al. (2009) [c]
M. rosenbergii		416/437	472-14,5/458-15,6	-	Cheng et al. (2003) [d]
M. rosenbergii		460	500-17	28	Funge-Smith et al. (1995)
M. rosenbergii		400	-	28	Soegianto (2016)
M. rosenbergii		450	500	27	Stern et al. (1987)
M. rosenbergii		433	640-15	27	Huong et al. (2010)
M. rosenbergii		435	475-12	28	Wilder et al. (1998)

[a] Valores da osmolalidade em água doce próximo ao encontrado no presente estudo
[b] Concentrações da osmolalidade calculada
[c] Estudo realizado em mudanças do ciclo de muda (ecdise)
[d] Estudo realizado com machos e fêmeas
FONTE: Dutra (2017)

No presente estudo não foi observado aumento na osmolalidade em juvenis e adultos de *M. amazonicum* com o aumento da concentração de amônia total e nitrito. Entretanto, a fase de pós larva apresentou menores valores de osmolalidade nas concentrações de 20 e 40 $mg.L^{-1}$ de amônia total e nas concentrações de 1 a 4 $mg.L^{-1}$ de nitrito. Queda na osmolalidade pode ocorrer em função do aumento na absorção de água influenciada pela presença de compostos

nitrogenados (Romano e Zeng, 2013), devido a este processo estar relacionado à regulação de volume celular. Isto pode ocasionar inchaço da célula ou tecido, como observado no estudo de Dutra et al. (submetido), onde reporta inchaço em células e em tecidos branquiais de juvenis de *M. amazonicum* expostos à amônia e nitrito. Concentrações de amônia total e nitrito não interferiram no processo isosmótico de juvenis e adultos da espécie, entretanto, a osmorregulação de pós larvas foi influenciada por estes compostos. Isto pode estar relacionado ao status evolutivo de espécies diádromas, onde juvenis e adultos têm seus mecanismos de regulação mais desenvolvidos por viverem em ambientes de água doce, ao contrário de estágios mais jovens (pós larvas), que têm seus mecanismos de regulação menos adaptados a água doce por estarem em ambiente estuarino ou iniciando seu processo de migração para água doce (Augusto et al., 2007).

Portanto, podemos concluir que pós larvas de *M. amazonicum* tem menor capacidade de manter sua AAC quando submetida ao aumento da concentração de amônia total. Para nitrito, todas as fases mantiveram AAC constante, mesmo apresentando pequena variação na AAC entre as concentrações.

Pós larvas também apresentaram menor capacidade de manter sua concentração osmótica quando há aumento na concentração de nitrito. Para amônia, todas as fases apresentaram capacidade de manter alta osmolalidade na hemolinfa.

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2. CONCLUSÃO GERAL

O camarão-da-amazônia *M. amazonicum*, a cada ano que passa, vem se tornando alvo de novas pesquisas por ser uma espécie nativa e de ampla distribuição, bem como, por apresentar grande potencial para produção em cativeiro. Entretanto, pesquisas para a compreensão da biologia são necessárias para qualquer organismo com potencial econômico, a fim de proporcionar condições adequadas para a produção do mesmo.

Observando a necessidade de se conhecer os efeitos da exposição do camarão *M. amazonicum* à amônia e ao nitrito é que propusemos avaliar o efeito das diferentes concentrações de amônia e nitrito em diferentes estágios de vida da espécie. Os experimentos possibilitaram estabelecer relações diretas entre a exposição dos organismos a compostos nitrogenados como amônia e nitrito e suas subsequentes respostas biológicas. Os resultados gerados forneceram uma compreensão acerca dos efeitos causados à espécie *M. amazonicum* quando exposta a compostos nitrogenados.

Por meio dos resultados aqui obtidos foi possível determinar o nível seguro para amônia total e nitrito para cada estágio de vida de *M. amazonicum*, apresentando implicações importantes para a produção deste camarão, possibilitando o manuseio e manejo desses animais em sistemas de produção, principalmente em sistemas de larvicultura e recirculação.

A avaliação histológica das brânquias confirmou que quanto maiores as concentrações de amônia total e nitrito, maiores os danos causados à estrutura branquial, correspondendo aos dados de mortalidade relatados nos capítulos 1 e 2 desta tese. Também foi observado que concentrações baixas de nitrito causaram danos semelhantes às alterações encontradas em camarões submetidos a altas concentrações de amônia total, o que reflete a menor capacidade dos camarões em tolerar nitrito.

Ao avaliar os resultados obtidos no capítulo 4, observamos que, quando exposto a diferentes concentrações de amônia e nitrio, *M. amazonicum* não apresentou inibição no seu processo osmótico, tanto para atividade da enzima Anidrase Carbônica branquial, quanto para a osmolalidade da hemolinfa. Isto é um forte indicativo de que a espécie tem capacidade de manter a atividade da enzima AC constante, mantendo também sua osmolalidade na hemolinfa. A maioria dos trabalhos avaliando o processo osmótico é realizada principalmente com aumento ou diminuição da salinidade do meio, o que possibilita boa compreensão do mecanismo osmótico de enfrentamento quando animais de água doce são submetidos a estresse salino. Porém, quando analisamos esse mesmo mecanismo em animais expostos a compostos nitrogenados, poucas informações estão disponíveis, e muitas destas são superficiais.

Assim, acreditamos que o presente estudo trouxe informações importantes para a compreensão dos efeitos tóxicos de amônia e nitrito sobre *M. amazonicum*, podendo ser utilizado na gestão e manejo da espécie no meio ambiente e em sistema de produção, bem como, pode ser replicado para uma gama de outras espécies. Acreditamos também que os estudos aqui realizados, poderão servir como ponto de partida para novos estudos com foco nesta temática, respondendo a questões ainda pouco compreendidas.

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APÊNDICE:

SUSCETIBILIDADE DOS ESTÁGIOS ONTOGENÉTICOS: PADRÃO OSMORREGULATÓRIO EM CAMARÕES PENEÍDEOS E PALEMONÍDEOS

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DUTRA, F. M.; FORNECK S. C.; BALLESTER, E. L. C. Suscetibilidade dos estágios ontogenéticos: padrão osmorregulatório em camarões peneídeos e palemonídeos. **Arq. Ciênc. Vet. Zool. UNIPAR**, Umuarama, v. 17, n. 3, p. 207-213, jul./set. 2014.

RESUMO: A invasão da água doce por crustáceos ocorreu repetidamente ao longo dos anos, pressionando um ajuste em seus processos fisiológicos. A habilidade de sobreviver aos diferentes ambientes foi encontrada em traços osmorregulatórios, e estão relacionadas à capacidade de regular o fluido extracelular. Portanto, o objetivo deste manuscrito é explorar os processos osmorregulatórios de camarões palemonídeos e peneídeos, visualizando um padrão na capacidade osmorregulatória em ambas as famílias e, em particular, tratar de assuntos pertinentes aos efeito no ciclo ontogenético e o efeito ocasionado pelo nitrito na hemolinfa de crustáceos. Comparando palemonídeos e peneídeos, percebe-se que o sucesso da conquista do ambiente dulcícola, e a não necessidade de hiporregulação, levou à perda dessa capacidade na maioria dos palemonídeos. Dessa forma, se supõem um ancestral com capacidade hiporregulatória, uma vez que invertebrados marinhos são, parcimoniosamente, osmoconformadores. Compara-se também que o nitrito apresenta efeito nas proteínas presentes na hemolinfa dos crustáceos quando submetidos à exposição natural ou induzida deste composto, e que a capacidade osmorregulatória é, portanto, variada de acordo com seus estágios.
PALAVRAS-CHAVE: Crustáceos. Decápodes. Fisiología. Ontogenia. Toxicologia.

SUSCEPTIBILITY OF ONTOGENETIC STAGES: OSMOREGULATORY STANDARD IN PENAEID AND PALAEMONID SHRIMPS

ABSTRACT: The invasion of freshwater by crustaceans has occurred repeatedly over the years, pressing for an adjustment in their physiological processes. The ability to survive in different environments was found in osmoregulatory traits and is related to the ability to regulate the extracellular fluid. Therefore, the purpose of this manuscript is to explore the osmore-gulatory processes in palaemonid and penaeid shrimps, which display a pattern of osmoregulatory capacity in both families and, in particular, to deal with matters related to the effects on ontogenetic cycle and the effect raised by nitrite in crustacean hemolymph. By comparing palaemonid and peneid shrimps, it is possible to note the success of conquering freshwater envi-ronment, and not the need for hypo-regulation, has led to the loss of this capability in most palaemonids. Thus, it is assumed that they share an ancestor with hypo-regulatory capacity, since marine invertebrates are sparsely osmoconformers. The article also compares that nitrite has effect on proteins present in the hemolymph of crustaceans when subjected to natural or induced exposure to these compound, and osmoregulatory capacity is therefore varied according to their stages.
KEYWORDS: Crustaceans. Decapods. Physiology. Ontogeny. Toxicology.

SUSCEPTIBILIDAD DE ETAPAS ONTOGENÉTICAS: ESTÁNDAR OSMORREGULATORIO EN CAMARONES PENEÍDEOS Y PALEMONÍDEOS

RESUMEN: La invasión del agua dulce por crustáceos ocurre repetidamente a lo largo de los años, pulsando un ajuste en sus procesos fisiológicos. La capacidad de sobrevivir en diferentes ambientes se ha encontrado en rasgos osmorregulatorios y están relacionados a la capacidad de regular el fluido extracelular. Por lo tanto, el objetivo de este manuscrito es el de explotar los procesos osmorregulatorios de camarones palemonídeos y peneídeos, se muestra un estándar en la capacidad osmorregu-latoria en ambas las familias y, en especial, trata de cuestiones relativas a los efectos sobre los ciclo ontogenético y el efecto causado por nitrito en la hemolinfa de crustáceos. Comparando palemonídeos y peneídeos, se da cuenta de que el éxito de la conquista del ambiente dulcícola, y sin necesidad de hipo regulación, condujo a la pérdida de esa capacidad en la mayoría de los palemonídeos. Así, se supone un ancestral con capacidad hipo regulatoria, una vez que los invertebrados marinos son, parsimoniosamente, osmoconformadores. Se compara también que el nitrito presenta efectos en las proteínas presentes en la hemolinfa de los crustáceos cuando sometidos a la exposición natural o inducida por estes compuesto, y que la capacidad osmorregulatoria es, por lo tanto, variada de acuerdo con sus etapas.
PALABRAS CLAVE: Crustáceos. Decápodo. Fisiología. Ontogenia. Toxicología.

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Introdução

Os camarões da família Penaeidae, pertencentes à ordem Decapoda, são distribuídos por todos os continentes (LOPES et al., 2010), habitando estuários quando juvenis e ambientes marinhos quando adultos (BAUER, 2004). A família Palaemonidae, também pertencente à ordem Decapoda, distribuise por todos os continentes, em regiões tropicais e temperadas, habitam água doce ou salobra (HOLTHUIS, 1980), são popularmente conhecidos como pitus ou camarões de água doce e vivem junto a pedras e a vegetação aquática (SOUSA et al., 2014).

Essas duas famílias apresentam importância comercial por ser um produto nobre e de alto valor, com centenas de toneladas de camarões sendo exportadas por ano (PÉREZ-RAMIREZ; LLUCHCOTA, 2010), tornando esse recurso, em termos de valor, a mais importante *commodity* pesquei-ra comercializada internacionalmente, com o mercado concentrado nos Estados Unidos, Japão e Europa (FAO, 2009). Além disso, apresentam fundamental importância ecológica por constituírem itens alimentares importantes para várias espécies da fauna aquática, contribuindo para o equilíbrio trófico desse ambiente (COSTA, 2002).

Os crustáceos apresentaram capacidade de sair do mar para colonizar ambientes dulcícolas. Esta característica, por meio de pressão seletiva solicitou a estes animais um ajuste nos seus processos fisiológicos, destacando-se o aumento na absorção de íons, diminuição da permeabilidade de íons pelo exoesqueleto, aumento da produção de metabólitos como urina e diminuição nos níveis de pequenas moléculas orgânicas que atuam como osmólitos no controle de volume celular (PÉQUEUX, 1995).

As fases ontogenéticas dos animais aquáticos, susceptíveis a variações ambientais e muitas vezes à pressão de seleção natural, refletem na diferença de cada espécie quanto ao estilo de vida e estratégia reprodutiva (INMAN; LOCKWOOD, 1977). Isto fica mais evidente quando pensamos na estratégia reprodutiva. Alguns crustáceos passam certa parte de seu ciclo de vida migrando entre águas com diferentes salinidades, expondo seus sucessivos estágios ontogenéticos aos diferentes regimes nas variáveis físicas e químicas presentes no ambiente, necessitando de maior eficiência no processo osmorregulatório a certos tempos de exposição (CHARMANTIER, 1998; SHORT, 2004).

Vários estudos também têm demonstrado as causas dos efeitos tóxicos decorrentes da presença antrópica e de fatores naturais sobre o equilíbrio osmótico, o que pode levar a redução e até mesmo a inibição da capacidade dos crustáceos de realizar seu processo osmótico, provocando, assim, altas taxas de mortalidade de acordo com a eficácia da espécie em se osmorregular e o tempo de exposição do animal a tal estresse (INMAN; LOCKWOORD, 1977; TARAZONA et al., 1987; LIN et al., 1993).

Dessa forma, o presente manuscrito tem como objetivo explorar os processos osmorregulatórios de camarões palemonídeos e peneídeos, visualizando um padrão na capacidade osmorregulatória em ambas as famílias e, em particular, tratar de assuntos pertinentes aos efeitos no ciclo ontogenético e o efeito ocasionado pelo nitrito na hemolinfa de crustáceos.

Desenvolvimento

Osmorregulação: Palemonídeos versus Peneídeos

A invasão da água doce ocorreu repetidamente ao longo dos anos (LEE; BELL, 1999). A adaptação provocada nessa transição foi encontrada em traços osmorregulatórios que permitem aos organismos sobreviverem em ambientes aquáticos, onde há menor concentração de íons no ambiente externo do que nos fluidos corporais dos animais (LEE; BELL, 1999).

Em organismos como crustáceos, a habilidade de sobreviver em vários ambientes e com diferentes concentrações osmóticas está relacionada com a capacidade de regular o fluido extracelular. Portanto, crustáceos de ambientes marinhos apresentam baixo gasto energético na regulação da osmolalidade de seu fluido, por apresentar isosmoticidade em relação ao meio em que se encontram (CROGHAN, 1976). Entretanto, crustáceos de água doce são hipersmóticos ao meio e estão à mercê dos movimentos passivos de entrada corpórea de água e perda de sais (MANTEL; FARMER, 1983).

A maioria dos peneídoes são fortes osmorreguladores (CHARMANTIER-DAURES et al., 1988) por possuírem mecanismos para regular a concentração de seu fluido corporal em ambientes com altas flutuações de salinidade (LIN et al. 1993). Muitos estudos sobre o efeito da salinidade nas propriedades osmóticas e iônicas foram realizados (CASTELA; LAWRENCE, 1981; LIGNOT et al., 1998; SANG; FOTEDAR, 2004; GONG et al., 2008), comprovando que cada espécie de camarão peneídeo apresenta uma determinada tolerância à diferentes salinidades. Mas parece ser um padrão para os peneídeos se hiposmorregularem quando a salinidade do ambiente for acima do ponto isosmótico, e hiperosmorregularem quando a salinidade for inferior ao ponto isosmótico. Entretanto, quando expostos a poluentes, os peneídeos podem encontrar dificuldades no processo de se osmorregular (HUNTER, 1949; JONES, 1975). No estudo realizado por Lin et al. (1993) observou-se que a amônia prejudicou a capacidade de hipo e hiper-regulação em juvenis de *Penaeus japonicus*. No mesmo estudo foi observado que a capacidade hipoosmótica em 36% de salinidade (1050 mosm kg^{-1}) e hiperosmótica em 15% de salinidade (450 mosm kg^{-1}) diminuiu acentuadamente com o aumento da amônia no ambiente, demonstrando que a capacidade osmótica é extremamente sensível ao efeito tóxico.

Os camarões palemonídeos tiveram sucesso ao saírem do ambiente ancestral marinho para habitar ambientes de água doce (JALIHAL et al., 1993), mas trabalhos realizados sobre a osmorregulação em camarões de água doce, demostram uma forte capacidade de hiper-osmorregulação em água doce e em baixas salinidades, perdendo essa capacidade em ambientes de alta salinidade (CASTELA; LAWRENCE, 1981; MOREIRA et al., 1983; FREIRE et al., 2003). A maioria dos decápodas de água doce se tornam hipoconformadores em água com alta concentração de sal (MOREIRA et al., 1983; WILDER et al., 1998; SIGNORET; BRAILOVSKY, 2004), enquanto alguns camarões do gênero *Macrobrachium*, como *M. equidens* e *M. olfersii* são exceções por possuírem a capacidade de hipo-osmorregular em altas salinidades (DENNE, 1968; MOREIRA

et al., 1983; FREIRE et al., 2003). Para tal afirmação, o tempo de exposição deve ser considerado, uma vez que a avaliação da participação do mecanismo de redução de permeabiliadde nem sempre é evidente. Estudos também demostraram que a alteração osmótica tem como base a mudança nos teores de Na / K na hemolinfa (CASTELA; LAWRENCE, 1981).

Portanto, o que parece é que o sucesso da conquista do ambiente dulcícola e a não necessidade de hiporregulação, levou à perda dessa capacidade na maioria dos palemo-nídeos.

Ação do nitrito sobre a oxihemocianina

Os compostos nitrogenados aparecem sob três formas no ambiente aquático. O nitrato é a principal forma de nitrogênio encontrada nas águas e, quando em elevadas concentrações, pode conduzir a um processo de produção primária exagerada, denominada eutrofização. O nitrogênio amoniacal (amônia) é uma substância tóxica não persistente e não acumulativa e que, em concentrações baixas, não causa nenhum dano fisiológico aos animais. O nitrito, que é uma forma química do nitrogênio, normalmente encontrada em quantidades diminutas nas águas superficiais, e é instável na presença do oxigênio. Sua presença indica processos biológicos ativos, influenciados por poluição orgânica (GORSEL; JENSEN, 1999). As concentrações desses compostos nitrogenados nas águas superficiais contribuem para a degradação dos ecossistemas aquáticos. Consequentemente, os organismos aquáticos sofrem os efeitos tóxicos desse processo de eutrofização (SMITH et al., 1999; HOWARTH et al., 2000).

O nitrito é o composto intermediário na nitrificação bacteriana de amônia a nitrato, podendo apresentar alta toxicidade, dependendo de sua concentração no meio e do estágio de desenvolvimento do organismo (MIRANDA-FILHO et al., 1995). Conhecido por se difundir na hemolinfa (CHENG et al., 2002) de crustáceos, o nitrito provoca uma elevação do O_2 livre e um decréscimo de O_2 ligado a hemocianina (oxi-hemocianina) (CHEN; CHENG,1996; CHENG et al., 2002). O mesmo efeito foi observado em *P. enaeus japonicus* (CHEN; CHENG, 1995), *Penaeus monodon* (CHENG; CHEN, 1999) e em *Macrobrachium rosembergii* (CHEN; LEE, 1997). Segundo Needham (1961; ARMSTRONG et al., 1976), a hemocianina é menos afetada pelo nitrito que a hemoglobina.

A hemocianina é uma proteína que tem como função o transporte de oxigênio na hemolinfa de crustáceos (DEEN; HOVING, 1977), se ligando a molécula de dioxigênio por meio do sítio ativo binuclear de Cobre (BROWN et al., 1980), representando em oxihemocianina a transferência de um elétron de cada cobre cuproso de desoxihemocianina ao dioxigênio limite (BROWN et al., 1980). Portanto, a cor azul da proteína é oriunda da presença do dioxigênio, enquanto a desoxihemocianina possui proteína incolor (BUBACCO et al., 1995). Chen e Cheng (1995) observaram no estudo da alteração no nível de oxihemocianina em *P. enaeus japonicus* exposto a nitrito, que no tratamento controle a oxihemociani-na constituía 84,6% da proteína total na hemolinfa. No entanto, a proporção de oxihemocianina diminuiu significamente para 72,4% em animais expostos a 5,12 mg/L de nitrito após 24h. Camarões marinhos, quando expostos a ambientes com nitrito, incorporam o composto na hemolinfa, reduzindo o nível da oxihemocianina e aumentando o nível da desoxihe-mocianina (CHENG; CHEN, 1999). *Astacus astacus* expostos ao nitrito por 48h apresentaram aumento deste composto na hemolinfa de 0,8mM até 10mM (JENSEN, 1990). *P. leniusculus* expostos por 24h apresentaram acúmulo de nitrito na hemolinfa de 1,0mM para 25mM (HARRIS; COLEY, 1991). Harris e Coley (1991) e Jensen (1996) relatam que o nitrito inibiu a captação de Cl^- na água doce para *Pacifastacus leniusculus* e *A. astacus*. Cheng e Chen (1998) afirmam que o nitrito é um inibidor competitivo na absorção de Cl^- e vice-versa. Estudo *in vitro* utilizando *Astacus leptodactylus* mostrou que a reação do nitrito com desoxihemocianina é 15 vezes maior do que com a oxihemocianina, e que produz metahemocianina em pH 5,7 (TAHON et al., 1988).

De acordo com Jensen (1995), duas vias são envolvidas no influxo de nitrito. Uma é que o íon de nitrito penetra pelo sistema de captação do cloreto pela brânquia. Então, a troca que está ligada ao Cl^- influxo e HCO_3^- efluxo (Cl^-/ HCO_3^-) regula o transporte de Cl^- na hemolinfa, bem como, o equilíbrio ácido-base (HENRY; WHEATLY, 1992). A outra via é por meio da entrada do nitrito nos organismos aquáti-cos através da difusão de ácido nitroso por ser solúvel em lipídios. Portanto, sua entrada nas brânquias ocorre por meio das células epiteliais (COLT; TCHOBANOGLOUS, 1976).

As informações acima comprovam que o nitrito apresenta efeitos nas proteínas presentes na hemolinfa dos crustáceos quando submetidos à exposição natural ou induzida deste composto.

Sensibilidade a compostos nitrogenados em diferentes estágios ontogenéticos

Resíduos nitrogenados em ambientes aquáticos naturais são cada vez mais enfocados dentro de uma visão global, podendo ser mais ou menos tóxicos decorrente da especificidade da espécie e o seu desenvolvimento ontogenético (ROMANO; ZENG, 2013). Além dos compostos nitrogenados presentes no ambiente, os crustáceos apresentam participação ativa com a excreção de amônia, correspondendo de 40% a 90% na concentração deste composto (PARRY, 1960). Já o nitrito, por se tratar de um produto intermédiario da amônia durante a nitrificação, está presente invulgarmente em condições naturais (CHEN et al., 1989), o que pode causar retardo no crescimento e até a morte dos organismos aquáticos quando em elevada concentração (CHEN; CHEN, 1992). Portanto, o acúmulo de compostos nitrogenados em ambiente aquático tem sido uma das causas mais comuns de morte em crustáceos (TARAZONA et al., 1987).

Estudos em laboratórios têm revelado diferenças significativas nos padrões de osmorregulação (CHARMATIER; ANGER, 2011). Por exemplo, juvenis e adultos pare-cem apresentar mecanismos eficientes para se osmoregular por um determinado tempo quando expostos a poluentes, mas o mesmo parece não ocorrer na fase de pós-larvas, o que pode influir o mau funcionamento no processo fisiológico do organismo, decorrente do efeito tóxico (INMAN; LOCKWOOD, 1977). Dessa forma, o rompimento do equilibrio osmótico e iônico normal é provocado após a exposição a poluentes como pesticidas e metais pessados, ou condições físico-químicas em crustáceos (YOUNG-LAI et al., 1991).

Um estudo realizado em diferentes fases de vida da espécie *Homarus americanus*, observou que a maior tolerância à toxicidade aguda da amônia foi encontrada com aumento do desenvolvimento ontogenético após 96 horas de exposição, comprovando que a diminuição na capacidade de pós-larvas e adultos de hiperregular em ambientes com bai-xa salinidade após a exposição à amônia é diferenciada pela fase de vida (YOUNG-LAI et al., 1991). Em *P. japonicus*, a tolerância à amônia aumentou com o desenvolvimento ontongenético, como observado por Peng et al. (1993). Em trabalhos com *P. monodom* e *Penaeus indieus* foi observado tolerância crescente da amônia com o aumento da fase de vida (JAYASANKAR; MUTHU, 1983; CHIN; CHEN, 1987). Alta suscetibilidade também foi relatada por Armstrong et al. (1976) e Rainbow (1990) para crustáceos em processo de muda submetidos a outras substâncias tóxicas. Essa maior suscetibilidade em animais jovens demostra um padrão, indicando estar relacionada com a elevada permeabilidade da cutícula recém formada, com aumento das atividades metabólicas e com a troca de hidrominerais no momento da muda (PENG et al., 1993).

Outros estudos também mostram que a capacidade de suportar maiores concentrações de nitrito está diretamente ligada ao aumento do desenvolvimento ontogenético. Jensen (1990) aponta que o nitrito pode afetar o equilíbrio ácido-base de *A. astacus* (JENSEN, 1990). Chen e Chin (1988) obser-varam que a resistência ao nitrito aumentou com o aumento da fase de vida de náuplio para pós-larva em *P. monodon*. Armostrong et al. (1976) relatam aumento na resistência ao nitrito com o aumento da fase ontogenética de *Penaeus indicus,* sendo de 10 mg/Le 23 mg/L nitrito para náuplios, de 20,43 mg/L nitrito para protozoe e de 33,87 mg/L nitrito para misis. Catedral et al. (1977) relatam que os níveis de toxicidade de nitrito para *P. monodon* variou de acordo com os estágios larvais (Zoé – 5 mg/L de nitrito e Misis 10 mg/L nitrito). Portanto, já é sabido que a presença do nitrito na hemocianina de crustáceos provoca menos danos do que em moléculas de hemoglobina de peixes (SMITH; RUSSO, 1975), mas apesar de vários estudos comprovarem a capacidade da hemocianina se ligar ao oxigênio na presença de ni-trito (CONANT et al., 1933; NEEDHAM, 1961; WICKINS, 1976), isso pode afetar a muda de *Callinectes sapidus*e e *M. rosenbergii* (ARMSTRONG et al., 1976; MANTHE et al., 1984).

Dessa forma, o desempenho osmorregulatório é, portanto, uma potencial ferramenta indicadora para detectar pressões fisiológicas, avaliando os efeitos de compostos contaminantes em que são submetidos os animais em ambientes naturais ou experimentais.

Considerações finais

Considerando o sucesso na colonização de ambien-te dulcícola, observa-se que a maioria dos palemonídeos não necessitaram mais hiporregulacão, levando a perda dessa capacidade. Dessa forma, supõese um ancestral com capacidade hiporregulatória, uma vez que invertebrados marinhos são, parcimoniosamente, osmoconformadores. Considerase também, que o nitrito apresenta efeitos nas proteínas presentes na hemolinfa dos crustáceos quando submetido à exposição natural ou induzidas destes compostos, e que a capacidade osmorregulatória é, portanto, variada de acordo como os estágios ontogenéticos.

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ANEXOS:

Normas das Revistas Científicas

(Instruções aos autores)

Anexo I: Normas da Revista "Aquaculture"

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The *Letters to the Editor* section is intended to provide a forum for discussion of aquacultural science emanating from material published in the journal.

Contact details for submission

Papers for consideration should be submitted via the electronic submission system mentioned below to the appropriate Section Editor:

Nutrition:
D.M. Gatlin

The Nutrition Section welcomes high quality research papers presenting novel data as well as original reviews on various aspects of aquatic animal nutrition relevant to aquaculture. Manuscripts addressing the following areas of investigation are encouraged:

1) determination of dietary and metabolic requirements for various nutrients by representative aquatic species. Studies may include environmental/stress effects on animal's physiological responses and requirements at different developmental stages;
2) evaluation of novel or established feedstuffs as well as feed processing and manufacturing procedures with digestibility and growth trials. Such studies should provide

comprehensive specifications of the process or evaluated ingredients including nutrients, potential anti-nutrients, and contaminants;
3) comparison of nutrient bioavailability from various ingredients or product forms as well as metabolic kinetics of nutrients, food borne anti-nutrients or toxins;
4) identification of key components in natural diets that influence attractability, palatability, metabolism, growth reproduction and/or immunity of cultured organisms;
5) optimization of diet formulations and feeding practices;
6) characterization of the actions of hormones, cytokines and/or components in intracellular signaling pathway(s) that influence nutrient and/or energy utilization.
7) evaluation of diet supplementation strategies to influence animal performance, metabolism, health and/or flesh quality.

Manuscripts concerning other areas of nutrition using novel or advanced methods are also welcome. Please note that in regard to various diet additives such as probiotics, prebiotics, herbal extracts, etc., a very large number of papers have already been published. Therefore, Aquaculture will not continue to accept manuscripts that present initial and preliminary investigations of such additives. Manuscripts addressing these and other feed additives will be accepted for review only if they are of the highest scientific quality and they represent a significant advance in our knowledge of the mechanisms involved in their metabolism. Manuscripts may also be considered if they present clinical efficacy data generated in large-scale trials and economic cost-benefit analysis of these applications.

Aquaculture Production Science:
B.Costa-Pierce

AQUACULTURE PRODUCTION SCIENCE (PS) is one of 5 sections of the international journal AQUACULTURE dedicated to research on improvements and innovations in aquatic food production.

This section supports worldwide dissemination of the results of innovative, globally important, scientific research on production methods for aquatic foods from fish, crustaceans, mollusks, amphibians, and all types of aquatic plants. Contributions are encouraged in the following areas:
1) Improvement of production systems that results in greater efficiencies of resource usage and sustainability of aquaculture; 2) Effective applications of technologies and methods of aquaculture production for improved stocking regimes; 3) The use of new species and species assemblages; and,
4) Investigations to minimize aquaculture wastes and improve water quality, including technologies for nutrient recycling in aquaculture ecosystems, and potential synergy of aquaculture and other food production systems using methods such as polyculture and integrated aquaculture. Aspects of seafood processing and technology will not be considered in this section although aquaculture techniques that may influence the nutritional value of aquatic food products may be considered in the Nutrition Section.

Physiology:
Fish: A. P. (Tony) Farrell
Invertebrates: J. Benzie

The Physiology Section welcomes high quality papers that present either novel research data or original reviews. The content must be relevant to solving aquaculture problems on all aspects of the physiology of cultured aquatic animals and plants.

Submitted manuscripts must have a valid hypothesis or objective, clearly state the relevance to aquaculture, have proper experimental design with appropriate controls and utilize appropriate statistical analysis. Mention of trade names is limited to the main text.

Relevant physiological topics include, but are not limited to: Reproductive and endocrine physiology, including control of development and sex differentiation, induced ovulation and spermiation, gamete quality, storage and cryopreservation, physiology of gynogenetic, and triploid and transgenic organisms Cardiorespiratory, muscle and exercise physiology Osmoregulatory physiology Digestive physiology, including endocrine and environmental regulation of growth Larval physiology and ontogeny, including metamorphosis, smolting and molting Performance under variable culture conditions, including temperature, water quality, rearing density, and stress and disease physiology Physiology of harvest and handling techniques

Genetics:
G. Hulata

The Genetics Section welcomes high-quality research papers presenting novel data, as well as critical reviews, on various aspects of selective breeding, genetics and genomics. Submitted manuscripts must have a valid hypothesis or objective, clearly state the relevance to aquaculture, have proper experimental design with appropriate sample size and controls and utilize appropriate statistical analysis.

Relevant genetics topics include, but are not limited to: Breeding programs using classic selection procedures, markers or combining marker assisted selection with classic selection Applications of crossbreeding and interspecific hybridization Evaluation of commercially important phenotypes among cultured strains, populations or stocks Applications of biotechnology and genetic manipulation methods Development of linkage maps, identification of QTL or association of commercially important traits with specific gene(s). Where appropriate, linkage maps should include co-dominant markers, such as microsatellite DNA and SNP markers, to enable application to other populations and facilitate comparative mapping. Aquaculture will NOT accept manuscripts dealing with the application of well- described techniques to yet another species, unless the application solves a specific biological problem important to aquaculture production; or manuscripts dealing with gene cloning, characterizing of microsatellites, species identification using molecular markers, EST papers with small collections, or mapping papers with a small number of markers, unless the papers also deal with solving a biological problem that is relevant to aquaculture production.

Aquaculture will not accept manuscripts focusing mainly on population genetics studies that are based on RAPD and AFLP markers, since the dominance and multilocus nature of the fingerprints are not suitable for making inferences about population genetic diversity and structure.

Sustainability and Society:
D.C. Little

The Sustainability and Society section of the journal Aquaculture invites articles at the interface of natural and social sciences that address the broader roles of aquaculture in global food security and trade.

Aims and scope of the Sustainability and Society section are the: global dissemination of interdisciplinary knowledge regarding the management of aquatic resources and resulting impacts on people. Interconnections with other sectors of food production; resource management and implications for societal impact. Going beyond a narrow techno-centric focus, towards more holistic analyses of aquaculture within well-defined contexts. Enquiry based on understanding trajectories of change amid the global challenges of climate change and food security. Mixed methods and approaches that incorporate and integrate both social and natural sciences. Relevance for the diverse range of policy makers, practitioners and other stakeholders involved. Articles that take a value chain approach, rather than being wholly production orientated, are encouraged.

Disease
B. Austin

The Disease sections welcomes critical reviews and high quality articles containing novel data on all aspects concerning diseases of farmed aquatic species. The aims of the section are: description of new and emerging diseases including characterization of the causal agent(s), development in the understanding of fish pathogens for example including new methods of growth where this has been a problem for fastidious organisms, pathogenicity and epizootiology, developments in the diagnosis of disease going beyond the use of standard well used methods, and methods of disease control, notably new developments in vaccines, immunostimulants, dietary supplements, medicinal plant products, probiotics, prebiotics and genetically-disease resistant stock. Relevance to aquaculture must be demonstrated. Articles, which adapt well known methods without further refinement of those methods, are unlikely to be accepted.

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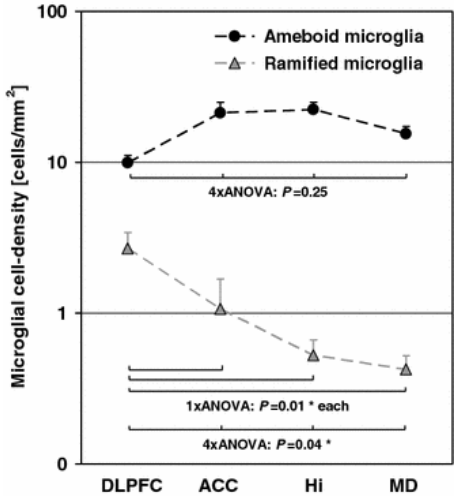

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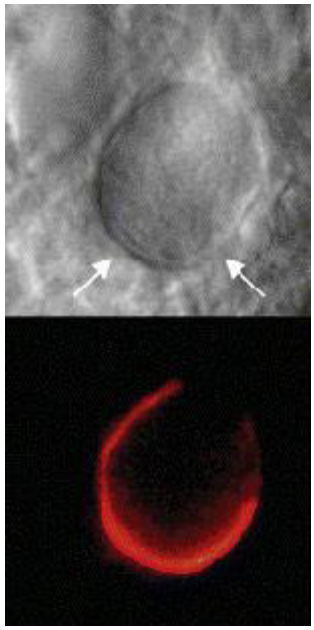

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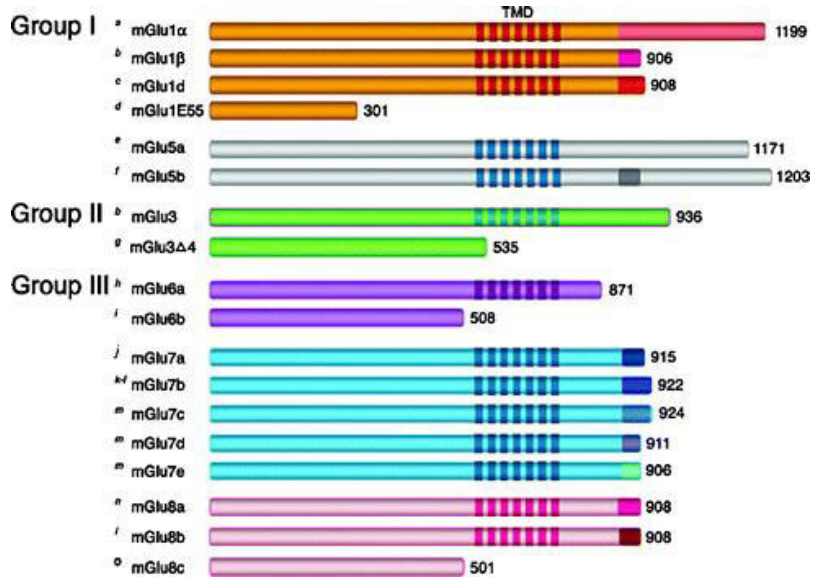

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Anexo III: Normas da Revista "Aquatic Toxicology"

AQUATIC TOXICOLOGY

AUTHOR INFORMATION PACK

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DESCRIPTION

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Environmental Toxicologists, Marine Biologists, Ecotoxicologists, Biochemical Toxicologists, Conservationists.

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