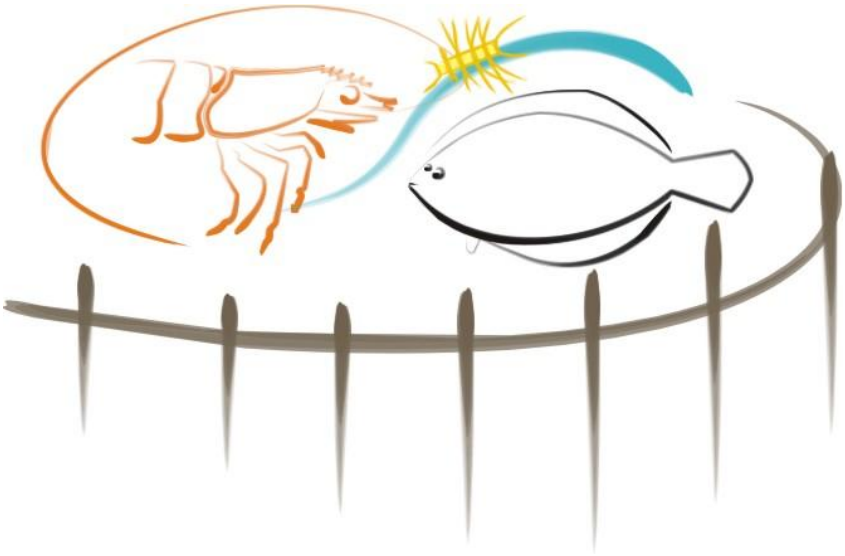
UNIVERSIDADE FEDERAL DO RIO GRANDE

INSTITUTO DE OCEANOGRAFIA

PROGRAMA DE PÓS-GRADUAÇÃO EM AQUICULTURA



Efeito da temperatura e suas implicações no sistema antioxidante e parâmetros imunológicos de camarões peneídeos

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

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Indice

Dedicatória

Dedico este trabalho:

A Deus, por abençoar o meu caminho.

Aos meus pais Air e Mara

À minha esposa

Aos meus amigos e

A toda minha família.

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MUITO OBRIGADO!!!

Resumo geral

A espécie exótica *Litopenaeus vannamei* é a mais cultivada mundialmente, enquanto que espécies de camarões nativos como o *Farfantepenaeus brasiliensis* já demonstraram potencial de cultivo. Fatores ambientais, como a temperatura, podem influenciar diretamente o cultivo de camarões. A temperatura da água é um dos mais importantes fatores a serem considerados na aquicultura. Mudanças na temperatura da água podem promover alterações no consumo de oxigênio e disponibilização aos tecidos, alterar a solubilidade de gases na água e alterar de forma geral o metabolismo dos organismos. No Sul do Brasil há uma ampla variação térmica ao longo do ano por isso a temperatura é um fator limitante na região. Portanto essa tese teve como objetivo avaliar o efeito da temperatura em parâmetros fisiológicos e imunológicos tanto da espécie exótica *Litopenaeus vannamei* quanto do camarão nativo *F. brasiliensis*. Foram realizados experimentos a curto e longo prazo expondo camarões da espécie *L. vannamei* a diferentes temperaturas e aos sistemas de cultivo em bioflocos e água clara para análise do sistema antioxidante e parâmetros imunológicos. Nesses experimentos com a espécie *L. vannamei*, os animais dos tratamentos com as temperaturas mais baixas 15 e 21ºC apresentaram maior atividade das enzimas antioxidantes. Essa maior atividade das enzimas é uma tentativa de neutralizar as espécies reativas de oxigênio, porém uma grande energia é necessária devido à condição de estresse, portanto menor crescimento e sobrevivência foram verificados, pois em muitos casos os animais não suportaram a condição térmica a que foram expostos. Foi realizado um experimento de simulação de transporte com o camarão-rosa *Farfatepenaeus brasiliensis* em diferentes temperaturas para avaliar a influencia da temperatura no sistema antioxidante durante o transporte. Os resultados apontaram que a melhor temperatura foi a de 19.3ºC, pois com a temperatura reduzida, aumenta a solubilidade de oxigênio e reduz o metabolismo dos animais o que pode resultar em uma menor produção de espécies reativas de oxigênio. Nessa condição os animais apresentaram uma melhor atividade de enzimas antioxidantes demonstrando um melhor estado fisiológico para suportar o transporte. Portanto a temperatura recomendada para o transporte de *F. brasiliensis* foi de 19.3ºC enquanto que com relação à espécie *L. vannamei* recomenda-se o cultivo no sistema de bioflocos na temperatura de 27ºC nos quais os animais apresentaram melhor estado fisiológico.

Abstract

The exotic species *Litopenaeus vannamei* is the most cultured species worldwide; however, some indigenous species such as *Farfantepenaeus brasiliensis* have shown potential for culture. Environmental factors, such as temperature can influence the shrimp culture. The water temperature is one of the most important factors to be considered in aquaculture. Changes in water temperature can promote alterations in oxygen consumption and tissues disponibilization, alter the solubility of gases in water and change in general the organism metabolism. In the South of Brazil there is a wide thermal variation throughout the year, by this reason the temperature is a limit factor in this region. Therefore, the aim of this thesis was evaluate the temperature effect in physiological and immunological parameters not only of the exotic species *L. vannamei*, but also the indigenous species *F. brasiliensis*. Experiments, short and long exposure in bioflocs and clear water systems were realized with *L. vannamei* at different water temperatures to analyze of the antioxidant system and immunological parameters. In these experiments with *L. vannamei*, the animals of treatments 15 and 21ºC shown higher enzymatic activities. These higher enzyme activity is an attempt to counteract the reactive oxygen species, however higher energy expenditure is necessary due to the stress condition, therefore lower growth and survival were verified, in some cases the animals did not tolerate the thermal condition that they were exposed. An experiment of transport simulation was performed with the pink shrimp *F. brasiliensis* at different water temperatures to evaluate the temperature effect in the antioxidant system during transport. The results revealed that the best temperature was 19.3ºC, because the reduction in water temperature increases the oxygen solubility and reduces the general metabolism which can result in a lower reactive oxygen species generation. In this condition the animals presented a better physiological status to resist the transport. Therefore, the recommended temperature for the transport of *F. brasiliensis* was 19.3ºC while in relation to *L. vannamei* we recommend to culture in biofloc system at 27ºC due to the animals presented better physiological status.

1. Introdução Geral

1.1. Histórico da Pesca e Aquicultura

A aquicultura, definida como o cultivo de organismos aquáticos, incluindo peixes, moluscos, crustáceos, anfíbios e plantas aquáticas (Bardach et al. 1972) é uma atividade que tem crescido substancialmente nas últimas décadas, com um suprimento de alimentos de origem aquícola aumentando em uma taxa anual de 3,2%. Além disso, atualmente fornece quase metade de todo pescado destinado ao consumo humano (FAO 2014).

Segundo dados da FAO (2012) a população mundial em 2006 era de 6,6 bilhões de pessoas, passou para 7 bilhões em 2011 e projeções apontam que até o ano de 2050 a população mundial poderá alcançar a marca de 9,2 bilhões de pessoas. Estimativas revelam que no mundo em torno de 1 bilhão de pessoas estão em estado crônico de fome (FAO 2009). Portanto o desafio da humanidade é fornecer alimento a todas essas pessoas que se encontram em estado alimentar crítico e duplicar a produção de alimentos para que se essas estimativas de crescimento populacional forem confirmadas, a produção de alimento seja suficiente para suprir essa demanda (FAO 2009). A necessidade de geração de alimento e a importancia da aquicultura como atividade fornecedora deste podem ser observadas nos dados de consumo per capta de pescado. Na década de 1960 esse consumo per capta era cerca de 9,9 kg enquanto que em 2012 foi de 19,2 kg (FAO 2014).

A pesca e aquicultura forneceram ao mundo cerca de 158 milhões de toneladas de pescado em 2012, sendo que 66,6 milhões de toneladas provenientes da aquicultura e 91,3 milhões da pesca (FAO 2014). Desses 158 milhões de toneladas produzidas, 136 milhões foram destinadas ao consumo humano. Esse desenvolvimento da aquicultura é fruto de pesquisas e da melhoria do comércio e distribuição dos produtos aquícolas (FAO 2014).

Este aumento potencial na aquicultura contrapõe a atividade da pesca extrativa que está estagnada há muitos anos (cerca de 90 milhões de toneladas/ano) devido à super-exploração dos estoques pesqueiros, o que vai além da capacidade de suporte do sistema. Para suprir à demanda alimentar, a aquicultura será uma das responsáveis por esse suprimento, uma vez que a pesca se mantém constante (FAO 2014). O aumento anual da produção da aquicultura é devido ao desenvolvimento de pesquisas para a otimização da produção de organismos de interesse comercial.

A aquicultura ainda depende da captura de pescado para utilização como ingredientes (incluindo óleo e farinha de peixe) das rações com elevado teor proteico (Tacon 2004). Embora haja uma progressiva redução anual com relação ao percentual desses ingredientes incorporados nas rações, mais esforços devem ser concentrados para diminuir cada vez mais o uso de pescado para alimentar organismos aquáticos (FAO 2014). Devido a esse déficit de alimento produzido mundialmente e o número de pessoas em estado alimentar crítico, esse pescado deve ser utilizado para o consumo humano na tentativa de minimizar a fome mundial.

A carinocultura é uma atividade rentável e muito importante para a aquicultura. O camarão continua sendo um dos mais valorizados produtos aquícolas, representando cerca de 15% do valor total de produtos comercializados internacionalmente. Em 2010 a produção aquicola de crustáceos foi de 9,6%, representando 5,7 milhões de toneladas (FAO 2012). No Brasil o cultivo de camarões é responsável por mais de 80.000 toneladas (ABCC 2011).

1.2. Carcinicultura no Brasil

A carcinicultura iniciou no Brasil na década de 70, sendo o estado do Rio Grande do Norte considerado o berço da carcinicultura brasileira. Na época, o Governo

do Rio Grande do Norte criou o "Projeto Camarão" como alternativa para substituir a extração do sal – atividade tradicional que se encontrava em crise. Nesse período, a Região Sul também apostava no crustáceo. No estado de Santa Catarina foram desenvolvidas pesquisas nas áreas de reprodução, larvicultura e engorda do camarão cultivado e então foi possível produzir as primeiras pós-larvas em laboratório da América Latina. O primeiro projeto de produção comercial do camarão cultivado ocorreu no período entre 1978 e 1984 (ABCC 2011).

Nesse período, o Governo do Rio Grande do Norte importou o camarão *Penaeus japonicus* e começou a desenvolver pesquisas para adaptação da espécie às condições locais. As primeiras pesquisas foram promissoras, período que coincidiu com o de seca no nordeste, favorecendo o cultivo da espécie. Após o ano de 1984 quando encerrou a época de seca e iniciou um período de intensas chuvas as condições foram desfavoráveis, limitando a possibilidade de cultivar essa espécie em larga escala (ABCC 2011).

Após, iniciou-se a segunda fase, na qual foi realizado o cultivo de espécies nativas como o *Farfantepenaeus brasiliensis*, *Farfantepenaeus paulensis* e *Litopenaeus schimitti*. Aproximadamente foram dez anos de esforços para domesticação e tentativa de cultivo dessas espécies. A conclusão foi de que a rentabilidade era baixa devido aos requisitos nutricionais, foi quando ainda na década de oitenta buscaram solução na espécie exótica *Litopenaeus vannamei* (ABCC 2011).

No início da década de noventa surgiram os primeiros resultados de estudos feitos com o *L. vannamei* e a partir de 1995 se demonstrou sua viabilidade comercial. Atualmente é a única espécie cultivada em larga escala.

1.2.1 Litopenaeus vannamei

O primeiro registro dos peneídeos aconteceu em 1759. Em 1815, Rafines reconheceu que os peneídeos representavam um grupo distinto dentro dos decápodas e os nominou Penedia, o que foi alterado para Penaeidae em 1955. Em 1997, Isabel Perez Farfante e Brian Kensley propuseram uma mudança na grafia dos gêneros de alguns peneídeos para *Litopenaeus*, *Fenneropenaeus*, *Marsupenaeus* e *Farfantepenaeus*. Após a mudança, a espécie de camarão marinho *Penaeus vannamei* passou a chamar-se *Litopenaeus vannamei* (Pérez-Farfante & Kensley 1997; Treece 2000; Carvalho 2011). O camarão marinho *Litopenaeus vannamei* (Boone 1931), popularmente conhecido como camarão branco do pacífico, pertence ao filo Artthropoda, a classe Crustacea, à Ordem Decapoda, a Subordem Dendrobranchiata, a Superfamília Penaeoidea, a família Penaeidae e ao gênero *Litopenaeus* (Pérez-Farfante & Kensley, 1997).

O camarão branco *Litopenaeus vannamei* é uma das espécies mais cultivadas no mundo e está naturalmente distribuída na costa do Pacífico e América do Sul (Zhou et al. 2009). O seu ciclo de vida inicia com a migração dos adultos para o mar aberto em busca de água com características oceânicas com salinidade e temperatura estáveis para a maturação e a desova. Os náuplios eclodem em águas oceânicas e à medida que passam para os estágios larvais seguintes se deslocam em direção aos estuários que funcionam como berçários naturais ricos em alimento onde os animais crescem e fazem sucessivas metamorfoses até o estágio juvenil quando então iniciam a migração para o mar aberto para um novo ciclo (Treece 2000; Carvalho 2011).

As excelentes características zootécnicas de rápido crescimento, eficiente conversão alimentar, rusticidade, alta taxa de sobrevivência, ampla tolerância do *L. vannamei* à diferentes salinidades, aliada à expansão dos cultivos em águas interiores salinas, tornaram esta espécie muito relevante para a aquicultura (Maicá et al. 2012; Krume nauer et al. 2011; Ostrensky 2002; McGraw et al. 2002). Por se tratar da espécie

de camarão mais cultivada mundialmente, as tecnologias de cultivo já estão bem difundidas devido a inúmeras pesquisas desenvolvidas com essa espécie. Portanto a carcinocultura está cada vez aumentando mais a produção e auxiliando a aquicultura no fornecimento de alimento de origem animal.

1.2.2 Farfantepenaeus brasiliensis

Embora a carcinicultura brasileira esteja fundamentada na espécie exótica *L. vannamei*, espécies de camarões nativos já demonstraram potencial de cultivo como é o caso dos camarões do gênero *Farfantepenaeus* (*F. paulensis*, *F. brasiliensis* e *F. subtilis*) (Lopes 2009). O camarão-rosa, como são chamados os camarões do gênero *Farfantepenaeus*, pode ser encontrado desde as águas costeiras da província de Buenos Aires - Argentina até a Carolina do Norte - EUA (D'Incao 1991). Porém, entre essas três espécies o *F. brasiliensis* é o que apresenta uma distribuição mais ampla, podendo ser encontrado desde o sul do Rio Grande do Sul (Brasil) até a Carolina do Norte (EUA) (D'Incao 1999).

Diferentemente do cultivo de espécies exóticas, o cultivo de camarão nativo permite a realização desta atividade em estruturas de baixo custo (gaiolas e cercados) os quais podem ser instalados em corpos de águas naturais, possibilitando outra fonte de renda para a população da região (Wasielesky 2000; Lopes 2009). Além disso, tornam viáveis programas de repovoamento de camarão, principalmente em áreas onde, o declínio das capturas vem sendo historicamente registrado, como é o caso do estuário da Lagoa dos Patos, Rio Grande, RS, afetando diretamente as comunidades de pescadores artesanais da região (D'Incao et al. 2002). Além da sua utilização para consumo, o cultivo de camarões nativos também é realizado com a finalidade de produção de isca

viva para a pesca esportiva, mercado crescente principalmente no litoral dos estados do Rio de Janeiro, São Paulo, Paraná e Santa Catarina (Preto et al. 2009; Beccato 2009).

Alguns estudos mostram que essa espécie também utiliza o biofloco como alimento melhorando as taxas de sobrevivência (Wasielesky et al. 2006; Kim et al. 2014), melhora seu desempenho zootécnico em cultivo no sistema de bioflocos tanto relacionado a presença de probióticos (Souza et al. 2012) quanto na adição de melaço para estimular a formação dos bioflocos (Souza et al. 2014).

1.3. Biofloc Technology System (BFT)

Devido ao desenvolvimento da aquicultura, uma das preocupações mundiais é com relação ao impacto ambiental que a atividade pode ocasionar. Portanto boas práticas de manejo e de biossegurança são necessárias para uma atividade sustentável e ambientalmente amigável. A aquicultura ainda está restrita a regiões costeiras, onde a especulação imobiliária é muito alta e à farinha e óleo de peixe são ainda muito utilizados como ingredientes de rações (Emerenciano et al. 2013). Os custos com alimentação representam cerca de 50–60% dos custos totais da produção (Shiau 1998; Epp 2002; Emerenciano et al. 2013). Além disso, no cultivo de camarões apenas 15-30% do alimento fornecido é convertido em biomassa dos organismos cultivados (Tacon et al. 1999), o restante acaba sendo perdido para o sedimento, efluente e a atmosfera (Boyd 2003).

A maioria da produção de camarões e peixes é realizada em viveiros escavados, e estes extravasam efluentes após chuvas fortes, quando são drenados para a despesca e quando há renovação de água. Este fato muitas vezes pode causar severos danos ao meio ambiente, através da eutrofização dos corpos de água naturais, que recebem os efluentes e também degradam o sedimento do próprio local do cultivo. Dentre os

impactos negativos, a poluição da água por efluentes é provavelmente o mais comum, portanto, tem atraído atenção mundial para que esse problema seja minimizado (Boyd 2003).

Uma eficiente alternativa para solucionar e/ou amenizar os impactos negativos da aquicultura é o sistema de bioflocos, pois consiste em uma forma eficaz de ciclagem de nutrientes no ambiente (Emerenciano et al. 2013). Este sistema reduz a emissão de efluentes e possibilita tanto o aumento da produtividade quanto melhoria da qualidade de água, reduzindo o volume de água utilizado, além de não serem feitas renovações, apenas adicionando o volume perdido por evaporação (Mishra et al. 2008; Wasielesky et al. 2006). Nesse sistema é adicionado uma fonte de carbono para elevar a relação C:N, auxiliando o desenvolvimento de bactérias heterotróficas. Esses microrganismos são capazes de utilizar a matéria orgânica dissolvida e capturar o nitrogênio da água para aumentar sua biomassa e converter amônia em proteína celular microbiana, favorecendo não só a qualidade de água, mas também fornecendo uma fonte de alimento secundária para os camarões, uma vez que eles também se alimentam dos microrganismos presentes no biofloco (Burford et al. 2004; Wasielesky et al. 2006; Souza et al. 2012; Souza et al. 2014).

Outro benefício extremamente importante no sistema BFT é a troca zero de água, ou seja, um sistema onde não há renovações e, portanto reduz drasticamente o custo de bombeamento de água, aumenta a biossegurança e diminui a emissão de efluentes, pois os microrganismos presentes nos bioflocos reduzem a concentração de nitrogênio possibilitando que se utilize a mesma água em diferentes ciclos de cultivo (Timmons & Losordo 1994; Chamberlain et al. 2001; McAbee et al. 2003; Krummenauer et al. 2014). Isso significa dizer que a água drenada na despesca pode ser re-utilizada para o próximo ciclo de cultivo otimizando desta forma o sistema e

colaborando para uma atividade sustentável e ambientalmente amigável (Krummenauer et al. 2014). Como os compostos nitrogenados são assimilados pela comunidade microbiana, a água pode ser reutilizada por vários ciclos sem praticamente nenhum impacto negativo. Nesse sistema de cultivo em bioflocos visando o reuso da água, o foco é aumentar a concentração de carbono no sistema favorecendo a multiplicação de bactérias heterotróficas que utilizarão o nitrogênio proveniente da amônia excretada pelos camarões e assim melhorando a qualidade de água, uma vez que, o nitrogênio na forma de amônia é extremamente prejudicial aos camarões (Vinatea 2002; Lin & Chen 2003). Portanto o reuso da água abre a perspectiva de que a carcinicultura poderá ser realizada em outras regiões que não precisam estar necessariamente em locais costeiros com corpos de água próximos. Isso possibilita que novos empreendimentos possam ser construídos e operados fora de regiões litorâneas onde geralmente a especulação imobiliária é alta, reduzindo significativamente o custo de implementação e operação desse sistema (Ray et al. 2009; Vinatea et al. 2010; Samocha et al. 2011; Krummenauer et al. 2014).

1.4. Temperatura

A temperatura por definição é o grau de agitação das moléculas. Não é simples classificarmos as variações térmicas ambientais. Atualmente, para diferenciarmos os animais quanto à temperatura corpórea, separamos em dois grupos: Endotérmicos e ectotérmicos. Animais endotérmicos são capazes de manter a temperatura do corpo pela produção interna de calor, enquanto que os ectotérmicos dependem de fontes externas, ou seja, sua temperatura interna varia de acordo com as condições ambientais a que se encontra (Schmidt-Nielsen 1996).

Os camarões são animais ectotérmicos, e, ao contrário dos endotermicos, a sua temperatura não está internamente regulada. Sendo assim, a temperatura ambiental exerce um forte efeito sobre o crescimento, a taxa de alimentação e o metabolismo destes animais (Laevastu & Hayes 1984; Qiu et al., 2011). Os invertebrados aquáticos e os peixes apresentam uma zona restrita de tolerância térmica (específico para cada espécie) e temperaturas letais, que podem ser variadas por meio de aclimatação ou pela adaptação a longo prazo a habitats com diferentes limites térmicos (Vinatea 2002).

Segundo Morales (1986), quanto mais constante a temperatura, mais previsível será o comportamento dos animais e, portanto, mais fácil será seu cultivo. De acordo com este mesmo autor, os efeitos biológicos das variações de temperatura são complexos porque dependem de numerosas variáveis. Estas variações podem afetar a reprodução, o crescimento e a sobrevivência. À medida que aumenta a temperatura, produz-se um aumento no metabolismo de forma geral até um ponto limite, caso siga aumentando pode levar o animal a morte. Já a diminuição de temperatura produz uma queda da atividade fisiológica, sendo que abaixo de uma certa temperatura o animal morre. O aumento da atividade fisiológica e metabólica implica em um maior consumo de oxigênio e incremento das necessidades nutritivas. A temperatura torna-se um fator crítico, pois seu aumento no ambiente produz uma diminuição na solubilidade dos gases na água, e no oxigênio dissolvido, e um aumento de sua demanda por parte dos organismos. (Vinatea 2002).

No Sul do Brasil há uma ampla variação da temperatura da água ao longo do ano. Garcia et al. (2008) constataram que no inverno a temperatura da água coletada em diversas estações de tratamento de água no Rio Grande do Sul foi de 9ºC, enquanto que no verão chegou a 32ºC. Essa ampla variação térmica requer adaptações fisiológicas para que os animais suportem e se desenvolvam nessas condições que muitas vezes são

desfavoráveis. Em crustáceos, como em outros animais, mudanças na temperatura podem afetar a capacidade de captar oxigênio do ambiente, promover mudanças a nível fisiológico que podem alterar a taxa de consumo de oxigênio e disponibilização aos tecidos (Powell & Watts 2006). Por essa razão a temperatura é um fator muito importante que tem que ser levado em consideração na carcinicultura, pois influencia diretamente o crescimento e a sobrevivência dos animais (Wang et al. 2006; Zhou et al. 2011). Li et al. (2014) testaram o efeito da temperatura no camarão branco *Litopenaeus vannamei*, diminuindo a temperatura em 1ºC/h em uma amplitude térmica de 26-17ºC e observaram que a 17ºC diminuiu o número de hemócitos e aumentou a concentração de espécies reativas de oxigênio (ERO). Além disso, ocorreu apoptose nos hemócitos dos camarões expostos a 17ºC. Esses resultados claramente evidenciam o efeito da temperatura nos animais, prejudicando não só o sistema imunológico quanto o fisiológico.

1.5. Estresse oxidativo

No metabolismo normal dos organismos, incluindo camarões peneídeos, vários processos metabólicos geram radiais livres e espécies reativas de oxigênio (ERO) e nitrogênio (ERN). O estresse oxidativo é definido como o desbalanço entre altas concentrações de pro-oxidantes em relação aos antioxidantes (Matés 1999). Esse conceito foi aceito por décadas, porém atualmente a definição de estresse oxidativo não pode ser caracterizada apenas como o desbalanço entre pró e antioxidantes a favor dos oxidantes. A existência de vias de sinalização redox discretas sugere que uma definição mais adequada seria que o estresse oxidativo é o desequilíbrio entre oxidantes e antioxidantes a favor dos oxidantes, porém em uma condição que perturbe o controle e a sinalização redox, mais do que apenas o desbalanço, uma condição potencialmente

causadora de dano oxidativo (Jones 2006). O sistema antioxidante é o responsável pela neutralização desses compostos que em concentrações elevadas podem causar dano oxidativo, ocasionando a inativação de enzimas, dano ao DNA e dano nas membranas fosfolipídicas (Cossu et al. 2000; Qiu et al. 2011).

Durante a respiração mitocondrial, por exemplo, de 0,1 a 0,2% do oxigênio consumido é convertido em ERO (Fridovich 2004; Parrilla-Taylor & Zenteno-Savín 2011). Em pequenas concentrações essas ERO são importantes em algumas vias de sinalização, porém em altas concentrações podem ser extremamente prejudiciais aos animais (Amado et al. 2009). Em um estado fisiológico normal esse excesso de ERO são neutralizados pelo sistema antioxidante que apresenta enzimas e moléculas com capacidade de interceptar e neutralizar estas ERO (Blanchette et al. 2007; Souza et al. 2014). O radical livre hidroxila (-OH) é o mais prejudicial, pois apenas moléculas antioxidantes não enzimáticas conseguem atuar nesse radical, portanto não há enzimas que atuem na hidroxila, por isso é importante que seja evitado a sua formação (Borg & Schaich 1984; Winston & Di Giulio 1991). Outro radical comum é o ânion superóxido (O_2^-) o qual é convertido em peróxido de hidrogênio (H_2O_2) pela enzima superóxido dismutase (SOD). Esse peróxido de hidrogênio é por fim convertido em $H_2O + O_2$ pela ação de enzimas como a catalase e a glutationa peroxidase (Di Giulio et al. 1995; Halliwell & Gutteridge 2001). Outra enzima importante é a GST que está envolvida na detoxificação de xenobióticos e proteção ao dano oxidativo (Monteiro et al. 2006; Zhou et al. 2009). As GSTs compreendem uma família de enzimas citosólicas multifuncionais, microssomais ou mitocondriais que catalisam o ataque nucleofílico de glutationa reduzida (GSH) a compostos que possuem um átomo eletrofílico de carbono, de nitrogênio ou de enxofre (Hayes et al. 2005; Huber et al. 2008).

Glutamato cisteína ligase (GCL) é um tripeptídeo que atua no primeiro passo para a síntese de GSH; o segundo passo é catalisado pela glutationa sintetase (liga a glycina formando o GSH) (Franklin et al. 2009). GSH é um tripeptídeo e nos eucariotos 90% da concentração de GSH está no citosol, 10% na mitocôndria e uma pequena porcentagem no retículo endoplasmático e sua função está envolvida na detoxificação de xenobióticos, neutralização de radicais livres, espécies reativas de oxigênio e nitrogênio, modulação da síntese do DNA e funções imunológicas. É uma molécula multifuncional sem dúvida (Lu 2009). Pode reduzir o peróxido de hidrogênio, principalmente na mitocôndria, onde ocorre a respiração celular, pois nesta organela não há catalase (Fernández-Checa et al. 1997; Garcia-Ruiz & Fernández-Checa 2006). Esta molécula também modula a apoptose, pois a concentração de GSH influencia na morte programada da célula (Lu 2009).

A avaliação do dano peroxidativo de lipídeos, via TBARS (substâncias reativas ao ácido tiobarbitúrico), avalia o estresse oxidativo devido à ação causada pelos radicais aos lipídios, a qual resulta na produção de MDA (malondialdeido). O MDA reage com o TBA (2- ácido tiobarbitúrico) em algumas condições como altas temperaturas e acidez gerando um cromógeno que pode ser medido por espectrofotometria ou espectrofluorimetria. Portanto a técnica de TBARS nos permite quantificar o dano oxidativo aos lipídios (lipoperoxidação) (Oakes & Van Der Kraak 2003).

Em um ambiente com condições desfavoráveis, os animais podem sofrer estresse e desestruturar os mecanismos fisiológicos alterando a demanda energética, atividade de enzimas podendo acarretar danos fisiológicos, imunológicos resultando em baixo crescimento e desenvolvimento e inclusive leva-los a morte. Portanto é necessário que se conheça o padrão do metabolismo, as condições ótimas para a espécie cultivada e os

problemas que serão enfrentados quando algum fator possa estar influenciando negativamente, como por exemplo, extremos de temperatura.

1.6. Transporte de organismos aquáticos

O comércio de animais vivos é comum na Ásia e em outros locais onde aquários e tanques dispõem de organismos vivos para o fornecimento de restaurantes e supermercaados o que está se tornando cada vez mais frequente (Fotedar & Evans 2011). No Brasil, o sistema mais usual de transporte de organismos aquáticos é o fechado, utilizando sacos plásticos (Golombieski et al. 2003). As limitações desses sistemas fechados são o suprimento de oxigênio e o aumento da concentração de amônia e dióxido de carbono durante o transporte (Amend et al. 1982). A qualidade de água é afetada de acordo com a densidade de animais colocada em cada saco plástico e o tempo de transporte, tornando-se um fator limitante dependendo do estágio de vida do animal (Hamman 1981; Berka 1986). A temperatura determina a taxa das reações químicas no metabolismo animal (Hazel 1993), influenciando o consumo de oxigênio e sua disponibilidade na água (Ross & Ross 1984). A sobrevivencia ou aumento da densidade podem ser favorecidas reduzindo a temperatura da água (Bercka 1986). Portanto, otimizar a saúde e as condições de estocagem e transporte de animais vivos é fundamental para otimizar a sobrevivência dos animais e o lucro desse comércio emergente. Estudos da resposta ao estresse são fundamentais e formam a base do entendimento de como podemos reduzir o estresse sofrido pelos animais e maximizar a qualidade do produto. Sendo assim, é importante que se estabeleçam métodos que avaliem os parâmetros de estresse de forma simples e eficaz para que se tenha uma boa aplicabilidade no comércio de animais vivos (Fotedar & Evans 2011).

1.6. Sistema imunológico de Crustáceos

O sistema imunológico é fundamental para a sobrevivência e para a proteção do organismo frente a patógenos, vírus, bactérias e parasitas que causam doenças (Male et al. 2013). Os camarões apresentam como primeira linha de defesa a carapaça externa e rígida (exoesqueleto), funcionando como uma barreira física protetora. Assim como outros invertebrados, os crustáceos apresentam apenas um sistema imune inato, diferentemente dos vertebrados que possuem além do sistema inato, um sistema imune adaptativo (Barracco et al. 2008; Male et al. 2013).

O sistema imune inato (filogeneticamente mais antigo) é encontrado em todos os organismos multicelulares, incluindo invertebrados e plantas, enquanto o sistema adaptativo ocorre apenas nos vertebrados. Este último caracteriza-se pela presença de receptores e anticorpos específicos e pela indução de células de memória, que garantem uma resposta de defesa eficiente, rápida e específica contra patógenos (Barracco et al. 2008).

Esta resposta decorre da presença de uma linhagem celular linfocítica, presente apenas nos vertebrados, e nos quais todos os mecanismos de especificidade e de memória imunológica estão interligados. O sistema imune adaptativo depende do reconhecimento do antígeno pelos linfócitos. Sendo assim, sua ausência nos invertebrados inviabiliza o desenvolvimento de vacinas, diminuindo substancialmente a possibilidade de se prevenir e controlar infecções em crustáceos (Barracco et al. 2008).

O sistema imune não adaptativo é baseado em componentes humorais e celulares (Schmid-Hempel, 2003) divididos em aferente (sensitivo) e eferente (efetor) (Beutler 2004). O aferente involve o reconhecimento do patógeno associado a padrões moleculares, os quais são altamente conservados dentro das espécies microbianas e geralmente ausentes no hospedeiro (Janeway & Medzhitov 2002). Isto permite que

receptores do hospedeiro se liguem a patógenos e desencadeiem uma resposta imunológica através da via eferente (Beutler 2004; Bosch 2008). Através de uma variedade de células que são capazes de realizar fagocitose, respostas citotóxicas e inflamatórias, um amplo conjunto de possíveis respostas imunológicas inatas, são acionados quando o organismo é imunologicamente desafiado (Roch 1999). Entender como o sistema imunológico de invertebrados responde a mudanças ambientais é vital para nos ajudar a entender como as interações entre patógeno e hospedeiro são afetadas por essas mudanças, o que pode nos auxiliar no entendimento e previsão de mudanças na imunocompetencia causados pelas variabilidades ambientais que podem impactar os organismos (Morley 2010; Ellis et al. 2011).

O mecanismo imunológico celular predominante dos invertebrados marinhos é a fagocitose desencadeada por células imunológicas. As células imunológicas ou fagócitos podem ser divididos quanto a características funcionais (Pipe 1990; Noël et al. 1994; Pipe et al. 1995). Na fagocitose de patógenos os fagócitos são capazes de reconhecer o não-próprio através das lectinas (Pipe 1990; Mitta et al. 2000) anexando, fagocitando o patógeno e matando o invasor pela produção de compostos citotóxicos e enzimas antimicrobianas (Bachère 1991; Carballal et al. 1997; Ellis et al. 2011).

Com relação ao sistema imune humoral, esse caracteriza-se por moléculas que apresentam efeito destruindo bactérias pela ação de opsoninas e/ou efeito aglutinante no invasor, neutralizando o agente invasor ou causando lise e desestabilização do metabolismo, alteração da permeabilidade da membrana celular dentre outros. Nos invertebrados o sistema imune humoral é avaliado através da habilidade dos organismos combaterem as bactérias pela produção de substâncias antimicrobianas (Elis et al. 2011).

Esses componentes imunológicos estão presentes na hemolinfa dos camarões. Esta hemolinfa circula em um sistema circulatório do tipo aberto ou semi-aberto, correspondente ao sangue dos vertebrados. Na hemolinfa são transportados continuamente nutrientes, excretas, oxigênio, hormônios e outras moléculas importantes para os diferentes órgãos desses animais. Devido à sua fluidez e pela capacidade de atingir diretamente todos os tecidos dos crustáceos, a hemolinfa contém ainda todos os componentes do sistema imunológico (Barracco et al. 2008).

A hemolinfa é composta por uma fração celular, representada pelas células circulantes ou hemócitos, e por uma fração líquida, constituída pelo plasma que contém diferentes fatores humorais. Os hemócitos são envolvidos principalmente com reações imune celulares, tais como fagocitose e encapsulação de patógenos e sua posterior destruição pela produção de moléculas citotóxicas e microbicidas. Os fatores humorais incluem moléculas de não reconhecimento e auto-imunes efetores tais como lectinas, fatores de coagulação e os componentes do sistema profenoloxidase (proPO) (Millar & Ratcliffe 1994; Söderhäll & Cerenius 1992; Roch 1999; Sritunyalucksana & Söderhäll 2000). As respostas imunes celulares e humorais atuam de forma integrada nos crustáceos, protegendo-os contra a invasão de microrganismos e parasitas e garantindo assim sua integridade.

Os parâmetros hemato-imunológicos como o hemograma, atividade da Profeniloxidase, o índice fagocitário e produção de espécies reativas de oxigênio (ERO), têm sido utilizados para monitorar as condições de saúde de crustáceos (Hauton et al. 1997; Le Moullac et al. 1997; Hennig et al. 1998; Sritunyalucksana et al. 1999; Muñoz et al. 2000; Sànchez et al. 2001; Perazzolo et al. 2002). Portanto é importante que seja estudada a imunologia de crustáceos, pois como não fazem parte do sistema

adquirido, seu sistema é menos eficaz, então se o sistema inato for favorecido, os animais terão uma condição melhor de enfrentar adversidades ambientais.

Apesar de ter ocorrido um aumento no número de pesquisas que avaliaram o impacto de fatores estressantes ambientais no sistema imune de invertebrados, ainda existem muitas lacunas no entendimento do sistema imunológico desses animais. Para avançar no conhecimento é necessário que se estabeleçam modelos experimentais de fatores estressantes e avaliar tanto o efeito de cada um, como a combinação desses fatores. Além disso, testar indivíduos de outras famílias e outros filos para que se tenha uma maior abrangência dos resultados e auxilie a compreensão dos impactos das variáveis ambientais (Ellis et al. 2011).

Objetivos:

Avaliar o efeito da temperatura no sistema antioxidante e nos parâmetros imunológicos de camarões peneídeos.

Objetivos específicos:

- Avaliar a atividade enzimática da catalase, GST e dano lipídico de camarões expostos a diferentes temperaturas;

- Avaliar a influencia da temperatura na atividade de GCL, conentração de GSH, dano lipídico e parâmetros imunológicos de camarões cultivados em diferentes temperaturas no sistema de bioflocos;

- Avaliar as diferenças entre os sistemas de cultivo em bioflocos e água clara nos parâmetros fisiológicos e imunológicos;

- Avaliar o efeito da temperatura na simulação de transporte do camarão-rosa *Farfantepenaeus brasiliensis* em diferentes temperaturas.

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Capítulo I

Effect of temperature on antioxidant enzymatic activity in the Pacific white shrimp *Litopenaeus vannamei* in a BFT (Bio-Floc Technology) system

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Abstract

A 10-day trial was conducted to evaluate the effect of temperature on the antioxidant system of *Litopenaeus vannamei* in a Bio-floc technology system. Four treatments in triplicate tanks were assigned to the following temperatures: 15, 21, 27 (control) and 33°C and the water quality parameters were monitored every day. For all enzyme assays, the hemolymph cell lysate of six shrimp per treatment was collected. Lipid peroxidation was assessed by determining the content of thiobarbituric acid reactive substances (TBARS), the activities of catalase (CAT) and glutathione-*S*-transferase (GST) were also evaluated. The results of TBARS showed that shrimp reared at 15 and 21°C presented an increase of 407% (240h) and 339% (120h), respectively. *L. vannamei* exposed to 15°C augmented in 186% (24 h) the activity of catalase. Moreover shrimp of 21°C group also increased CAT activity in 228% (6 h). GST presented the strongest variation reaching 1,437% in shrimp of 15°C at 6 h and 1425% on the 21°C treatment at the same time. All these results were compared to the initial time (27°C). Moreover, the antioxidant system was not sufficient to counteract lipid peroxidation; therefore, the animals reared in 15 and 21°C presented higher enzyme activities, suggesting that cold water can induce oxidative stress in *L. vannamei*.

Keywords: shrimp, oxidative stress, TBARS, GST, CAT, antioxidant system

Introduction

Aquaculture is the fastest growing food-producing sector in the world (FAO 2007). The white shrimp *Litopenaeus vannamei* is one of the most important economic species cultured worldwide and is naturally distributed along the Pacific coast of Central and South America (Zhou et al. 2009). The intensification of *L. vannamei* culture was able through the system with zero water exchange called BFT (Biofloc technology) with microbial flocs, which provides benefits by improving the water quality through the bacterial uptake of nitrogen and conversion of ammonia into cellular proteins that serve as a supplemental source of nutrition (Burford et al. 2004, Wasielesky et al. 2006).

Penaeid shrimp such as *L. vannamei* may be affected by environmental stress factors, leading to problems in growth, survival, osmotic capacity, immune response and the homeostatic regulation of metabolism (Zhou et al. 2009). Water temperature is an environmental factor in aquaculture farming that affects growth and survival (Wang et al. 2006) of aquatic animals. This factor affects many chemical and biological processes, including the amount of dissolved oxygen in the water, the velocity of chemical reactions, photosynthesis, aerobic respiration, the mobility and metabolism of organisms, as well as their sensitivity to toxic substances, parasites and disease (Belló et al. 2000, Jee et al. 2000, Bolton and Havenhand 2005, Mubiana and Blust 2007, Garcia et al. 2011).

In crustaceans, as in other animals, changes in temperature can affect their ability to obtain oxygen from the environment and may promote changes at the physiological level that alter the rate of oxygen consumption and its delivery to tissues (Powell and Watts 2006). In the south of Brazil, there is a wide range of water temperature from 10 (winter) to 30°C (summer) in freshwater and estuary (Garcia et al. 2003, Garcia et al. 2008). This temperature variation occurs due to the temperate climate of southern Brazil, which influences the water temperature and moreover can interfere on physiological conditions of the animals. These variations induce adaptations of the metabolic rate, caused by temperature fluctuations which can be a result of changes of the chemical composition of the hemolymph (Truchot 1975).

In aquatic animals, antioxidant enzymes are believed to play roles in detoxification and protection from oxidative damage, as occurs in other aerobic organisms (Blanchette et al. 2007). Endogenous antioxidants include an enzymatic and a non-enzymatic system, which contain molecules that scavenge reactive oxygen species (ROS) and/or degrade them. The antioxidant enzymes are the first line of

defense against ROS and include superoxide dismutase (SOD), which converts the superoxide radical ($O_2^{\bullet-}$) to peroxide (H_2O_2); catalase (CAT), which reduces H_2O_2 to water; glutathione peroxidase (GPx), which degrades H_2O_2; and organic hydroperoxides (Di Giulio et al., 1995; Halliwell and Gutteridge, 2001). Another important enzyme is glutathione-S-transferase (GST), which is involved in the detoxification of xenobiotics and protection from oxidative damage (Monteiro et al. 2006, Zhou et al. 2009). Evaluation of the antioxidant system has been consistently used as a potential indicator of oxidative stress in several marine invertebrates (Parrilla-Taylor et al. 2013).

During respiration, approximately 0.1–0.2% of the oxygen consumed by aerobic cells is converted to ROS (Fridovich 2004, Parrilla-Taylor and Zenteno-Savín 2011), which in high concentrations can be extremely harmful to cell constituents (Amado et al. 2009). Further damage induced by ROS includes lipid peroxidation, a form of oxidative damage that is usually quantified by determining the tissue content of thiobarbituric acid reactive substances (TBARS). Under normal physiological states, ROS are rapidly eliminated by antioxidant enzymes. All these metabolic antioxidants directly detoxify harmful reactive oxygen species and other compounds involved in ROS generation (Yu 1994, Parodi et al. 2012). Therefore, the aim of this work was to evaluate the effect of water temperature on the antioxidant system of *L. vannamei* reared in a BFT system.

Material and methods

Experimental design

A 10-day trial was conducted at the Marine Aquaculture Station (EMA) at the Universidade Federal do Rio Grande - FURG, in southern Brazil. The experimental system consisted of 12 tanks (250 L) with a bottom area of 0.20 m^2. *Litopenaeus*

vannamei (6.5 g ± 1.8) were stocked in the tanks at a density equivalent to 150 shrimp/m^2 (30 shrimp/tank). Three replicate tanks were assigned to each of the following temperatures: 15; 21; 27 (control) and 33°C. The control temperature was chosen according to the *L. vannamei* physiology in order to provide a preferendum temperature to the animals. To promote the development of the microbial flocs, all experimental tanks received an inoculum of 75 L from a heterotrophic shrimp culture system. The water in the tanks of treatments 21, 27 and 33°C was heated using submerged heaters with thermostat, while the 15°C tanks were cooled using plastic bags containing salt ice (marine water), which was immersed directly in the experimental tanks.

Prior to the experiment, the shrimp were acclimatized for one week at 27°C and 30‰ salinity. The animals were fed twice daily with a commercial diet containing 38% crude protein via a specially designed feeding tray (Wasielesky et al. 2006). The feeding rate was 15% of the total tank biomass and was adjusted daily according to shrimp consumption during the acclimation and the experimental conditions.

Water quality analysis

Throughout the experimental period, the water temperature (mercury thermometer, precision ± 0.5°C), salinity (optical refractometer model RTS – 101, Atago® US, Bellevue, WA, USA, precision: ± 1 g/L), pH (digital pH meter model Handylab 2 BNC, Schott®, Hattenbergstr, Germany, precision: ± 0.01 precision) and dissolved oxygen (dissolved oxygen meter model Handylab/ OXI/set, Schott® Cambridge, UK, precision: ± 0.01 mg L^{-1}) were measured every day. Water samples were collected four times to determine the concentrations of total ammonia (Unesco 1983) and nitrite (Bendschneider and Robinson 1952).

Enzyme assays

For each experimental temperature and sampling point (initial time or 0, 2, 6, 12, 24, 120 and 240 h), the hemolymph of two shrimp from each tank was collected directly from the heart of the shrimp using sterile syringes containing an anticoagulant solution (glucose 0.1 M, sodium citrate 30 M, citric acid 0.026 M, NaCl 0.45 M, EDTA 0.02 M and pH 7.4) (Sotherhall and Smith 1983) to avoid hemolymph coagulation.

For protein quantification and antioxidant enzyme analysis, the hemolymph was centrifuged twice at 500 and 900 x g at 4°C for 35 and 15 min, respectively, to obtain a pellet or cell lysate. After the centrifugation process, the cell lysate was re-suspended in a 4°C buffer solution containing Tris base (20×10^{-3} M), EDTA (1×10^{-3} M), dithiothreitol (1×10^{-3} M), KCl (150×10^{-3} M), and PMSF (0.1×10^{-3} M), with the pH adjusted to 7.6, transferred to 1.5 mL polyethylene tubes and stored at -80°C in an ultra-freezer. All enzymatic measurements were performed at least in triplicate. Total protein content was determined through the Biuret method using a commercial kit (Doles Reagents Ltd., Goiânia, GO, Brazil) and read at 550 nm using a microplate reader (Victor 2, Perkin Elmer).

Catalase (CAT) catalyzes the dismutation of $2H_2O_2$ to O_2 and $2H_2O$. The measurement of catalase activity was based on Beutler (1975). The cell lysate (10 µL) was added to 990 µL of a reaction mixture (5 mM EDTA, 1 M Tris-HCl solution, pH 8.0) and read spectrophotometrically at 230 nm. One CAT unit represents the amount of enzyme needed to degrade 1 µmol of H_2O_2 per min and per mg of total protein present in the cell lysate at 30°C and pH 8.00.

GST catalyzes the conjugation of glutathione to the electrophilic center of lipophilic compounds, increasing their solubility and excretion from the cell. GST activity was performed monitoring the conjugation of 1-chloro-2,4dinitro benzene

(CDNB) with glutathione (GSH) read at 340 nm using a microplate reader (Victor 2, Perkin Elmer). The enzyme assay was performed according to the methods of Habig et al. (1974) and Habig and Jakoby (1981). The 1.0 ml of the enzyme assay mixture contained 50 Mm CDNB ml), 25 mM GSH, and 1.1×10^{-1} M phosphate buffer (K_2HPO_4/KH_2PO_4; pH = 7) was diluted in ethanol (100%). The CDNB was pre mixed with the phosphate buffer before use. The phosphate buffer-CDNB mixture was pre-incubated for 15 min at 25°C and the reaction started by adding GSH, followed immediately by an aliquot (15 µl) of the cell lysate. One GST unit is the amount of enzyme necessary to conjugate 1 µmol of 1-chloro-2,4-dinitrobenzene (Sigma) per min and per mg of total protein present in the cell lysate at 25°C and pH 7.00.

Lipid peroxidation was measured through determination of thiobarbituric acid reactive substances (TBARS), following the methodology of Oakes and Van Der Kraak (2003). The cell lysate (10 µL) was added to a reaction mixture made with 150 µL of 20% acetic acid, 150 µL of thiobarbituric acid (0.8%), 50 µL of Milli Q water and 20 µL of sodium dodecyl sulfate (SDS, 8.1 %). Samples were heated at 95°C for 30 min and after cooling for 10 min, 100 µL of Milli Q water and 500 µL of n-butanol were added. After centrifugation (3,000 x g for 10 min at 15°C), the organic phase (150 µL) was placed in a microplate reader and the fluorescence was registered after excitation at 515 nm and emission of 553 nm. Tetramethoxypropane (TMP, ACROS Organics) was used as standard and TBARS results are expressed in nanomoles of TMP equivalents per mg of protein.

Statistical analysis

Data are expressed as the mean ± SEM and were analyzed through one-way analysis of variance (ANOVA), followed by Newman-Keuls post-hoc mean

comparisons. Assumptions of normality and variance homogeneity were previously checked. In all cases the significance level was fixed in 0.05.

Results

The physicochemical parameters of water quality are shown in Table 1. Although we observed significant differences in the temperature and dissolved oxygen, the water quality parameters analyzed during the experimental period remained at concentrations suitable for shrimp culture at all assayed temperatures.

After 6 h of exposure to 21°C, the hemolymph cells lysate of these shrimp exhibited significantly higher TBARS levels when compared to that of shrimp kept at 15°C (28.1%). After the same exposure period, the hemolymph cells lysate of shrimp maintained at low temperatures (15 and 21°C) showed a significant increase in the level of TBARS when compared to shrimp maintained at 27 and 33°C (766.7%). After 24 h, the only significant result was observed in the hemolymph cells lysate of shrimp maintained at 33°C, which exhibited TBARS levels approximately 229.8, 324.6 and 675.0% higher than the shrimp kept at 15, 21 and 27°C, respectively (P <0.05). After 240 h, the hemolymph cells lysate of shrimp exposed to 15°C presented rises in 100.0, 1,584.2 and 5,233.3% compared to those of the treatments at 21, 27 and 33°C, respectively (P < 0.05). Over the same period, the shrimp maintained at 21°C exhibited approximately 742.1 and 2,566.6% higher TBARS levels than those maintained at 27 and 33°C, respectively (P < 0.05) (Fig. 1A).

CAT activity showed significant differences over the 24 h period in white shrimp exposed to 15 and 33°C compared to those kept at 21°C, exhibiting increases of 28.7 and 57.5%, respectively. At the same extreme temperatures (15 and 33°C), increases of 58.5 and 69.1% were observed, respectively, when compared with the CAT

levels of the hemolymph cells lysate from shrimp maintained at 27°C. After 120 h, white shrimp submitted to 15°C presented CAT levels 127.2% higher than those of shrimp kept at 27°C. After 240 h, the hemolymph cells lysate of shrimp maintained at the control temperature of 27°C exhibited the highest statistical difference in CAT activity, 177.5, 119.3 and 98.5% higher than the CAT activity of shrimp maintained at 15, 21 and 33°C, respectively (Fig. 1B).

After exposure for 2 h, the hemolymph cells lysate of shrimp maintained at 27°C exhibited a significant rise of GST activity of 215.5, 1,134.8 and 158.2% over those of shrimp maintained at 15, 21 and 33°C, respectively. After 6 h, significant differences in GST activity were registered in shrimp maintained at 15 and 21°C when compared to shrimp maintained at 27 and 33°C (916.7 and 1,255.5%, respectively). White shrimp exposed to 33°C for 120 h showed significant increases in GST activity of approximately 27.0 and 487.5% when compared to those of shrimp maintained at 15 and 27°C, respectively. The activity of this enzyme after 240 h presented statistical difference on the hemolymph cells lysate of shrimp maintained at 27°C, which showed increases of 133.3, 110.0 and 162.5% relative to the GST activities registered in shrimp maintained at 15, 21 and 33°C, respectively (Fig. 1C).

The results of each treatment were also analyzed throughout the experimental period, where 15°C treatment produced increased TBARS levels after 2, 6, 120 and 240 h (169.8, 296.8, 344.4 and 407.9%, respectively) compared to the initial time (0 h). White shrimp maintained at 21°C for 2, 6 and 120 h showed an increase in TBARS levels of approximately 339.0% when compared to the initial time. TBARS levels in *L. vannamei* submitted to 27°C after 2 and 120 h exhibited a rise of 352.3% when compared to the initial time. Finally, shrimp submitted to 33°C exhibited increases in

TBARS levels over 2 h (146.0%), 24 h (377.0%), 120 h (192.3%) and a decrease of (90.7%) after 240 h compared to the initial time (Fig. 1A).

Catalase activity in shrimp maintained at 15°C presented an increase in the first 24 h (186.5%) and after 240 h (88.5%) when compared to shrimp from the initial time. Shrimp reared at 21°C exhibited a rise of catalase activity at 6 h (228.0%) when compared to the initial time. *Litopenaeus vannamei* maintained at 27°C exhibited increases in CAT activity at 2 and 6 h (approximately 125.0%) and 240 h (332.0%) when compared to the initial time. The shrimp maintained at 33°C exhibited higher CAT activity levels at 6 h (162.0%), 24 h (216.0%) and 240 h (174.0%) compared to shrimp from initial time (Fig. 1B).

Higher GST activity was registered in the hemolymph cells lysate of shrimp maintained at 15 and 21°C after 6 h (1,437.0 and 1,425.0%, respectively) when compared to initial time. At 21°C, an increase was also observed at 24 h (87.5%) when compared to the initial time. Shrimp maintained at 27°C demonstrated a rise in GST activity at 2 h (255.0%) and 240 h (162.5%) when compared to the initial time. The only difference at 33°C was observed at 120 h, which increased by 488.75% compared to the initial time (Fig. 1C).

Discussion

Environmental factors such as temperature, pH and salinity are known to affect antioxidant responses in *L. vannamei* (Li et al., 2008; Wang et al., 2009; Qiu et al., 2011), but little is known about antioxidant defenses and oxidative stress in *L. vannamei* reared in BFT system and this knowledge is important for understanding the physiological and metabolic responses in shrimp.

In the present study, we observed clear effects of temperature through the experimental period (240 h) on the oxidative stress responses in *L. vannamei* reared in BFT system. Lipid peroxidation (LPO) is one of the major problems associated with failure of the antioxidant system (Castex et al. 2010), furthermore, malondialdehyde (MDA) is a marker of membrane lipid peroxidation that results from the interaction of ROS and cellular membranes (Aslan et al. 1997). In this study, the exposure to low temperatures (15 and 21°C) induced higher LPO levels at 6 h. This increase on LPO levels might have enhanced a system for its detoxification. This can be inferred because shrimp maintained at the lowest temperatures exhibited the highest GST activity, enzymes involved in the detoxification of lipid hydroperoxides (Castex et al. 2010). Schvezov et al. (2013) evaluated the daily variations of the antioxidant system of the crab *Lithodes santolla* and observed that LPO increases in the dark phase (at night), whereas later in the same phase LPO levels decreased following the increase of CAT and GST activities, indicating an important role in the antioxidant defense mechanism.

Qiu et al. (2011) exposed *L. vanammei* to low water temperatures (12°C) and observed that LPO increased in the hemolymph after 3 h, decreased after 6 h, and reached control levels after 12 h. In our experiment, we also found higher LPO levels in hemolymph cells lysate after few hours of exposure (2 h), but the values did not reach the levels found in the control shrimp until 24 h. Therefore, some results, in our work, may be viewed as an endogenous attempt to attenuate the stress condition in relation to the water temperature. Our results revealed, in some cases, an increase of CAT activity at lower temperatures. These results are in agreement with those of Li et al. (2008), who exposed the white shrimp *L. vannamei* to various salinities (3 and 32‰) and found an increase in CAT activity, suggesting that some stress factors may activate this enzyme to scavenge hydrogen peroxide. Therefore, these lower temperatures may induce stress

in animals and could lead to radical accumulation under these conditions. If the radicals induced by low temperature were not scavenged, the organisms would suffer oxidative damage. High activity of CAT under temperature stress can enable shrimp to maintain health by scavenging the radicals produced by these conditions.

In this work, we observed a significant increase in GST activity after 6 h (15 and 21°C). The same was observed for Schvezov et al. (2013), who recorded in *Lithodes santolla* the highest GST activity at 12 and 20 h. Particularly, GST is a phase II enzyme catalyzing GSH conjugation with several molecules, including some by-products coming from lipid peroxidation and it is considered to ameliorate oxidative damage. However, our work opens a new field for studies relating enzyme activity according to the water temperature in the BFT system, which can also be related to the circadian rhythm of animals and should be observed in future studies.

Conclusion

Our results revealed that shrimp submitted to 15 and 21°C exhibited higher enzyme activities, moreover this adverse condition may direct or indirectly promotes stress for the animals. Although it is known that *L. vannamei* can be reared in lower temperatures than 28°C, our results indicate that cold temperatures generate stress in shrimp. This stressful scenario could be unfavorable because greater energy expenditure would be needed to attenuate ROS production. Further studies should be performed to evaluate the endogenous circadian rhythmicity in order to elucidate the daily variations of the antioxidant enzyme activities. These results can contribute to animal welfare and provide useful information to improve shrimp health and performance.

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Figure legends

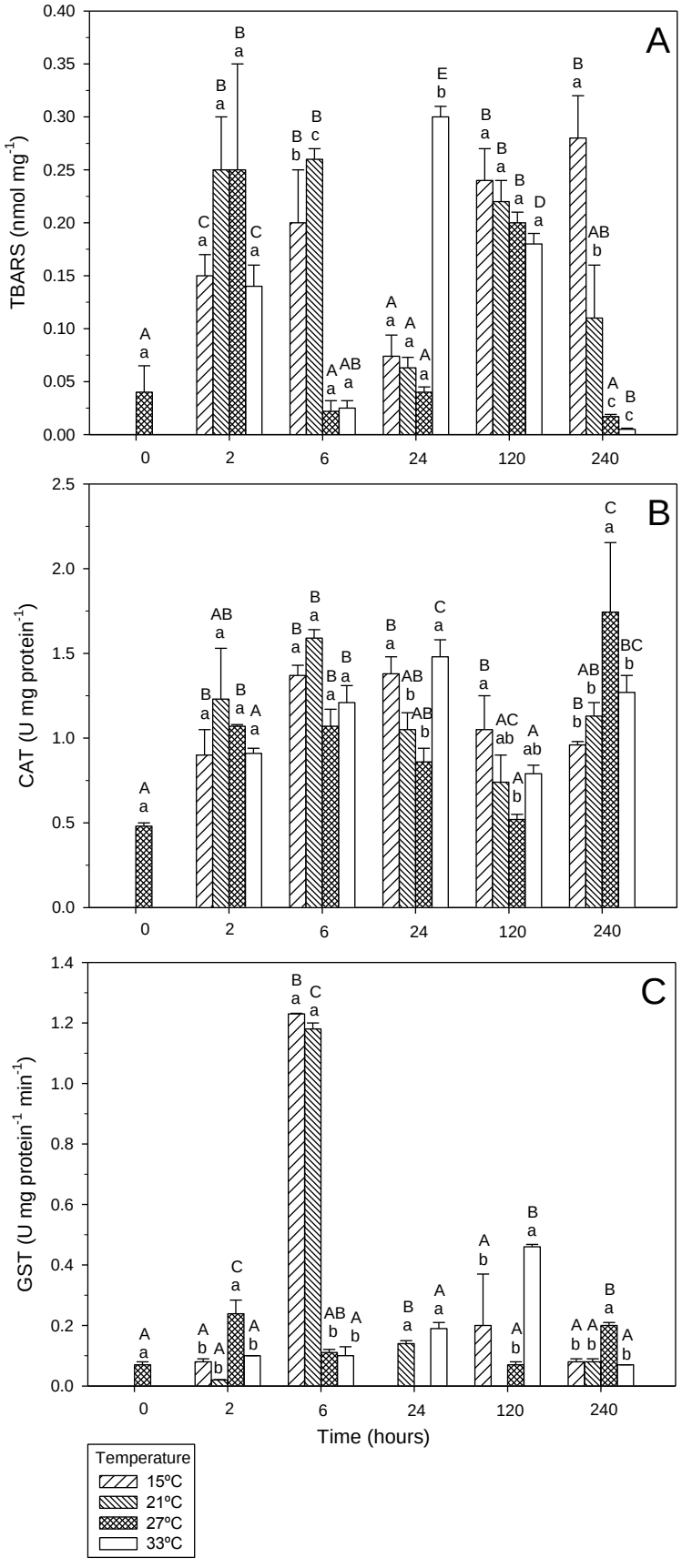
Figure 1. Concentration of thiobarbituric acid reactive substances (TBARS) (A), catalase (CAT) activity (B) and glutathione-S-transferase (GST) activity (C) in the hemolymph cells lysate of *L. vannamei* exposed to different temperatures. The data are expressed as the means ± SEM. Different lower case letters indicate significant differences ($P < 0.05$) among treatments over the same time period. Different capital letters indicate significant differences ($P < 0.05$) in the same treatment at different sample times throughout the experimental period. All bars that are not shown in the graphs are due to a lack of recordable activity.

Table 1. Physicochemical parameters of the water in the experimental tanks. Data are reported as the mean ± SEM. Different letters in the lines indicate significant differences among treatments by one-way ANOVA ($P < 0.05$). Between parentheses, the minimum and maximum values observed throughout the experiment.

Water quality parameters	**Treatments**			
	15°C	**21°C**	**27°C (Control)**	**33°C**
Temperature (°C)	16.1 ± 0.12	21.2 ± 0.08	27.3 ± 0.09	32.7 ± 0.1
	(15.2 – 17.0)	(20.0 – 22.1)	(25.5 – 28.0)	(31.5 – 33.5)
pH	8.1 ± 0.01	8.1 ± 0.01	8.1 ± 0.01	8.2 ± 0.02
	(8.0 – 8.2)	(7.9 – 8.2)	(7.9 – 8.2)	(7.9 – 8.40)
Salinity (‰)	27.8 ± 0.2	29.2 ± 0.06	29.4 ± 0.1	29.5 ± 0.17
	(26.6 – 30.0)	(28.6 – 30.0)	(28.0 – 29.9)	(27.1 – 30.0)
DO (mg L^{-1})	7.6 ± 0.13[a]	6.6 ± 0.12[b]	5.6 ± 0.1[c]	5.0 ± 0.09[d]
	(7.1 – 8.4)	(6.3 – 7.2)	(5.3 – 6.2)	(5.1 – 5.7)
TAN (mg L^{-1})	0.04 ± 0.01	0.06 ± 0.01	0.08 ± 0.02	0.04 ± 0.008
	(0.01 – 0.1)	(0.02 – 0.1)	(0.01 – 0.2)	(0.01 – 0.07)
Nitrite (mg L^{-1})	0.02 ± 0.001	0.02 ± 0.0007	0.05 ± 0.03	0.03 ± 0.005
	(0.01 – 0.03)	(0.02 – 0.03)	(0.03 – 0.5)	(0.01 – 0.06)

DO: Dissolved oxygen, TAN: total ammonia nitrogen

Figure 1


A
B
C
TBARS (nmol mg-1)
CAT (U mg protein-1)
GST (U mg protein-1 min-1)
Time (hours)
0
2
6
24
120
240
Temperature
15ºC
21ºC
27ºC
33ºC

Capítulo II

Antioxidant enzyme activities and immunological system analysis of *Litopenaeus vannamei* reared in BFT (Bio-Floc Technology) at different water temperatures

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Abstract

The purpose of this study was to evaluate the effect of water temperature on the antioxidant (gills, hemolymph and hepatopancreas) and immunological systems (hemolymph) of *L. vannamei* reared with zero water exchange. For this, a 60-day trial was conducted in different temperatures (15; 21; 27 – control and 33 ºC) with 3 replicates tanks each. The immunologic parameters analyzed were: hyaline and granular hemocyte count, total protein and apoptosis. The enzymatic and lipid peroxidation assays consisted of glutamate cysteine ligase (GCL), reduced glutathione (GSH) and thiobarbituric acid-reactive substances (TBARS). The immunological parameters did not show significant differences ($p>0.05$) among treatments throughout the experimental period. The results revealed that shrimp exposed to 15 ºC at 30 days exhibit significantly higher GCL activity in gills compared to 21 ºC (1,166.28%), 27 ºC (422.96%) and 33 ºC (809.37%). *L. vannamei* reared at 15 ºC showed the highest GSH concentration at 30 days. The increase in gills was 390.29; 376.94 and 361.75% compared to 21; 27 and 33 ºC, respectively. The TBARS results at 30 days shown that shrimp submitted to 33 ºC presented higher levels (6,157.14; 8,620.00 and 14,336.66% compared to 15; 21 and 27 ºC, respectively). Our results demonstrated that shrimp reared in 15 ºC exhibited higher activity of the rate-limiting for GSH production (GCL) and higher concentration of this non-enzymatic antioxidant, an attempt to counteract the overproduction of reactive oxygen species generated by temperature stress and at 33 ºC the animals shown higher lipid peroxidation. The best results were observed in shrimp reared at 27 ºC (control). Therefore, the extreme temperatures should be avoided.

1. Introduction

Shrimp culture worldwide has grown substantially on the last decade; moreover the white shrimp *Litopenaeus vannamei* is one of the most important economic species cultured and is distributed along the Pacific coast of Central and South America (Zhou et al. 2009). The intensification of *L. vannamei* culture was achieved through the system with zero water exchange called BFT (Biofloc technology), with microbial flocs. This system provides benefits by improving the water quality through nitrogen concentration reduction and conversion of ammonia into cellular proteins that serves as a supplemental source of nutrition (Burford et al. 2004, Wasielesky et al. 2006). In addition, BFT system could enhance immune cellular response and antioxidant status of cultured shrimp (Xu and Pan, 2013). The circulating hemocytes play an important role in the immune defense system (cellular and humoral) (Bachère et al., 2004) and the hemocyte count has been considered a functional indicator of immune capability (Rodríguez and Le Moullac, 2000).

In terms of the immune responses, apoptosis is described as an effector mechanism in invertebrates (Clem and Miller, 1993; 1994). Apoptosis is essential in both development and the homeostasis of multicellular organisms (Chang et al., 2009). Apoptosis has been known as an effector mechanism in invertebrates (Clem and Miller, 1993, 1994), but in mammals the apoptosis regulates the adaptive response (Koyama et al., 2000). If apoptosis happens at the early stage of viral infection, there is an increase in the number of apoptotic cells to block viral replication in shrimp (Everett and McFadden, 1999). Nevertheless, if viruses induce apoptosis at the late stage of viral infection, the viral replication may be facilitated and the virus may be spread (Best, 2008). In addition, oxidative stress is known to be an important induction factor for cell apoptosis (Ermak and Davies, 2001). Reactive oxygen species (ROS) overproduction

paralleled with hemocyte apoptosis was observed in shrimp exposed to some environmental stress factors (Xian et al. 2010; Xian et al. 2011).

Penaeid shrimp such as *L. vannamei* may be affected by environmental stress factors as temperature, leading to problems in growth, survival, osmotic capacity, immune response, and the regulation of metabolism (Zhou et al., 2009; Souza et al. 2014). Any change in the water temperature can trigger negative effects on the growth and survival of aquatic organisms (Hennig and Andreatta, 1998; Wang et al. 2006). In addition, this factor can affect chemical and biological processes, including the amount of dissolved oxygen in the water and the velocity of chemical reactions (Belló et al., 2000; Bolton and Havenhand, 2005; Jee et al., 2000; Mubiana and Blust, 2007; Garcia et al., 2011).

In the south of Brazil, there is a wide variation of water temperature from 10 to 30 °C (Garcia et al., 2003; Garcia et al., 2008) throughout the year. These changes on temperature can promote stress to the animals according to the time of exposure to these conditions. To counteract the stress and prevent the oxidative damage, the animals must have some mechanisms as the antioxidant system.

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen. They form as a natural by-product of the normal mitochondrial metabolism of oxygen and have important roles in cell signalling and homeostasis (Cadenas, 1989). Low levels of reactive oxygen species are important for maintaining and regulating homeostasis (Ames et al., 1993), signaling mechanisms and diverse cellular functions, such as secretion, growth and gene expression (Halliwell and Gutteridge, 1997). However, in stress situations, ROS levels can increase (Cadenas, 1989). When the production and accumulation of ROS is beyond the organism's capacity to counteract

these reactive species then occur oxidative damage in several biomolecules, including lipids, proteins and deoxyribonucleic acid (DNA) (Chang et al., 2009).

The protection against the damages caused by ROS is due to the activities of some antioxidant as glutathione (GSH), an endogenously synthesized tripeptide thiol, with important biochemical and antioxidant properties. GSH is part of numerous basic cellular processes including protein synthesis, DNA synthesis and repair, cell proliferation, and redox signaling (Meister, 1983; Wu et al., 2004; Townsend, 2007). As an antioxidant, GSH scavenges ROS, RNS (reactive nitrogen species), and other free radicals (Rahman et al., 2004; Haddad and Harb, 2005; Rahman et al., 2005). The GSH biosynthetic capacity is controlled by multiple factors, including substrate availability (especially cysteine) and the activity of glutamate cysteine ligase (GCL), the rate-limiting enzyme in GSH synthesis (Griffith, 1999; Griffith and Mulcahy, 1999). Because GCL is a major determinant of cellular GSH levels, many studies have investigated the factors that regulate GCL expression and activity. Moreover, oxidative stress has been shown to stimulate GCL activity (Lu, 2009; Franklin et al., 2009). As a result, GSH levels may increase and the antioxidant defense become stronger to counteract the ROS. Further damage induced by ROS includes lipid peroxidation (LPO), a form of oxidative damage that is usually quantified by determining the tissue content of thiobarbituric acid-reactive substances (TBARS). Therefore, the purpose of this study was to evaluate the effect of water temperature on the antioxidant and immunological systems of *L. vannamei* reared in BFT system.

2. Material and methods

2.1 Experimental design

A 60-day trial was conducted at the Marine Aquaculture Station at the Universidade Federal do Rio Grande – FURG, in southern Brazil. The experimental system consisted of 12 tanks (250 L) with a bottom area of 0.20 m^2. *L. vannamei* (1.91 g ± 0.22) were stocked in the tanks at a density equivalent to 200 shrimp/m^2 (40 shrimp/tank). Three replicate tanks were assigned to each of the following temperatures: 15, 21, 27 (control) and 33 °C. The control temperature was chosen according to the *L. vannamei* physiology in order to provide an optimum temperature to the animals. To promote the development of the microbial flocs, all experimental tanks received an inoculum of 75 L from a heterotrophic shrimp culture system before start the experiment. When the ammonia concentration reached values of 1 mg/L or higher, these tanks received a molasses dose calculated according to the equations proposed by Ebeling et al. (2006) and Avnimelech (1999). The water in the tanks of treatments 21, 27, and 33 °C was heated using submerged heaters with thermostat, while the 15 °C tanks were cooled using plastic bags containing salt ice (marine water), which was immersed directly in the experimental tanks (Souza et al. 2014).

Prior to the experiment, the shrimp were acclimated for 10 days at 27 °C and 30‰ salinity. The animals were fed twice daily with a commercial diet containing 38% crude protein via a specially designed feeding tray (Wasielesky et al. 2006). The feeding rate was 15% of the total tank biomass and was adjusted daily according to shrimp consumption during the acclimation and the experimental conditions. At the end of experimental period the survival (final number of shrimp/initial number of shrimp) x 100; final weight (FW) and specific growth rate (SGR) ((ln(final weight) – ln(initial weight))/trial duration x 100 were evaluated.

2.2 Water quality analysis

Throughout the experimental period, the water temperature (mercury thermometer, precision ± 0.5 °C), salinity (optical refractometer model RTS – 101, Atago® US, Bellevue, WA, USA, precision: ± 1 g L^{-1}), pH (digital pH meter model Handylab 2 BNC, Schott®, Hattenbergstr, Germany, precision: ±0.01 precision), and dissolved oxygen (dissolved oxygen meter model Handylab/OXI/set, Schott® Cambridge, UK, precision: ± 0.01 mg L^{-1}) were measured every day. Water samples were collected three times a week to determine the concentrations of total ammonia (UNESCO, 1983) and nitrite (Bendschneider and Robinson, 1952). Alkalinity and nitrate were monitored once a week according to the methods of Baumgarten et al. (1996) and Aminot and Chaussepied (1983), respectively.

2.3 Imunological assays

Granular and hyaline hemocyte count was determined using a Neubauer chamber, after collecting the hemolymph at 30 and 60 days of six shrimp/treatment directly into an anticoagulant solution (1:4) (modified Alsever solution or MAS: 27 mM sodium citrate, 336 mM sodium chloride, 115 mM glucose, 9 mM EDTA, pH 7.0) (Maggioni et al. 2004). Total protein concentration (TPC) in shrimp serum (six animals per treatment) was determined according to the Bradford method (1976) using bovine serum albumin (BSA) as standard (Maggioni et al. 2004). For the detection of apoptosis, shrimp hemolymph (5 μL) was smeared onto a clean glass slide and air-dried, with 5 clean slides per treatment. The hemocytes in apoptosis were evaluated by the TUNEL method using the ApopTag® Plus Peroxidase In Situ Apoptosis Detection kit (Millipore) according to Charriaut-Marlangue and Ben-Ari (1995) and Wang and Zhang (2008).

2.4 Enzyme assays

The hemolymph and tissues (gills and hepatopancreas) of nine shrimp per treatment (three from each tank) were sampled randomly at 15; 30; and 60 days. Hemolymph was collected directly from the heart of the shrimp using sterile syringes containing an anticoagulant solution (glucose 0.1 M, sodium citrate 30 M, citric acid 0.026 M, NaCl 0.45 M, EDTA 0.02 M, and pH 7.4) (Sötherhall and Smith, 1983) to avoid hemolymph coagulation. For protein quantification and antioxidant enzyme analysis, the hemolymph was centrifuged twice at 500 and 900 × g at 4 °C for 35 and 15 min, respectively, to obtain a pellet or cell lysate. After the centrifugation process, the cell lysate was re-suspended in a 4 °C buffer solution containing Tris base (20×10^{-3} M), EDTA (1×10^{-3} M), dithiothreitol (1×10^{-3} M), KCl (150×10^{-3} M), and PMSF (0.1×10^{-3} M), with the pH adjusted to 7.6, transferred to 1.5 mL polyethylene tubes and stored at −80°C in an ultra freezer. The gills and hepatopancreas samples (for protein quantification and antioxidant enzyme analysis) were weighed, then added 1:5 of buffer solution Tris base (20.0×10^{-3} M), EDTA (1.0×10^{-3} M), dithiothreitol (1.0×10^{-3} M), KCl (150.0×10^{-3} M), and Sucrose (8.0×10^{-3} M) and centrifuged for 30 min at 9,000 x *g* at 4 ºC. The supernatant was transferred to 1.5 mL polyethylene tubes and stored in an ultra freezer (-80 ºC) to perform the analysis. All enzymatic measurements were performed at least in triplicate. Total protein content was determined through the Biuret method using a commercial kit (Doles Reagents Ltd. Goiânia, GO, Brazil) and read at 550 nm using a microplate reader (Victor 2, Perkin Elmer).

2.4.1 GCL activity and GSH concentration

These analysis followed the methodology described by White et al. (2003). The GCL and GSH assays were performed at the same time, one plate for each analysis. The GSH plate was kept on ice during all process. The GCL reaction cocktail (40 mM ATP, 20 mM L-glutamic acid, 20 mM sodium borate, 2 mM serine, 40 mM $MgCl_2$) were diluted in Tris-HCl (400 mM) and EDTA (2 mM) solution. On a separate 96-well round-bottom reaction plate, 25 µl aliquots of this reaction cocktail were pipetted into wells (3 per sample). These plates were either prepared just prior to the assay to prevent degradation of ATP in the reaction cocktail. While the sample plate was kept on ice, 25 µl aliquots of sample were pipetted into the pre-warmed (25 °C) reaction plate (GCL) at 15-s time intervals. After a 5 min pre-incubation, the GCL reaction was initiated by adding 25 µl of 2 mM cysteine (dissolved in Tris-HCl 100 mM, EDTA 2 mM, $MgCl_2.6H_2O$ 5 mM) to each GCL activity well (cysteine was not added to the GSH-baseline wells at this time). The plate was then vortexed, covered, and incubated for one hour. The GCL reaction was stopped by adding 25 µl of 200 mM 5-sulfosalicylic acid to all wells, and then 25 µl of 2mM cysteine was added to the GSH-baseline wells. The plate was then vortexed and held on ice for at least 20 min. Following protein precipitation, the plate was centrifuged for 5 min at 2,500 rpm on a tabletop centrifuge. Following centrifugation, 20 µl aliquots of supernatant from each well of the reaction plate were transferred to a 96-well plate designed for fluorescence detection. Next, 180 µl of 2,3-naphthalenedicarboxaldehyde (NDA) derivatization solution (50mM Tris, pH 10, 500mM NaOH, and 10mM NDA- previously diluted in Me_2SO) according to the follow proportion v/v/v 1.4/0.2/0.2 was added to all wells of this plate. The plate was covered to protect the wells from room light and allowed to incubate at room temperature for 30 min. Following incubation, NDA–γ-GC or NDA–GSH fluorescence

intensity was measured (485 excitation/535 emission wavelengths) on a fluorescence plate reader (Victor 2, Perkin Elmer).

2.4.2 Lipid peroxidation (LPO) assay

LPO was measured through determination of TBARS, following the methodology of Oakes & Van Der Kraak (2003). The cell lysate (10 μL) was added to a reaction mixture made with 150 μL of 20% acetic acid, 150 μL of thiobarbituric acid (0.8%), 50 μL of Milli Q water, and 20 μL of sodium dodecyl sulfate (SDS, 8.1%). Samples were heated at 95 °C for 30 min and after cooling for 10 min, 100 μL of Milli-Q water and 500 μL of n-butanol were added. After centrifugation (3000x g for 10 min at 15 °C), the organic phase (150 μL) was placed in a microplate reader and the fluorescence was registered after excitation at 520 nm and emission of 580 nm. Tetramethoxypropane (TMP, ACROS Organics) was used as standard and TBARS results are expressed in nanomoles of TMP equivalents per mg of protein.

2.5 Statistical analysis

Data are expressed as the mean ± SEM and were analyzed through one-way analysis of variance (ANOVA), followed by Newman-Keuls post hoc mean comparisons. Assumptions of normality and variance homogeneity were previously checked. In all cases, the significance level was fixed in 0.05.

3. Results

3.1 Water quality

The physicochemical parameters of water quality are shown in Table 1. Although we observed significant differences in pH, alkalinity and nitrate, the water

quality parameters analyzed during the experimental period remained at concentrations suitable for shrimp culture at all assayed temperatures. As showed in Table 2, *L. vannamei* submitted to 21 ºC demonstrated significantly lower and SGR over those reared at 27 ºC. In addition, shrimp reared in extremes temperatures (15 and 33 ºC) presented lower survival (55.83 and 29.16%, respectively).

3.2 Immunological analysis

The immunological parameters evaluated in this work did not show significant differences ($p>0.05$) among treatments throughout the experimental period (Table 3).

3.3 Antioxidant system analysis

3.3.1 GCL activity in hemolymph

The GCL activity in the hemolymph of those shrimp kept at 33 ºC in 15 days presented a rise in 298.95; 335.62 and 382.27% in relation to the treatments 15, 21 and 27 ºC, respectively. At 60 days, the treatment 33 ºC presented significantly higher GCL activity 756.01 and 5,120.24% compared to 15 and 27 ºC, respectively (Fig. 1A). In hemolymph, shrimp reared at 15 e 21 ºC presented at 30 days a rise in GCL activity when compared to 15 and 60 days. At 27 ºC the shrimp hemolymph has shown at 15 and 30 days an increase of GCL activity (Fig. 1A).

3.3.2 GSH concentration in hemolymph

The concentration of GSH at 15 days in the hemolymph of shrimp reared at 27 ºC was 304.48, 234.18 and 248.52% higher compared to 15, 21 and 33 ºC, respectively (Fig. 1B). Shrimp exposed to 15 e 33 ºC presented at 60 days a rise in GSH concentration compared to 15 and 30 days. Shrimp reared at 27 ºC shown at 30 days a

decrease in GSH concentration (44.46 and 50.05% compared to 15 and 60 days, respectively Fig. 1B).

3.3.3 TBARS in hemolymph

The TBARS results at 30 days from hemolymph of shrimp reared at 27 ºC was 336.36 and 846.96% lower compared to 15 and 33 ºC, respectively. Shrimp of treatment 33 ºC at 30 days presented, in hemolymph, the highest result of TBARS (251.80, 417.16, 846.96% compared to 15, 21 and 27 ºC, respectively Fig. 1C). The TBARS results of shrimp exposed to 33 ºC at 30 days shown an increase of 1,552.77 and 191.43% compared to 15 and 60 days, respectively. In addition, at 60 days in the same conditions, the increase was 811.11% compared to 15 days (Fig. 1C).

3.3.4 GCL activity in gills

Analyzing the same period of exposure in the different treatments, shrimp exposed to 15 ºC at 30 days exhibit significantly higher GCL activity in gills compared to 21 ºC (1,166.28%), 27 ºC (422.96%) and 33 ºC (809.37%) (Fig 2A). Shrimp submitted to 21 ºC at 15 days presented a decrease of GSH concentration in gills compared to 15 ºC (49.57%) (Fig 2A). In gills, the treatment 15 ºC presented significantly differences among GCL activity throughout the experimental period. At 30 days this analyzes shown the highest result, being 4,786.97 and 614.42% higher when compared to 15 and 60 days, respectively. At the end of the experimental period (60 days) the GCL activity was 779.10% higher in relation to 15 days. *L. vannamei* reared at 27 ºC presented in 15 days 1,698.69% lower GCL activity compared to 60 days. Shrimp of 33 ºC group presented at 15 days approximately 1,357.76% lower GCL activity compared to 30 and 60 days (Fig 2A).

3.3.5 GSH concentration in gills

L. vannamei reared at 15 ºC showed the highest GSH concentration at 30 days. The increase in gills was 390.29; 376.94 and 361.75% compared to 21, 27 and 33 ºC, respectively (Fig 2B). White shrimp exposed to 21 ºC at 15 days demonstrated in gills a decrease of GSH concentration 49.57% in relation to 15 ºC (Fig 2B).

3.3.6 TBARS in gills

TBARS results in gills of *L. vannamei* exposed to 21 ºC (Fig 2C) presented a rise of 223.40; 284.02 and 705.17% in relation to 15, 27 and 33 ºC, respectively. At 30 days, shrimp submitted to 15 ºC presented an increase in lipid peroxidation of 481.67; 526.76 and 484.57% compared to 21, 27 and 33 ºC, respectively. The TBARS results of shrimp exposed to 15 ºC shown at 30 days a rise of 1,321.31 and 582.65% in relation to days 15 and 60, respectively. Shrimp reared at 33 ºC presented at 15 days a lower TBARS content, representing a decrease of 860.34 and 968.96% compared to 30 and 60 days, respectively (Fig. 2C).

3.3.7 GCL activity in hepatopancreas

The GCL activity in hepatopancreas of shrimp exposed at 33 ºC at 15 days presented a rise of 179.90; 228.80 and 401.29% compared to 15, 21 and 27 ºC, respectively (Fig. 3A). At 30 days, shrimp maintained at 27 ºC presented, in hepatopancreas, GCL activity 545.40% higher compared to 33 ºC (Fig. 3A). *L. vannamei* submitted to 21 ºC demonstrated an increase in GCL activity at 60 days of 2,120.53 and 282.26% when compared to 15 and 30 days, respectively. Shrimp reared at 27 ºC presented at 30 and 60 days a rise in GCL activity of approximately 1,450.77% in relation to 15 days (Fig. 3A). At 60 days, shrimp exposed to 21 ºC showed a rise in

GCL activity of 653.73, 294.60 and 273.13% in relation to 15, 27 and 33 ºC, respectively.

3.3.8 GSH concentration in hepatopancreas

The GSH concentration of shrimp kept at 15 ºC (60 days) was 349.52 and 589.87% lower compared to 27 and 33 ºC, respectively (Fig. 3B). According to the results of GSH concentration, shrimp submitted at 21, 27 e 33 ºC presented at 15 days approximately 3,000.00% lower GSH concentration compared to 60 days. At 27 ºC the animals also demonstrated at 15 days a decrease of GSH concentration of approximately 3,578.10% in relation to 30 days (Fig. 3B).

3.3.9 TBARS in hepatopancreas

The TBARS results at 30 days shown that shrimp submitted to 33 ºC presented an increase of 6,157.14; 8,620.00 and 14,336.66% compared to 15, 21 and 27 ºC, respectively (Fig. 3C). Shrimp submitted to 21 ºC presented higher level of lipid peroxidation at 15 days being 940.00 and 1,044.44% compared to 30 and 60 days, respectively. *L. vannamei* exposed to 33 ºC showed higher TBARS results at 30 days (3,591.66 and 8,795.91% compared to 15 and 60 days, respectively) (Fig. 3C).

4. Discussion

Environmental fluctuations associated with seasonal climatic changes are of major importance in triggering adjustments in the physiology and behavior of aquatic organisms. Many aquatic organisms are adapted to temperature variation allowing them to proceed efficiently at different temperatures (Brett and Groves, 1979; Akhtar et al., 2014). Changes in body temperature can compromise membrane integrity and function

because of alterations in their physical properties (Skalli et al., 2006). Some aquatic animals adjust the compositions of their membrane lipids to minimize the effects of temperature changes (Farkas et al., 1980). It is known that the fatty acid composition of animal tissues can be modified by a change in environmental temperature, and the proportions of unsaturated to saturated fatty acids varies when the environmental temperature changes (Hilditch and Williams, 1964; Akhtar et al., 2014). The decrease of environmental temperature may increase the insaturation of fatty acids favoring the lipoperoxidation and it is considered to be one of the key events in oxidative damage (Cossu et al., 2000; Doyotte et al., 1997; Livingstone et al., 1990). In fact, in this study it was observed higher lipid peroxidation levels in animals reared at 15 ºC.

In this work, hemocyte counts did not show significantly differences among treatments, when evaluated the long exposure (60 days) to different water temperatures. Li et al., (2014) also evaluated the effect of temperature decrease on hemocyte apoptosis of the white shrimp *Litopenaeus vannamei* and found that the total hemocyte count (THC) did not show significant difference when the water temperature decreased to 23 and 20 ºC, but at 17 ºC the THC decreased significantly. In that work the authors decreased 1 ºC/h the water temperature and evaluated the short exposure (9 h) sampling every 3 h in clear water system with salinity 5 ‰ which could be influenced because it is known that biofloc from shrimp culture water is rich in bioactive compounds including carotenoids, chlorophylls, polysaccharides, phytosterols, taurine and fat-soluble vitamins (Ju et al., 2008a) which can contribute to a healthy environment. Carotenoids have been reported to improve animal immune systems, increase stress tolerance and present antioxidant functions (Babin et al., 2010; Linan-Cabello et al., 2002). Li et al. (2014) found an increase of apoptotic cell ratio at 17 ºC. In our study, we evaluated the long exposure to different water temperatures that was constant during 60

days and we did not find differences, probably, by the same reason of THC. We evaluated the long exposure; in contrast, these authors evaluated the short one. Moreover, in shrimp without virus infection or that was not exposed to factors and substances that induce apoptosis, the number of apoptotic cells decreases meaning an improvement of immune system (Macias-Sancho et al., 2014)

Considering the results of survival, the animals of treatments 15 and 33 ºC could be suffered a strong selection remaining only the ones that presented better antioxidant competence that allowed them to survive in these conditions. The animals that suffered a strong oxidative damage, as a result, died. The reminiscent animals developed resistance to survive and throughout the experimental period did not show these differences. The results of survival rate of the extremes temperatures 15 ºC (55.83%) and 33 ºC (29.16%) suggest that the temperature selected the most prepared animals to survive in stressful conditions. These animals developed some mechanisms, as an example, the activity of the antioxidant system, to resist to those extremes temperatures. Probably the energetic expenditure of the antioxidant system was higher.The formation of the antioxidant GSH is ATP-dependent. Thus, there is energy cost in this process. Therefore, when the animals presented higher GSH concentration, as a result, higher energy was required (Chen et al., 2005); consequently, the animal growth was lower in these treatments.

Lower temperature showed increase in ROS content of those shrimp submitted to 17 ºC (Li et al., 2014). This evidence was confirmed, in our study, when *L. vannamei* exposed to 15 ºC since it demonstrated a rise in GCL activity and GSH concentration to counteract a pro-oxidant condition. In gills, these attempt to counteract the free radicals were not sufficient because the LPO was high at 30 days. In addition, the increase of antioxidant system activity at 30 days in gills, was not sufficient to reduce the LPO

levels, leading the animals to suffer an oxidative damage. At 60 days, the LPO result was lower compared to 30 days. These results can be explained because this treatment presented the lowest survival, suggesting that the reminiscent animals presented naturally a high antioxidant competence that allow them keep alive and reduce the lipid peroxidation. In hemolymph, the animals of both extremes temperatures (15 and 33 ºC) presented higher levels of LPO that was not completely compensated by the antioxidant system confirming that the oxidative stress generated by temperature. In hepatopancreas, shrimp reared at 33 ºC also presented oxidative damage.Seasonal variations in metabolic rate are assumed to induce alterations in reactive oxygen species (ROS) generation (for review see Abele and Puntarulo, 2004). In general, ROS production rates in marine invertebrates should be much reduced when compared to endotherms with a constant body temperature above 35 ºC (Abele and Puntarulo, 2004). Specifically, a decrease in the activity of antioxidant defenses in the digestive gland of *Mytilus edulis* has been observed in winter, accompanied by an increase in the lipid peroxidation (TBARS) (Viarengo et al., 1991). In the present study we observed higher lipid peroxidation in gills of *L. vannamei* reared at 15 ºC. Summer metabolic rates increased by approximately 3-fold over winter values (Malanga et al., 2007). Therefore, higher temperatures can induce higher ROS generation rate, resulting in augmented oxidative stress as could be observed high lipid peroxidation in hepatopancreas of shrimp reared at 33 ºC.

Souza et al. (2014) analyzed the antioxidant system of *L. vannamei* exposing them to low water temperatures at a short time and also observed an augment in antioxidant enzymes activities of shrimp submitted to 15 and 21 ºC. These results demonstrated that low water temperatures induce a stressful scenario that sometimes demonstrated a failure of antioxidant system to counteract the overproduction of ROS

as we can see in gills of shrimp reared at 15 ºC in the present work. Qiu et al. (2011) demonstrated that acute low temperature (12 ± 2 °C) can induce oxidative stress and elevate levels of DNA damage and malondialdehydes in plasma of the shrimp *L. vannamei*. Evaluation of seasonal variations in the antioxidant system and LPO of mussels (*Mytitus galloprouinciulis*) caused a reduction of the antioxidant defense systems, which are directly responsible for an increased susceptibility to oxidative stress during the winter (Viarengo et al., 1991), which are in agreement with our results.

Juvenile shrimps of *Macrobrachium nipponense* were submitted to different temperatures (16-32 ºC) and the results revealed their best growth at 25 °C, and their oxidative stress was the lowest (Wang et al. 2006). The results of their study also suggested that chronic exposure to colder and warmer temperature causes an increase in oxidative stress. The same authors mentioned that shrimp exposed to colder and warmer temperatures usually translate into a higher energy activity that reflects the energetic costs with maintenance or standard metabolism. Our results are in agreement with Wang et al. (2006). The best results of the present study were achieved by the animals reared at 27 ºC which presented better performance and the lowest oxidative stress.

5. Conclusion

Our results revealed that shrimp reared in 15 and 33 °C exhibited higher antioxidant enzyme activities as an attempt to counteract the overproduction of ROS generated by temperature stress.

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Figure legends

Figure 1. GCL activity (2A), GSH concentration (2B) and thiobarbituric acid reactive substances (TBARS) (2C) in hemolymph of *L. vannamei* exposed to different temperatures. The data are expressed as the means ± SEM. Different lower case letters indicate significant differences ($P < 0.05$) among treatments over the same time period. Different capital letters indicate significant differences ($P < 0.05$) in the same treatment throughout the experimental period.

Figure 2. GCL activity (1A), GSH (1B) concentration and thiobarbituric acid reactive substances (TBARS) (1C) in gills of *L. vannamei* exposed to different temperatures. The data are expressed as the means ± SEM. Different lower case letters indicate significant differences ($P < 0.05$) among treatments over the same time period. Different capital letters indicate significant differences ($P < 0.05$) in the same treatment throughout the experimental period.

Figure 3. GCL activity (3A), GSH concentration (3B) and thiobarbituric acid reactive substances (TBARS) (3C) in hepatopancreas of *L. vannamei* exposed to different temperatures. The data are expressed as the means ± SEM. Different lower case letters indicate significant differences ($P < 0.05$) among treatments over the same time period. Different capital letters indicate significant differences ($P < 0.05$) in the same treatment throughout the experimental period.

Table 1. Physicochemical parameters of the water in the experimental tanks. Data are reported as the mean ± SEM. Different superscript letters in the lines indicate significant differences among treatments by one-way ANOVA ($P < 0.05$).

	Treatments			
Parameter	**15°C**	**21°C**	**27°C (control)**	**33°C**
Temperature (°C)	15.63 ± 0.14^{a}	21.14 ± 0.06^{b}	26.89 ± 0.15^{c}	32.47 ± 0.06^{d}
pH	8.14 ± 0.01^{a}	7.73 ± 0.01^{b}	7.68 ± 0.02^{b}	7.74 ± 0.02^{b}
Salinity (g L^{-1})	29.14 ± 0.21^{a}	31.87 ± 0.14^{a}	31.88 ± 0.24^{a}	32.36 ± 0.25^{a}
DO (mg L^{-1})	7.97 ± 0.04^{a}	6.82 ± 0.03^{b}	5.91 ± 0.03^{c}	5.19 ± 0.02^{d}
Alkalinity (mg L^{-1})	113.4 ± 1.83^{a}	88.2 ± 5.49^{b}	84.63 ± 5.91^{b}	85.5 ± 5.48^{b}
TAN (mg L^{-1})	0.14 ± 0.01^{a}	0.14 ± 0.01^{a}	0.15 ± 0.01^{a}	0.14 ± 0.01^{a}
Nitrite (mg L^{-1})	0.07 ± 0.006^{a}	0.05 ± 0.005^{a}	0.07 ± 0.006^{a}	0.07 ± 0.008^{a}
Nitrate	18.18 ± 1.74^{a}	34.31 ± 3.12^{b}	42.02 ± 4.77^{bc}	49.07 ± 4.89^{c}

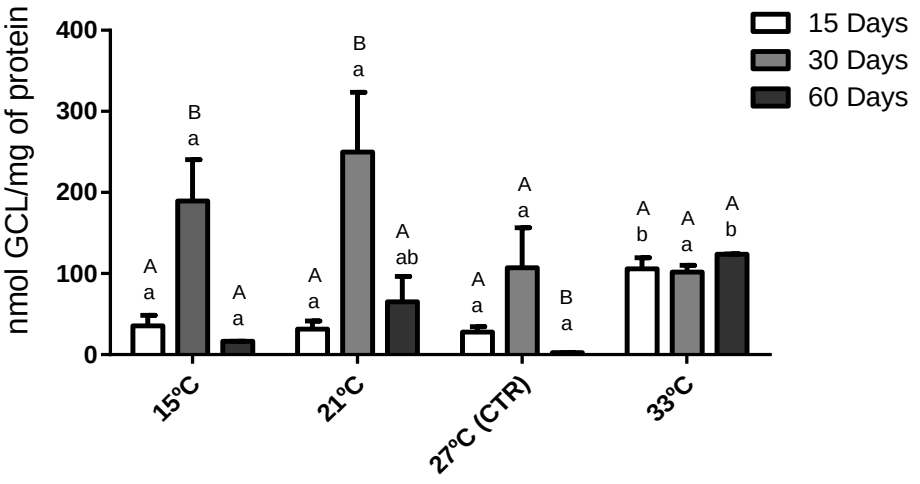
DO: Dissolved oxygen, TAN: total ammonia nitrogen

Table 2. Survival, final weight (FW) and specific growth rate (SGR) (mean ± SEM) of shrimp reared in different temperatures. Different superscript letters indicate significant differences among treatments by one-way ANOVA ($P < 0.05$).

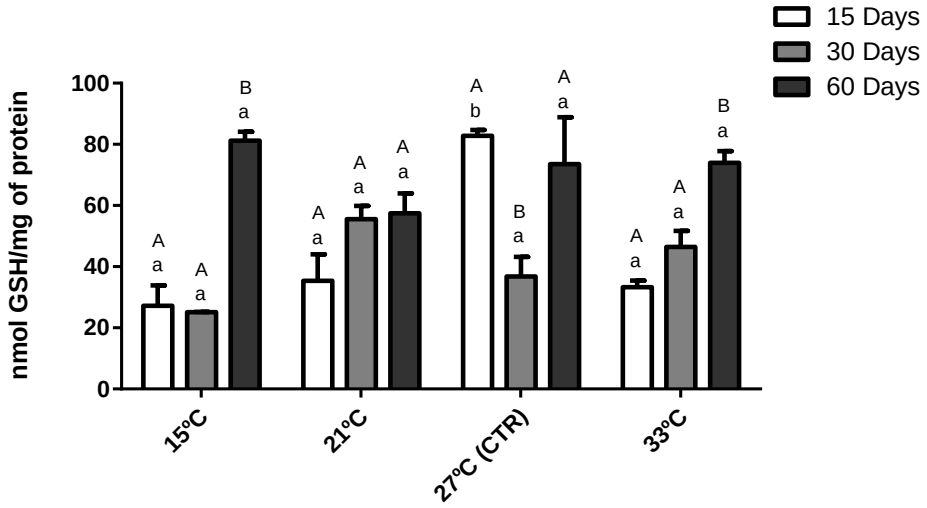
Treatments	**Survival (%)**	**FW (g)**	**SGR (%)**
15°C	55.83 ± 1.45^{b}	2.38 ± 0.19^{a}	0.65 ± 0.11^{a}
21°C	81.66 ± 0.66^{a}	4.2 ± 0.20^{b}	1.42 ± 0.07^{b}
27°C (Control)	73.33 ± 1.45^{a}	7.03 ± 0.34^{c}	2.3 ± 0.07^{c}
33°C	29.16 ± 0.33^{c}	7.79 ± 1.35^{c}	2.19 ± 0.39^{c}

Table 3. Imunological analysis of granular hemocytes (HG), hyaline hemocytes (HH), total protein (TP) and apoptosyis at 30 and 60 days of shrimp reared in different temperatures.

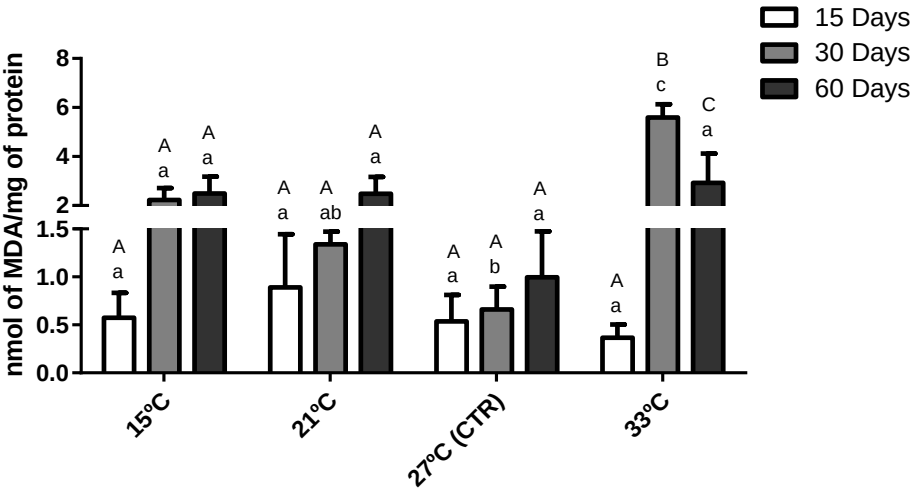
Treatments	Granular hemocytes (%HG) days		Hyaline hemocytes (%HH) days		Total protein (mg mL $^{-1}$) days		Apoptosis (%)	
	30	60	30	60	30	60	30	60
15°C	67.4±1.20	67.6 ± 1.72	32.6 ± 1.20	32.4 ± 1.72	118.8 ± 1.01	122.4 ± 1.32	2.2 ± 0.58	3.4 ± 0.50
21°C	64.6 ± 1.8	66.4 ± 2.37	37.4 ± 2.42	33.6 ± 2.37	118.2 ± 1.15	120.4 ± 1.4	2 ± 0.44	2.4 ± 0.50
27°C (control)	66.2 ± 2.22	67.8 ± 1.95	33.8 ± 2.22	32.2 ± 1.95	119.6 ± 2.11	119.4 ± 1.02	2 ± 0.31	3.2 ± 0.58
33°C	68.8 ± 1.59	68.5 ± 3.5	31.4 ± 1.50	31.5 ± 3.5	120.8 ± 0.8	119.5 ± 1.5	3 ± 0.70	1.5 ± 0.5


nmol GCL/mg of protein
400
300
200
100
0
15 Days
30 Days
60 Days
15°C
21°C
27°C (CTR)
33°C

(A)

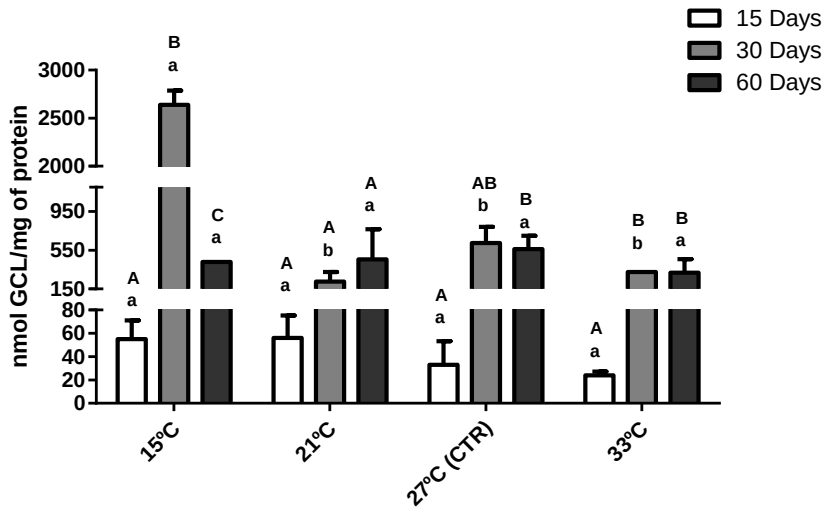

nmol GSH/mg of protein
100
80
60
40
20
0
15 Days
30 Days
60 Days
15°C
21°C
27°C (CTR)
33°C

(B)

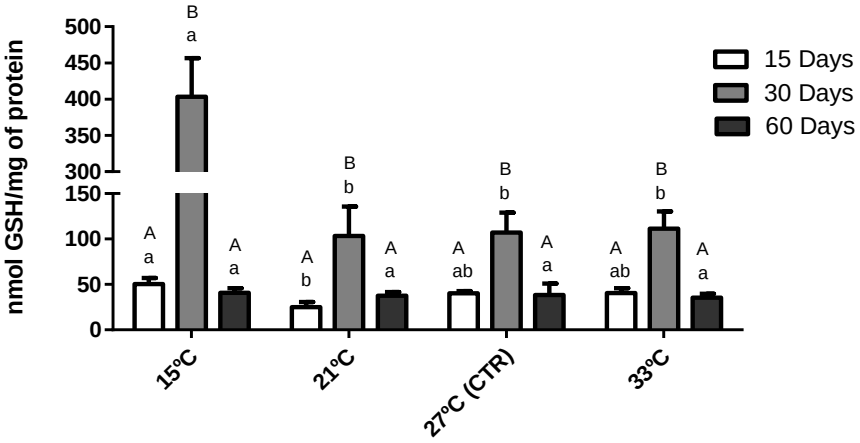

nmol of MDA/mg of protein
8
6
4
2
1.5
1.0
0.5
0.0
15 Days
30 Days
60 Days
15°C
21°C
27°C (CTR)
33°C

(C)

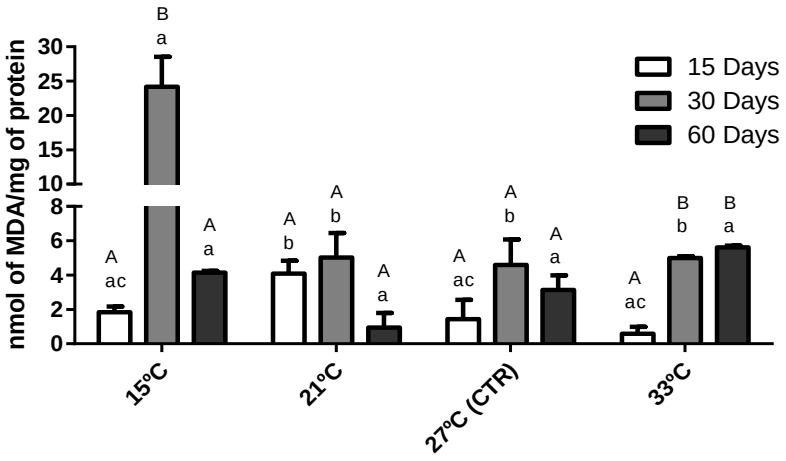
Figure 1


15 Days
30 Days
60 Days
nmol GCL/mg of protein
3000
2500
2000
950
550
150
80
60
40
20
0
15ºC
21ºC
27ºC (CTR)
33ºC

(A)

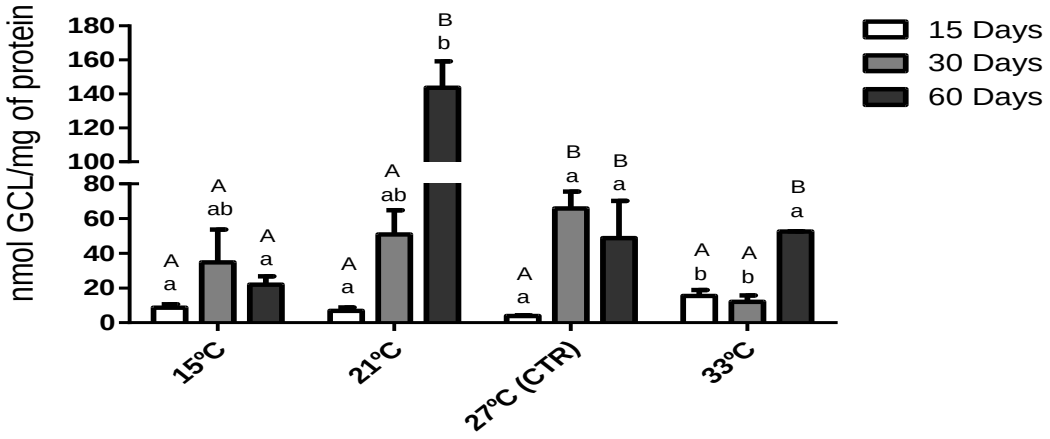

15 Days
30 Days
60 Days
nmol GSH/mg of protein
500
450
400
350
300
150
100
50
0
15ºC
21ºC
27ºC (CTR)
33ºC

(B)

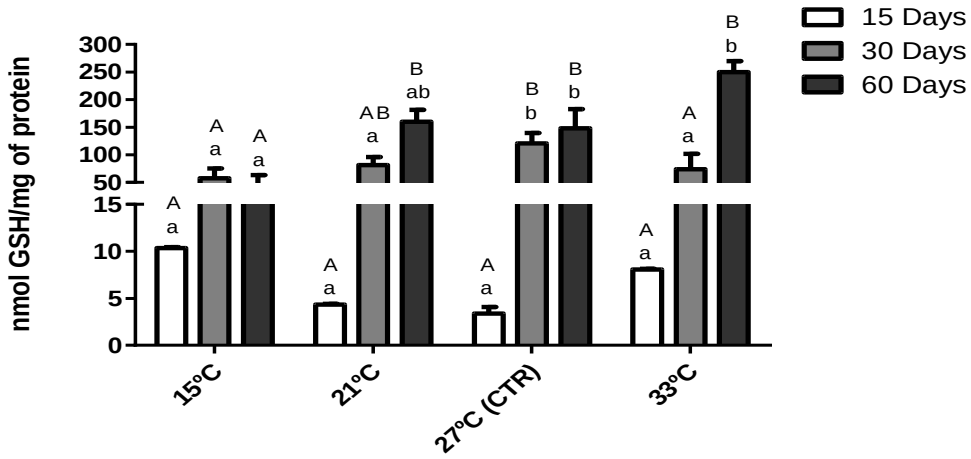

15 Days
30 Days
60 Days
nmol of MDA/mg of protein
30
25
20
15
10
8
6
4
2
0
15ºC
21ºC
27ºC (CTR)
33ºC

(C)

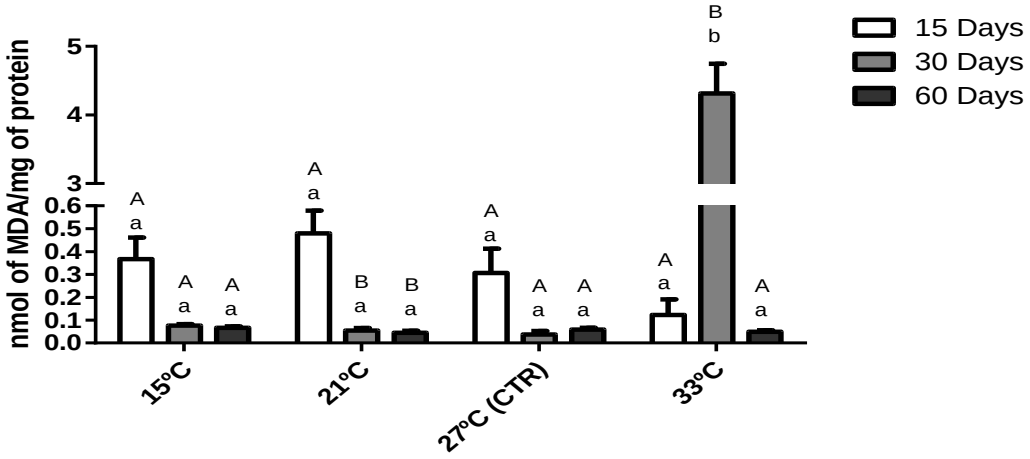
Figure 2


nmol GCL/mg of protein
180
160
140
120
100
80
60
40
20
0
15 Days
30 Days
60 Days
15°C
21°C
27°C (CTR)
33°C

(A)


nmol GSH/mg of protein
300
250
200
150
100
50
15
10
5
0
15 Days
30 Days
60 Days
15°C
21°C
27°C (CTR)
33°C

(B)


nmol of MDA/mg of protein
5
4
3
0.6
0.5
0.4
0.3
0.2
0.1
0.0
15 Days
30 Days
60 Days
15°C
21°C
27°C (CTR)
33°C

(C)

Figure 3

Capítulo III

Comparation between Bio-Floc Technology (BFT) and clear water systems on the antioxidant and immunological status of *Litopenaeus vannamei*

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Abstract

The Biofloc technology system (BFT) has been shown important benefits to shrimp culture. Therefore the aim of the present work was compare the traditional system of clear water (CW) and BFT with microbial flocs to evaluate the antioxidant and immunological status of shrimp reared in both systems. A 60-day trial was conducted in two different treatments: BFT and CW with 3 replicates tanks for each. The samples (gills, hemolymph and hepatopancreas) were collected at 15; 30 and 60 days. The immunologic parameters analyzed were: Hyaline and granular hemocyte count, total protein and apoptosis. The enzymatic and lipid peroxidation assays consisted of GCL, GSH and TBARS. The immunological parameters did not show significant differences between treatments throughout the experimental period. The results revealed that shrimp reared in CW system presented in gills a higher lipid peroxidation (656.25%) at 15 days compared to those of BFT treatment. At 30 days, *L. vannamei* reared in CW system presented higher level of lipid peroxidation (351.51%) compared to BFT. In hemolymph, shrimp reared in CW system at 15 days exhibited a rise in GCL activity (431.04%) over those reared in BFT. At 60 days GCL activity was higher (4,139.80%) of treatment CW in relation to BFT. Shrimp reared in BFT system presented higher survival rate and no water was renewed during the experimental period. Shrimp reared in clear water presented higher lipid peroxidation. Therefore, we recommend the use of BFT system in shrimp culture to improve not only the production, but also to provide a healthier environment to the animals.

Keywords: shrimp, BFT, clear water, TBARS, GCL, GSH

Introduction

The aquaculture is increasing substantially over the years. According to the data from FAO (2014), the total aquaculture production is more than 66 million tonnes. Shrimp continues to be the largest single commodity in value terms, being responsible for 15 percent of the total value of internationally traded fishery products in 2012 (FAO 2014). *L. vannamei* is the most cultured penaeid shrimp species worldwide, being distributed along the Pacific coast of Central and South America (Zhou et al. 2009).

The development of aquaculture requires strategies to reduce the negative impacts caused by high stocking densities, effluent discharge and limited options for disease treatment (Xie and Yu 2007, Moss et al. 2012). The aquaculture has developed managing practices to become even more an environmental friendly activity. In the 1980's decade, shrimp production was based primarily on high surface area with low stocking density in ponds. Subsequently, strategies were developed to increase shrimp production which includes: fertilization, use of feeding trays, increased stocking densities and the systems called BFT (Bio-floc technology) (Wasielesky et al. 2006). This system with zero water exchange reduces not only the water use, but also the issuance of effluent into the environment avoiding the environmental damage (Burford et al. 2003, Souza et al. 2014).

The BFT is based on the manipulation of microbial community through the addition of a carbon source that promotes the development of heterotrophic bacteria (Souza et al. 2014). There are several benefits of this system including the bacterial uptake of nitrogen, including ammonia (Burford et al. 2003), and its conversion into cellular protein providing a supplemental source of nutrition (Burford et al. 2004, Wasielesky et al. 2006) and possibly reducing the demand for protein on feed (Crab et al. 2007, Ballester et al. 2010). The microbial community of bioflocs can act as a supplemental food source and improve animal growth (Kim et al. 2014). It is known that biofloc from shrimp culture water is rich in bioactive compounds including carotenoids, chlorophylls and fat-soluble vitamins (Ju et al., 2008a) which can improve animal immune systems, increase stress tolerance and also present antioxidant functions (Babin et al., 2010; Linan-Cabello et al., 2002). Therefore, the aim of this work was compare the traditional system of culture in clear water (without microbial floc) and the

BFT system (with microbial floc) and also the influence of these systems in the immunological and physiological parameters.

Material and methods

Experimental design

A 60-day trial was conducted at the Marine Aquaculture Station at the Universidade Federal do Rio Grande – FURG, in southern Brazil. The experimental system consisted of 6 tanks (250 L) with a bottom area of 0.20 m^2. *L. vannamei* (1.91 g ± 0.22 for BFT and 2.26 ± 0.24 for CW) were stocked in the tanks at a density equivalent to 200 shrimp/m^2 (40 shrimp/tank). Three replicate tanks were assigned to each of the following treatments: BFT and clear water (CW) at 27ºC through the use of heaters with a thermostat and salinity at 30‰. The temperature was chosen according to the *L. vannamei* physiology in order to provide a preferendum temperature to the animals. To promote the development of the microbial flocs, BTF tanks received an inoculum of 75 L from a heterotrophic shrimp culture system before start the experiment. Prior to the experiment, the shrimp were acclimatized for 10 days at 27°C and 30‰ salinity. The water from treatment CW was renewed in approximately 70% every three days to avoid increasing ammonia or when the total ammonia nitrogen (TAN) level reaches 1mg/L. When the ammonia concentration in the experimental tanks of BFT treatment reached values of 1 mg/L or higher, these tanks received a molasses dose calculated according to the equations proposed by Ebeling et al. (2006) and Avnimelech (1999). The animals were fed twice daily with a commercial diet containing 38% crude protein via a specially designed feeding tray (Wasielesky et al. 2006).

The feeding rate was 15% of the total tank biomass and was adjusted daily according to shrimp consumption during the acclimation and the experimental conditions. At the end of experimental period the survival (final number of shrimp/initial number of shrimp) x 100; final weight (FW) and specific growth rate (SGR) ((ln(final weight) – ln(initial weight))/trial duration x 100 were evaluated.

Water quality analysis

Throughout the experimental period, the water temperature (mercury thermometer, precision ±0.5°C), salinity (optical refractometer model RTS – 101, Atago® US, Bellevue, WA, USA, precision: ±1 g/L), pH (digital pH meter model Handylab 2 BNC, Schott®, Hattenbergstr, Germany, precision: ±0.01 precision), and dissolved oxygen (dissolved oxygen meter model Handylab/OXI/set, Schott® Cambridge, UK, precision: ±0.01 mg L^{-1}) were measured every day. Water samples were collected three times a week to determine the concentrations of total ammonia (UNESCO 1983) and nitrite (Bendschneider and Robinson 1952). Alkalinity and nitrate were monitored once a week according to the methods of UNESCO (1983) and Baumgarten et al. (1996), respectively.

Imunological assays

Granular and hyaline hemocyte count was determined using a Neubauer chamber, after collecting the hemolymph at 30 and 60 days of six shrimp/treatment directly into an anticoagulant solution (1:4) (modified Alsever solution or MAS: 27 mM sodium citrate, 336 mM sodium chloride, 115 mM glucose, 9 mM EDTA, pH 7.0) (Maggioni et al. 2004). Total protein concentration (TPC) in shrimp serum (six animals per treatment) was determined according to the Bradford method (1976) using bovine

serum albumin (BSA) as standard (Maggioni et al. 2004). For the detection of apoptosis, shrimp hemolymph (5 μL) was smeared onto a clean glass slide and air-dried, with 5 clean slides per treatment. The hemocytes in apoptosis were evaluated by the TUNEL method using the ApopTag® Plus Peroxidase In Situ Apoptosis Detection kit (Millipore) according to Charriaut-Marlangue and Ben-Ari (1995) and Wang and Zhang (2008).

Enzyme assays

The hemolymph and tissues (gills and hepatopancreas) of nine shrimp per treatment (three from each tank) were sampled randomly at 15; 30; and 60 days. Hemolymph was collected directly from the heart of the shrimp using sterile syringes containing an anticoagulant solution (glucose 0.1 M, sodium citrate 30 M, citric acid 0.026 M, NaCl 0.45 M, EDTA 0.02 M, and pH 7.4) (Sotherhall and Smith 1983) to avoid hemolymph coagulation. For protein quantification and antioxidant enzyme analysis, the hemolymph was centrifuged twice at 500 and 900 × g at 4°C for 35 and 15 min, respectively, to obtain a pellet or cell lysate. After the centrifugation process, the cell lysate was re-suspended in a 4°C buffer solution containing Tris base (20×10^{-3} M), EDTA (1×10^{-3} M), dithiothreitol (1×10^{-3} M), KCl (150×10^{-3} M), and PMSF (0.1×10^{-3} M), with the pH adjusted to 7.6, transferred to 1.5 mL polyethylene tubes and stored at −80°C in an ultra freezer. The gills and hepatopancreas samples (for protein quantification and antioxidant enzyme analysis) were weighed, then added 1:5 of buffer solution and centrifuged for 30 min at 9,000 x *g* at 4ºC. The supernatant was transferred to 1.5 mL polyethylene tubes and stored in an ultra freezer to perform the analysis. All enzymatic measurements were performed at least in triplicate. Total protein content was determined through the Biuret method using a commercial kit

(Doles Reagents Ltd. Goiânia, GO, Brazil) and read at 550 nm using a microplate reader (Victor 2, Perkin Elmer).

GCL activity and GSH concentration

These analysis were followed the methodology described by White et al. (2003). The GCL and GSH assays were performed at the same time, one plate for each analysis. The GSH plate was kept on ice during all process. The GCL reaction cocktail (40mM ATP, 20mM L-glutamic acid, 20mM sodium borate, 2mM serine, 40mM $MgCl_2$) were diluted in Tris-HCl (400mM) and EDTA (2 mM) solution. On a separate 96-well round-bottom reaction plate, 25 µl aliquots of this reaction cocktail were pipetted into wells (3 per sample). These plates were either prepared just prior to the assay to prevent degradation of ATP in the reaction cocktail. While the sample plate was kept on ice, 25 µl aliquots of sample were pipetted into the prewarmed (37°C) reaction plate (GCL) at 15-s time intervals. After a 5 min preincubation, the GCL reaction was initiated by adding 25 µl of 2mM cysteine (dissolved in Tris-HCl 100 mM, EDTA 2 mM, $MgCl_2.6H_2O$ 5 mM) to each GCL activity well (cysteine was not added to the GSH-baseline wells at this time). The plate was then vortexed, covered, and incubated for one hour. The GCL reaction was stopped by adding 25 µl 200mM SSA to all wells, and then 25 µl of 2mM cysteine was added to the GSH-baseline wells. The plate was then vortexed and held on ice for at least 20 min. Following protein precipitation, the plate was centrifuged for 5 min at 2,500 rpm on a tabletop centrifuge. Following centrifugation, 20µl aliquots of supernatant from each well of the reaction plate were transferred to a 96-well plate designed for fluorescence detection. Next, 180 µl of NDA derivatization solution (50mM Tris, pH 10, 500mM NaOH, and 10mM NDA-previously diluted in Me_2SO) according to the follow proportion v/v/v 1.4/0.2/0.2 was

added to all wells of this plate. The plate was covered to protect the wells from room light and allowed to incubate at room temperature for 30 min. Following incubation, NDA–γ-GC or NDA–GSH fluorescence intensity was measured (472 excitation/528 emission wavelengths) on a fluorescence plate reader (Victor 2, Perkin Elmer).

Lipid peroxidation (LPO) assay

LPO was measured through determination of TBARS, following the methodology of Oakes and Van Der Kraak (2003). The cell lysate (10 μL) was added to a reaction mixture made with 150 μL of 20% acetic acid, 150 μL of thiobarbituric acid (0.8%), 50 μL of Milli Q water, and 20 μL of sodium dodecyl sulfate (SDS, 8.1%). Samples were heated at 95°C for 30 min and after cooling for 10 min, 100 μL of Milli-Q water and 500 μL of n-butanol were added. After centrifugation (3,000 x g for 10 min at 15°C), the organic phase (150 μL) was placed in a microplate reader and the fluorescence was registered after excitation at 515 nm and emission of 553 nm. Tetramethoxypropane (TMP, ACROS Organics) was used as standard and TBARS results are expressed in nanomoles of TMP equivalents per mg of protein.

Statistical analysis

Data are expressed as the mean ± SEM and were analyzed through one-way analysis of variance (ANOVA), followed by Newman-Keuls post hoc mean comparisons. Assumptions of normality and variance homogeneity were previously checked (Levene test). In all cases, the significance level was fixed in 0.05.

Results

Water quality, survival and growth

The physicochemical parameters of water quality are shown in Table 1. Although we observed significant differences in pH, salinity, alkalinity, total ammonia, nitrite and nitrate between treatments, the water quality parameters analyzed during the experimental period remained at concentrations suitable for shrimp culture in both treatments.

Shrimp reared in BFT presented higher survival rate (73.33%) over those reared in CW (59.16%). The final weight was higher in CW, nevertheless the SGR did not present difference between treatments.

Immunological analysis

The immunological parameters evaluated in this work did not show significant differences between treatments throughout the experimental period (Table 3).

Antioxidant system analysis

Hemolymph

Litopenaeus vannamei reared in CW system at 15 and 60 days exhibited an increase in GCL activity, 431.04 and 4,193.80%, respectively, over those reared in BFT. Shrimp submitted to BFT showed the lowest GCL activity at 60 days, approximately 1,068.18% compared to 15 and 30 days. The CW treatment presented the lowest GCL activity at 30 days, approximately 256.35% in relation to 15 and 60 days (Figure 1A). White shrimp reared in BFT system exhibit a decrease in GSH concentration at 30 days compared to 15 (224.89%) and 60 (199.78%) days. At 15 days, shrimp reared in BFT system demonstrated higher GSH concentration (252.70%) over those reared in CW system (Figure 1B). At 30 days, *L. vannamei* reared in CW system presented higher

level (351.51%) of TBARS compared to BFT system. White shrimp of CW treatment at 15 days presented lower TBARS level (483.33%) compared to 30 days (Figure 1C).

Gills

Shrimp reared in BFT system demonstrated in gills at 15 days a rise in GCL activity 1,889.00 and 1,698.69% compared to 30 and 60 days, respectively (Figure 2A). The GSH concentration follows the same pattern showing the highest level at 30 days when compared to 15 (266.56%) and 60 (279.22%) days (Figure 2B). Shrimp reared in CW system presented higher lipid peroxidation in gills at 15 days (656.25%) over those of BFT. *L. vannamei* reared in BFT presented at 30 and 60 days a rise in TBARS levels 1,434.37 and 978.12%, respectively, in relation to 15 days. Shrimp of treatment CW presented the highest lipid peroxidation at 30 days when compared to 15 (271.90%) and and 60 (165.02%) days (Figure 2C).

Hepatopancreas

L. vannamei reared in BFT system demonstrated higher GCL activity at 30 days compared to 15 (1,705.44%) and 60 (236.96%) days. At 60 days the GCL results of BFT treatment were higher when compared to 15 days (719.68%). Shrimp reared in BFT system showed at 15 and 30 days higher GCL activity (364.15 and 234.60%, respectively) over those reared in CW system (Figure 3A). The GSH concentration of shrimp reared in BFT system presented the lowest level at 15 days when compared to 30 (4,350.35%) and 60 (5,325.89%) days. Shrimp reared in CW system presented at 15 days an augmented in GSH concentration 275.89% over those reared in BFT system. White shrimp reared in CW system demonstrated at 30 days a rise in GSH concentration compared to 15 (2,844.58%) and 60 (1,876.79%) days (Figure 3B). The

TBARS results in 30 days presented an augmented of 1,200.00 and 7,320.00% in BFT and 696.77 and 4,320.00% CW systems compared to 15 and 60 days, respectively (Figure 3C).

Discussion

The water quality parameters analyzed during the experimental period remained at concentrations suitable for shrimp culture in both treatments. The pH and alkalinity in the BFT group were lower than CW, probably due to the respiration of heterotrophic organisms, which increased the carbon dioxide concentration in the water of the biofloc treatment and due to the nitrification (Tacon et al. 2002, Wasielesky et al. 2006), as demonstrated by the higher level of nitrate in the biofloc tanks. As expected, DO levels were lower in the BFT treatment than the CW because of the greater demand by the microorganisms. The TAN was lower in BFT tanks by the nitrification process which involves the microorganisms present in this system. Previous studies have shown that rearing *L. vannamei* in BFT systems can improve shrimp survival and growth performance, compared to clear water (Moss and Pruder 1995, Cohen et al. 2005, Azim and Little 2008, Mishra et al. 2008).

The microbial community associated with BFT not only detoxifies substances, but also can improve feed utilization and animal growth (Kim et al. 2014). In the present work the BFT treatment presented higher survival rate when compared to the clear water. Souza et al. (2014) evaluated the shrimp performance and immunological parameters of shrimp *Farfantepenaus brasiliensis* reared in systems with and without molasses addition and found that the carbon added in the system developed the microbial community of bioflocs earlier reaching higher survival rate, SGR and final weight. In our work we also observed a higher survival in BFT treatment. Souza et al.

(2014) did not find any differences in the immunological parameters tested (hyaline and granulous hemocytes count and total protein) between treatments. These results are in agreement with our findings, because in this study the immunological parameters did not show any difference. Biofloc system contains several bacteria of which cell wall presents various components such as bacterial lipopolysaccharide, peptidoglycan and b-1,3-glucans, and is known that these substances may act stimulating nonspecific immune activity of shrimp. Bioflocs, therefore, are assumed to enhance shrimp immunity because shrimp consume the bioflocs as additional food source (Kim et al., 2014).

The improved performance of *L. vannamei* reared in BFT systems can be related to the consumption of bioflocs by the shrimp, moreover, some studies reported that up to 29% of daily feed intake of this species can come from particles in heterotrophic culture system (Burford et al. 2004). However little is known about the mechanisms and the preferences of the animals about the groups of microorganisms present in bioflocs (Kim et al. 2014).

In the present study some immunological parameters (hemocyte count, total protein and apoptosis) were evaluated in juveniles of *L. vannamei* and no difference were detected. The development of the organisms in their life cycle presents different requirements in each phase and probably the requirements of shrimp postlarvae should be different to a juvenile or adult (Zhou et al. 2013). In the present study the water quality was monitored in both treatments, so the animals were reared in a healthy environment. By this reason the differences in immunological parameters analyzed in this work could not be detected. In addition, in intensive culture systems, the health status of shrimp is closely related to the living water environment (Xu and Pan 2013).

The hemocyte count in the hemolymph has been considered an indicator of immune capability (Rodríguez and Le Moullac 2000). Xu and Pan (2013) observed that mean values of total hemocyte count (THC) and hemocyte phagocytic activity increased in the shrimp of biofloc treatments as compared with the control group. In the present work we did not find these differences. However, the modes of action of biofloc on innate immune system of shrimp are complicated and unknown at present, and further studies is required for accumulating more detailed knowledge (Xu and Pan 2013). It is postulated that the microbial community associated with the bioflocs induces a permanent trigger towards the development and maintenance of the shrimp immune system and thus build up a defense mechanism in shrimp. This mechanism and its utilization may be a very important means to protect shrimp against disease outbreaks that often lead to collapse of shrimp production systems generating important losses (Kim et al. 2014). By this reason can be explained the improvement in growth and survival reported in several studies (Megahed 2010; Krummenauer et al. 2011, Viau et al. 2013, Souza et al. 2014).

Few researches investigated the immunological potential of the biofloc technology (de Jesús Becerra-Dorame et al. 2012, Xu and Pan 2013, Kim et al. 2014, Xu and Pan 2014), although it is widely known that microorganisms, their cell components or their metabolites can play a important role as immunostimulants that enhance the shrimp innate immune system and provide improved protection against pathogens (Smith et al. 2003, Vazquez et.al. 2009). Recent studies correlated and suggest that an enhanced antioxidant status facilitates shrimp immune defense functions (Chiu et al. 2007, Castex et al. 2010). Antioxidant research has attracted increasing focus in aquaculture, since aquatic animals are susceptible to oxidative stress as a result of pathogen pressure and environmental perturbations (Liu and Chen 2004, Castex et al.

2010). Like other aerobic organisms, shrimp possess an integrated antioxidant system, including enzymatic and non-enzymatic antioxidants, to maintain normal oxidant status and especially to cope with natural or induced stressors (Castex et al. 2009, Parrilla-Taylor and Zenteno-Savín 2011). Generally, the antioxidant capability of an organism under certain condition can reflect its health status.

Based on its composition characteristics, the biofloc may play a role in antioxidant activity because it contains an appropriate amount of antioxidants such as carotenoids and fat-soluble vitamins (Ju et al. 2008) and stimulate digestive enzyme activities and improve feed utilization (Xu and Pan 2012, Xu et al. 2013), which in turn can improve the assimilation of dietary antioxidants from the feed. In the present work can be observed in gills higher lipid peroxidation in CW compared to BFT treatment demonstrating a failure of the antioxidant system to counteract the free radicals.

The BFT system presents many advantages to be used in shrimp farming. The immunological and physiological reasons were discussed above. In addition, can be emphasized the extremely important advantage that is the zero water exchange. Currently, the world concern is developing strategies to increase biosecurity and more environmental friendly handling to reduce the negative impacts. Therefore, further studies should be done to evaluate the role of microorganisms in bioflocs about their mechanisms of action in enhancement of immunological and physiological status and their properties as a probiotic in order to even more increase the benefits of this system.

In conclusion, shrimp reared in BFT system presented higher survival rate and no water was renewed during the experimental period. In hemolymph, *L. vannamei* reared in clear water (CW) presented a rise in GCL activity and higher lipid peroxidation demonstrating that the antioxidant system was not sufficient to counteract the free radicals leading the animals of this treatment to oxidative damage, as a result,

presented lower survival rate. Therefore, we recommend the use of BFT system in shrimp culture to improve not only the production, but also to provide a healthier environment to the animals.

Acknowledgements

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Figure legends

Figure 1. GCL activity (1A), GSH (1B) concentration and thiobarbituric acid reactive substances (TBARS) (1C) in hemolymph of *L. vannamei* reared in BFT and CW systems. The data are expressed as the means ± SEM. Different lower case letters indicate significant differences ($P < 0.05$) among treatments over the same time period. Different capital letters indicate significant differences ($P < 0.05$) in the same treatment throughout the experimental period.

Figure 2. GCL activity (2A), GSH concentration (2B) and thiobarbituric acid reactive substances (TBARS) (2C) in gills of *L. vannamei* reared in BFT and CW systems. The data are expressed as the means ± SEM. Different lower case letters indicate significant differences ($P < 0.05$) among treatments over the same time period. Different capital letters indicate significant differences ($P < 0.05$) in the same treatment throughout the experimental period.

Figure 3. GCL activity (3A), GSH concentration (3B) and thiobarbituric acid reactive substances (TBARS) (3C) in hepatopancreas of *L. vannamei* reared in BFT and CW systems. The data are expressed as the means ± SEM. Different lower case letters indicate significant differences ($P < 0.05$) among treatments over the same time period. Different capital letters indicate significant differences ($P < 0.05$) in the same treatment throughout the experimental period.

Table 1. Physicochemical parameters of the water in the experimental tanks. Data are reported as the mean ± SEM. Different superscript letters in the lines indicate significant differences among treatments by one-way ANOVA ($P < 0.05$).

Parameter	Treatments	
	27°C BFT	**27ºC CW**
Temperature (°C)	26.89 ± 0.15[a]	26.73 ±0.09[a]
pH	7.68 ± 0.02[a]	8.23± 0.01[b]
Salinity (g L^{-1})	31.88 ± 0.24[a]	29.28 ±0.18[b]
DO (mg L^{-1})	5.91 ± 0.03[a]	6.13 ± 0.02[a]
Alkalinity (mg L^{-1})	93.14 ± 5.91[a]	148.70 ±1.15[b]
TAN (mg L^{-1})	0.15 ± 0.01[a]	0.33±0.03[b]
Nitrite (mg L^{-1})	0.07 ± 0.006[a]	1.40±0.23[b]
Nitrate (mg L^{-1})	42.02 ± 4.77[a]	7.77±1.78[b]

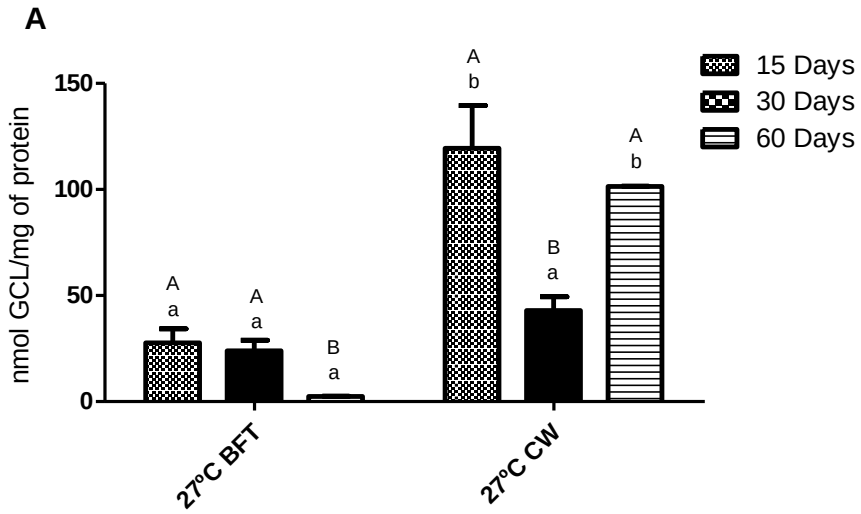
DO: Dissolved oxygen, TAN: total ammonia nitrogen

Table 2. Survival, final weight (FW) and specific growth rate (SGR) (mean ± SEM) of shrimp reared in BFT and CW systems. Different superscript letters indicate significant differences among treatments by one-way ANOVA ($P < 0.05$).

Treatments	Survival (%)	FW (g)	SGR (%)
27ºC BFT	73.33 ± 1.45[a]	7.03 ± 0.34[a]	2.30 ± 0.07[a]
27ºC CW	59.16 ± 3.75[b]	9.84 ± 0.61[b]	2.46 ± 0.08[a]

Table 3. Imunological analysis of granular hemocytes (HG), hyaline hemocytes (HH), total protein (TP) and apoptosyis at 30 and 60 days of shrimp reared in BFT and CW systems.

Treatments	Granular hemocytes (%HG) days		Hyaline hemocytes (%HH) days		Total protein (mg mL $^{-1}$) days		Apoptosis days	
	30	60	30	60	30	60	30	60
27°C BFT	66.2 ±2.22	67.8 ± 1.95	33.8 ± 2.22	32.2 ± 1.95	119.6 ± 2.11	119.4 ± 1.02	2 ± 0.31	3.2 ± 0.58
27ºC CW	64.8 ± 1.59	69.2 ± 2.10	35.2 ± 1.59	30.8 ± 2.10	119.2 ± 1.2	121.0 ± 1.70	2.4 ± 0.50	2.6 ± 0.68



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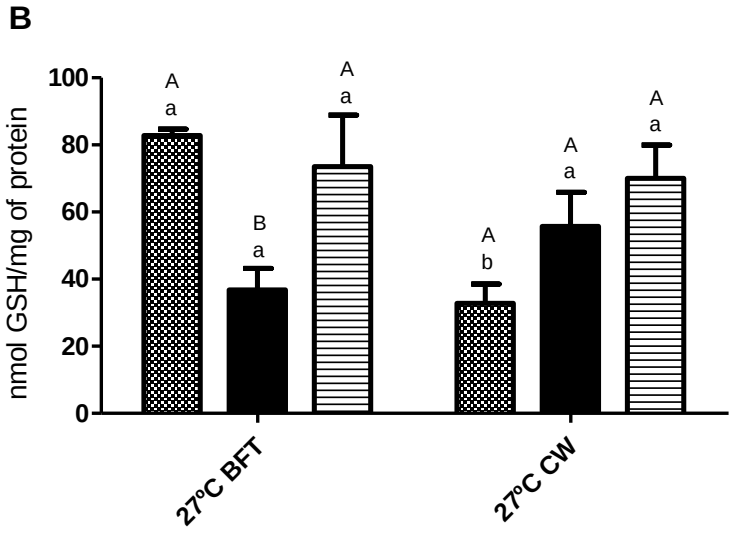

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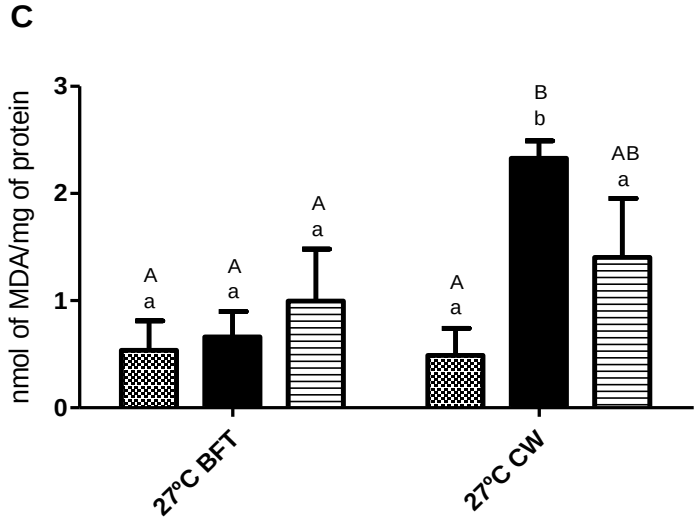

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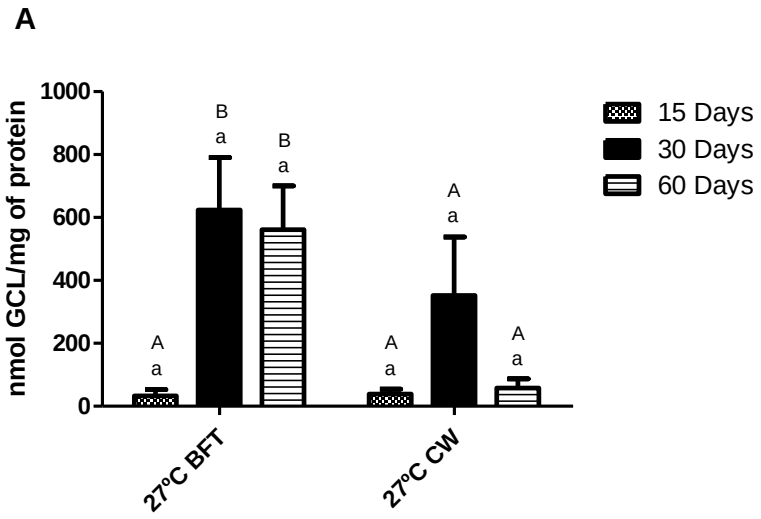
4 **Figure 1**



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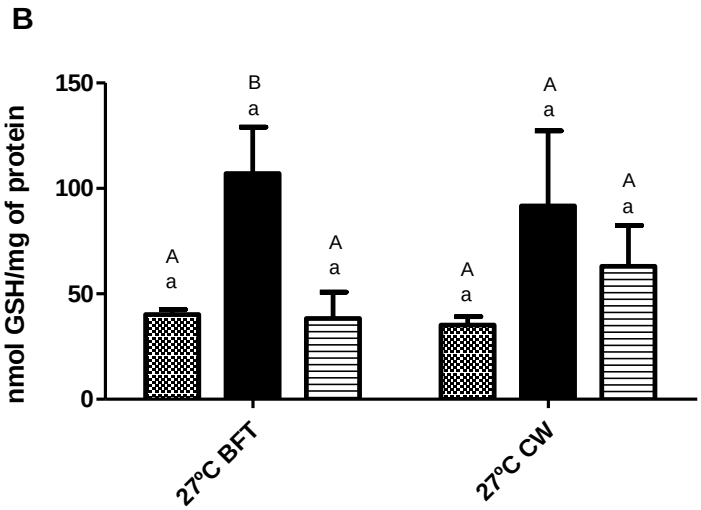

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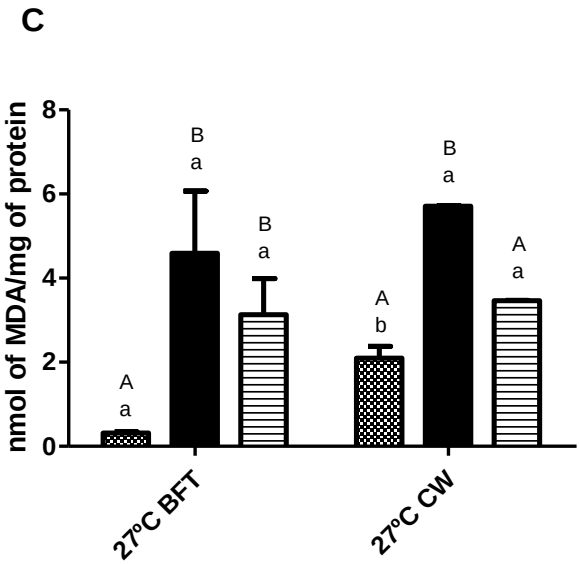

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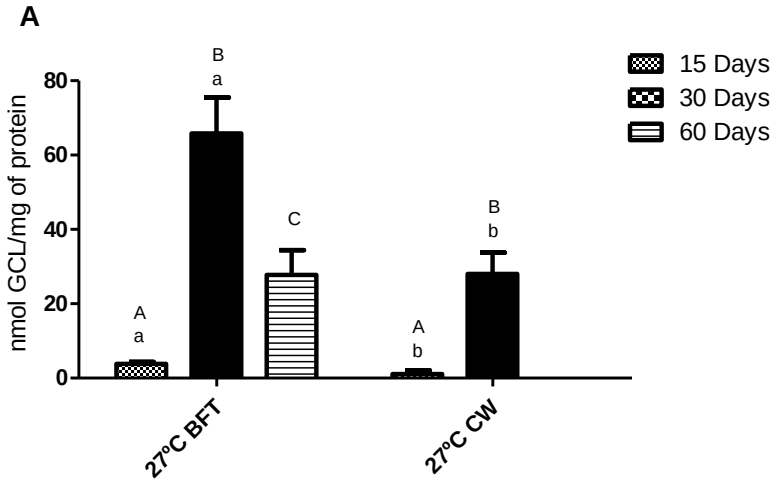
5 **Figure 2**



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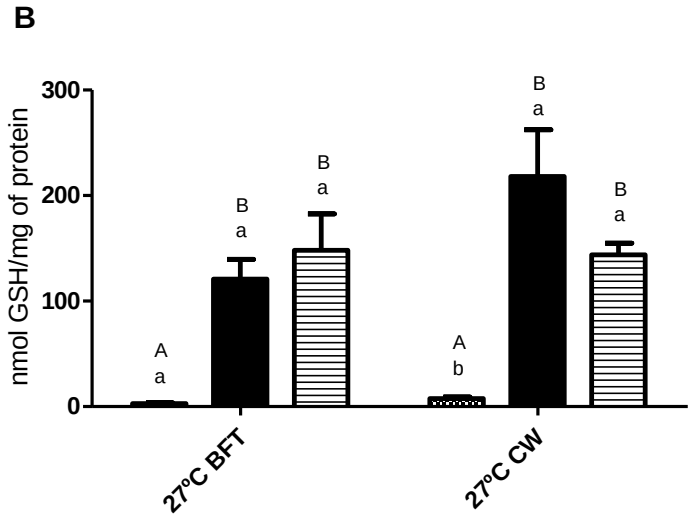

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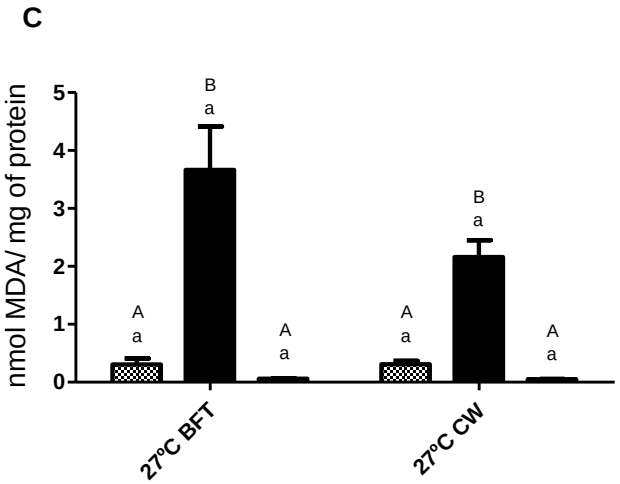

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6 **Figure 3**

Capítulo IV

Effects of water temperature on oxidative stress parameters in the pink shrimp *Farfantepenaeus brasiliensis* during transport

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Abstract

Farfantepenaeus brasiliensis juveniles (5.53 ± 1.20 g) were subjected to different temperatures to evaluate the optimal temperature for transport. The shrimp were acclimated in a 4,000 L tank at 22.4 °C and salinity of 22 ‰. The shrimp were then transported for 12 h at different temperatures (16.0, 19.3, 22.4 (control), 25.0 or 28.0 °C) in transparent plastic bags containing one-third water (10 L) and two-thirds pure oxygen, with three repetitions for each treatment. Analyses were performed at the beginning and the end of the transport. The hemolymph from five shrimp in each plastic bag was collected to measure catalase (CAT), glutathione-*S*- transferase (GST) and total antioxidant competence against peroxyl radicals. The results showed that shrimp transported in lower temperatures had higher antioxidant competence and the best water quality parameters. Therefore, the temperature we recommend for transporting *F. brasiliensis* is 19.3 °C, which enhanced the total antioxidant capacity against peroxyl radicals and the activity of CAT and GST, as well as providing better water quality conditions.

Keywords: antioxidant defenses, catalase, glutathione-S-transferase, shrimp, hemolymph, water quality

1. Introduction

Worldwide aquaculture production has grown substantially during the last two decades (FAO, 2010). The penaeid shrimp *Farfantepenaeus brasiliensis* is widely distributed from North Carolina (USA) to the coast of Rio Grande do Sul (D'Incao, 1999) and lives in a broad range of water temperatures. In Brazil, it is one of the most common species in the live bait trade, especially in the coastal states of Rio de Janeiro, São Paulo, Paraná and Santa Catarina (Preto et al., 2009; Jensen et al. *in press*).

The transport and storage of live crustaceans are becoming increasingly common and have been made possible through technological advances such as specially designed tanks and transport vehicles equipped with aeration and/or oxygenation inputs (Fotedar and Evans, 2011). Moreover, aquatic animals in Brazil are frequently transported in plastic bags (Golombieski et al., 2003).

The transportation of live organisms generally does not take into account the animal´s health and welfare and, in most cases, results in high mortalities. These mortalities occur because transport is a traumatic procedure involving a succession of adverse stimuli, including the capture, transport and storage of the animals (Robertson et al., 1988; NRC, 2006). Moreover, transportation for long periods may result in water quality deterioration, which can be exacerbated by increases in the temperature and in the ammonia excretion rate in a closed system (Teo et al., 1989; Jiang et al., 2000; Golombieski et al., 2003).

The effects of environmental stressors are also studied in aquatic organisms to elucidate the oxidative damage and physiological effects resulting from transport. Several techniques have been developed to maximize health and survival and to reduce stress during transport, including the addition of cold water inside the plastic bags (Fotedar and Evans, 2011).

Stress responses have been studied in many vertebrate and invertebrate species to identify the mechanisms involved (Selye, 1973; Moberg, 1985; Barton and Iwama, 1991; Buchanan, 2000; Barton, 2002). In general, crustaceans vary their tolerance to environmental perturbations (e.g., temperature fluctuations, high levels of ammonia and varying salinity). Therefore, the recommended practices are species specific (Fotedar and Evans, 2011). However, no literature is available for *F. brasiliensis* concerning oxidative stress and antioxidant systems.

Various protocols have been established to provide technological guidelines in the handling and transportation of live crustaceans (Codex Alimentarius, 1983; APEC, 1999; Aquatic Animal Welfare Guidelines). Contradictory results have been presented concerning the capacity of crustaceans to feel pain (Sample, 2007); however, recent reports suggest that crustaceans do, indeed, feel pain (RSPCA, 2001; Barr et al., 2008; Yue, 2009; Parodi et al., 2012). Although they do not have the classical pain receptors of higher order vertebrates, it is nevertheless important to minimize the possibility of pain by maintaining good husbandry and care (Bennison, 2000).

Temperature is a controlling factor in the physiology of poikilothermic species (Dove et al., 2005), and in temperate regions like the South of Brazil, water temperatures range from 12 ºC in the winter to 28 ºC in the summer (Garcia et al., 2008). Water temperature changes can stress living organisms and adversely affect their health (Yu et al., 2009). Stress induced by water temperature changes has also been associated with enhanced generation of reactive oxygen species (ROS) and oxidative stress (Lushchak and Bagnyukova, 2006a).

Oxidative stress is defined as an imbalanced state between elevated concentrations of pro-oxidants over antioxidants that results in the generation of ROS (Matés et al., 1999). ROS are naturally produced during oxidative metabolism. These

ROS include superoxide anions ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($HO^{\cdot}$), and singlet oxygen (Livingstone, 2001). The antioxidant defense system of shrimp includes enzymes such as catalase (CAT) and glutathione-*S*-transferase (GST), which are important for ROS scavenging and/or the prevention of oxidative damage.

Catalase is involved in the detoxification of hydrogen peroxide (H_2O_2) (Guemouri et al., 1991). Glutathione-*S*-transferases are a family of dimeric multifunctional enzymes involved in the detoxification of xenobiotics; protection from oxidative damage; the intracellular transport of hormones, endogenous metabolites and exogenous chemicals in diverse organisms (Eaton and Bammler, 1999; Sheehan et al., 2001; Frova, 2006; Goto et al., 2009).

Total antioxidant scavenging capacity assay against peroxyl radicals quantifies the capacity of animals to neutralize ROS, thus providing an index of biological resistance to oxidative stress (Winston et al., 1998). This assay has been used to evaluate the ROS scavenging capacity of several marine invertebrate species (Regoli and Winston, 1998; Regoli et al., 2000). In recent years, a new technique based on the fluorescence detection of ROS using a peroxyl radical generator has been proposed (Amado et al., 2009). The main objective of this study was to evaluate the best temperature for transporting *F. brasiliensis*.

2. Material and Methods

2.1 Experimental design

Farfantepenaeus brasiliensis juveniles (5.53 ± 1.20 g) were obtained from the Marine Station of Aquaculture, Universidade Federal do Rio Grande - FURG, Rio Grande do Sul State (RS), Southern Brazil. Shrimp were acclimated in tanks with 4,000 L of filtered water at 22.4 °C and 22‰ salinity. After acclimatization, the animals were

transported for 12 h at five different temperatures (16.0, 19.3, 22.4, 25.0 or 28.0 °C), with three repetitions of each treatment. The control temperature was that of the sample collected before starting the experiment at initial time (22.4 °C). Feeding was suspended 12 h before beginning the experiment.

The shrimp were transported in 50 L transparent plastic bags containing one-third water (10 L) and two-thirds pure oxygen and tied with rubber strings. To maintain different water temperatures, five 200 L tanks containing the experimental units were used as water baths. The water in these tanks was heated by submerged heaters with thermostats and was cooled with ice.

Before sealing, a transparent hose was inserted into the bags through which 60 mL water samples were collected every two hours to monitor the water quality during transport. The plastic bags were randomly arranged in the 200 L tanks at a density of three shrimp L^{-1} (30 shrimp per bag). The water temperature inside the plastic bags was slowly adjusted to match the temperature inside each tank (16.0 ± 0.2, 19.3 ± 0.3, 22.4 ± 0.2, 25.0 ± 0.3 or 28.0 ± 0.4 °C), which was maintained until the end of transport.

The water quality parameters (temperature, salinity, dissolved oxygen and pH) were monitored every two hours using a multi-parameter YSI 556 (Yellow Springs Instruments, Yellow Springs, OH, USA). Total ammonia and alkalinity were also monitored every two hours according to the methods of UNESCO (1983) and Baumgarten et al. (1996), respectively.

2.2 Enzymatic assay

The hemolymph of five shrimp from each plastic bag was collected at the beginning (initial time) and at the end of transport. The samples were obtained directly from the heart of each shrimp using sterile syringes containing an anticoagulant solution

(glucose 0.1 M, sodium citrate 30 M, citric acid 0.026 M, NaCl 0.45 M, EDTA 0.02 M and pH adjusted to 7.4) (Söderhall and Smith, 1983), transferred to 1.5 mL polyethylene tubes and stored at -80 °C in an ultra-freezer. For protein quantification and antioxidant enzyme analysis, the hemolymph was centrifuged twice at 500 and 900 × *g* at 4 °C for 35 and 15 min, respectively, to obtain a pellet. After centrifugation, the pellet was re-suspended in a cold 4 °C buffer solution containing Tris base (20×10^{-3}M), EDTA (1×10^{-3} M), dithiothreitol (1×10^{-3} M), KCl (150×10^{-3} M), and PMSF (0.1×10^{-3} M), with the pH adjusted to 7.6. All biochemical determinations were performed at least in triplicate.

The total antioxidant competence against peroxyl radicals was analyzed by quantifying ROS in the hemolymph as described by Amado et al. (2009). The catalase and GST activity measurements were based on McCord and Fridovich (1969) and Habig et al. (1974), respectively.

All results were expressed as enzyme units except the total antioxidant competence, which was expressed as the relative areas between the difference in the same sample with and without the addition of 2,2′-azobis-2-methylpropionamidine dihydrochloride (ABAP) and standardized to the ROS area without ABAP (background area). The relative difference between the ROS area with and without ABAP was considered a measurement of the total antioxidant capacity. A large area difference corresponded to a low antioxidant capacity because high fluorescence levels were obtained after the addition of ABAP, indicating low competency in neutralizing peroxyl radicals. The total fluorescence production was calculated by integrating the fluorescence units (FU) along the time (30 min) and measurement after adjusting the FU data to a second order polynomial function.

One CAT unit represents the amount of enzyme needed to degrade 1 µmol of H_2O_2 per min and per mg of total proteins present in the homogenates, at 30 °C and pH 8.00. One GST unit is the amount of enzyme necessary to conjugate 1 µmol of 1-chloro-2,4-dinitrobenzene (Sigma-Aldrich) per min and per mg of total protein present in the homogenates, at 25 °C and pH 7.00.

2.3 Statistical analysis

The statistics were expressed as the mean ± standard error. Assumptions of normality and homogeneity were previously verified (Levene test). All tests of enzyme activities and water quality parameters were performed by analysis of variance (two-way ANOVA) followed by Newman-Keuls post-hoc comparisons with a significance level of 0.05.

3. Results

3.1 Water quality and survival

The physicochemical water quality parameters are shown in Table 1. Water quality parameters analyzed during the experimental period remained at concentrations suitable for shrimp culture, except for dissolved oxygen due to increasing temperature. The treatment at 28.0 °C produced the lowest final survival (90.0%) and differed significantly from the other treatments (Table 1).

3.2 Antioxidant responses

Total antioxidant capacity was higher for shrimp transported at 22.4 °C, which demonstrated the highest relative area difference (277%) compared to the control and

other temperatures, indicating a significant loss of antioxidant competence against peroxyl radicals in shrimp maintained at 22.4 °C.

CAT enzymatic activity of shrimp transported at 19.3 °C was significantly higher compared with the initial time (36.6%); 16.0 °C (40.6%) and 22.4 °C (50%). The best result was observed in shrimp exposed to 19.3 °C, which resulted in the highest CAT activity (32.35%) relative to the control results. GST levels in shrimp stored in lower water temperature showed increased activity. The shrimp transported at 16.0 °C exhibited an increase of 172.9%, 336.7%, 1,771.4% and 991.7% compared to the initial time, 22.4, 25.0 and 28.0 °C groups, respectively. The pink shrimp transported at 19.3 °C showed the highest levels of GST activity, which were 375.0%, 74.0%, 660.0%, 3,157.1% and 1,800.0% higher than the initial time, 16.0, 22.4, 25.0 and 28.0 °C, respectively.

4. Discussion

Water temperature is one of the most important environmental factors to consider in aquaculture (Qiu et al., 2011). Maintaining low water temperature during transport and avoiding disturbances may maximize organism survival (Whiteley and Taylor, 1992) and reduce ROS production due to decreased shrimp metabolism. The effects of water temperature in this study showed that shrimp maintained at low water temperatures experienced better conditions for transport compared to warmer temperatures, and a water temperature of 28.0 °C negatively affects the shrimp survival rate (90%) during transport (12 h).

In agreement with our results, Tidwell and Coyle (2002) evaluated the effect of water temperature (20 and 25 °C) on the transport of *Macrobrachium rosenbergii* and verified that lower water temperatures had a positive impact on shrimp survival and

water quality during transport. These authors observed that survival was significantly lower at 25 °C (24.3%) compared to those organisms stocked at 20 °C (96.48%). During transportation, low water temperature reduced shrimp metabolism and also improved the water quality by reducing the excretion levels and oxygen consumption by shrimp, resulting in maximal survival.

In contrast, higher water temperatures stimulate metabolism and thus may increase ROS production (Lushchak, 2011). These conditions result in oxidative stress, which affects the enzyme activity of superoxide dismutase (SOD), CAT, glutathione peroxidase (GPx) and GST (Lushchak and Bagnyukova 2006a, 2006b). Our results are in accordance with these results because shrimp exposed to 19.3 °C had higher activities of CAT and GST. In general, the increased activities of CAT and GST are associated with increasing temperatures, which in turn further accelerate the metabolic rate of the organisms (Daoud et al., 2007).

In our study, although the total antioxidant capacity against peroxyl radicals of the treatment at 19.3 °C did not differ from the other treatments (except at 22.4 °C), this experimental condition showed higher antioxidant competence compared to the others.

Shrimp transported at 19.3 °C showed the highest CAT activity compared with the other treatments. Similar results were found in *Farfantepenaeus paulensis* and *Corbicula fluminea* (clam) when subjected to eyestalk ablation and hypoxia, respectively, which resulted in an increase in CAT activity, thus indicating an enhanced antioxidant capacity (Almeida et al., 2004; Lushchak, 2011). Increased CAT activity is important because it catalyzes the conversion of hydrogen peroxide (H_2O_2) into water and oxygen, thus preventing its conversion to hydroxyl radicals and reducing oxidative stress levels (Gonçalves-Soares et al., 2012).

The GST increase in shrimp stored at 19.3 °C may be a response aiming to neutralize the harmful free radicals generated either directly or indirectly by the transportation process. Zhou et al. (2009) mention that exposure to environmental stressors induces the expression of shrimp GST. After 12 h of exposure to a low pH (5.6), these authors found a significant elevation of total GST activities in *Litopenaeus vannamei* hepatopancreas and hemocytes. Furthermore, GST is involved in the neutralization of xenobiotics, harmful reactive oxygen species and other compounds involved in ROS generation, thereby conferring protection from oxidative damage (Yu, 1994; Kim et al., 2010).

Lowering water temperature promotes higher oxygen solubility and promotes a pro-oxidant environment. Reduced water temperature is also expected to produce a concomitant reduction in shrimp metabolism, resulting in lower endogenous ROS production. If shrimp simultaneously augment their antioxidant capacity, the balance of pro- and antioxidants should favor the antioxidants.

Our study demonstrated that shrimp transported in low water temperatures had better dissolved oxygen conditions and enhanced activity of antioxidant enzymes responsible for scavenging ROS. These results contribute to the improved survival and welfare of the animals and facilitate their handling during the transport. Therefore, we recommend 19.3 °C for transporting pink shrimp, *F. brasiliensis*, as this temperature results in the best antioxidant status and dissolved oxygen levels.

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Figure legends

Figure 1. Total antioxidant capacity against peroxyl radicals (A), catalase activity (B) and glutathione transferase activity (C) in the hemolymph of *Farfantepenaeus brasiliensis* juveniles transported at different temperatures.

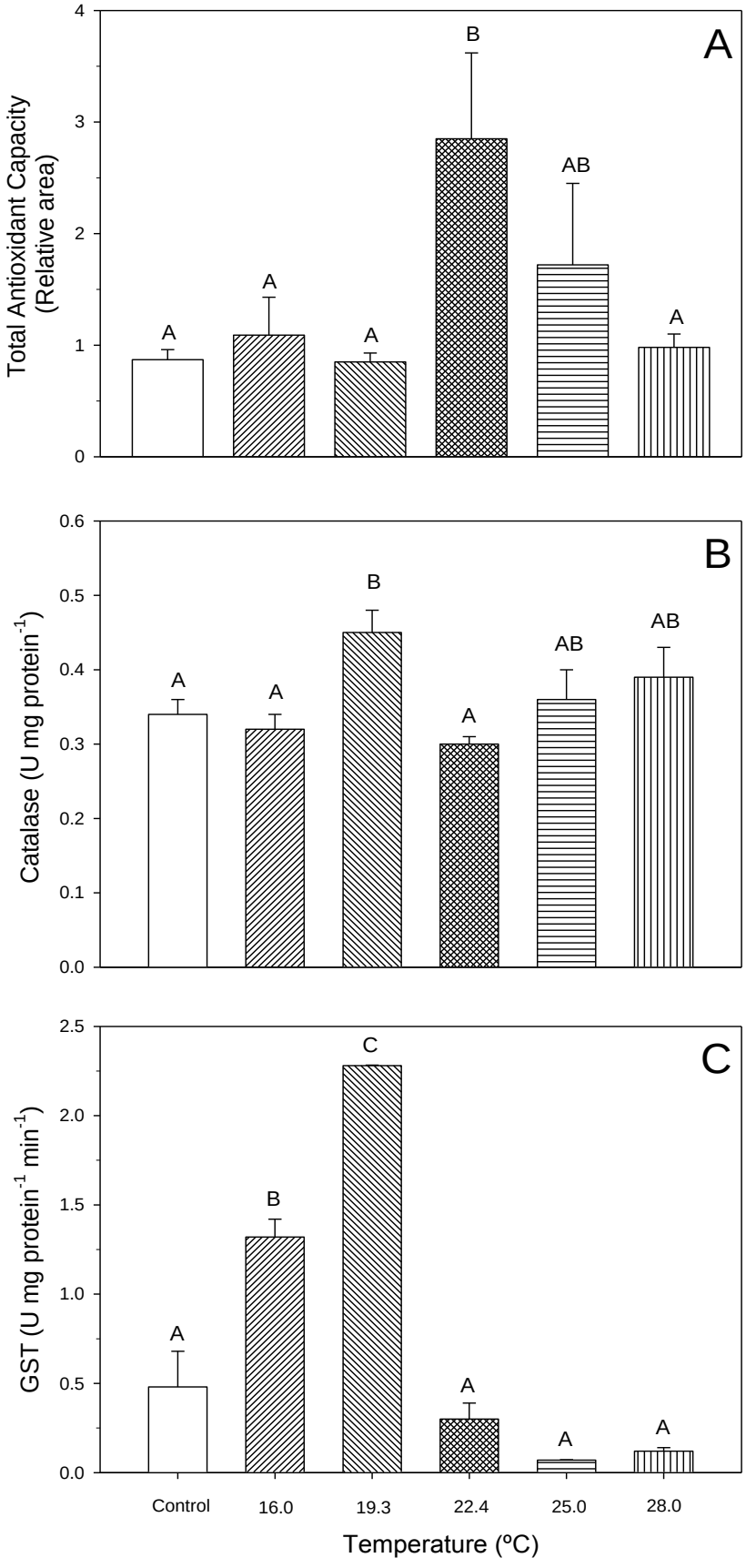
Tables

Table 1. Physicochemical parameters of transport water in experimental plastic bags during transport at different water temperatures. Data are reported as the means ± SEM. Different superscript letters indicate statistically significant differences between different treatments in the same parameters ($P < 0.05$).

	Water quality parameters					
Treatments	Temperature (°C)	DO (mg L^{-1})	pH	Alkalinity (mg L^{-1})	TAN (mg L^{-1})	Survival (%)
16ºC	16.0 ± 0.2^{a}	6.35 ± 1.6^{a}	7.13 ± 0.4^{a}	94.5 ± 14.4^{a}	1.61 ± 1.1^{a}	98.9 ± 1.9^{a}
19ºC	19.3 ± 0.3^{b}	6.91 ± 1.6^{a}	6.93 ± 0.5^{a}	91.4 ± 15.6^{a}	1.79 ± 1.1^{a}	100 ± 0.0^{a}
22ºC	22.4 ± 0.2^{c}	4.03 ± 0.2^{b}	6.87 ± 0.5^{a}	87.3 ± 17.0^{a}	1.63 ± 1.0^{a}	98.9 ± 1.9^{a}
25ºC	25.0 ± 0.3^{d}	3.28 ± 0.3^{b}	6.89 ± 0.5^{a}	91.2 ± 15.5^{a}	1.95 ± 1.3^{a}	100 ± 0.0^{a}
28ºC	28.0 ± 0.4^{e}	3.07 ± 0.3^{b}	6.92 ± 0.5^{a}	80.0 ± 20.1^{a}	1.82 ± 1.3^{a}	90.0 ± 8.8^{b}

DO, dissolved oxygen; TAN, total ammonia nitrogen

Figure 1


A
B
C
Total Antioxidant Capacity (Relative area)
Catalase (U mg protein-1)
GST (U mg protein-1 min-1)
A
A
A
B
AB
A
A
A
B
A
AB
AB
A
B
C
A
A
A
Control
16.0
19.3
22.4
25.0
28.0
Temperature (ºC)

Discussão geral

Os experimentos realizados nessa tese abordaram parâmetros fisiológicos e imunológicos de camarões peneídeos em diferentes temperaturas nos sistemas de cultivo em bioflocos e água clara.

No cultivo de *L. vannamei* por um curto período (10 dias), os resultados mostraram que em baixas temperaturas nas primeiras 6 h os camarões destes tratamentos (15 e 21ºC) apresentaram aumento da peroxidação lipídica (LPO) e por conta disso também foi observada aumento das enzimas antioxidantes Catalase e GST envolvidas na detoxificação lipídica. Fatores de estresse ambiental podem desencadear respostas antioxidantes para reverter o quadro oxidativo. Os camarões expostos a 15 e 21ºC apresentaram aumento da atividade enzimática para compensar o provável aumento de ERO e esse estresse gerado pela temperatura aumentou o gasto energético para atenuar a produção de ERO. Sendo assim é importante que o animal seja cultivado em ambiente com condições favoráveis para que seu desempenho zootécnico seja otimizado e o seu bem estar esteja assegurado.

Esses resultados foram confirmados quando foi realizado o experimento de longa duração, quando juvenis de *L. vannamei* foram cultivados durante 60 dias em cultivo de bioflocos e água clara em diferentes temperaturas. Os animais cultivados a 15 e 33ºC apresentaram maior atividade antioxidante para neutralizar a produção de ERO. Esses tratamentos apresentaram baixa sobrevivência confirmando o estresse oxidativo e a peroxidação lipídica dos animais, os quais o sistema antioxidante não foi suficiente para eliminar as ERO como pode ser observado nos resultados de brânquias e hemolinfa desses tratamentos. Os animais que apresentaram os melhores resultados de desempenho zootécnico e fisiológico foram os cultivados a 27ºC. Portanto extremos de

temperatura devem ser evitados para promover o bem estar animal e melhorar a produção de camarões.

No experimento comparando os dois sistemas de cultivo, ambos a 27ºC, os camarões cultivados em BFT apresentaram maior sobrevivência. O melhor desempenho dos animais deste tratamento pode ser relacionado ao consumo de bioflocos pelos camarões. É sabido que a comunidade microbiana presente no biofloco pode contribuir para melhorar parâmetros imunológicos e de desempenho zootécnico. De acordo com a composição, os bioflocos podem desempenhar função antioxidante uma vez que contém uma quantidade razoável de carotenoides e vitaminas que atuam como antioxidantes. No tratamento com água clara pode ser observado maior peroxidação lipídica nos camarões. Esse resultado demonstra que o sistema antioxidante não foi suficiente para neutralizar a ação de radicais livres e, portanto ocorreu o dano lipídico.

O sistema de bioflocos apresenta muitas vantagens frente ao sistema tradicional de cultivo em água clara. O BFT propiciou o cultivo de camarões em áreas menores, em sistema super-intensivo, ou seja, alta densidade de estocagem aumentando significativamente a produção de camarões e uma das consequências mais importantes foi desenvolver o cultivo com troca zero de água. Um ganho extremamente importante para a atividade que visa à sustentabilidade e à preservação ambiental. Dessa forma reduz drasticamente a emissão de efluentes, promove a ciclagem de nutrientes no sistema e beneficia toda a cadeia trófica, reduzindo os custos de produção e o impacto ambiental.

Fatores ambientais como temperatura, pH e salinidade podem afetar as respostas antioxidantes de organismos aquáticos. Durante o transporte de camarões a manutenção de uma temperatura reduzida, favorece a redução da produção de ERO pela diminuição do metabolismo dos animais, além de maximizar a sobrevivência. No experimento de

transporte foi evidenciado que a temperatura reduzida (19ºC) favoreceu o transporte enquanto que temperaturas mais altas (28ºC) apresentaram menor sobrevivência de *F. brasiliensis*.

Altas temperaturas estimulam o metabolismo animal e consequentemente aumentam a produção de ERO. No caso de transporte, o aumento da temperatura por aumentar o metabolismo, consequentemente também aumenta o consumo de oxigênio e a excreção por parte dos animais. Nesse cenário a qualidade de água fica prejudicada e afeta diretamente os animais transportados, reduzindo significativamente o tempo que os animais suportarão o transporte em virtude do prejuízo gerado na qualidade da água, bem como podendo comprometer de forma geral as atividades fisiológicas e imunológicas básicas dos animais.

Considerações finais

Os resultados obtidos nessa tese nos permitem concluir que o cultivo da espécie exótica *L. vannamei* é otimizado em sistema de bioflocos e a temperatura ideal para cultivo é de 27ºC. Nessa condição os animais apresentaram melhor desempenho zootécnico e seu sistema antioxidante foi favorecido. O transporte de camarões peneídeos da espécie *F. brasiliensis*, realizado em temperatura reduzida (19ºC) favorece os animais, pois apresentam maior taxa de sobrevivência quando comparados com o transporte em temperaturas mais elevadas (28ºC). Os animais apresentaram uma melhor condição antioxidante em temperaturas mais baixas, em relação aos transportados em temperaturas maiores (28ºC). Por essas razões, extremos de temperatura tanto para o transporte de camarões da espécie *F. brasiliensis* quanto para o cultivo de *L. vannamei* devem ser evitados.

Perspectivas futuras

Nessa tese podemos observar que a temperatura exerce importante influencia no sistema antioxidante de camarões. A produção de antioxidantes como, por exemplo, a GSH é ATP-dependente, ou seja, sua formação demanda gasto energético, consequentemente menor será a disponibilidade de ATP (Adenosina tri-fosfato) para o crescimento dos animais. Novos estudos devem ser feitos para avaliar o efeito da temperatura no gasto energético, com intuito de quantificar a demanda de ATP requerida pelos animais expostos ao estresse térmico. Outro aspecto importante é avaliar o dano lipídico e a atividade enzimas antioxidantes nos animais sujeitos ao transporte, pois nos sacos plásticos (utilizados para o transporte de animais) é adicionado oxigênio puro e pode desencadear um cenário pró-oxidante. Portanto estudos dessa natureza também são importantes, pois ainda não estão elucidados os possíveis danos que a adição de oxigênio puro pode desencadear nos animais.

O cultivo em bioflocos revolucionou a carcinocultura. O refino final do pacote tecnológico do sistema BFT será estudar a comunidade microbiana do floco. Identificar as espécies, avaliar o seu mecanismo de ação, identificar o potencial relacionado à qualidade da água na captura do nitrogênio e o potencial microbiano como probiótico para melhoria do sistema imunológico e nutricional dos animais. O enfoque microbiológico do sistema fará impulsionar ainda mais a atividade no país, fazendo com que a tecnologia de cultivo de camarões marinhos no Brasil avance ainda mais.