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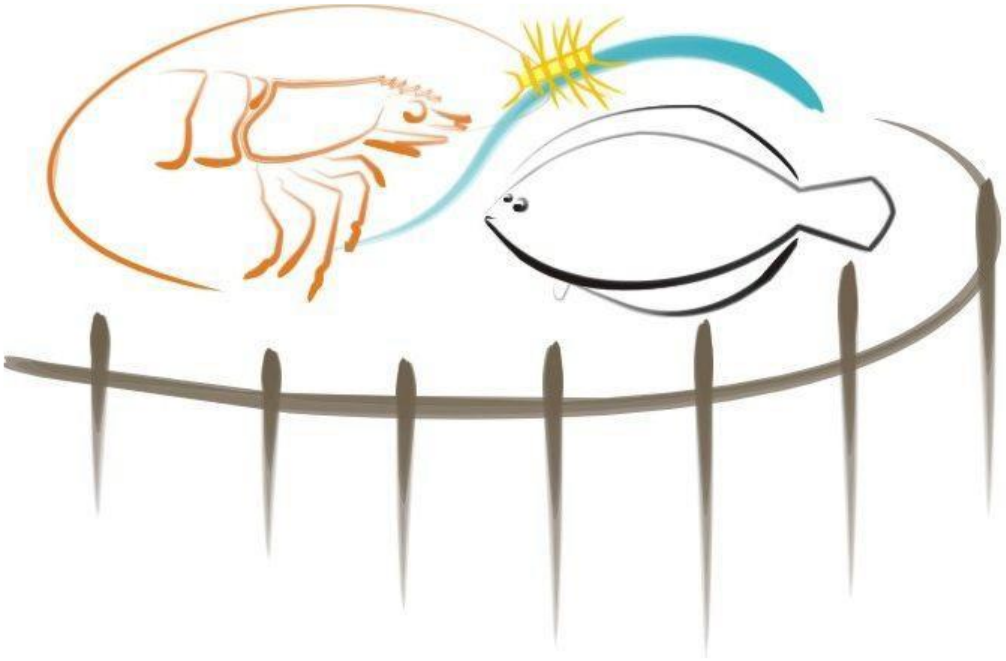

RESPOSTAS ANTIOXIDANTES E DANO OXIDATIVO NO CAMARÃO *Litopenaeus vannamei*: EFEITOS DA SUPLEMENTAÇÃO COM ANTIOXIDANTE E USO DE TECNOLOGIA DE BIOFLOCOS

ÁTILA CLIVEA DA SILVA MARTINS

RIO GRANDE, RS
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TESE DE DOUTORADO

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Tese apresentada ao Programa de Pós-graduação em Aquicultura da Universidade Federal do Rio Grande, como requisito parcial à obtenção do título de DOUTOR.

Orientador: Dr. José María Monserrat (FURG)
Coorientador: Dr. Wilson Wasielesky Jr. (FURG)

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SUMÁRIO

DEDICATÓRIA

À minha mãe (Célia Santana) e a meu pai (Antônio Carlos Martins), entre erros e acertos não permitiram que o amor incondicional provocasse vícios e dependências, me deram autonomia, confiança e independência para traçar rumos, fazer escolhas, superar frustações e cometer meus próprios erros e acertos. Em cada nova fase de minha vida tivemos perdas e ganhos dos dois lados, porque o amor de pai e mãe é processo de libertação permanente e esse vínculo não para de se transformar ao longo da vida. Dando-me dando a certeza a cada dia de que eles, até quando puderem, estarão lá firmes na concordância ou na divergência, no sucesso ou no fracasso, com o peito aberto para um aconchego, abraço apertado e conforto em todas as horas. A natureza nos ajuda a enxergar e compreender a importância das raízes. Quanto mais vigorosas são, maior e mais forte é a árvore. Para voar alto e livre é preciso ter um terreno firme de onde decolar. É tudo isso que meus pais representam para mim.

À minha vó (Maria Madalena Santana – em memória), que estava sempre preocupada com as horas que estávamos sem comer, se andávamos descalços. A mulher que a seu modo através de olhares e sorrisos conseguia dizer o quanto nos amava. Ainda tenho vivo na memória nossos últimos momentos juntas. Presenciei alguns sorrisos, alguns olhares atentos, e percebi, ela ainda estava ali, estava vendo e observando todos nós, por trás daquela mulher fraca estava ela: A minha vó, a mesma de sempre!

À minha irmã (Carla Martins), que me ajudou a enfrentar o mundo com inteligência, coragem e sabedoria. Inteligência para encontrar no mundo a oportunidade de mudança e aprendizado. Coragem para aceitar mudanças. Sabedoria para sorrir, chorar, sem perder a linha, sem perder o passo. Ensinou-me que algumas situações na vida servem como cinzel que esculpe, que talha, que faz o bloco amorfo de mármore se transformar em estátua, em obra de arte.

A Carlos Eduardo Winievisck, quem tem sorriso mais contagiante que já conheci. Carrega consigo a alegria de viver, que ainda conserva um sotaque irresistivelmente encantador. E quando estamos juntos a paz chega bem pertinho de mim, os problemas que embalam os dias e que roubam as energias misteriosamente se escondem como se

por aqueles instantes não mais existissem. Somos apenas nós diante de um cenário escolhido, jogando palavras, formando conversas e confissões. Quem me trouxe os mais lindos instantes e me completou com a magnitude de cada olhar. "Como tudo o que não pode ser tocado com a mão e nem visto como os olhos, e ainda se torna mais forte. As únicas coisas que importam são as feitas de verdade e alegria, não as de lata e vidro..." (Fernão Capelo Gaivota).

A Ivis Winievisck, meu amigo foram tantas as vezes que você apareceu no momento certo, e que mesmo sem querer dizia exatamente o que eu precisava ouvir, como se pudesse me ler. Nos conhecemos sem que sejam necessárias as palavras. É tudo tão grande que as diferenças se tornaram pequenos detalhes. Homem de frases lindas que tanto respondi com brincadeiras tão sinceras, mas que hora ou outra me faz dizer olhando nos olhos frases tão difíceis de serem ditas pessoalmente por mim. Que me ensinou a arte de ver a mim mesmo com minhas forças e fraquezas, mas sem máscaras, sem ilusões. A arte de perceber que as feridas cicatrizam sempre, e que ali a pele se torna mais resistente.

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RESUMO

Em aquicultura o aumento da intensificação está diretamente ligado a aplicação de novas tecnologias que aumente o volume de produção ao mesmo tempo que causa o mínimo de impacto ao meio ambiente que circunda a produção. Deste modo a tecnologia de biofloco desponta como método no qual o tratamento de qualidade de água é efetuado dentro do tanque de criação de modo que organismos fotoautotrofico (microalgas), autotróficos (bactéria nitrificante) e heterotróficos (bactéria heterotrófica) reciclam compostos nitrogenados que podem vir a ser tóxicos para o camarão *Litopenaeus vannamei*, além de serem capazes de transformar amônia em biomassa bacteriana que servirá como fonte proteica e lipídica para o camarão, podendo reduzir custo com ração. Com aumento da densidade no sistema de criação faz-se necessário aumentar a resistência bioquímica do animal criado, para tanto este trabalho apresenta em três capítulos meios de suplementação com antioxidante ácido lipóico (AL) que auxilia na resposta bioquímica antioxidante como mecanismo de melhorar o bem estar do camarão *Litopenaeus vannamei*. Primeiramente, através de análises bioquímicas como atividade da glutationa S-transferase (GST), concentração de glutationa reduzida (GSH), capacidade antioxidante total contra radicais peroxil (ACAP) e níveis de peroxidação lipídica (TBARS) em brânquias, hepatopâncreas e músculo de camarão, observando-se que o biofloco induz aumento da atividade da GST em brânquias, aumento da concentração de GSH em músculo, aumenta a capacidade antioxidante total em músculo e reduz níveis de peroxidação lipídica em hepatopâncreas. O segundo trabalho, aplicou-se ácido lipóico nanoencapsulado (NCLA) e cápsula vazia (NC) na ração, em água clara e em água com biofloco no qual foi observado que NCLA induzida aumento da atividade de GST nos hepatopâncreas. A concentração de GSH foi maior no músculo do que em brânquias e hepatopâncreas. A capacidade antioxidante também mostrou um padrão tecido-específico, tendo hepatopâncreas com maior capacidade antioxidante nenhuma ação evidente do desempenho do NCLA contra os radicais peroxil. Níveis de peroxidação lipídica foram menores no músculo, com acentuado efeito do NCLA. Nos grupos com NCLA houve um aumento na porcentagem de hemócitos granulares, células com maiores quantidades de componentes imunocompetentes. No trabalho 3, foi observado que o AL é capaz de aumentar a capacidade antioxidante no biofloco, analisado através da determinação da capacidade

antioxidante total contra radicais peroxil (ACAP), principalmente para concentração de 10 µM (2.06 mg de AL in 1 L de água destilada).

Palavras chave: Biofloco, *Litopenaus vannamei*, antioxidante, ácido lipoíco, análises bioquímicas, nanotecnologia, contagem diferencial de hemócitos.

ABSTRACT

In aquaculture, the increased intensification is directly linked to application of new technologies to increase the volume of production while causing minimal impact to the environment surrounding the production. Thus, the biofloc blunts as a new technology method in which the treatment water quality is made inside the tank, so that creation of photoautotrophic organisms (microalgae), autotrophs (nitrifying bacteria) and heterotrophic (heterotrophic bacteria) recycle nitrogen compounds that may to be toxic to shrimp *Litopenaeus vannamei*. These microorganisms are able to turn ammonia into bacterial biomass that will serve as protein and lipid source for shrimp and may reduce feed cost. With increased density in the build system it is necessary to increase the resistance biochemistry of the animal created, therefore this work presents in four chapters how of supplementation with the antioxidant lipoic acid (LA) can be assist in antioxidant biochemical response as a mechanism to improve the wellness of shrimp *Litopenaeus vannamei*. Firstly, by biochemical analyzes as gluthatione S-transferase (GST) activity, reduced gluthatione (GSH) concentration, total antioxidant capacity against peroxyl radicals (ACAP) and levels of lipid peroxidation (TBARS) in gills, hepatopancreas and shrimp muscle, observing that the biofloc induces increased in GST activity in gills, increased GSH concentration in muscle, increases the total antioxidant capacity in muscle and reduces lipid peroxidation levels in hepatopancreas. The second work was applied nanoencapsulated lipoic acid (NCLA) and empty nanocapsules (CN) in feed in clean water (SW) and biofloc (BFT) in which it was observed that NCLA induced increased GST activity in hepatopancreas. The GSH concentration was higher in muscle than in gills and hepatopancreas. The antioxidant activity also showed a specific pattern of tissue having higher antioxidant capacity in hepatopancreas, without no obvious action of NCLA in performance against peroxyl radicals. Lipid peroxidation levels were lower in the muscle, with marked effect of NCLA. In groups with NCLA there was an increase in the percentage of granular hemocytes, cells with higher amounts of immunocompetents components. In the work 3, it was observed that LA can increase the antioxidant capacity on biofloc, analyzed by determining the total antioxidant capacity against peroxyl radicals (ACAP), especially for concentration 10 µM (2.06 mg AL in 1 L of water distilled).

Keywords: Biofloc, *Litopenaus vannamei*, antioxidant, lipoic acid, biochemistry, nanotechnology, differential count of hemocytes.

1 INTRODUÇÃO GERAL

Várias pesquisas no setor de aquicultura são destinadas ao aumento da intensificação da produção. Estes esforços englobam maximizar as atividades na relação aquicultura e ambiente externo, como também o aumentar a sustentabilidade (econômica, social e ambiental). Ainda deve ser considerada a relação entre aquicultura e ambiente interno, que corresponde a potencializar o crescimento e sobrevivência do camarão, em menores ciclos de produção e com rendimento e qualidade do produto final. A carcinicultura se tornou uma grande atividade aquícola e sua expansão em todo mundo tem aumentado a preocupação ambiental em torno desta atividade (Xu et al. 2013). Tendo o camarão branco do Pacífico, *Litopenaeus vannamei* (Boone 1931), a espécie com maior volume de produção (Kim et al. 2014) por apresentar rápido crescimento, maior índice de sobrevivência e ser tolerante a alta densidade e estocagem (Xu et al. 2012).

A produção mundial cresceu a uma taxa de 15,1% entre 2000 e 2008, que se deve a intensificação da produção de *Litopenaeus vannamei* na China, Tailândia e Indonésia (FAO 2012). Em 2014, a produção mundial de camarão em cativeiro aumentou para 3.680.404 toneladas, um aumento de 7% em relação aos 3.436.918 de toneladas produzidas em 2013, com base em estimativas obtidas de fontes oficiais e na sua ausência de fontes oficiais, as estimativas fornecidas por fontes da indústria, a produção brasileira se estabilizou entre os anos de 2013 e 2014 em 90 mil toneladas (Shrimp News International 2015).

O camarão branco *Litopenaeus vannamei* é a espécie de camarão mais comercializada em muitas partes do mundo e sua produção em sistemas intensivos de biofloco com pouca ou nenhuma troca de água tem demostrado ser uma prática sustentável (Avnimelech 2012; Xu e Pan 2014), especialmente em aquicultura intensiva que está ligada com a poluição da água por um excesso de materiais orgânicos e nutrientes que são susceptíveis de causar efeitos tóxicos agudos e riscos ambientais de longo prazo (Piedrahita 2003). Em um sistema aquícola convencional o método mais comum para lidar com este tipo de poluição tem sido a substituição contínua da água do tanque com água fresca externo (Gutierrez-Wing e Malone, 2006). No entanto, o

volume de água necessário para pequeno a médio tanque aquícola pode chegar a várias centenas de metros cúbicos por dia.

Uma nova abordagem é uso da tecnologia de biofloco (BFT), que é a formação e estimulação de um microecossistema que incluem microalga, bactérias autotróficas, bactérias heterotróficas, detritos orgânicos e inorgânicos. Desta forma, processos de renovação de água neste sistema é mínima ou zero, havendo, portanto, reutilização da água e alguns riscos, como a introdução de agentes patogénicos, escapamento de espécies exóticas e descarga de águas residuais (poluição) são reduzidos ou mesmo eliminados (Ray 2012).

Estes microrganismos (biofloco) tem como funções principais: (i) manutenção da qualidade da água, pela absorção de compostos nitrogenados e sua transformação em proteína microbiana e (ii) nutrição que aumenta viabilidade econômica da produção, reduzindo a conversão alimentar e uma diminuição dos custos de alimentação (Emerenciano 2013). Os macroagregados (biofloco) é fonte natural rica em lipídeos e proteínas, disponível *in situ* 24 horas por dia (Avnimelech 2007). Na coluna de água ocorre uma complexa interação entre matéria orgânica, substrato físico e grande variedade de microrganismos, como fitoplâncton, bactérias livres e aderidas, agregados de partículas de matéria orgânica e herbívoros, como os rotíferos, ciliados e flagelados protozoários e copépodes (Ray 2010).

Esta produtividade natural tem um papel importante na reciclagem de nutrientes e na manutenção da qualidade de água. Bactérias autotróficas fazem a conversam de amônia a nitrito e depois convertem nitrito a nitrato e, as bactérias heterotróficas conseguem compostos nitrogenados e transformam em proteína microbiana. As bactérias autotróficas são mais eficientes nesta conversão, porém o processo é feito de forma lenta, e as bactérias heterotróficas tem crescimento mais rápido e, portanto, retiram de forma mais rápida os nitrogenados e transformam em proteína microbiana, por isso há a manipulação da taxa de carbono e nitrogênio na proporção 20:1 para favorecer crescimento e domínio de bactérias heterotróficas (Avnimelech 1999).

O consumo de biofloco por camarão ou peixe tem demonstrado inúmeros benefícios tais como a melhoria da taxa de crescimento, diminuição da taxa de conversão alimentar e os custos associados em alimentos para animais (Buford et al.

2004; Wasielesky et al. 2006). A melhoria do crescimento tem sido atribuída a bactérias e algas como componentes nutricionais, pelo qual até 30% da ração comercial pode ser reduzido devido ao consumo de biofloco pelo camarão (Buford et al. 2004). E o uso de biofloco pode ser uma alternativa para substituir o uso de proteínas alternativas como a farinha de peixe (Azim 2008).

Estudo tem demostrado que além do uso manutenção da qualidade de água e como fonte de proteína e lipídio, o biofloco também tem o efeito de manter o equilíbrio das funções fisiológicas como sistema antioxidante, que é essencial para a manutenção do bem estar do camarão e assim garantir crescimento e sobrevivência satisfatórios (Castex et al. 2010; Xu e Pan 2014; Martins et al. 2014, 2015) em um ambiente de criação. Estudos efetuados com *Litopenaues vannamei* indicaram que o biofloco pode aumentar o estado antioxidante do camarão, com causa provável do biofloco ser rico em microrganismos naturais e compostos bioativos de natureza antioxidante (Ju et al. 2008; Xu e Pan 2013; Martins et al. 2015).

A espécie *Litopenaeus vannamei* vem largamente sendo afetada por doenças (Kim et al. 2014). Muitas doenças são agravadas pela alteração do equilíbrio bioquímico celular e pouco se conhece sobre os benéficos bioquímico/fisiológicos do biofloco e seus efeitos no camarão. O desequilíbrio bioquímico, em espécies aeróbicas, pode ocorrer quando há maior produção de espécies reativas de oxigênio (ERO), que são produtos intermediários da redução parcial dos quatro elétrons do oxigênio resultando em água (H_2O), ânion superóxido ($O_2\bullet^-$), radical hidroxila (OH•) e espécies não radicalar como o peróxido de hidrogênio (H_2O_2) (Abele e Pintarulo 2004). Por definição redução é a perda de oxigênio ou ganho de elétrons, desta forma o O_2 sofre redução tetravalente com ganho de quatro elétrons e formando H_2O (Gutteridge e Halliwell 2010) e neste processo os produtos intermediários reativos ($O_2\bullet^-$, H_2O_2, OH•) podem ser nocivos quando o sistema antioxidante não é capaz de controlá-los, situação que pode derivar em estresse oxidativo (Sies 1985; Abele e Pintarulo 2004).

O estresse oxidativo é um estado de desbalanço entre a produção intra e extracelular de ERO e o sistema antioxidante, resultando em dano oxidativo de muitos tipos de moléculas como lipídios, proteínas e DNA. Desta forma, a necessidade de prevenir, interceptar ou retardar as ações das ERO, no decorrer da evolução, acarretou

no desenvolvimento de defesas antioxidantes, comumente divididas em enzimáticas e não-enzimáticas (Anderson 1998; Dickinson e Forman 2002). O sistema antioxidante enzimático é o primeiro mecanismo de defesa celular e é composto por superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx) e glutationa-S-transferase (GST) que são moléculas de maior peso molecular, somado a estes há a ação de outros compostos antioxiantes como vitaminas A, C e E que têm menor peso molecular (Hellou et al. 2012). Dentre as defesas antioxidantes não-enzimáticas o tripeptídeo glutationa (γ-L-glutamil–L-cisteinil–glicina) é considerado a primeira ação de defesa contra ERO (Anderson 1998; Dickinson e Forman 2002) e está presente nos organismos nas formas reduzidas (GSH) e oxidada (GSSG) (Hellou et al. 2012). Além deste, inclui-se também α-tocoferol, carotenóides e flavonoides (Barreiros et al. 2006) e o ácido lipóico (AL) que é um dos focos desta Tese.

Acredita-se que as mitocôndrias consumam 90% do oxigênio celular em células átonas (jovens) e são os principais locais de produção de ERO em células aeróbicas (Lenaz 1998; Abele e Pintarulo 2004). Na redução univalente, o O_2 (oxigênio) é convertido a $O_2•^-$ (radical superóxido), que por ação da enzima superóxido dismutase (SOD) é convertido em H_2O_2 (peróxido de hidrogênio), que é um ERO mas não é um radical livre, este composto é difundo livremente através da mitocôndria de forma espontânea (Abele e Pintarulo 2004). Caso o H_2O_2 não seja decomposto enzimaticamente, este pode ser convertido em OH• (radical hidroxila), que tem curta vida por ser altamente reativo (Halliwell e Gutteridge 1985; Abele e Pintarulo 2004). Alguns componentes (poluentes) podem ser difíceis de oxidar e, portanto, é necessário que o H_2O_2 seja ativado por catalizadores (ferro, cobre, manganês), sendo mais comum utilizar o ferro como catalizador que quando reage com H_2O_2 caracteriza a reação de Fenton que requer pH ácido e produz radicais hidroxila (OH•) que são altamente reativos que degradam poluentes orgânicos (Wang et al. 2012). O H_2O_2 é convertido em água e oxigênio, cuja reação é catalisada pela enzima catalase (CAT) ou é utilizado para oxidar substratos, como por exemplo peroxidases como glutationa peroxidase (GPx) (Hellou et al. 2012).

Um estado redox pró-oxidante é caracterizado com uma queda relativa na proporção de glutationa reduzida (GSH/GSSG) e da relação NADH/NAD (Abele e

Pintarulo 2004). A glutationa é uma molécula produzido naturalmente pelo fígado, também é encontrada em frutas, verduras e carnes, sendo uma combinação de três blocos de proteína ou aminoácidos (tripéptido – cisteína, glicina e glutamina) que contém um grupo químico de enxofre (SH), que atua como atrativo para moléculas que podem causar dano ao organismo como espécies reativas de oxigênio e xenofibiótico (Nuttall et al. 1998, Huber et al. 2008). Normalmente a glutationa é reciclada no corpo, exceto quando há sobrecarga com muito estresse oxidativo ou muitas toxinas, a glutationa se esgota e diminui a proteção contra os radicais livres ou toxinas (Nuttall et al. 1998, Huber et al. 2008). A rede antioxidante é composta por vários componentes que incluem vitaminas, minerais e produtos químicos especiais chamados tióis (glutationa e o ácido alfa-lipóico) (Huber et al. 2008).

A glutationa é um antioxidante intracelular que tem a capacidade de maximizar a atividade de todos os outros antioxidantes, incluindo vitaminas C, vitamina E e ácido lipóico, removendo toxinas das células e protegendo contra os efeitos nocivos da radiação, produtos químicos e poluentes ambientais (Schafer e Buettner 2001; Dickinson e Forman 2002; Huber et al. 2008). O AL é uma molécula com características hidro e lipossolúvel, com múltiplos efeitos benéficos em doenças como diabetes, Alzheimer e hipertensão, o que mostra seu grande potencial biomédico (Packer et al. 1995). O AL e sua forma reduzida, o ácido dihidrolipóico (DHLA), preenchem todos os critérios avaliados na análise do potencial antioxidante de um composto: quelam metais, são varredores de ERO, participam da reciclagem de outras moléculas antioxidantes e do reparo de moléculas danificadas pelo estresse oxidativo (Packer et al. 1995). O ácido lipóico a princípio foi classificado como vitamina, contudo posteriormente foi constatado que este composto é sintetizado em células animais (Carreau 1979), atuando como cofator em complexos multi-enzimáticos que catalisam reações de descarboxilação oxidativa no ciclo de Krebs (Packer et al. 1995).

Um antioxidante pode ser definido como qualquer substância que quando presente em baixas concentrações em relação ao substrato oxidável (que causa dano), atrasa significativamente ou impede a ação danosa do referido substrato (Halliwell e Getturidge 1995). Nas últimas décadas o ácido lipóico tem recebido atenção devido a sua função antioxidante em organismos aquáticos (Monserrat et al. 2008). O papel do

ácido lipóico no reestabelecimento dos níveis de ácido ascórbico em pacu (*Piaractus mesopotamicus*) foi importante do ponto de vista bioquímico e fisiológico, o pacu, assim como outros teleósteos, não sintetiza o ácido ascórbico, sendo este, portanto, uma vitamina. A deficiência dela pode induzir uma redução de crescimento e maior suscetibilidade a vários tipos de doenças (Terjesen et al. 2004; Trattner et al. 2007). Em truta arco-íris (*Oncorhynchus mykiss*) e carpas (*Cyprinus carpio*) a deficiência de vitamina C provoca anorexia, lordose, escoliose, hemorragias, deformações em brânquias, exoftalmia (Trattner et al. 2007).

A vitamina C quela o ferro e o reduz a Fe^{2+}, subsequentemente, o Fe^{2+} pode transferir um elétron ao oxigênio ou para outro ERO e induzir o estresse oxidativo, porém em quantidades equimolares de ferro e vitamina C, ácido lipóico é capaz de competir com a vitamina C para a quelação e, consequentemente, haver proteção contra a peroxidação de lípidos (Biewenga et al. 1997). O ácido lipóico tem sido referido como um antioxidante universal por atuar tanto na membrana quanto na fase aquosa das células, proporcionando proteção a membrana, devido a sua interação com os antioxidantes vitamina C e glutationa, as quais por sua vez podem reciclar a vitamina E (Flora 2009). As propriedades do ácido lipóico incluem também a capacidade de varrer ERO, além de regenerar antioxidantes endógenos (Packer et al. 1995; Flora 2009; Külkamp-Guerreiro et al. 2009).

O trabalho de Amado et al. (2011) avaliou o efeito quimioprotetor do ácido lipóico contra a toxidade de microcistina em carpa *Cyprinus carpo*. Estes autores observaram o tempo necessário para indução na expressão de genes que codificam três classes da glutationa-S-transferase (alfa, mu e pi). Os resultados constataram que o AL foi eficaz em promover aumento na transcrição de genes da GST no fígado após duas injeções de AL dadas com intervalo de 24 h. Os dados sugerem que AL pode ser útil como agente quimioprotetor contra indução toxica da microcistina, estimulando a desintoxicação através do incremento da atividade da GST (cérebro) ou por meio da reversão da inibição da GST (fígado).

O trabalho de Monserrat et al. (2008), considerou os efeitos do AL em diferentes órgãos (brânquias, cérebro, músculo e fígado) no peixe *Corudoras paleatus* (Callychthyidae). O AL, na dose de 70 mg/kg de massa corporal foi adicionada na

ração, alimentados diariamente (1% do peso). Os resultados mostraram redução da concentração de espécies reativas de oxigênio no cérebro e aumento da atividade do glutamato-cisteína ligase (GCL) no cérebro e no fígado do mesmo grupo experimental. A GCL é uma enzima que controla a velocidade da síntese de GSH. Organismos suplementados com AL apresentaram maior atividade da glutationa-S-transferase no cérebro, indicando que o AL melhora a capacidade de desintoxicação nas reações de fase II. Foi observado também notável redução da oxidação de proteínas no músculo e no fígado dos peixes suplementados com AL, indicando que o tratamento foi eficaz na redução de parâmetros de estresse oxidativo.

O organismo do camarão, como qualquer organismo aeróbico, é susceptível a estresse oxidativo, resultado da ação de ERO que podem ser provenientes do meio ambiente (exógenas) ou gerada no próprio organismo (endógenas). No camarão *Litopenaeus vannanmei*, foi verificado o efeito de 3 doses de AL (35, 70 e 140 mg de AL por 1 kg de ração), que foi suplementada na ração durante 45 dias e análises de espécies reativas de oxigênio foi efetuada em brânquias e hepatopâncreas. Constatou-se que das 3 doses aplicadas a dose de 70 mg/kg foi a que melhor resultou no aumento da atividade antioxidante, principalmente em brânquias (Martins et al. 2014).

A utilização do ácido lipóico é promissora, contudo esta substância é lábil e sem estabilidade química e, portanto, sujeito a degradação por ação térmica, fotoquímica, meio ácido e oxidação. Sendo assim, existem pesquisas desenvolvendo um complexo de ácido lipóico e ciclodextrina, que apresentam maior estabilidade à temperatura e a luz, o que reduz a dispersão do AL em água, aumenta a biodisponibilidade e reduz seu odor, no entanto, estes complexos apresentaram ampla distribuição de tamanho e aumento de tamanho das partículas após duas semanas de armazenamento à temperatura ambiente (Külkamp-Guerreiro et al. 2009). A alternativa ainda pouco explorada para a estabilização do ácido lipoico é o emprego de nanocápsulas poliméricas, as quais consistem em sistemas vesiculares nanoestruturados carreadores de substâncias que apresentam diâmetros entre 200 e 300 nm e baixa polidispersão, e que se destacam devido às suas potencialidades no controle da liberação de substâncias e à capacidade de aumentar a estabilidade do composto, tanto no armazenamento quanto nos fluídos biológicos (Külkamp-Guerreiro et al. 2009). O termo nanotecnologia foi inserido em 1974, para

descrever a manipulação de partículas de menos de um micrômetro. Em particular, o processo de nanoencapsulado é eficiente no desenvolvimento de produtos funcionais e pode auxiliar a combater a perda de funcionalidade dos antioxidantes ou produtos bioativos durante o processamento ou armazenamento, geração de maus odores e sabores, entre outros problemas (Quintanilla-Carvajal et al. 2010).

A utilização de nanocápsula é descrito para proteção de diferentes sistemas aplicados em fármacos e cosméticos, especialmente em substâncias que degradam em temperaturas acima de 40 ºC ou são sensíveis à oxidação em presença de água, por variação de pH ou por efeito de luz ultravioleta (Müller et al. 2004, Külkamp-Guerreiro et al. 2009). A membrana polimérica da nanocápsula possui efeito protetor de substâncias contra danos causados por agentes externos, prevenindo a degradação. (Bauchemal et al. 2006, Weiss-Angeli et al. 2008, Külkamp-Guerreiro et al. 2009). Para este estudo, a análise das condições do sistema antioxidante do biofloco e do camarão *Litopenaues vannamei* envolveram as análises da atividade da glutationa S-transferase (GST), concentração da glutationa reduzida (GSH), níveis de peroxidação lipídica (TBARS), análise da capacidade antioxidante contra radicais peroxil (ACAP) e contagem diferencial de hemócitos para análise do estado imune.

A glutationa-S-transferase é uma enzima multifuncional que está envolvida na desintoxicação de xenobióticos, oferecendo proteção contra danos oxidativos e efetúa também transporte intracelular de hormônios, metabólitos endógenos e exógenos de produtos químicos em diversos organismos. Assim a GST é componente importante de várias vias de desintoxicação e tolerância ao estresse, uma vez que protege contra lesões induzidas por substâncias químicas ambientais (Zhou et al. 2009).

A glutationa (GSH) é um antioxidante muito importante na preservação do estado redox celular, na defesa contra ERO e detoxificação de xenobióticos. Está molécula é um tripeptídeo composto por ácido glutâmico, cisteína e glicina, que sob condições normais e niveis de cisteína adequados a taxa limitante para sua sintese é determinada pela atividade da enzima glutamato cisteína ligase (GCL) (White et al. 2003).

Quando em condições normais ou anormais a produção de ERO ultrapassa a proteção endógena de enzimas específicas e vitaminas antioxidantes ocorre dano

celular, fenômeno este chamado de estresse oxidativo (Oakes e Van Der Kraak 2003). As EROs atacam radicais livres como ácidos graxos poliinsaturados (PUFAs) que são um substrato rico em elétrons (Esterbauer 1996; Oakes e Van Der Kraak 2003). Este procedimento então, é realizado pela quantificação de compostos como o malondialdeído (MDA), que é um subproduto da peroxidação lipídica (Janero 1990; Oakes e Van Der Kraak 2003). Portanto, a reação do MDA com o ácido 2-tiobarbitúrico (TBA), ensaio este conhecido como TBARS, é um dos mais amplamente utilizados como estimadores de estresse oxidativo que analise os níveis de peroxidação lipídica dos tecidos (Liu et al. 1997; Oakes e Van Der Kraak 2003).

Embora seja importante medir a eficência de antioxidante individuais para combater a produção de oxiradicais, faz-se necessário compreender a resistência dos tecidos a toxidade causada por ERO e não somente medir um numero limitado de antioxidantes (Amado et al. 2009). Por isto usa-se a análise da capacidade antioxidante contra radicais peroxil (ACAP), que é um método simples, rápido e confiável na detecção de ERO por fluorometria, se valendo do 2,7´ dicloroflurescéína diacetato (H_2DCF-DA) como substrato o qual, após sua deacetilação, que irá a interagir com radicas peroxil que são gerados pela decomposição térmica a 37°C do 2,2- azobis (2metilpropianoamidina) dihidrocloreto (ABAP). Uma queda na fluorescência nestas condições é interpretada como um efeito antioxidante da amostra, através da interceptação ou redução dos peroxi radicais, gerando um menor sinal de fluorescência emitida pela reação entre EROs e H_2DCF (Amado et al. 2009).

2 HIPÓTESES

- Camarões criados em sistema de biofloco terão sua maior capacidade antioxidante, em função dos antioxidantes presentes no biofloco.

- O ácido lipóico aumentará a capacidade antioxidante e diminuir o dano oxidativo no camarão branco do pacífico *Litopenaus vannemei*, visto os efeitos já descritos deste antioxidante em espécies aquáticas.

- O ácido lipóico aumentará a competência antioxidante do biofloco, vista suas caraterísticas descritas e definidas como de "antioxidante ideal".

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar se a suplementação com ácido lipoíco na ração e no biofloco altera do estado antioxidante do camarão *L. vannemei* e do biofloco através de análise bioquímicas que contemplem respostas antioxidantes, de detoxificação e de dano oxidativo.

3.2 OBJETIVOS ESPECÍFICOS

- Avaliar os efeitos antioxidantes do biofloco em brânquia, hepatopâncreas, músculo do camarão *L. vannemei* por meio da determinação a atividade da glutationa S-transferase (GST) e glutationa reduzida (GSH), bem como avaliar o dano oxidativo dos tecidos pela análise dos níveis de peroxidação lipídica (TBARS) e capacidade antioxidante total contra radicais peroxil (ACAP).

- Avaliar o estado redox dos tecidos (brânquia, hepatopâncreas, músculo) após suplementação do ácido lipóico (AL) na ração do camarão *L. vannemei* por meio da determinação a atividade da glutationa S-transferase (GST) e glutationa reduzida (GSH), bem como avaliar o dano oxidativo dos tecidos pela análise dos níveis de peroxidação lipídica (TBARS) e contagem diferencial de hemócitos (CDH).

- Avaliar os efeitos do ácido lipóico no biofloco através da análise da capacidade antioxidante total contra radicais peroxil, capacidade dos compostos em atuar como varredores de espécies reativas de oxigênio (ERO).

4 METODOLOGIA GERAL

4.1 DESENHO EXPERIMENTAL

Os camarões utilizados foram juvenis da espécie *Litopenaeus vannamei*, provenientes dos viveiros da Estação Marinha de Aquacultura (EMA), Universidade Federal do Rio Grande – FURG. Os animais foram aclimatados em 2 tanques de fibra de vidro (1000 L) com volume útil de 800 L, 150 animais em cada tanque foram estocados a aclimatados durante 25 dias (6 a 30 de maio).

Após estes dias, foram submetidos a biometria e transferidos para tanques de plástico (70 L) com volume útil de 50 L. Foram separados para primeira publicação em tratamentos: (i) água clara e (ii) biofloco (Figura 1). Para o segundo manuscrito os tratamentos foram: (i) água clara/nanocapsula vazia, (ii) água clara/AL nanoencapsulado, (iii) biofloco/nanocapsula vazia e (iv) biofloco/AL nanoencapsulado (Figura 2). Os camarões foram estocados a uma densidade de 15 camarões por tanque (cerca de 300 camarões por m^3) e passaram 7 dias aclimatados nessa condição (31 de maio a 6 de junho), a partir de então foram ofertadas as respectivas rações. Nos tanques com água clara os animais apresentaram peso médio inicial de 5,91 ± 0,07 g, nos tanques com biofloco o peso médio inicial foi de 5,01 ± 0,05 g e quando houve aumento de amônia acima de 1 mg/L foi adicionado melaço.

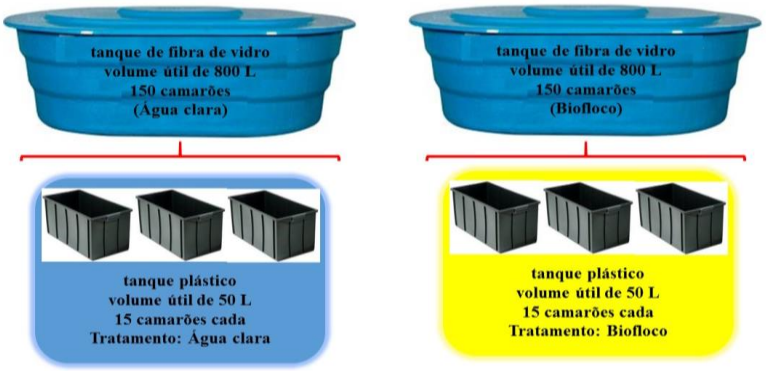

Figura 1 – Desenho experimental trabalho 1.

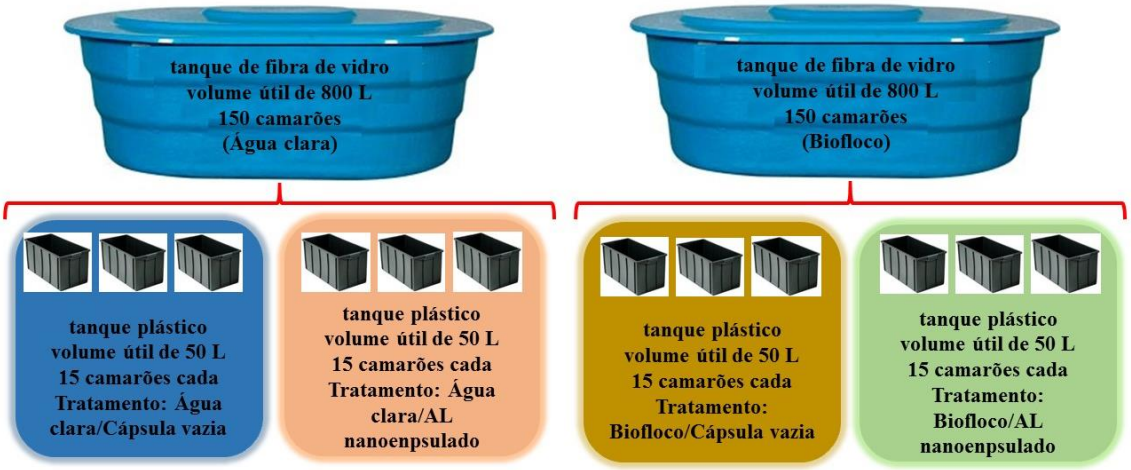

Figura 2 – Desenho experimental trabalho 2.

A ração comercial SUPRA® (35% de proteína bruta) foi pesada, em seguida trituradas, misturadas a respectivas soluções (Tabela 1) para obtenção de uma massa homogenia, passada em seringa de 5 mL e produto foi colocado em estufa a 50°C e depois peleitizada. A dieta foi administrada três vezes ao dia (8:00, 15:00 e 22:00 h) a uma taxa alimentar de 3% da biomassa do camarão. O experimento teve duração de 30 dias (7 de junho a 6 de julho), nos quais nos tanques com água clara houve renovação de 80-90 % de água a intervalo de 1 dia e nos tanques com biofloco não houve renovação de água.

Tabela 1 – Quantidades de ração e solução em cada tratamento.

Tratamento	Ração (g)	Solução (mL)
Água clara	500	200 (água destilada)
Biofloco	500	200 (água destilada)
Água clara/cápsula vazia	500	200 (cápsula vazia)
Água clara/AL nanoencapsulado	500	200 (AL nanoencapsulado)
Biofloco/cápsula vazia	500	200 (cápsula vazia)
Biofloco//AL nanoencapsulado	500	200 (AL nanoencapsulado)

Para o terceiro trabalho, foi realizado a suplementação do ácido lípoico na água com biofloco. Neste caso foram utilizados 15 tanques de plásticos (70 L), com volume útil de 50 L cada. Os tratamentos foram fixados em: Controle (0 mg de AL/L de biofloco), Concentração de 1,25 µM (0,26 mg/L), Concentração de 2,5 µM (0,51 mg/L), Concentração de 5 µM (1,03 mg/L) e Concentração de 10 µM (2,06 mg/L); todos em triplicada (Figura 2).

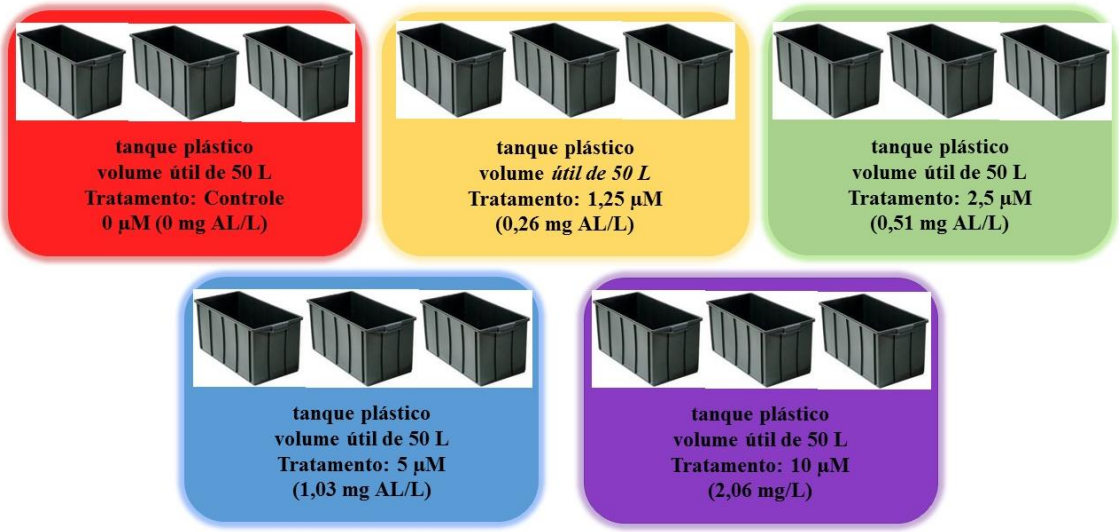

Figura 2 – Desenho experimental do trabalho 3.

O primeiro trabalho, comparando água clara e biofloco foi publicado na revista Marine and Freshwater Behaviour and Physiology, com título "Antioxidant and oxidative damage responses in different organs of Pacific white shrimp *Litopenaeus vannamei* (Boone 1931) reared in a biofloc technology system". O segundo trabalho comparando tratamentos com nanocapsula vazia e ácido lipóico nanoencapsulado foi submetido a revista Comparative Biochemistry and Physiology e é intitulado: "Antioxidant effects of nanoencapsulated lipoic acid in tissues and immune condition in hemolymph of shrimp Pacific *Litopenaeus vannamei* (Boone, 1931)". O terceiro trabalho, com título de "Effects of lipoic acid in the total antioxidant capacity in biofloc", será submetido a revista Aquaculture.

4.1.1 Preparação das Nanocapsulas e Nanocapsulas vazias

Foi realizado segundo Longaray-Garcia et al. (2013), no qual a suspensão de nanocápsulas com ácido lipóico foram preparados pelo método de precipitação do polímero pré-formado. O ácido lipóico foi pesado (70 mg) e dissolvido na fase orgânica composta por triglicéridos caprílico (0,33 mL), monoestearato de sorbitano (76,6 mg), poli (ξ-caprolactona) (100 mg), acetona (26,7 mL) e butil-hidroxi-tolueno (BHT) (0,01 g). A fase orgânica foi injetada em fase aquosa contendo polissorbato 80 (76,6 mg), diazolidinil ureia (0,01 g) e água Milli-Q (53,3 mL), através de um funil e mantida sob agitação magnética moderada durante 10 min. A suspensão foi preparada protegida da luz, e os solventes foram evaporados em evaporador rotativo (Bu chi R-114) a uma temperatura de aproximadamente 30 °C até um volume final de 10 mL, para dar uma concentração final de 70 mg mL-1. As suspensões foram nanocápsulas por espessão com o emulsionante de silicone DC RM2051® (4 g) e Unistab S69® (0,5g). Somente suspensão foi utilizada para o tratamento com nanocapsula vazia.

4.1.2 Diluição do Ácido Lipóico em Hidróxido de Sódio

As concentrações fixadas de α-ácido lipóico sintético (≥ 99% pureza, Sigma-Aldrich), foram dissolvidas individualmente para cada tanque, em solução de hidróxido de sódio e água destilada para garantir a diluição total do ácido lipóico seguindo a metodologia de Amado et al. (2011). Após a diluição o pH foi ajustado para 7,90. As concentrações utilizadas foram adicionadas a cada 24 horas (Tabela 2)

Tabela 2: Preparação de solução de ácido lipóico, NaOH (hidróxido de sódio; 2 mM), ddH_2O (água destilada), AL (ácido lipóico). A partir do cálculo geral de 300 mL de NaOH para 1g de AL e 1000 mL de H_2Odd para 80 g de NaOH.

Tratamento	**NaOH (g)**	**ddH_2O (mL)**	**AL (g)**
Controle	0	0	0
1,25 µM	0,62	7,8	0,26
2,5 µM	1,22	15,3	0,51
5 µM	2,47	30,9	1,03
10 µM	4,94	61,8	2,06

4.2 ANÁLISE DA ÁGUA

Diariamente os parâmetros físicos e químicos da água foram monitorados, incluindo oxigênio dissolvido (mg/L) e temperatura da água (°C) com multiparâmetro YSI, salinidade com refratômetro ótico (Atago 103, ±1 ppt), pH com eléctrodo Mettler Toledo FEP20 – FiveEasy Plus™. As determinações de alcalinidade (mg de $CaCO_3$/L) e nitrato (mg/L) foram feitas pelo método APHA (1985) e as de amônia total (NH_3 + NH_4^+ mg/L; UNESCO 1983) e nitrito (mg/L) de acordo com Benderschneider e Robinson (1952) (Trabalho 1, 2 e 3). Quando a amônia foi superior a 1 mg/L foi adicionado melaço de cana de açúcar como fonte de carbono para ajustar a relação C/N de 20:1, segundo métodos de Ebeling et al. (2006) e Avnimelech (1999), nos quais determinaram que 6 g de carbono é necessário para converter 1 g de nitrogênio amoniacal total em biomassa bacteriana.

Os sólidos sedimentáveis (material particulado orgânico e inorgânico) foram determinados pela sedimentação dos sólidos em cones Imhoff, onde coloca-se 1 L de água e deixa-se repousar por 1 h, momento o qual são lidos os sólidos suspensos em mL/L, caracterizado neste estudo como biomassa do biofloco (Tovar e Erazo 2009) (Trabalho 3).

4.3 COLETA E HOMOGENEIZAÇÃO DAS AMOSTRAS

Para o trabalho 1 e 2, após 30 dias, foi efetuada a biometria final e os camarões foram colocados em água com gelo para serem eutanaziado. Depois foram armazenados em ultrafreezer (-80 ºC) para no dia seguinte ser retirado brânquias, hepatopâncreas e músculo. As amostras foram homogeneizadas (1:5, peso/volume) em solução tampão de crustáceos (pH 7,2), contendo Tris-base (20 mM), EDTA (1 mM), MgCl2 (0,05 mM); DTT (Ditiotreitol – 1 mM); Sacarose (5 mM), KCl (Cloreto de potássio – 1 mM), dissolvidos em água Milli Q. Posteriormente os extratos foram centrifugados a 9000 x g, durante 30 minutos, em temperatura de 4 ºC e retirado o sobrenadante que foi congelado a -80 ºC (Trabalho 1 e 2).

Para o trabalho 3, as amostras foram coletadas após leitura do cone Imhoff, no qual a água foi retirada por sifonamento, e o floco colocados em falcon de 50 mL e armazenado em gelo, para depois serem transferidos para eppendorf de 2 mL e serem centrifugadas a 800 x g, a 4 °C, por 10 minutos e armazenado a -80°C. Para homogeneização, as amostras de biofloco foram pesadas em eppendorff, adicionados metanol (100%) sobre a relação 1000mg/1000µL, homogeneizadas e agitadas durante 3 horas, em seguida centrifugadas por 10 minutos a 10000 rpm e 4°C, então retirado o sobrenadante que foram utilizados para determinar a capacidade antioxidante contra radicais peroxil.

4.4 DETERMINAÇÃO DA ATIVIDADE DA ENZIMA GLUTATIONA S-TRANSFERASE (GST)

O ensaio da atividade da GST seguiu o método de Habig e Jakobi (1981). Neste processo a absorbância é gerada pela conjugação de 1 mM de GSH (glutationa reduzida, Sigma-Aldrich) com 1 mM de CDNB (1-chloro-2,4-dinitrobenzene, Sigma-Aldrich), em absorbância de 340 nm, a 25 °C. Para leitura foi adicionado em microplaca transparente de fundo chato, 15 µL do sobrenadante em 235 µL de meio de reação (tampão fosfato 0,1 M e CNDB 50 mM, pH 7,0), mais 10 µL de GSH 25 mM. Nas amostras de branco foi utilizado 15 µL tampão de homogeneização de crustáceo. As leituras foram em espectrofluorímetro com leitora de placas (Víctor 2, Perkin Elmer). A

atividade específica da GST foi expressa em nanomoles de produto CDNB-GSH por minuto por mg de proteína.

4.5 DETERMINAÇÃO DA ATIVIDADE DA GLUTATIONA REDUZIDA (GSH)

Foi efetuado pelo método de White et al. (2003) que mensura a concentração de GSH pela sua reação com NDA (2,3 naftalenedicarboxialdeido), gerando um complexo fluorescente (GSH-NDA) que detectado a 485 nm de excitação e 530 nm de emissão. Para o ensaio primeiro foi feita uma curva padrão em diferentes diluições de GSH (40, 20, 10, 5 e 2,5 µM). Depois foram adicionados a microplaca transparente de fundo cônico 25 µL de sobrenadante e 25 µL de tampão de homogeneização para o branco, 25 µL de ácido sulfosalicílico (200 mM) e incubado por 20 minutos. Em seguida a placa foi centrifugada a 2500 rpm por 5 minutos. Foram então transferidos para microplaca branca 20 µL de sobrenadante e 180 µL de solução de reação (Tris-base 50 mM, NaOH 500 mM e NDA 10 mM). As leituras foram feitas em espectrofluorímetro com leitora de placas (Víctor 2, Perkin Elmer), em temperatura ambiente. A concentração foi expressa em µomoles de GSH por mg de proteína.

4.6 DETERMINAÇÃO DOS NÍVEIS DE PEROXIDAÇÃO LIPÍDICA

A determinação de dano oxidativo segui o protocolo descrito por Oakes & Van der Kraak (2003), este método envolve a reação do malondialdeído (MDA), um subproduto da peroxidação lipídica (Hermes-Lima, 2004), com o ácido tiobarbitúrico (TBA) sob condições de alta temperatura e acidez, gerando um cromógeno que é quantificado por fluorometria. Para o ensaio foi feito a curva padrão com 6,25; 3,125; 1,5625; 0,78; 0,39; 0,195; 0,0975; 0,04887; 0,0243 e 0,0121 nmol de TMP (1,1,3,3-tetramethoxypropano). Em tubos de vidro (em duplicata) foi colocado 10 µL de amostra e 41,2 µL de tampão de homogeneização de crustáceo nos brancos. Depois 20 µL de solução estoque de BHT (hidroxitoluenobutilado, 1,407 mM), apenas nos tudos das amostras. Em seguida 150 µL de solução de ácido acético 20%, 150 µL da solução de TBA 0,8%; 50 µL de água MilliQ, 20 µL de SDS 8,1%. A mistura foi vortexada e colocada em banho-maria a 95 ºC por 30 minutos. Logo após os tubos foram esfriados

por 10 minutos a temperatura ambiente, para então adicionar 100 µL de água MilliQ. O contudo dos tubos foi transferido para eppendors de 1,5 mL e adicionou-se 500 µL de n-butanol, sendo logo vortexado e centrifugado a 3.000 x g por 10 minutos a 15 ºC. Finalmente foi removida 150 µL da fase orgânica (sobrenadante) e transferida a microplacas brancas. As leituras são feitas em fluorímetro, com comprimento de excitação de 520 nm e emissão de 580 nm (Víctor 2, Perkin Elmer). Os resultados foram expressos em nmol de TMP (Acros Organics) por mg de tecido fresco.

4.7 DETERMINAÇÃO DA CAPACIDADE ANTIOXIDANTE TOTAL CONTRA RADICAIS PEROXIL (ACAP)

Foi efetuada de acordo com o protocolo de Amado et al. (2009), a dosagem tem início com fixação a concentração de proteína em 2 mg/ml das amostras. Para o trabalho 3, não houve a fixação da concentração de proteína. Depois é adicionado a microplaca branca 127,5 µL de tampão de reação, composto por 0,3575 g de ácido etanosulfônico 4.2-hidroxietil piperazina-1 (HEPES), 0,7455 g de cloreto de potássio (KCl), 0,0102 g de cloreto de magnésio ($MgCl_2$) dissolvidos em 50 ml de água Milli Q com pH ajustado em 7,2; 10 µL de extrato de tecido; 7,5 µL de água MilliQ para as amostras sem ABAP (2,2- azobis 2metilpropianoamidina dihidrocloreto) ou 7,5 µL de solução de ABAP para amostras com ABAP (gerador de radicais peroxil) e 10 µL de solução de H_2DCF-DA (diacetato de ´2,7 diclorofluresceína). A leitura é realizada no tempo zero e depois a cada 5 minutos até completar 30 minutos em fluorímetro de placas (Víctor 2, Perkin Elmer) utilizando comprimento de onda de 530 nm de emissão e 485 nm de excitação, a 37 °C, temperatura que favorece a termólise do ABAP. Este método quantifica a capacidade que o tecido possui em neutralizar as ERO geradas pela decomposição do ABAP, incluindo as defesas antioxidantes enzimáticas e/ou não enzimáticas, através do cálculo da área relativa. A área relativa apresenta uma relação inversa com a capacidade antioxidante, onde menores valores da área relativa indicam uma maior capacidade antioxidante e vice-versa. Para o trabalho 3, as leituras foram feitas em fluorímetro de placas (FILTERMAX F5, Multi-mode microplate reader) utilizando comprimento de onda de 530 nm de emissão e 485 nm de excitação, a 37 °C e o cálculo da área relativa foi realizado segundo Monserrat et al. (2014).

4.8 CONTAGEM DIFERENCIAL DE HEMÓCITOS

A hemolinfa foi recolhido por punção cardíaca utilizando uma seringa de 3 mL contendo solução anticoagulante de crustáceo, contendo NaCl (450 mM), glucose (100 mM), citrato de sódio (30 mM), ácido cítrico (23 mM), EDTA (20 mM) diluídos em água MilliQ, com pH fixado em 7,4. Depois, um esfregaço de hemolinfa foi feita em lâminas de vidro que foram imersas em metanol durante 5 minutos e coradas com May-Grunwald-Giemsa. Em seguida, hemócitos foram quantificados por microscópio de lente ocular Integrando Disc 1, 25 pontos-G49 (Carl Zeiss), seguindo a metodologia de Weibel (1980).

4.9 ANÁLISE ESTATÍSTICA DOS RESULTADOS

No primeiro trabalho os dados foram expressos em média ± desvio padrão. Cada variável (atividade GST, capacidade antioxidante total, concentração de GSH, peroxidação lipídica) foi analisada através de bi-fatorial ANOVA, sendo os fatores o tratamento (água clara e biofloco) e órgãos (brânquias, hepatopâncreas e músculos). Anteriormente, os pressupostos de normalidade e homogeneidade de variância foram analisados e transformações matemáticas aplicada se a menos uma suposição foi violado. Comparações de médias foram feitas usando a teste Newman-Keuls ou contrastes ortogonais. Foram analisados os parâmetros físico e químicos da água por meio do teste t de Student para variâncias desiguais (Zar, 1984). Em todos os casos foi utilizado um nível de significância de 5%.

Para o segundo trabalho, os dados foram expressos em média ± desvio padrão. Cada variável de cada órgão, incluindo a hemolinfa (atividade GST, peroxidação lipídica, capacidade antioxidante total e contagem total de hemócitos) foi analisada através de bi-fatorial ANOVA, sendo os fatores os tratamentos (NC ou NCLA) e condição de criação (SW ou BFT). Anteriormente foram analisados os pressupostos de normalidade e homogeneidade de variância. Comparações de médias foram feitas usando o teste de Newman Keuls. Parâmetros físico e químicos da água foram analisados pelo teste t de Student para variâncias desiguais (Zar 1984). Em todos os casos foi utilizado um nível de significância de 5%.

Para o trabalho 3, os dados foram expressos em média ± desvio padrão. Com análise feita por meio de bi-fatorial ANOVA (diferentes concentrações de ácido lipóico e tempo). Anteriormente, os pressupostos de normalidade e homogeneidade de variância foram analisados. Comparações de médias foram feitas usando a teste Newman-Keuls. Em todos os casos foi utilizado um nível de significância de 5%.

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CAPÍTULO I

Antioxidant and oxidative damage responses in different organs of Pacific white shrimp *Litopenaeus vannamei* (Boone, 1931) reared in a biofloc technology system

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ABSTRACT

Shrimp (*Litopenaeus vannamei*) reared in a conventional seawater (SW) aquarium system SW were compared with those raised in a biofloc technology (BFT) system. After 30 days, the *L. vannamei* shrimp were euthanized and samples of gills, hepatopancreas and muscle were dissected. Statistical analysis was performed using bi-factorial ANOVA, with the factors of the treatment (shrimp reared in SW or in a BFT system) and organs (gills, hepatopancreas and muscles). No differences ($p>0.05$) in glutathione-S-transferase activity were observed between shrimp reared in SW and shrimp reared in BFT ($p< 0.05$). Glutathione levels were lower ($p<0.05$) in the gills and hepatopancreas of shrimp reared in the BFT system, suggesting changes in the antioxidant composition of these organs. Lipid peroxidation levels were higher in the hepatopancreas than in muscle in shrimp reared in SW ($p<0.05$) and this difference was not observed in organisms reared in the BFT system ($p>0.05$). Overall, the results showed that rearing shrimp in the BFT system altered both their antioxidant and oxidative damage responses. This indicates that some microbial communities in BFT systems can influence the redox state of *L. vannamei*.

Keywords: *Litopenaeus vannamei*; BFT; bioflocs; antioxidant responses; lipid peroxidation; total antioxidant capacity.

1 INTRODUCTION

Among many aquatic species that are cultured in the world, the white shrimp *Litopenaeus vannamei* has a number of advantages. For example, the species has a high tolerance to variations in temperature, salinity, pH, and dissolved oxygen, possesses in efficient feed conversion ratio, is resistant to several types of disease and is tolerant to crowding (ABCC 2010).

Aquacultural activity can have some negative environmental outcomes such as the release of toxic nitrogen compounds like ammonia and nitrite (Xu et al. 2013). The use of bioflocs technology (BFT) systems has provided a useful alternative in which the presence of aerobic and heterotrophic organisms together with vigorous aeration allows for the rearing of aquatic organisms at a high density without the need for constant water exchange (Furtado et al. 2011; Kim et al. 2014). Recent studies have shown that the benefits of BFT systems are not restricted to the reduction of the need for water exchanges. Additionally, the microorganism communities are beneficial for the organisms being cultured. Xu et al. (2013) showed that BFT improved activity in digestive enzymes such as proteases, amylase and lipase in *L. vannamei*. These authors proposed that the high digestive enzyme activity could be related to exogenous enzymes released from microorganisms lysed in the bioflocs. Improvement in weight gain, specific growth rate and the feed conversion rate of *L. vannamei* reared in a BFT system were reported by Xu and Pan (2012). These results led the authors to postulate that bioflocs are a food supplement that improves digestion and growth. Kim et al. (2014) observed augmented gene expression in the prophenol oxidase system in white shrimp reared in BFT, which reinforces the idea that several biological benefits are linked by this rearing strategy.

When considering bioflocs as an additional food source (Xu et al. 2013), it is important to note that they contain several biomolecules including carotenoids, amino sugars and phytosterols, among others (Ju et al. 2008). Molecules such as carotenoids are known to possess antioxidant properties (Hermes-Lima 2004; Halliwell & Gutteridge 2007). It is thus possible that BFT may improve not only digestive, imune and growth parameters, but also antioxidant responses. Several conditions can generate oxidative stress in aquatic organisms, and some of these conditions are important for

aquaculture practices. Crustaceans exhibit daily variations in their enzymatic antioxidant system (Fanjul-Moles et al. 2003; Maciel et al. 2004). This means that they may be more susceptible to oxidative stress during certain periods. Infection by the white spot virus was also shown to reduce the antioxidant competence of *Fenneropenaeus indicus* (Mohankumar & Ramasamy 2006) and the crustacean immune response itself promotes a pro-oxidant condition (Cornet et al. 2007).

Taking into account the properties of BFT systems that led Xu et al. (2003) to consider providing a natural food supplement to organisms such as *L. vannamei*, this study aimed to evaluate the effect of a BFT system on the antioxidant and oxidative damage responses of this species and to record other beneficial changes elicited by the use of this technology. To address this aim we compared the antioxidant response of the shrimp *L. vannamei* reared in a SW system (without biofloc) and in a BFT system.

2 MATERIALS AND METHODS

This study employed juvenile shrimp (*L. vannamei*) from the nursery sector of the Aquaculture Marine Station at the Federal University of Rio Grande – FURG (Brazil). During acclimation period (seven days) shrimp were dispersed in six plastic tanks with 50 L of sea water (SW) each. Measured physico–chemical parameters of SW were: 7.79 ± 0.15 (pH), 6.5 ± 0.12 mg/L (dissolved oxygen), 26.26 ± 0.38 °C (temperature), 31.00 ± 0.01 (salinity), 0.78 ± 0.47 mg/L (ammonia), 0.07 ± 0.04 mg/L (nitrite) and 0.39 ± 0.53 mg/L (nitrite). Organisms were stocked at a density of 15 shrimp per tank (approximately 210 shrimp per m^3). The diet consisted of commercial food with 35% crude protein supplied three times daily (08:00, 15:00 and 22:00 h) at a feeding rate of 3% of shrimp biomass. When the concentration of total ammonia exceeded 1 mg/L, molasses was added to the bioflocs (Avnimelech 1999). The experiment lasted for 30 days and no water exchanges were performed in the BFT system treatment. In SW, 80–90% of the water was replaced every 48 h.

During the experiment, the physical and chemical parameters of water, including dissolved oxygen (mg/L), water temperature (°C), salinity and pH, were measured daily using an YSI multiparameter apparatus (Yellow Springs Instruments, model 55). Shrimp

from both treatments were weighed at the end of the experiment, frozen, and the gills, hepatopancreas and muscle from the second abdominal somite were dissected.

Organ and tissue samples were homogenized (1:5, w/v) in a buffer, previously used for crustacean species (Pinho et al. 2005; de Souza et al. 2014). The composition was Tris-Base (20 mM), EDTA (1 mM), dithiothreitol (1 mM; Sigma), sucrose (500 mM) and KCl (150 mM) dissolved in Milli Q water with the pH adjusted to 7.2. The homogenates were centrifuged (9000 × g, 30 min at 4 °C), and the supernatants were kept and divided into four aliquots for subsequent analysis of glutathione-S-transferase (GST) activity, levels of lipid peroxidation (TBARS assay), levels of reduced glutathione (GSH) and total antioxidant capacity against peroxyl radicals.

2.1 DETERMINATION OF GST ACTIVITY

GST was measured according to the protocol described by Habig et al. (1974). Briefly, in the assay the conjugation of 1 mM 1-chloro-2,4-dinitrobenzene (CDNB, Sigma) with 1 mM reduced glutathione (GSH, Sigma) in the presence of 10 µl of sample homogenates (source of GST enzyme) generated a CDNB-GSH complex. The absorbance of this complex was measured at 340 nm using a spectrofluorometer with a microplate reader (Victor 2, Perkin Elmer).

2.2 DETERMINATION OF TOTAL ANTIOXIDANT CAPACITY

Total antioxidant capacity against peroxyl radicals was measured by determining the reactive oxygen species in aliquot homogenates according to the protocol of Amado et al. (2009). Peroxyl radicals were generated through thermal decomposition, at 37 °C of 2,2′-azobis 2 methylpropionamidine dihydrochloride (ABAP; Aldrich). The probe dichlorofluorescein diacetate (H_2DCF-DA, Invitrogen; 40 µM) was added to a white ELISA 96-well microplate, which was immediately placed in a fluorometer with a microplate reader (Victor 2, Perkin Elmer) heated at 37 °C. The fluorescence was read (excitation: 485 nm; emission: 520 nm) every 5 min over a 30-min period, and the relative area was calculated according to Amado et al. (2009). According to these authors, a high relative area means a low antioxidant capacity indicating a low capacity

of antioxidants present in the biological sample to degrade or intercept the peroxyl radicals.

2.3 DETERMINATION OF CONCENTRATION OF REDUCED GLUTATHIONE (GSH)

The procedure was based on that described by White et al. (2003) with modifications proposed by da Rocha et al. (2009). The substrate 2,3 naphthalene dicarboxialdehyde (NDA, Invitrogen) reacts with GSH to form a fluorogenic compound. Aliquots of organ homogenates were transferred to white ELISA 96-well microplate. The NDA solution was subsequently added to all wells and, after 30 min of incubation, the fluorescence intensity of the NDA–GSH complex was read at excitation and emission wavelengths of 485 and 530 nm, respectively, using a plate reader fluorometer (Victor 2, Perkin Elmer). A standard curve of GSH was prepared to express the data in terms of μM equivalents of GSH per mg of total proteins.

2.4 DETERMINATION OF LIPID PEROXIDATION

Lipid peroxidation was determined by a TBARS (thiobarbituric acid reactive substances) assay following Oakes and Van der Kraak (2003), adapted to microplate by Da Rocha et al. (2009). Aliquots (10 μl) of sample homogenates were added to glass tubes, together with thiobarbituric acid (0.8%, Sigma), acetic acid (20%), Milli Q water and sodium dodecyl sulfate (8.1%). The mixture was incubated at 95 °C over 30 min and, after cooling, n-butanol was added following a centrifugation step (3000 × g for 10 min at 15 °C). The n-butanol phase was pipetted to a white ELISA 96-well microplate and the fluorescence read (excitation: 520 nm; emission: 580 nm) in a fluorometer with a microplate reader (Victor 2, Perkin Elmer). The standard employed was 1,1,3,3-tetramethoxypropane (TMP, Across Organics). The results were expressed as nmol equivalents of TMP per mg of fresh tissue.

2.5 DETERMINATION OF TOTAL AMMONIA

Total ammonia determination (NH_3+ NH_4^+) follows the UNESCO (1983) method in which ammonia in basic pH (between 10.8 and 11.5) reacts with bleaching solution, forming monochloramine. In the presence of phenol and an excess of hypochlorite and the addition of sodium nitroprusside as a catalyst, indophenol blue is formed, a molecule that absorbs at 630 nm.

2.6 STATISTICAL ANALYSIS

Data were expressed as the mean ± standard deviation. Each variable (GST activity, lipid peroxidation, GSH levels and total antioxidant capacity) was analyzed through bifactorial ANOVA, with factors composed of the treatment (shrimp reared in SW or BFT and organs (gills, hepatopancreas and muscles). Prior to application of ANOVA the assumptions of normality and homogeneity of variance were tested and mathematical transformations were applied if at least one assumption was violated. Means comparisons were done using the Newman–Keuls test or orthogonal contrasts. Water physico–chemical parameters were analyzed using Student's t test for unequal variances (Zar 1984). In all cases, a significance level of 5% was used.

3 RESULTS

The average initial weight, final weight and average weight gain were not significantly different ($p>0.05$) for SW and BFT reared shrimp. In both cases, survival was 100% (Table 1). Several differences in physico–chemical parameters between SW and water from BFT system were observed (Table 2). Water from the BFT system showed significantly higher values for pH, salinity and nitrate ($p<0.05$) compared with SW whereas oxygen concentration and nitrite levels were lower ($p<0.05$).

Table 1. Mean concentration (±1 standard error) of morphological and physiological parameters measured in shrimp *L. vannamei* maintained in a SW or in a BFT system.

Parameters	SW	BFT
Initial weight (IW) (g)	5.91 (±0.07)	5.01 (±0.05)
Final weight (FW) (g)	10.03 (±0.18)	9.61 (±0.13)
Weight gain (FW-IW) (g)	4.12 (±0.30)	3.70 (±0.17)
Survival	100%	100%

Note: No significant differences were detected between treatments ($p>0.05$). Number of samples analyzed in each treatment: 45.

No significant differences were observed in GST activity between treatments ($p>0.05$ and Table 3) although GST activity in the gills showed an upward trend in BFT shrimp. When comparing the different organs, the gills showed the highest GST activity, followed by the muscle and the hepatopancreas ($p<0.05$).

The muscles of BFT shrimp showed significantly higher total antioxidant capacity (low relative area) when compared with SW shrimp (high relative area) ($p<0.05$ and Table 4). In SW shrimp the hepatopancreas had the highest antioxidant capacity ($p<0.05$) when compared with the muscle and gills, which presented similar values ($p>0.05$). BFT shrimp showed differences in every organ: highest antioxidant capacity in the hepatopancreas, followed by the muscle with the lowest mean value in the gills ($p<0.05$ and Table 4).

A significant GSH reduction was observed in the gills and hepatopancreas of BFT shrimp ($p<0.05$) indicating that glutathione levels were affected by the rearing environment. In each system, the differences in GSH content among the different organs were the same: muscle showed the highest level, followed by the hepatopancreas and then the gills ($p<0.05$ and Table 5A).

Table 2. Mean concentration (±1 standard error) of the physico-chemical parameters measured in SW or in a BFT system.

Parameter	SW	BFT
pH ($n = 186$)*	7.90 ± 0.001	7.98 ± 0.001
[O_2] (mg/L) ($n = 186$)*	6.44 ± 0.004	5.50 ± 0.007
Temperature (°C) ($n = 186$)	27.09 ± 0.12	27.08 ± 0.12
Salinity ($n = 186$)*	31.01 ± 0.01	33.49 ± 0.03
Ammonia (mg/L) ($n = 66$)	1.74 ± 0.19	1.52 ± 0.28
Nitrite (mg/L) ($n = 66$)*	0.56 ± 0.10	0.26 ± 0.04
Nitrate (mg/L) ($n = 28–30$)*	0.42 ± 0.19	72.38 ± 10.08

Note: Asterisks (*) indicate significant differences ($p<0.05$) between the experimental groups after performing Student's t-test assuming unequal variances. Number of samples analyzed in each treatment: 27–186.

Table 3. Mean activity (±1 standard error) of GST (nmol CDNB-GSH conjugate/min/mg of proteins) in the gills, hepatopancreas and muscle of shrimp *L. vannamei* reared in SW or in a BFT system.

Organ	SW	BFT
Gills	26.35 ± 2.31 a	51.08 ± 7.66 a
Hepatopancreas	1.02 ± 0.56 b	0.40 ± 0.12 b
Muscle	2.15 ± 0.40 c	2.83 ± 0.32 c

Note: Different letters indicate significant differences ($p<0.05$) between experimental groups after performing a Student–Newman–Keuls post hoc test. Number of samples analyzed in each treatment: 15. GSH: reduced glutathione. CDNB: 1-chloro-2,4-dinitrobenzene.

Table 4. Mean values (±1 standard error) of the total antioxidant capacity against peroxyl radicals (expressed in relative area) in the gills, hepatopancreas and muscle of *L. vannamei* reared in SW or in a BFT system.

Organ	SW	BFT
Gills	5.82 ± 0.82 a	7.40 ± 1.18 a
Hepatopancreas	0.25 ± 0.06 b	0.33 ± 0.13 b
Muscle	12.81 ± 4.69 a	2.12 ± 0.49 c

Note: Different letters indicate significant differences ($p<0.05$) between experimental groups after performing a Student–Newman–Keuls post hoc test. Number of samples analyzed in each treatment: 10–15.

Table 5. Mean concentration (±1 standard error) of: (A) reduced glutathione (GSH) (µM of GSH/mg of total proteins) and (B) thiobarbituric reactive substances (nmol of TMP/mg of tissue) in the gills, hepatopancreas and muscle of *L. vannamei* reared in SW or in a BFT system.

Organ	SW	BFT
(A) GSH		
Gills	61.52 ± 8.07 a	33.84 ± 4.56 c
Hepatopancreas	224.52 ± 57.58 d	77.13 ± 21.46 a
Muscle	400.33 ± 36.55 b	457.08 ± 56.30 b
(B) TBARS		
Gills	2.44 ± 0.19 ab	2.37 ± 0.20 ab
Hepatopancreas	3.20 ± 0.40 b	2.51 ± 0.34 ab
Muscle	1.65 ± 0.27 a	1.41 ± 0.20 a

Note: Different letters indicate significant differences ($p<0.05$) between experimental groups after performing a Student–Newman–Keuls post hoc test. Number of samples analyzed in each treatment: 10–15. TMP: 1,1,3,3-tetramethoxypropane, the standard employed in TBARS assay.

Oxidative damage measured in terms of TBARS was similar between the organs of BFT shrimp and SW shrimp ($p>0.05$ and Table 5B). The TBARS concentration in each organ was, however, different depending on the rearing environment. In SW shrimp, the highest TBARS concentration was observed in the hepatopancreas, followed by the gills, with the lowest TBARS concentration observed in the muscle ($p<0.05$ and Table 5B). In BFT shrimp no differences in TBARS levels were detected between organs ($p>0.05$ and Table 5B).

4 DISCUSSION

Nitrate levels can vary from 4.52 to 26 to mg/L during the rearing of commercial shrimp (Muir et al. 1991) and can reach 500 mg/L in recirculation systems (Pierce et al. 1993). In studies with juvenile *Penaeus monodon*, however, the highest safe nitrate level at a salinity of 35 was reported to be 158 mg/L (Tsai & Chen 2002). Lower nitrate levels were observed during the present study in both SW, where 80–90% of water was renewed every 48 h and in BFT, where no water renewal was performed.

Safe nitrite levels were estimated for *L. vannamei* to be 15.2 mg/L (Lin & Chen 2003), a value much higher than those registered in both our treatments. The study of Zhang et al. (2006) into oxygen tolerance determined that in *L. vannamei*, the lethal levels were below 0.5 mg/L of dissolved oxygen. This shows again that the measured O_2 levels in both our treatments were far below a level that could be considered stressful. The lower O_2 concentration observed here with BFT was also reported in a previous study from our group (Emerenciano et al. 2012) and is consistent with the high density of aerobic organisms.

The measured parameters of water quality (temperature, salinity, pH, dissolved oxygen, ammonia, nitrite and nitrates) in a BFT system and in SW in the study of Wasielesky et al. (2006) were close to those registered in the present study. In summary, according to values cited in the literature the water quality parameters in our study were within acceptable limits for the survival and growth of *L. vannamei*.

Different organs of aquatic species differ in antioxidant competence and oxidative damage, as found by Monserrat et al. (2008) in the fish *Corydoras paleatus*, where, for example, GSH levels were higher in the muscle compared with the brain or gills. In *L. vannamei* shrimp, Lobato et al. (2013) found higher TBARS levels in the hepatopancreas compared to the muscle. In the present study, some differences were observed between the gills, hepatopancreas and muscle of *L. vannamei*. For example, higher GST activity was observed in the gills of shrimp reared in both systems. Some GSTs possess peroxidase activity, catalyzing the degradation of organic hydroperoxides (Halliweel & Gutteridge 2007), and this most likely explains why the gills have moderate TBARS levels even when the total antioxidant capacity is lower than that in the other organs. The differences among the organs in terms of lipid peroxidation for

SW reared shrimp were not observed in BFT reared shrimp which suggests that the microbial community of this system could be influencing the redox state of L. *vannamei* resulting in the organs presenting similar levels of oxidative damage expressed in terms of TBARS levels.

Other evidence of the positive influence of BFT on the antioxidant system of *L. vannamei* included the higher total antioxidant competence against peroxyl radicals (lower relative area) in the muscle of shrimp reared in the BFT system compared with SW reared shrimp. The observed reduction in GSH levels in the gills and hepatopancreas of shrimp reared in BFT deserves careful consideration. These results a priori can be interpreted as a pro-oxidant action induced by BFT, lowering the concentration of the antioxidant. However, the total antioxidant capacity in these two organs was similar in shrimp reared in both systems, and the TBARS levels in the gills and hepatopancreas were also similar between shrimp reared in the two systems. It is possible that rearing the shrimp in the BFT system altered their antioxidant composition. According to Regoli and Winston (1999) and Regoli (2000), antioxidants such as GSH, ascorbic acid, uric acid and α-tocopherol accounts for almost 70% of antioxidant capacity against peroxyl radicals. In this sense, a reduction in GSH content can be compensated by other antioxidants, leaving the total antioxidant capacity unchanged.

Several examples exist in nature that show how biological interactions modify the antioxidant defense system. Moraes et al. (2006) showed that the mucus secretion of the polychaete *Laeonereis acuta* is colonized by bacteria communities that confer antioxidant properties. In the present study, the results indicated altered antioxidant responses and ameliorated lipid peroxidation in shrimps maintained in the BFT system. Future studies will need to consider the influence of microbial communities with different species compositions on the antioxidant competence of cultured organisms. It will also be important to learn how a microbial community of a BFT system can vary its antioxidant properties such as by adding exogenous antioxidants to the system.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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CAPÍTULO II

Antioxidant effects of nanoencapsulated lipoic acid in tissues and differential hemocyte count in hemolymph of shrimp Pacific *Litopenaeus vannamei* (Boone, 1931) created in biofloc system

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Submetido a:

Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology

ABSTRACT

One of the limiting factors for the intensification of production in aquaculture is the welfare of reared organisms. In this study, we evaluated antioxidant and oxidative damage responses and immune parameters in shrimp *Litopenaeus vannamei* reared in saline water (SW) and in biofloc system (BFT). Animals were fed with ration supplemented with nanocapsules containing the antioxidant lipoic acid (NCLA) or with empty nanocapsules (NC). Oxidative stress analysis was evaluated through measurement of glutathione S-transferase activity (GST), reduced glutathione (GSH) concentration and lipid peroxidation (TBARS) in gills, hepatopancreas and muscle. Immune parameters were determined through differential hemocyte count in the hemolymph. There was an increase in the average final weight of shrimps treated with NCLA ($p < 0.05$). In gills, increased GST activity ($p < 0.05$) was verified in shrimps reared in SW in both treatments (WNC and NCLA). In the same organ, higher GSH levels were observed in shrimps maintained in BFT ($p < 0.05$), and decreased levels of lipid peroxidation was observed in shrimps supplemented with NCLA ($p < 0.05$). In hepatopancreas, both in SW and BFT, NCLA induced an increase of GST activity and GSH concentration, the lipid peroxidation was increased in BFT and NCLA treatment. The muscle was low levels of GST activity, there was no significant difference between treatments in GSH concentration, and there was an increased lipid peroxidation. There was a decrease in the percentage of hyaline hemocytes and increased granular hemocytes in all treatments. The nanoencapsulated lipoic acid had tissue-specific action, with increased antioxidant activity in gills and hepatopancreas.

Keywords: Biofloc System, Nanotechnology, Shrimp, Antioxidants supplements, Antioxidant defenses, Oxidative damage.

1. INTRODUCTION

Intensification of production in carciniculture demand priority attention in welfare of reared organisms, as well as an integrated water management to avoid or minimize pollution of water bodies (i.e.: release of nitrogenous compounds) and a greater control over infection of pathogens (Xie and Yu, 2007; Moss et al., 2012; Xu and Pan, 2013) These actions are particularly important for the rearing of the white shrimp *Litopenaeus vannamei,* one of the most marketed aquatic organisms worldwide (Xu and Pan, 2013).

Biofloc technology systems (BFT) appears as a sustainable solution that enables the improvement of water quality with the removal of toxic nitrogen (ammonia, nitrite), highest growth performance through food supplement and digestive stimulant by biofloc organisms (Xu and Pan, 2013). BFT are rich in natural microorganisms and bioactive compounds, forming a microbial culture through heterotrophic aggregates of organic particulate in suspended matter (Ray et al., 2010; Xu and Pan, 2013).

Animal welfare is compromised by several factors, including reactive oxygen species (ROS) that are generated as oxygen metabolism products in aerobic organisms (Vergely et al., 2003; Rochette et al., 2013). However, conditions of oxidative stress are generated when exist a redox imbalance between pro-oxidants and antioxidants causing damage to macromolecules. Several conditions can lead to a scenario of oxidative stress, including factors that simultaneously increase ROS generation and reduce antioxidant activity (Jones, 2006; Oudot et al., 2006; Rochette et al., 2013). To prevent

or ameliorate this imbalance, organisms have developed defense mechanisms like antioxidants.

An antioxidant (enzymatic and non-enzymatic) is any substance that, when present in low concentrations compared to an oxidable substrate, significantly delays or inhibits oxidation of this substrate (Halliwell and Gutteridge, 1995; Rochette et al., 2013). Lipoic acid (LA), also known as thioctic acid, is a naturally compound synthesized in small amounts by plants and animals. LA makes covalent bonds with specific proteins, acting as co-factor for mitochondrial enzyme complexes (Carreau, 1979; Reed, 2001). In addition, LA neutralize ROS, chelates metals and regenerates antioxidants (Rochette et al., 2013; Monserrat et al., 2014).

LA is a hydro and liposoluble antioxidant molecule, extensively distributed in cell membranes, cytosol, and extracellular spaces. When supplemented in the diet may accumulate in several tissues and a fraction is reduced to DHLA (dihydrolipoic acid), where the amount of this reduction is tissue-specific (Rochette et al., 2013; Monserrat et al., 2014). LA administered in the diet can reduce oxidative stress and restore levels of other antioxidants that may be at low levels (Monserrat et al., 2008; Martins et al., 2014).

However LA is an unstable compound, and being subject to degradation by thermal and photochemical degradation, acidic oxidation and thus the use of polymeric nanocapsules (lipid) for the protection and slow release of the antioxidant has been considered and applied in aquatic organisms as carp *Cyprinus carpio* (Longaray-Garcia et al., 2013). Nanocapsules are nanostructured vesicular systems carriers of with diameters between 200 and 300 nm and low polydispersity, which stand out due to their capabilities in

controlling the release of substances and the ability to increase the stability of the compound, both in storage and in the biological fluid (Külkamp-Guerreiro et al., 2009).

The cellular stress leads to the activation of defense mechanisms resulting in increase in the detoxification capacity (Kültz, 2005; Roncalli et al., 2015). One of the detoxification enzymes belongs to the glutathione S-transferase (GST) family, proteins of 200-250 amino acids that are activated in response to oxidative damage and/or exposure to a variety of xenobiotics (Frova, 2006; Sheehan et al., 2001; Roncalli et al., 2015). GST enzymes catalyze the conjugation of reduced glutathione (GSH) with hydrophobic molecules, enhancing their solubility and further excretion (Ranson and Hemingway, 2005; Roncalli et al., 2015).

Reduced glutathione (GSH) is an intracellular antioxidant, which assists in detoxification and elimination of ROS and maintains the cellular redox equilibrium (Rochette et al., 2013). Studies in rats suggest that LA can increase GSH synthesis in aged rats via increased expression of γ- glutamate cysteine ligase (GCL), the limiting enzyme for GSH generation (Suh et al., 2004a).

According to Amado et al. (2009), it is also necessary to evaluate oxidative damage and the ability of each tissue in neutralizing reactive oxygen species. Therefore, non-specific responses as thiobarbituric acid reactive substances (TBARS) have been measured in this work. Measurement of lipid peroxidation through TBARS assay offers information about phospholipid peroxidation of cell membranes (Gorbi et al., 2008; Diaz-Jaramillo et al., 2013).

The shrimp immune defense is performed by hemocytes present in the hemolymph and the measurement of number and kind of hemocytes may indicate the shrimp welfare

(Perazzolo et al., 2002). Hyaline cells have the immune function perform phagocytosis, the semigranular cells perform encapsulation, phagocytosis (limited), storage and release of prophenoloxidase system and cytotoxic activity, and granule cells has the function of storing and releasing the prophenoloxidase system and cytotoxic activity (Johansson et al., 2000). In this study was the analysis of the immune response of the shrimp *L. vannmaei* by differential hemocyte count in the hemolymph, by hyaline and granular hemocyte percentage and contribute to the wellness paranorama in front of the use of nanoencapsulated lipoic acid (NCLA) or nanocapsule without lipoic acid (empty nanocapsule – NC) in shrimp.

The objective of the study was therefore to determine the animal welfare conditions using NCLA in clean water and in a biofloc system, through biochemical analysis of antioxidant and oxidative damage parameters in gills, hepatopancreas and muscle, plus immune responses determined by differential hemocyte count in the hemolymph of Pacific white shrimp *L. vannmaei*.

2. MATERIAL AND METHODS

It was used juvenile shrimp (*L. vannamei*) reared at the Marine Aquaculture Station (EMA) of the Federal University of Rio Grande – FURG (Brazil). The shrimps were stored at a density of 15 shrimp per tank (about 210 shrimp per m^3), arranged in 12 plastic tanks with 50 L (useful volume) each. The shrimps were submitted to four different treatments: (i) shrimps maintained in saline water and fed with ration supplemented with nanocapsules without lipoic acid (SW-NC), (ii) shrimps mantained in saline water and fed with ration supplemented with nanoencapsulated lipoic acid

(SW-NCLA), (iii) shrimps maintained in water with bioflocs and fed with ration supplemented with nanocapsules without lipoic acid (BFT-NC, (iv) shrimps reared in water with bioflocs and fed with ration supplemented with nanoencapsulated lipoic acid (BFT-NCLA). In the two paragraphs below are described the methodologies used to prepare the nanocapsules suspensions and how they were included in the ration.

Nanocapsules containing lipoic acid were prepared according to Longaray-Garcia et al. (2013). Briefly, lipoic acid (70 mg/ml) was dissolved in the organic phase composed of triglycerides of caprylic and capric acid, sorbitan monostearate, poly (ε-caprolactone), acetone and butylated hydroxytoluene. The organic phase was disposed over an aqueous phase in the dark that contained polysorbate 80, diazolidinilureia and Milli-Q water and stirred for 10 min. The formulation was evaporated at 35 °C until a final volume of 10 ml, obtaining nanocapsules, which included lipoic acid (NCLA). Nanocapsules containing all the components cited above except the inclusion of lipoic acid were also prepared and represented the nanocapsules control (NC) (Külkamp-Guerreiro et al., 2009). The mean diameter and polydispersion of suspended nanocapsules were measured through dynamic light scattering employing the equipment Zetasizer Nano series Nano-Zs (Malvern Instruments). Samples were diluted (1:500 v/v) in Milli-Q water, and the size distribution by intensity was analyzed in triplicate. Determination of lipoic acid in diet was performed through liquid chromatography with mass spectrometric detection, following the procedures described in Martins et al. (2014).

Commercial diet SUPRA® with 35% crude protein was grinded and mixed with dispersions of NC or NCLA. The homogeneous mass was passed through a 5 mL syringe, and the obtained mass filaments were placed in oven at 50 °C, for 24 h before obtaining the pellets. The diet was provided three times a day (8:00, 15:00 and 22:00 h)

at a feeding rate of 3% of shrimp biomass. The concentration of LA present in the ration was estimated in 68 ± 8.6 mg LA/Kg.

Physical and chemical parameters of water were measured daily, including dissolved oxygen (mg/L), water temperature (°C), salinity and pH. Ammonia and nitrite was measured at intervals of one day and nitrate once a week. When the total ammonia concentration exceeded 1 mg/L, molasses were added to BFT water (Avnimelech, 1999). The experiment lasted 30 days, where there was no water changes in BFT treatment. In the treatment of saline water, 80-90% of water was renewed every 48 h. Parameters of water quality presented significant differences ($p < 0.05$) for the concentration of dissolved oxygen, being lower at BFT. Nitrite concentration also was lower at BFT, although nitrate levels were augmented in BFT (Table 1).

At the beginning and the end of the experiment, shrimps from the four treatment were weighed. At the end of the experiment, the hemolymph was collected from shrimp heart using a 3 mL syringe (see details in section 2.6). After, shrimps were frozen and in the next day the tissues gills, hepatopancreas and muscle from the second abdominal somite were dissected and stored in ultrafreezer at -80 ºC.

Organ samples were homogenized (1:5, w/v) in a buffer previously employed for crustacean species (Pinho et al., 2005; Sousa et al., 2014) composed of Tris-Base (20 mM), EDTA (1 mM), dithiothreitol (1 mM; Sigma), sucrose (500 mM), KCl (150 mM) and dissolved in Milli-Q water, with pH adjusted to 7.2. Homogenates were centrifuged (9,000 x g, 30 min at 4°C) and the supernatants were kept and divided into three aliquots for later analysis of glutathione-S-transferase (GST) activity, levels of lipid peroxidation (TBARS assay) and levels of reduced glutathione (GSH).

2.1. DETERMINATION OF GLUTATHIONE S-TRANSFERASE ACTIVITY (GST)

Glutathione-S-transferases was measured according to the protocol of Habig et al. (1974). The conjugation of 1 mM of 1-chloro-2,4-dinitrobenzene (CDNB, Sigma) with 1 mM of reduced glutathione (GSH, Sigma) in presence of 20 µL of sample homogenates (source of GST enzyme) generated a CDNB-GSH complex. The absorbance of this complex was measured at 340 nm in a spectrofluorometer with a microplate reader (Victor 2, Perkin Elmer).

2.2. DETERMINATION OF CONCENTRATION OF REDUCED GLUTATHIONE (GSH)

The procedure was based on White et al. (2003). The substrate 2,3 naphthalene dicarboxialdehyde (NDA, Invitrogen) reacts with GSH to form a fluorogenic compound. Aliquots from each homogenates were transferred to white ELISA 96-well microplates. After, it was added to all wells the NDA solution, and after 30 min of incubation, the fluorescence intensity of the NDA-GSH complex was read at excitation and emission wavelength of 485 and 530 nm, respectively, using a plate reader fluorometer (Victor 2, Perkin Elmer). A standard curve of GSH was prepared to express the data in terms of µM of GSH/mg of total proteins.

2.4. DETERMINATION OF LIPID PEROXIDATION

Lipid peroxidation was determined by TBARS (thiobarbituric acid reactive substances) assay following Oakes and Van der Kraak (2003). Aliquots (20 µl) of sample homogenates were added to glass tubes, allowing the reaction with thiobarbituric acid

(0.8%, Sigma), 20% acetic acid, Milli Q water and 8.1 % of sodium dodecyl sulfate. The mixture was incubated at 95 ºC during 30 min and after cooling it was added n-buthanol. Following a centrifugation step (3,000 x g during 10 min at 15 ºC), the n-buthanol phase was pippeted to a white ELISA 96-well microplate and the fluorescence read (excitation: 520 nm; emission: 580 nm) in a fluorometer with a microplate reader (Victor 2, Perkin Elmer). As standard it was employed 1,1,3,3-tetramethoxypropane (TMP, Across Organics). The results were expressed as nmol equivalents of TMP per mg of fresh tissue.

2.5. DETERMINATION OF TOTAL AMMONIA

Total ammonia determination (NH_3 + NH_4^+) follows the UNESCO (1983) method in which ammonia in basic pH (between 10.8 and 11.5) reacts with a bleaching solution, forming monochloramine. In the presence of phenol and an excess of hypochlorite, addition of sodium nitroprusside as a catalyst generates indophenol blue, a molecule that absorbs at 630 nm.

2.6. DIFFERENTIAL HEMOCYTE COUNT (DHC)

The hemolymph was collected by heart puncture using 3 mL syringe containing anticoagulant crustacean solution, containing NaCl (450 mM), glucose (100 mM), sodium citrate (30 mM), citric acid (23 mM), EDTA (20 mM) diluted in MilliQ water, with pH fixed at 7.4. After, a smear of hemolymph was made on glass slides that were fixed in metanol for 5 minutes a stained with May-Grunwald-Giemsa. Then, hemocytes were quantified by microscope eyepiece lens Integrating Disc 1, 25 points-G49 (Carl Zeiss), following methodology of Weibel (1980).

2.7. STATISTICAL ANALYSIS

Data were expressed as mean ± standard deviation. Each variable from each organ, including hemolymph (GST activity, lipid peroxidation, total antioxidant capacity and total hemocyte count) was analyzed through bi-factorial ANOVA, being the factors the treatments (NC or NCLA) and rearing condition (SW or BFT). Previously the assumptions of normality and homogeneity of variance were analyzed. Means comparisons were done using the Newman-Keuls test. Water physic-chemical parameters were analyzed using Student t test for unequal variances (Zar 1984). In all cases it was used a significance level of 5%.

3. RESULTS

The initial weight was not significantly different between treatments ($p > 0.05$), but after 30 days of experiment, shrimps that were supplemented with NCLA showed higher weight, both in SW and BFT, although the maximum increase was observed in BFT-NCLA treatment. In every case survival was of 100% (Table 2).

The GST activity was higher in gills of shrimp of SW-NC in SW and lower in BFT-NC when compared with the other two treatments ($p < 0.05$) (Figure 1a). In hepatopancreas the highest GST activity was observed in BFT-NCLA and the minimum in the treatment SW-NC ($p < 0.05$). In muscle, there was no significant difference in GST activity between treatments ($p > 0.05$) (Figure 1a).

In gills, the peak of GSH concentration was observed in treatment BFT-NCLA, being different from BFT-NC treatment ($p < 0.05$), whereas no differences were observed in SW (Figure 1b). In hepatopancreas, treatment with NCLA induced higher GSH levels

(p< 0.05) both in SW and in BFT. However, the BFT-NCLA showed the highest GSH level (Figure 1b). Muscle was the organ that presented highest GSH levels, although no influence of treatments was verified (p> 0.05) (Figure 1b).

In gills, shrimps from SW-NC presented the highest TBARS levels, an effect that was lowered in SW-NCLA treatment (p< 0.05). Shrimps maintained in BFT presented the lowest TBARS levels, both for NC and NCLA (p< 0.05; Figure. 1c). In hepatopancreas a significant (p< 0.05) peak of TBARS was registered in BFT-NCLA, being different from the rest of the treatments (Figure 1c). In muscle, the treatment SW-NC showed the highest TBARS levels, being different from the other treatments (p< 0.05) (Figure 1c).

Hemocytes counts were different in the four treatments. The percentage of hyaline hemocytes was highest in SW-NC treatment, and lowest in BFT-NCLA. Values for SW-NCLA and BFT-NC remained intermediate between those registered in SW-NC and BFT-NCLA (p< 0.05; Figure 2). An opposite response was observed for percentage of granular hemocytes, being highest for BFT-NCLA and lowest for SW-NC treatment, where SW-NCLA and BFT-NC responses remained intermediate (p< 0.05; Figure 2).

4. DISCUSSION

Water quality parameters were in accordance with proposed parameters for the *Litopenaeus vannamei* species proposed by Samocha et al. (2010) and Ray et al. (2014). These parameters were also in according with previous experiment Martins et al. (2015) with saline water and biofloc, except that salinity in such biofloc experiment was 33.

Ammonia and nitrite concentrations remained below of the levels considered toxic for this species (Lin and Chen, 2001; Lin and Chen, 2003). The low concentration of nitrite

and high concentration of nitrate in BFT indicate heterotrophic bacteria activity in the conversion of nitrogen products (Ebeling et al., 2006). There was an increase in the average final weight of the treatments with LA, indicating that this zootechnical parameter is improved with supplementation of LA for *Litopenaueus vannamei* shrimp in clean water and biofloc, as previously reported by Martins et al. (2014) for the same species reared in saline water when supplemented with lipoic acid at doses higher than 190 mg LA/kg of ration.

Shrimps as any other aerobic organism, possess an integrated and coordinated enzymatic and non-enzymatic antioxidant systems that maintain the redox equilibrium of the organism (Ren et al., 2014). Under this context, it is expected that lipoic acid supplementation should aid in the establishment of the redox system and thus for the organism welfare.

Previous studies have showed several beneficial effects of lipoic acid in aquatic organisms (for a review: Kütter et al., 2014), although the responses are strongly organ-dependent (Monserrat et al., 2008; Kütter et al., 2013). Some of the positive effects include: high GST activity, low concentration in thiobarbituric reactive substances in different organs, higher levels of GSH and of the limiting enzyme in their synthesis (glutamate cysteine ligase) (Monserrat et al., 2008; Martins et al., 2014; Monserrat et al., 2014; Enamorado et al., 2015). The lower GST activity observed in SW-NCLA when compared with SW-NC fits to the previous observation of Monserrat et al. (2014), where LA also reduced GST activity in gills of *Jenynsia multidentata.* These authors postulated that high levels of the antioxidant in these organs should favor a reductive redox state that should inhibit the up-regulation of genes related to antioxidant and detoxification processes. This idea is also supported by the results of Kütter et al.

(2013), where in liver of pompano fish *Trachinotus marginatus*, high LA doses (40 and 60 mg LA/kg of body weight) lowered GST activity and at a lower one (20 mg LA/kg of body weight) it was registered a significant increase of enzyme activity.

By the other side, the hepatopancreas presented high GST activity in shrimps reared in SW and BF and submitted to NCLA, a result similar to that found by Kütter et al. (2013) with low doses of free LA. A previous study of Lobato et al. (2013) also showed a similar result in the same species, *L. vannamei*, even when the free LA dose employed by those authors was higher than in present study. The comparison of GST results in gills and hepatopancreas suggest that the biodistribution and entry of LA in the different organs should determine strong or moderate reductive redox state and this should influence GST activity. The tripeptide-reduced glutathione (GSH) is used in conjugation reactions catalyzed by GST and also acts as a first barrier against reactive oxygen species (ROS). In this way, increased levels of this antioxidant in hepatopancreas of shrimps from experimental groups SW-NCLA and BFT-NCLA indicate the antioxidant responses triggered by LA. Up-regulation of genes that code for both modulatory and catalytic sub-units of glutamate cysteine ligase (the limiting enzyme for GSH synthesis) by LA has been described in rats (Suh et al., 2004). In gills, the same effect was observed only in shrimps reared in BFT indicating that in this case some components, including antioxidants present in bioflocs as reported by Xu et al. (2013), should aid to obtain a better antioxidant response that in saline water. Also Martins et al. (2015) reported that shrimps reared in BFT presented different antioxidant responses than shrimps maintained in saline water. In the case of muscle, the so much higher GSH levels indicate that the antioxidant system is expressed near its maximum and few

regulations can occur even for the antioxidants present in the bioflocs or by the exogenous LA offered through ration.

In terms of oxidative damage, the antioxidant treatment with LA was effective only in SW, where a reduction of TBARS levels were observed in gills and muscle, as previously reported by Lobato et al. (2013) in muscle of the same species *L. vannamei*. Coincident with this previous study no effect was observed in hepatopancreas. The results in BFT were quite different, since a pro-oxidant condition was verified in hepatopancreas when exposed to NCLA. The antioxidant/pro-oxidant duality of LA has been recognized by several authors like Çakatay et al. (2005), Atukeren et al. (2010), and Valdecantos et al. (2010). The pro-oxidant condition of LA is related to its ability to reduce Fe^{3+} to Fe^{2+}, favoring the generation of hydroxyl radical and thus the triggering of lipid peroxidation (Goralska et al., 2003). The reduced form of lipoic acid, dihydrolipoic acid (DHLA) possess the ability to remove iron from proteins like ferritin, again favoring the Fenton reaction and hydroxyl radical generation (Çakatay et al., 2005). Several enzymes, including glutathione reductase that employs GSH as reductive power, favor the reduction of LA to DHLA (for a review: Kütter et al., 2014). Note that hepatopancreas GSH levels are higher in shrimps from BFT-NCLA (824.75±34.64 µM of GSH mg^{-1} of total proteins) than the values presented by shrimps from SW-NCLA group (526.36 µM of GSH mg^{-1} of total proteins). Thus, a greater reductive power in shrimps from BFT-NCLA group should favor the generation of DHLA and thus promoting iron release from proteins.

On granular hemocytes (GH) are found degradative and coagulation enzymes, antimicrobial peptides, apopitotics factors and prophenoloxidase system, these have higher amount immunoefetoras and immunoregulatory molecules are considered more

immunocompetent (Barracco et al., 2008). Some immune responses are associated with the production of reactive oxygen species (ROS) and are highly microbicides (Bachère et al., 1995; Muñoz et al., 2000; Campa-Cordova et al., 2002). The process begins when stimulation leads to an increase in oxygen consumption, whose reduction, catalyzed by NADPH-oxidase bound to membrane yields superoxide anion (Campa-Cordova et al., 2002). The antioxidant superoxide dismutase (SOD) converts this metabolite microbicide in hydrogen peroxide which passes freely through membranes (Campa-Cordova et al., 2002). The antioxidant catalase (CAT) and glutathione peroxidase (GPx) remove hydrogen peroxide from the cells (Campa-Córdova et al., 2002). A significant quantity passes into the extracellular environment and can cause damage to cells (Warner, 1994; Campa-Cordova et al., 2002). To avoid this damage, cells and organisms use three defense strategies; one involves low molecular weight antioxidants such as ascorbic acid, α-tocopherol and glutathione, which can directly interact with ROS and neutralizing those (Campa-Cordova et al., 2002). The other two involve a variety of enzymes that metabolize ROS (SOD, catalase, glutathione peroxidase) or macromolecular damage repair as nucleic acids, proteins and lipids (DNA repair enzymes, proteases, lipases, etc.) caused by ROS (Warner, 1994; Campa-Cordova et al., 2002).

In NCLA treatments was increased in HH that is explained by the fact in this study were not exposed to any pathogen so no requiring activation of components associated with phagocytosis and/or coagulation. Thus, there is an increased percentage of GH with a greatest amount of immunoefetoras cell components and immunoregulatory to protect the shrimp against possible pathogenic attacks. There little research with differential hemocyte count, being more found total hemocyte count, and other study with compounds supplemented becomes delicate comparisons by the difference in composition and functionality of the compound. In conclusion, NCLA treatments improved the immune response by increasing the number of granular hemocytes.

5. CONCLUSION

The GST activity was tissue-specific, with greater activity in gills. However, the NLA induced increase in GST activity in the hepatopancreas. Likewise that the GST activity the GSH concentration is tissue-specific with greater performance in the muscle although not presenting significant difference between treatments had higher concentration in this tissue has direct antioxidant activity and not as a substrate for detoxification activity. Antioxidant capacity also showed a standard tissue-specific, with higher antioxidant capacity hepatopancreas no evident action of the performance of NLA against peroxyl radicals. Lipid peroxidation levels were lower in muscle, with obvious performance NLA action. It can say that the groups with NLA there was an increase in the percentage of granular hemocytes, cells with higher amounts of components immunocompetents, but cannot say that the NLA is a compound with immunostimulatory behavior.

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Table captions

Table 1 – Parameters physical and chemical water quality on average function (± 1 standard deviation). In saline water (SW) or in bioflocs system (BFS). Animals were fed with ration suplemented with nanocapsules containing lipoic acid (NCLA) or without the antioxidant (empty nanocapsules - NC). Asterisk (*) indicate significant differences ($p < 0.05$) between groups.

Parameter	**SW-NC**	**SW-NCLA**	**BFT-NC**	**BFT-NCLA**
Temperature °C (n=186)	27.01 (± 0,01)	27.01 (± 0,03)	27.03 (± 0,01)	27.01 (± 0,02)
[DO] (mg L^{-1}) (n=186)*	6.45 (± 0.01)	6.51 (± 0.01)	5.56 (± 0.05)	5.61 (± 0.04)
Salinity (n=186)	31.00 (± 0.01)	31.00 (± 0.01)	31.98 (± 0.01)	31.96 (± 0.01)
pH (n=186)	7.89 (± 0.06)	7.94 (± 0.02)	8.00 (± 0.01)	7.82 (± 0.07)
Ammonia (mg L^{-1}) (n=66)	1.72 (± 1.36)	1.71 (± 1.43)	1.84 (± 2.53)	1.90 (± 2.86)
Nitrite (mg L^{-1}) (n=66)*	0.55 (± 0.75)	0.53 (± 0.69)	0.27 (± 0.39)	0.25 (± 0.33)
Nitrate (mg L^{-1}) (n=30)*	3.64 (± 1.88)	0.18 (0.69)	84.60 (± 5.57)	80.90 (± 5.55)

Table 2 – Average (± 1 standard deviation) initial and final weight in shrimp *Litopenaeus vannamei* reared in saline water (SW) or in bioflocs system (BFS). Animals were fed with ration suplemented with nanocapsules containing lipoic acid (NCLA) or without the antioxidant (white nanocapsules - WNC). Statistical differences between treatments were represented by different lower case letters ($p<0.05$) for both initial and final weight. Number of samples analyzed per treatment: 45.

Treatments	**Average initial weight (g)**	**Average final weight (g)**	**Survival**
SW-NC	5.90 (± 0.08)^{a}	9.72 (± 0.06)^{c}	100%
SW-NCLA	5.91 (± 0.07)^{a}	11.85 (± 0.11)^{b}	100%
BFT-NC	5.91 (± 0.04)^{a}	9.43 (± 0.04)^{d}	100%
BFT-NCLA	5.91 (± 0.01)^{a}	12.34 (± 0,05)^{a}	100%

Figure captions

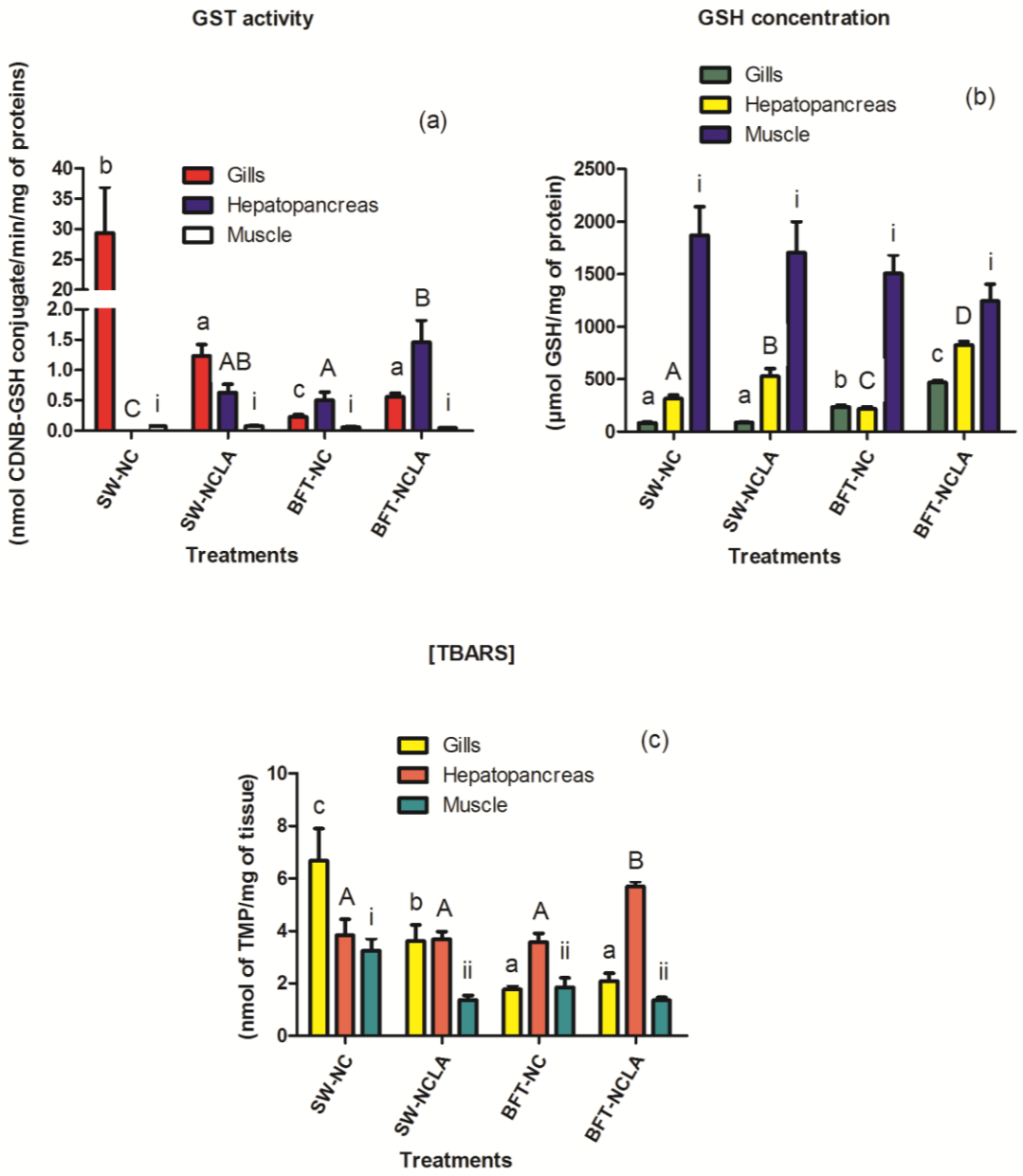

Figure 1. **(a)** Mean activity (± 1 standard error) of glutathione S-transferase (GST) (nmol of CDNB-GSH conjugate/min/mg of protein). **(b)** Mean (± 1 standard error) of reduced glutathione (GSH) concentration (nmol GSH/mg protein). **(c)** Mean concentration (± 1 standard error) of thiobarbituric acid reactive substances (TBARS)

(TMP nmol/mg tissue). In all cases, measurements were performed in gills, hepatopancreas and muscle of *L. vannamei* reared in saline (SW) or in biofloc system (BFT) and exposed to empty nanocapsules or nanoencapsulated lipoid acid. Different letters indicate significant differences ($p < 0.05$) between the experimental groups. Similar letters (lowercase in gills; uppercase in hepatopancreas and Roman numerals in muscle) means absence of statistical differences ($p > 0.05$). Number of samples analyzed per treatment: 11 – 15.

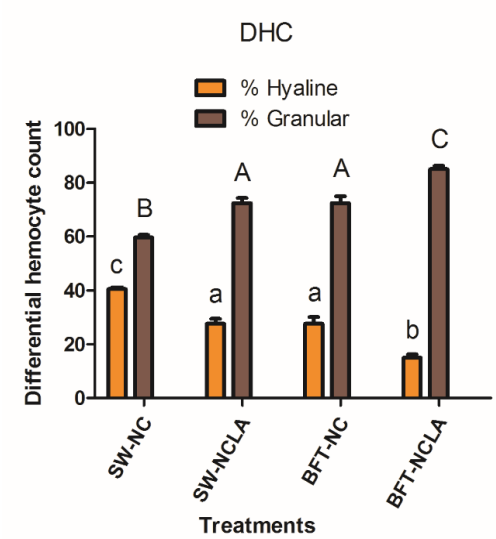

Figure 2. Mean (± 1 standard error) of percentage of hyaline and granular hemocytes in hemolymph from shrimps *L. vannamei* submitted to different experimental conditions in the same treatments. Shrimps were reared in saline water (SW) or in bioflocs system (BFT) and fed with ration supplemented with nanocapsules containing lipoic acid (NCLA) or empty nanocapsules (NC). Similar letters (lowercase in hyaline hemocytes and uppercase in granular hemocytes) means absence of statistical differences ($p>0.05$). Number of samples analyzed per treatment: 7.

CAPÍTULO III

Effects of lipoic acid in the total antioxidant capacity in biofloc

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Será submetido a revista Aquaculture

ABSTRACT

Several studies prove the effectiveness of biofloc in improving growth, survival and feed conversion to shrimp species *Litopenaues vannamei*, and the presence of bioactive compounds that help the immune and antioxidant defense. In this study, was observed the total antioxidant capacity of biofloc with application of antioxidant lipoic acid in different concentrations (control, [1,25μM], [2,5μM], [5μM] and [10μM]). Using the method of total antioxidant capacity against peroxyl radical (ACAP), for which was found increase in antioxidant capacity with the application of lipoic acid, especially in the treatment [10μM].

Keywords: biofloc, bioactive compound, lipoic acid, total antioxidant capacity.

1 INTRODUCTION

The biofloc Technology (BFT) is a production system with minimal water exchange, which includes the development of microorganisms aggregate belonging to different taxonomic groups (bacteria, micro-algae, protozoa and other) together with debris (organic and inorganic particles) that are suspended in the water column with constant aeration (Avnimelech, 2009; Cardona et al., 2015). The aggregates or biofloc of this microbial community in this system helps to maintain the water quality by setting of the recycling of nutrients (nitrogen compounds) and can be used as a source of proteins and lipids to aquatic organisms including fish and shrimp (Burford et al., 2004; Avnimelech, 2009).

From the ecology point of view, the biofloc can be understood as a microecosystem constituted by a great diversity of microorganisms (bacteria, phytoplankton, zooplankton), which develop at the expense of biological processes occurring in environment of raised. Thus, the nutritional value of biofloc is directly linked to the composition of the microbial communities and bioactive compounds present in these organisms. In the studies by Ju et al. (2008), Xu and Pan (2013) and Cardona et al., (2015), they proposed that animals raised in biofloc system showed an

increase in immune response and antioxidant status, probably for the intake of bioactive compounds present in these organisms.

In an intensive rearing system may increase the action of bioactive compounds by exogenous addition of other compounds with bioactive properties to assist in the wellness of animals raised, in this sense lipoic acid (LA) is presented as a multifaceted bioactive agent. It is known that the physiological level, this compound chelates free radicals, induces increase in the reduced glutathione concentration and restores the levels of other antioxidants; in this way also assists in maintaining the intracellular redox state (Shay et al., 2009).

The quantification of biofloc is monitored through the settleable solids in BTF system (Samocha et al., 2010; Ray et al., 2010; Schveitzera et al., 2013) and the presence of these solids over 10–14 mL L^{-1}, may increase consumption of dissolved oxygen limiting their concentrations and may change other variables such as alkalinity and pH (Tovar and Erazo, 2009; Samocha et al., 2010; Ray et al., 2010). The measurement of settleable solids may be a staunchly useful to interpret the interactions among the AL and biological processes that lead to increased or decreased of the biofloc quantity.

The application of AL in biofloc can increase the antioxidant capacity of biofloc, providing a means of reducing the damaging effects of reactive oxygen species (ROS) (Ghiselli et al., 2000). The ROS such as superoxide anion ($O_2^{\bullet -}$); hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2) are produced in cells of the organisms in normal or pathologic physiological conditions (Wajner et al., 2004), and can also be generated by cyclically dissolved oxygen and practice use of hydrogen peroxide to establish the oxygen levels in the water with biofloc by reduction or absence of mechanical aeration (Cardona et al., 2015). These ROS act on biomolecules that comprise cell membranes, proteins, DNA and RNA and the negative effects of this process are counteracted or mitigated by the action of endogenous or exogenous antioxidants (Barreiros et al., 2006).

An antioxidant may be defined as any substance that, when present at low concentrations compared with an oxidizable substrate, significantly delays or prevents oxidation of said substrate (Dickinson and Forman, 2002). The sum of the antioxidant

activity of an antioxidant compound is known as total antioxidant capacity (Bartosz, 2003). The term antioxidant activity is restricted to characterize the rate constant of a single antioxidant at a specific oxidant (generally free radical), whereas the antioxidant capacity should refer to the number of moles of oxidant eliminated by a given sample (Ghiselli et al., 2000).

Therefore, the objective of this study is to evaluate the effects of lipoic acid in biofloc over time (8 days), by analyzing the total antioxidant capacity against peroxyl radicals.

2 MATERIAL AND METHODS

2.1 EXPERIMENTAL DESIGN

It was held at the Green House I (GHI), in Marine Aquaculture Station of the Federal University of Rio Grande - FURG (Brazil), from 26 October to 2 November of 2014. In 15 tanks plastics (70 L), with a useful volume of 50 L each. Treatments were set at: Control (0 mg LA L^{-1} of biofloc), Concentration of 1.25 µM (0.26 mg L^{-1}), Concentration of 2.5 µM (0.51 mg L^{-1}), Concentration of 5 µM (1.03 mg L^{-1}) and Concentration of 10 µM (2.06 mg L^{-1}); all in triplicate. The collections of biofloc were made every 24 hours. After the collection of biofloc, were added the AL concentrations.

2.2 DILUTION LIPOIC ACID IN SODIUM HYDROXIDE

The fixed concentrations of synthetic α-lipoic acid (> 99% purity, Sigma-Aldrich) were dissolved individually for each tank, with sodium hydroxide solution and distilled water to ensure full dilution of lipoic acid following the methodology Amado et al. (2011). After dilution, the pH was adjusted to 7.5. The concentrations of LA were added every 24 hours (Table 1).

Table 1: Preparation of lipoic acid solution. NaOH (sodium hydroxide; 2mM), dwH_2O (distilled water), LA (lipoic acid). From the overall calculation of 300 mL of NaOH to 1 g of LA and 1000 mL of dwH_2O to 80 g of NaOH.

Treatment	**NaOH (g)**	**dwH_2O (mL)**	**LA (g)**
Control	0	0	0
1.25 µM	0.62	7.8	0.26
2.5 µM	1.22	15.3	0.51
5.0 µM	2.47	30.9	1.03
10.0 µM	4.94	61.8	2.06

2.3 WATER QUALITY ANALYSIS

Daily physical and chemical parameters of water were monitored, including dissolved oxygen (mg L^{-1}) and temperature (°C) with YSI multiparameter, salinity with optical refractometer (Atago 103, ± 1 ppt), pH with electrode Mettler Toledo FEP20 - FiveEasy Plus™. Determination of alkalinity (mg $CaCO_3$ L^{-1}) was made by the method APHA (1985), and calculating the concentration of sodium bicarbonate was performed according Furtado et al. (2011). The analyzes of total ammonia (NH_3 + NH_4^+ mg L^{-1}) by method UNESCO (1983) and nitrite (mg L^{-1}) according Benderschneider and Robinson (1952) (Figure 1).

The settleable solids (organic and inorganic particulate matter) were determined by sedimentation of solids in Imhoff cones where placed 1 L of water and allowed to stand for 1 h, time which is made to read the settleable solids mL L^{-1} (Tovar and Erazo 2009) (Figure 1).

2.4 COLLECTION AND HOMOGENIZATION OF SAMPLES

Samples were collected after reading the Imhoff cone, in which the water was removed by siphoning and flake placed in falcon (50 mL) and stored on ice, and then they were transferred to eppendorf (2 mL) and centrifuged at 800 x g being the 4 °C for 10 minutes and stored at -80 °C. For homogenization, the biofloc samples were weighed

in eppendorff, added methanol (100%) on relationship 1000 mg 1000 μL^{-1}, homogenized and stirred for 3 hours, then centrifuged for 10 minutes at 10000 rpm and 4 °C, the supernatant removed and it used to determine the antioxidant capacity against peroxyl radicals.

2.5 DETERMINATION OF TOTAL ANTIOXIDANT CAPACITY AGAINST PEROXYL RADICAL (ACAP)

It was conducted in accordance with the protocol Amado et al. (2009), but for this work was not the fixation of the protein concentration. After white microplate was added to 127.5 µL of a reaction buffer consisting of 0.3575 g of ethanesulfonic acid, 4.2 hydroxyethyl-piperazine-1 (HEPES), 0.7455 g of potassium chloride (KCl), 0.0102 g magnesium chloride (MgCl2) dissolved in 50 mL of MilliQ water with pH adjusted to 7.2; 10 µL tissue extract; 7.5 µL of water MilliQ to samples without ABAP (2,2 azobis 2metilpropianoamidina dihidrocloreto) or 7.5 µL ABAP solution for samples with ABAP (peroxyl radical generator) and 10 µL of H_2DCF-DA solution (diacetate '2,7 dicloroflurescein). Reading is performed at time zero and then every 5 minutes to complete 30 minutes in fluorimeter plates (FilterMax F5, Multi-mode microplate reader) using a wavelength of 530 nm emission and 485 nm excitation, at 37 °C, the temperature that favors ABAP thermolysis. This method quantifies the ability of the tissue to neutralize the ROS generated by the decomposition of ABAP, including enzymatic antioxidant defenses and/or non-enzymatic, by calculating the relative area performed according Monserrat et al. (2014). The relative area has an inverse relationship with the antioxidant capacity, the area where lower values indicate a higher relative antioxidant capacity and vice-versa.

2.6 STATISTICAL ANALYSIS

Data were expressed in mean ± standard deviation. On analysis by two-factor ANOVA (different concentrations of lipoic acid and time). Previously, the assumptions of normality and homogeneity of variance were analyzed. Mean comparisons were made using the Newman-Keuls test. In all cases, we used a 5% significance level.

3 RESULTS

The water quality parameters such as temperature got overall average of 28.48 ± 0.09 °C. The dissolved oxygen was not significantly different ($p>0.05$) between treatments or over time, getting between minimum 7.68 ± 0,05 and maximum 7.99 ± 0,02 mg L^{-1}, both in control at time 0 and time 2, respectively. The pH showed changes in time 4, 5 and 6 in the control treatment, with values of 7.81 ± 0.04, 7.69 ± 0.04 e 7.62 ± 0.07 respectively and have minimum of 7.62 and maximum of 8.10 on 3 (time 3) both in the control treatment. The lowest salinity was measured on time 1 in the treatment [1.25 µM] with 26.27 ± 2.99 and from time 4 was in 30 for all treatments. For alkalinity, the minimum value of 126.67 ± 53.64 mg $CaCO_3$ L^{-1} occurred at time 5 in the treatment [10 µM], with a maximum of 213.33 ± 38.33 at time 3 for the same treatment. The ammonia presents change in time 4 in treatment [5 µM], with maximum value of 0.41 ± 0.32 mg L^{-1} and have minimum of 0.02 ± 0.00 mg L^{-1} at time 3 in treatment [10 µM]. The minimum levels of nitrite was among 0.03 ± 0,00 mg L^{-1} in the control treatment at times 2, 6 and 7 and maximum levels at time 5, 6 e 7 with value 0.34 ± 0.23, 0.31 ± 0.15 and 0.34 ± 023 mg L^{-1} respectively in treatment [1.25 µM]. The settleable solids increased over time and showed significant differences ($p>0.05$) when compared to control, with a minimum value of 2.33 ± 0.33 mL L^{-1} at time 2 in the treatment control and up to 216.67 ± 16.67 mL L^{-1} in time 6 in treatment [1.25 µM].

For total antioxidant capacity, at time 0 was there is significant difference ($p<0.05$) between control and of the treatments 1.25 µM (0.26 mg L^{-1}), 2.5 µM (0.51 mg L^{-1}), 5 µM (1.03 mg L^{-1}) and 10 µM (2.06 mg L^{-1}). At time 1, noted increase in total antioxidant capacity with significant difference ($p<0.05$) between control and of the 2.5 µM (0.51 mg L^{-1}), 5 µM (1.03 mg L^{-1}) and 10 µM (2.06 mg L^{-1}). At time 2, there was a significant difference ($p<0.05$) between control and the other treatments, following this pattern for the times 3, 4, 5, 6 and 7 (Figure 2).

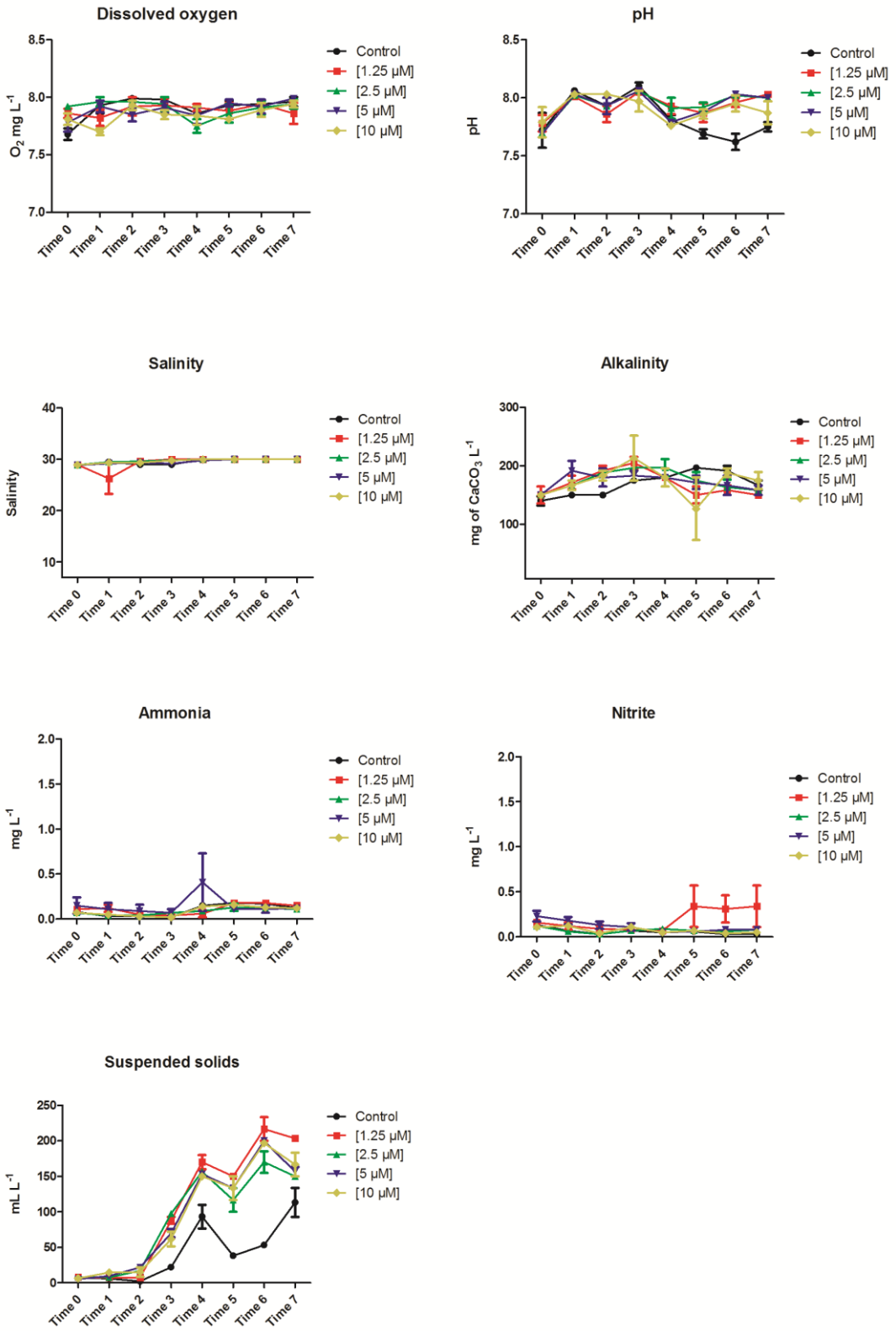

Figure 1 – Mean concentration (±1 standard error) of the physico and chemical parameters. Number of samples analyzed in each treatment: 3

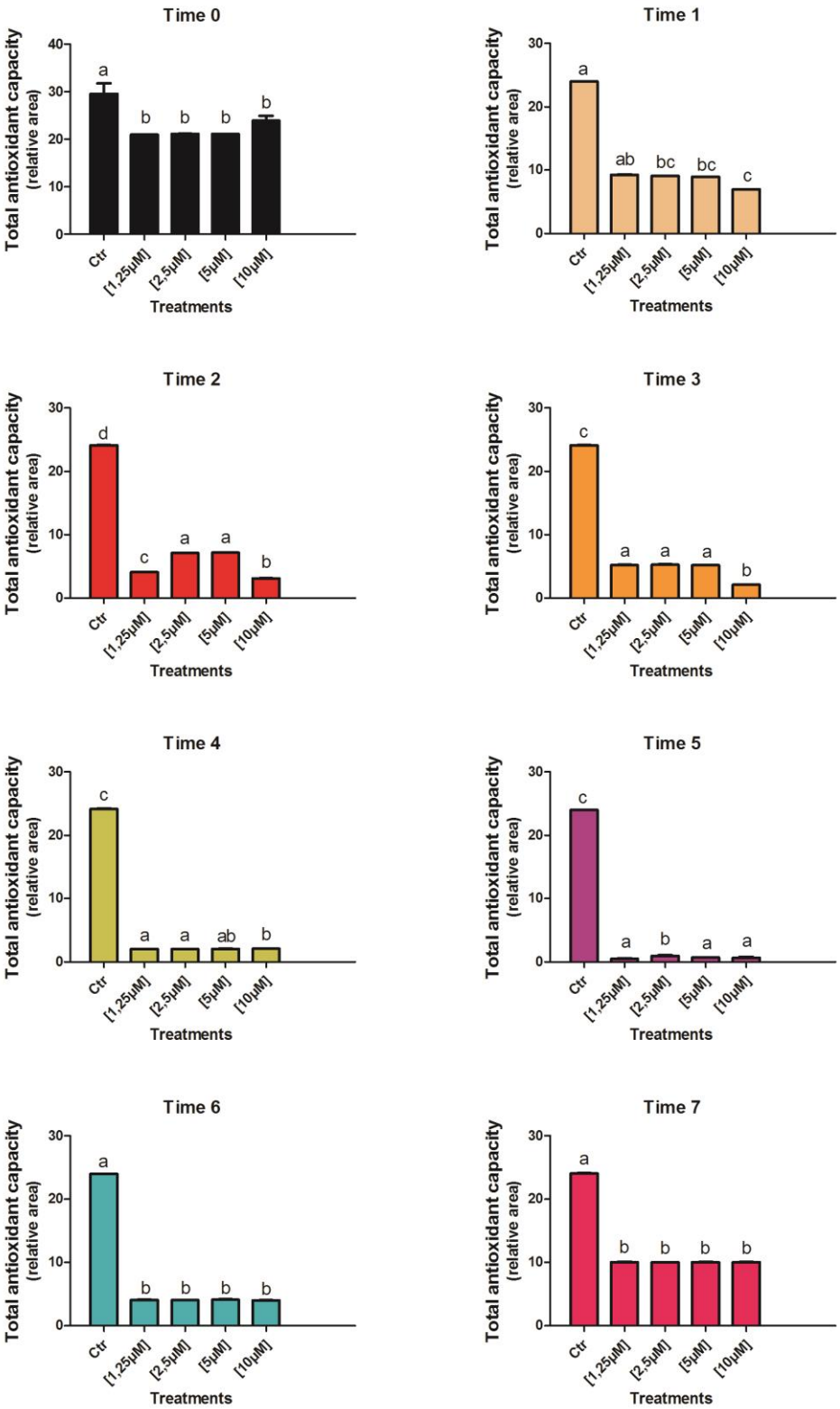

Figure 2 – Mean values (± 1 standard error) of total antioxidant capacity against peroxyl radicals (expressed in relative area) in biofloc. Different letters indicate significant

differences ($p<0.05$) between experimental groups after performing Newman-Keuls post hoc test. Number of samples analyzed in each treatment: 9.

4 DISCUSSION

Water quality parameters were monitored to comply with requirements physical and chemical of the water for species *Litopenaues vannamei*, following values proposed by Lin and Chen (2001; 2003), Ray et al. (2010) and Samocha et al. (2010), that recommended: (i) temperature should be between 28–30 °C, because low temperature decreases growth and high temperature cause stress; (ii) dissolved oxygen greater than 5 mg L^{-1}, reduces stress on the shrimp; (iii) pH in 7.5, preferably greater than 7; (iv) salinity greater than 10 with a tolerance of 4–35, high salinity help in buffering capacity of water; (v) alkalinity greater than 160 mg L L^{-1}, assists in balancing the pH; (vi) ammonia in 3.35 mg L^{-1} with salinity of 25 and 3.95 mg L^{-1} in salinity 35; (vii) nitrite levels between 5–25 mg L^{-1}, thresholds rise with increased salinity; (viii) settleable solids 10–14 mg L^{-1}, increase in the floc concentration may result in anaerobic mean. For this work, on time 1 the salinity had decreased to 26.67 ± 2.99, but is still in the recommended range for *L. vannamei*. On time 5, when the alkalinity has reached minimum 126.67 ± 53.64 mg $CaCO_3$ L^{-1}, was added sodium bicarbonate to increase the alkalinity levels. On time 4, showed an increase of ammonia remaining with value of 0.41 ± 0.32 mg L^{-1}, below the recommended amount. On time 5, 6 and 7, an increase in nitrite levels getting the value of 0.34 ± 0.23 mg L^{-1}; 0.31 ± 0.15 mg L^{-1} and 0.34 ± 0.23 mg L^{-1}, below the recommended values. At the end of the experiment, only settleable solids were beyond to standard recommended for shrimp *L. vannamei*, with a significant increase in treatments with LA, which may suggest that the AL induces increased amount of floc or the increase was due to the absence of shrimp in the tank, providing an imbalance between production and consumption floc.

The total antioxidant capacity of biofloc increased since the first day of application of AL, highlighting to treatments [2.5 µM], [5 µM] and [10 µM]. On the second day of the experiment, the increase occurred in treatments [1.25 µM] and [10 µM]. On the third day, the increase was observed in treatment [10 µM]. On the fourth day, increased total antioxidant capacity was observed [10 µM]. On the fifth day, the

increase occurred in treatments [1.25 µM] [µM 5] and [10 µM]. Thus, AL induces increasing the capacity of floc cells to neutralize ROS. On day 6 and 7 was observed decrease in antioxidant capacity with respect to treatment with AL, suggesting that under experimental conditions the biofloc has limited capacity to absorb AL or increase the amount of floc, evidenced by the increase in settleable solids, reduces the concentration and the LA effects. For the control treatment, no significant change over time.

5 CONCLUSION

The lipoic acid induces increase in total antioxidant capacity in biofloc, having the concentration of 10 µM AL per liter of water at most the total antioxidant capacity over time.

6 FUTURE PERSPECTIVE

Lipoic acid can convey their bioactive properties without causing damage to biofloc, but these benefits can be apply to the community that makes up the biofloc or it benefits are community-specific?

Once lipoic acid increases the total antioxidant capacity of the flake, the dose of 10 µM of LA per liter of water can influence the nutritional composition of biofloc?

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CONCLUSÃO GERAL

Os camarões criados em sistema de biofloco, em 30 dias de exposição, tiverem aumento na competência antioxidante, sendo a resposta tecido-específica para camarão branco do pacífico *Litopenaus vannemei*. Deste modo, algumas comunidades de microrganismo do sistema BFT influencia no estado redox de tecidos de camarão (brânquias, hepatopâncreas e músculo), como por exemplo: diminuição da concentração de glutaiona reduzida em brânquias e hepatopâncreas e níveis de peroxidação lipídica menores em hepatopâncreas.

Com suplementação com ácido lipóico nanoencapsulado, aumento atividade gluationa S-transferase em hetaopâncreas, aumento da concentração de glutationa reduzida em músculo, maior capacidade antioxidante no hepatopâncreas e níveis de peroxidação lipídica menos em músculo. E aumento dos hemóctios granulares em camarões exposto ao ácido lipóico. E, portanto, o ácido lipóico foi capaz de aumentar capacidade antioxidante e diminuir o dano oxidativo em tecido do camarão branco do pacífico *Litopenaus vannemei* com ação antioxidante tecido-específica.

O ácido lipóico foi capaz de aumentar a capacidade antioxidante no biofloco em concentração de [10 µM] (2,06 mg de AL/L de biofloco), com aumento gradual da capacidade antioxidante durante 5 dias.