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Determinação de parâmetros físico-químicos para experimentos com hipóxia e avaliação dos efeitos do antioxidante ácido lipóico associados à hipóxia/reperfusão no camarão *Litopenaeus vannamei*.

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Sumário

Resumo Geral

A concentração de oxigênio dissolvido (OD) em ambientes aquáticos é uma variável de extrema importância para o desenvolvimento e sobrevivência dos animais, sendo sua solubilidade influenciada diretamente por fatores abióticos como salinidade e temperatura. A concentração reduzida/ausência de OD caracteriza-se com hipóxia/anóxia (H) e diversos danos são produzidos nesta situação nos sistemas biológicos, entretanto a retomada do oxigênio (reperfusão) (R) aos níveis normais é tão prejudicial quanto a hipóxia. O presente trabalho foi dividido em dois capítulos. O primeiro capítulo teve como objetivo verificar a influência da salinidade (5, 25 e 35‰), temperatura (20, 25 e 30ºC) e OD (zero, 0.5, 1.5, 3 e 6 mg/O_2L^{-1}) na sobrevivência do camarão branco *Litopenaeus vannamei*. Nos cruzamentos da temperatura de 20 °C com as salinidades de 25 e 35‰ foi verificado o menor valor de $CL_{50\text{-}96h}$ (1.92 mg/O_2L^{-1}), em contrapartida, a maior $CL_{50\text{-}96h}$ foi encontrada na salinidade de 5‰ em 30 ºC (4.81 mg/O_2L^{-1}). Já o segundo capítulo teve como objetivo verificar a quimioproteção do antioxidante ácido lipóico (AL) suplementado na ração de camarões submetidos a hipóxia (1.5 mg/O_2L^{-1}) por 6 e 24 horas e reperfundido (6 mg/O_2L^{-1}) por 1 e 3 horas. Observou-se que os níveis de glicose na hemolinfa aumentaram enquanto o inverso foi verificado em músculo de animais suplementados durante a H/R. Porém a atividade da lactato desidrogenase e os níveis de lactato diminuíram ou permaneceram inalterados nesta mesma situação. Além disso, tanto na capacidade antioxidante como na peroxidação lipídica, o AL teve efeito protetor evidenciado durante a reperfusão. Em tempos diferentes durante a H/R a atividade da glutationa-*S*-transferase foi modulada de forma a concluir que a enzima não é um bom biomarcador para H/R. Os capítulos dessa tese direcionam a concluir que parâmetros abióticos devem ser levados em consideração para declarar situações de hipóxia, situações estas que uma ração enriquecida com antioxidantes pode ser de substancial ajuda para os indivíduos, principalmente de cultivo.

Palavras Chave: *Litopenaeus vannamei, $CL_{50\text{-}96h}$, ácido lipóico, metabolismo energético, hipóxia/reperfusão, capacidade antioxidante.*

Abstract

The concentration of dissolved oxygen (DO) in aquatic environments is a variable that is extremely important for the development and survival of animals, being its solubility directly influenced by abiotic factors such as salinity and temperature. The low concentration/absence of DO is characterized with hypoxia/anoxia (H) and various damage are produced in this situation in the biological systems, however the resumption of oxygen (reperfusion) (R) to normal levels is as harmful as hypoxia. The present study was divided into two chapters, the first chapter aimed to verify the influence of salinity (5, 25 and 35‰), temperature (20, 25, and 30 ºC) and DO (0, 0.5, 1.5, 3 and 6 mg/O_2L^{-1}) in the survival of white shrimp *Litopenaeus vannamei*. In the crossings of the temperature of 20 °C with the salinities of 25‰ and 35‰ is the lowest value of $CL_{50\text{-}96h}$ (1.92 mg/O_2L^{-1}), on the other hand, the largest $CL_{50\text{-}96h}$ was found in salinity of 5‰ is at 30 ºC (4.81 mg/O_2L^{-1}). The second chapter aimed to verify chemoprevention of antioxidant lipoic acid (LA) supplemented in the diet of shrimps submitted to hypoxia (1.5 mg/O_2L^{-1}) for 6 to 24 hours and posteriorly perfused (6 mg/O_2L^{-1}) for 1 and 3 hours. It was observed that the levels of glucose in the hemolymph increased, while the inverse was observed in muscle of animals supplemented during the H/R. However, the activity of lactate dehydrogenase and lactate levels decreased or remained unchanged in this same situation. In addition, both the antioxidant capacity as the lipid peroxidation, LA had a protective effect observed during reperfusion. At different times during the H/R the activity of glutathione-*S*-transferase was modulated in order to conclude that the enzyme is not a good biomarker for H/R. The chapters of this thesis lead to conclude that abiotic parameters should be taken into account to state situations of hypoxia, situations where a ration enriched with antioxidants can be of substantial help to individuals mainly shrimp farmed.

Keywords: *Litopenaeus vannamei,* $CL_{50\text{-}96h}$, lipoic acid, energetic metabolism, hypoxia/reperfusion and antioxidant capacity.

1. Introdução

1.1 Hipóxia

Os organismos vivos necessitam de energia para realizar processos metabólicos e fisiológicos, sendo o oxigênio um dos fatores limitante do processo por ser o aceptor final de elétrons durante a respiração mitocondrial. As moléculas de ácidos graxos, carboidratos e proteínas são oxidados em processos bioquímicos gerando coenzimas reduzidas que são reoxidadas na cadeia transportadora de elétrons que culminarão na redução do oxigênio (O_2) à água (H_2O). Este processo libera energia, a qual será utilizada na produção de moléculas de adenosina trifosfato (ATP) que é utilizada na maioria das reações bioquímicas pelos organismos (**Figura 1)** (Halliwell e Gutterridge, 1999).

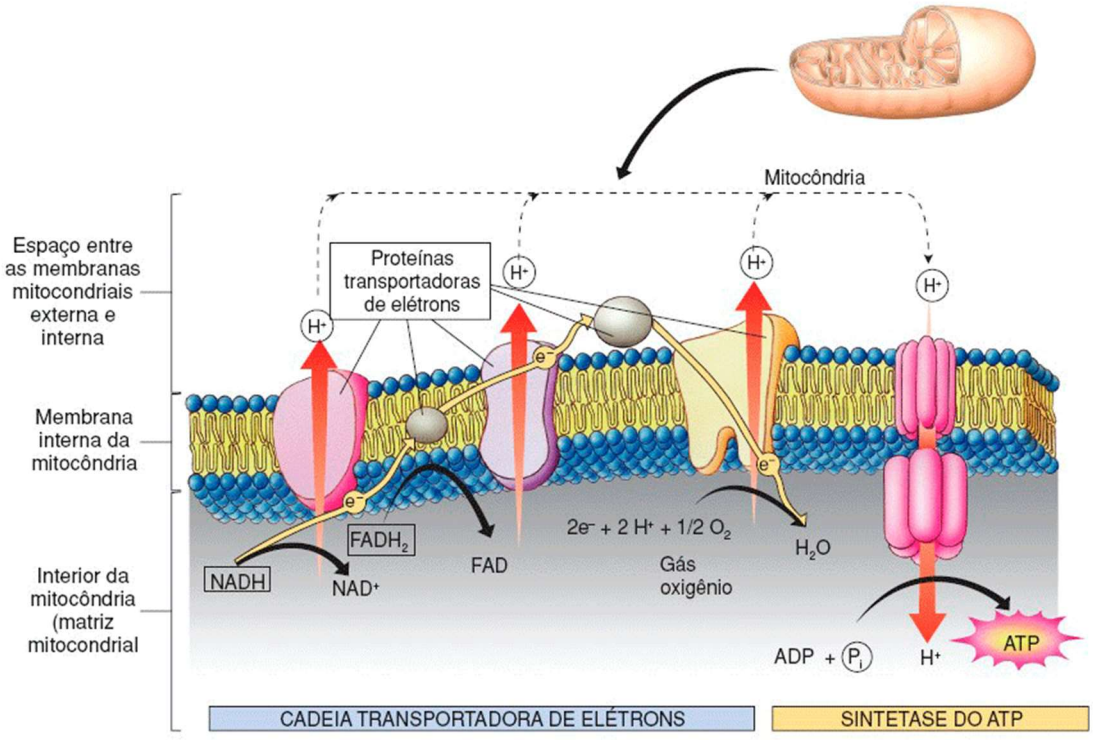

Figura 1: Detalhe dos transportadores de elétrons da cadeia respiratória e a síntese do ATP (membrana interna mitocondrial). Fonte: Amabis e Martho. Fundamentos da Biologia Moderna, 4ª edição.

Na diminuição e/ou ausência do oxigênio, ocorre a parada da progressão dos elétrons na cadeia transportadora, interrompendo ou diminuindo a fosforilação oxidativa. Paralelo a isso, quando os níveis de oxigênio são limitados, as células podem redirecionar o metabolismo aeróbico desviando para o metabolismo anaeróbico (glicólise anaeróbica), permitindo a continuação da glicólise, porém, com uma eficiência menor na produção de ATP (Ferreira, 2010).

Os tecidos com alta demanda metabólica, submetidos a períodos de hipóxia severa, podem ter sua atividade mitocondrial danificada, comprometendo e diminuindo os níveis de ATP de forma permanente. De fato, Geihs *et al.* (2013) verificaram que mesmo após a retomada dos níveis de oxigênio, danos permanentes podem ser gerados no músculo do crustáceo *Neohelice granulata*, visto que a progressão dos elétrons na cadeia transportadora do caranguejo ocorria com atrasos, comprometendo a obtenção de energia desses animais. Desta forma, os processos que demandam grande quantidade de energia, como regulação osmótica, síntese proteica, crescimento e reprodução ficam comprometidos, podendo levar o animal a morte no caso de longos períodos de privação do oxigênio.

O oxigênio em ambientes aquáticos em parte se dá por dissolução do ar atmosférico e é determinado por um conjunto de variáveis, como pressão atmosférica, temperatura e salinidade (Weiss, 1970). A pressão influência de formas diferentes a presença do oxigênio na água, que é proporcional à pressão parcial de O_2 na atmosférica (Lei de Henry). Outro fator é a temperatura, visto que a solubilidade dos gases na água diminui com a elevação da temperatura, pois o calor aumenta a mobilidade das moléculas levando-as para a superfície, o mesmo acontecendo com a salinidade que quando aumentada também influência a dispersão do oxigênio para a atmosfera (Hill *et al.,* 1993).

Baixos níveis de OD são considerados como hipóxia enquanto níveis normais de OD é referido com normoxia que por sua vez está estabelecida entre 6 ± 7 mg O_2 L^{-1} para ambientes marinhos (Welker *et al.,* 2013; Lushchak e Bagnyukova, 2006). A hipóxia tida como moderada

(± 3 mg O_2 L^{-1}) pode acarretar em distúrbios fisiológicos em crustáceos como, por exemplo, interferir nos ciclos de muda, taxa de alimentação, crescimento, reprodução e osmorregulação (Welker *et al.,* 2013). Além disso, com o metabolismo alterado pela privação do oxigênio, pode ocorrer a degradação de glicogênio e alta taxa de glicólise anaeróbica, que leva a maior produção de lactato, resultando em diminuição de pH, que pode afetar a estrutura de proteínas e enzimas (Pérez-Jimenéz, 2012). Entretanto, com o restabelecimento dos níveis de oxigênio, o metabolismo energético novamente é direcionado, porém desta vez para o metabolismo aeróbico ativando processos como gliconeogênese, glicólise aeróbica e também de outros combustíveis como ácidos graxos, por exemplo. De fato, Maciel *et al.* (2008), observou que uma maior quantidade de glicose estava sendo formada no músculo mandibular do caranguejo *N. granulata*, sugerindo que o processo de gliconeogênese poderia estar ocorrendo neste tecido.

Outro efeito direto causado pela hipóxia é a ativação de fatores que respondem ao estresse gerado pela privação de oxigênio como o Fator Induzido por Hipóxia (HIF-1), (Sonãnez-Organis *et al.,* 2009). Na ausência do oxigênio, vários processos de sinalização são ativados, e o HIF- 1 sofre dimerização no núcleo das células, ativando sequências de transcrição de genes alvos que são denominados elementos responsivos à hipóxia (HERs) (Cohen e McGovern, 2005; Sonãnez-Organis *et al.,* 2009).

Os genes alvos HER estão relacionados principalmente como os processos de angiogênese, eritropoietina, transporte de glicose e glicólise, a afim de permitir os a produção de ATP mesmo em condição anaeróbica, portanto sem a presença de oxigênio. Embora, com um baixo rendimento, esses processos tem a finalidade de ajudar na reperfusão do oxigênio ainda presente nos tecidos, embora essas respostas tenham sido estudadas na sua grande maioria em mamíferos, genes homólogos já foram descritos em crustáceos, peixes e anfíbios (Semenza, 2011; Handy *et al.,* 2012).

Sonãnez-Organis e colaboradores (2010), mostraram que o HIF-1 em situação de hipóxia acarreta em alterações nas concentrações de glicose e lactado na hemolinfa do camarão *L. vannamei*. Os crustáceos podem lidar com a hipóxia de formas diferentes como: aumentar a concentração de glicogênio e de arginina fosfato, utilizar vias anaeróbicas para produção de ATP, manutenção do sistema redox e reduzir a atividade metabólica (Childress e Seidell, 1998).

Como mencionado anteriormente, na presença de oxigênio (normoxia), os animais utilizam-se da glicólise aeróbica como estratégia de obtenção de energia onde a glicose é utilizada como substrato da via, resultando na produção de ATP (36 moles de ATP por mol de glicose). Porém, durante períodos prolongados de hipóxia/anóxia a estratégia de ativação da via de fermentação anaeróbica é de extrema importância, pois permite que os animais lidem por um maior período de tempo com a privação do oxigênio. Nesta situação, os organismos utilizam basicamente carboidratos armazenado sob a forma de glicogênio e alguns aminoácidos resultando em uma produção menor de ATP (2 moles de ATP por mol de glicose na conversão a lactato) quando comparado com o metabolismo aeróbico, porém, esta via é crucial para sobrevivência dos animais principalmente quando o aporte de oxigênio é limitado (Storey , 2007).

O principal produto do metabolismo anaeróbico é o lactato, sendo este principalmente acumulado na hemolinfa como resultado da mobilização do glicogênio tecidual após episódios de hipóxia/anóxia. A relação entre lactato e glicose neste tecido sugere que o lactato é um importante substrato energético para mobilização de energia (Maciel *et al.,* 2004).

O processo da retomada de oxigênio após um período de hipóxia é de grande importância, pois neste momento o organismo tem que lidar com a chamada "injúria da reperfusão", pois todos os elétrons acumulados durante o período de hipóxia são liberados de forma abrupta, ligando-se ao oxigênio (Storey, 1996; Hermes-Lima, 2004; Garcia *et al.,* 2008). O fluxo de oxigênio na cadeia transportadora de elétrons é retomado e ocasiona a liberação dos

produtos gerados e acumulados na mitocôndria pelo metabolismo anaeróbico, que podem causar extensos danos aos sistemas biológicos dos organismos (Hermes-Lima e Zenteno-Savín, 2002; Zenteno-Savín *et al.,* 2006; Lushchak, 2011; Hermes-Lima *et al*., 2015).

Vários estudos de hipóxia e reperfusão nos animais (Smith *et al.,* 2003; Peréz-Rostro *et al.*, 2004; Sañanez-Organis *et al*., 2011; Parrilla-Taylor *et al.,* 2011; Felix-Portillo *et al.,* 2014; Cota-Ruiz *et al.,* 2016; García-Triana *et al*., 2016; Felix-Portillo *et al*., 2016; Li *et al*., 2016) já verificaram a ocorrência de danos aos organismos, dentre elas, a produção de espécies reativas de oxigênio (ERO) que podem causar o desequilíbrio do sistema redox das células. Dentre estas ERO podem-se destacar o radical ânion superóxido (O_2^-), o peróxido de hidrogênio (H_2O_2) e o radical hidroxila ($^{\cdot}OH$). Adicionalmente existe a liberação/formação de metabólitos produzidos durante o período da restrição de oxigênio, como a xantina e a hipoxantina que subsequentemente também produz ERO, podendo afetar diretamente as macromoléculas biológicas (Fridovich, 2004; Halliwell e Gutteridge, 2007; Chandel, 2010).

A enzima xantina oxidase (XO) é importante fonte de radicais livres em tecidos reoxigenados. Durante a hipóxia, a xantina produzida pela quebra de AMP acumula-se nos tecidos juntamente com a hipoxantina. A enzima XO usa o oxigênio para converter hipoxantina em xantina produzindo superóxidos no processo **(Figura 2)**.

Também, durante a reperfusão, superóxidos e peróxido de hidrogênio são produzidos promovendo a liberação do íon ferroso, de forma que grande quantidade de ferro pode estar disponível para catalisar a conversão como peróxido de hidrogênio em radicais livres (Tabima *et al.,* 2005).

Proteínas, lipídios e DNA são macromoléculas biológicas passíveis de sofrer oxidação pela ação das ERO. Devido a esta produção de ERO, inerentes ao metabolismo aeróbico, os organismos possuem um sistema de defesa antioxidante (SDA) cuja função é inibir/reduzir ou

reverter os possíveis danos causados pela ação das ERO, além de atuar como “scavenges” de radicais ou ainda favorecendo o reparo das moléculas oxidadas (Halliwell e Gutteridge, 2007).

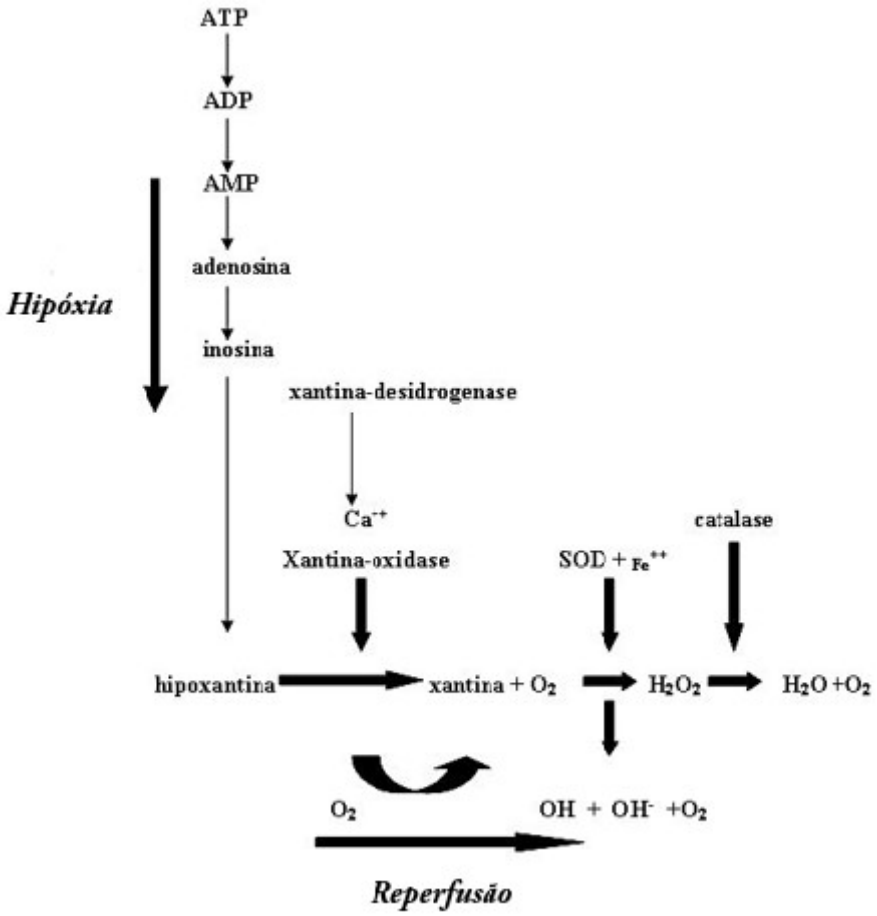

Figura 2: Mecanismo de produção de radicais livres pela ação da xantina oxidase. Fonte: Tabima *et al.,* 2005.

O SDA pode ser classificado como enzimático ou não-enzimático (Shila *et al.,* 2005). Entre o SDA enzimático estão as enzimas superóxido dismutase (SOD), catase (CAT), glutationa-*S*-transferase (GST), glutationa redutase (GR), glutationa peroxidase (GPx), sendo estes importantes na proteção contra danos oxidativos celulares em respostas a ambientes estressores (Maher, 2005). Dentre os antioxidantes não enzimáticos estão as vitaminas C e E, o

tripeptidio glutationa reduzida (GSH), carotenoides e o ácido lipóico (AL) (Packer *et al.,* 1995; Packer *et al.,* 1998; Ngueyn *et al.,* 2012).

Por definição, antioxidantes são substâncias com capacidade de interagir nas células reduzindo ou prevenindo a oxidação de macromoléculas pelas ERO, tanto de origem endógena a partir do metabolismo aeróbico como de fonte exógena como metais e agentes tóxicos (Aldini *et al.,* 2010).

1.2 O Ácido Lipóico

O ácido lipóico (AL) ou ácido 1,2-ditiolano-3-pentanóico é derivado do ácido octanóico, possuindo cadeia linear com pontes dissulfeto entre os carbonos 6 e 8 (White, 1980). Este antioxidante tem a capacidade de atuar nos distintos compartimentos celulares, pois é considerado lipo e hidrossolúvel, sendo capaz de ser digerida, absorvida e transportada para os tecidos, além de atravessar facilmente as membranas biológicas incluindo a barreira hematoencefálica (Muthuswamy *et al.,* 2006).

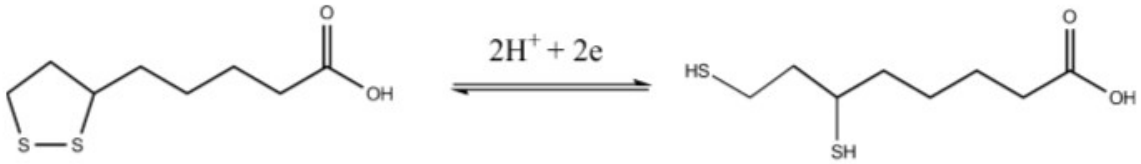

Figura 3: Estrutura química do par redox ácido α-lipóico (ALA) e ácido dihidrolipóico (DHLA). Fonte: Szelag *et al.,* 2012.

Esta molécula foi primeiramente classificada como sendo uma vitamina, após ser isolada por Reed e colaboradores em 1957. Posteriormente, esta classificação foi desconsiderada devido a síntese desta molécula também ter origem endógena nas células mitocôndrias de mamíferos

(Carreau, 1979). A síntese envolve a atividade da enzima ácido lipóico sintetase, ainda dentro da mitocôndria, onde o AL é então reduzido em parte a ácido diidrolipoico (DHLA) pela ação da enzima lipoamida desidrogenase ou pelo sistema da tioredoxina redutase, processos estes dependente de NADPH (Haramaki *et al.,* 1997). O AL é um cofator de complexos multienzimáticos do ciclo do ácido cítrico (α-cetoglutarato desidrogenase e piruvato desidrogenase) sendo portanto, fundamental para o metabolismo energético. (Packer *et al.,* 1998; Shila *et al.,* 2005). Além da produção endógena como cofator de enzimas, também é possivel absorção do AL através da ingesta de alimentos ou suplementos contendo este antioxidante (Morikawa *et al.,* 2001). De fato, os níveis do antioxidante produzido e livre nos tecidos e fluídos corporais são muito baixa, o que torna quase impossível que ele exerça efeito antioxidatante "*in vivo*" somente pela sintese endógena (Goraca *et al.,* 2011).

O AL pode ser normalmente encontrado nos alimentos, como a carne (coração, fígado e rins), vegetais (espinafre, ervilha e brócolis) e em alguns farelos (germe de trigo, levedo de cerveja, entre outros) (Samanthi *et al.,* 2012).

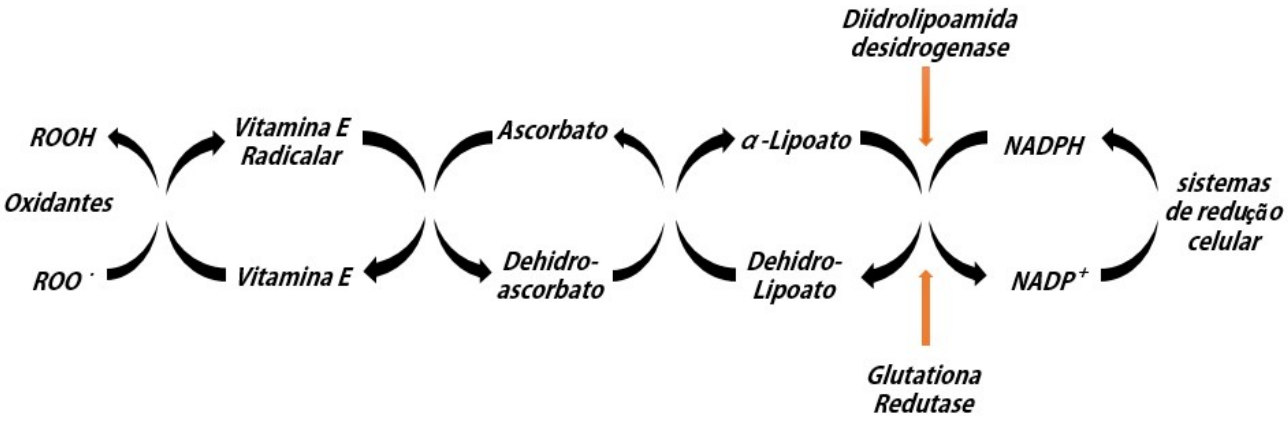

Figura 4: O papel do ácido lipóico na regeneração da vitamina C e da vitamina E.

Fonte: Packer e Suzuki, 1993.

Uma das características mais marcantes do ácido lipóico é a capacidade de atuar tanto na fase aquosa como na fase lipídica, além disso, esta molécula tem capacidade antioxidante tanto na forma oxidada ou reduzida (**Figura 3**) (Szelag *et al.,* 2012) (Packer *et al.,* 1995). O grupamento tiol (-SH) presente na molécula é capaz de neutralizar espécies radicalares a partir da interação direta com as mesmas (Perez e Castaneda, 2006). Também de maneira indireta atuando sobre outras moléculas antioxidantes nos sistemas biológicos. Adicionalmente, o AL pode quelar metais, atuar como "scavenger" de radical livres, prevenir danos oxidativos, além de, regenerar outros antioxidantes como o tripeptidio glutationa (GSH) bem como vitaminas E e C (Packer e Suzuki, 1993; Packer *et al.,* 1995; 1998) (**Figura 4**).

O AL como um suplemento dietético ou um agente terapêutico é capaz de modular circuitos redox distintos, devido à sua capacidade de equilíbrio entre diferentes compartimentos subcelulares, bem como extracelular. Estas propriedades despertam interesse nas mais diversas áreas e linhas de pesquisa principalmente na área clínica. Por exemplo, estudos contra o diabetes mostram que o AL é capaz de aumentar a ação do hormônio insulínico em ratos obesos devido a um aumento da translocação dos transportadores GLUT-1 e GLUT-4 nas membranas das células (Khamaisi *et al.,* 1997; Saengsirisuwan *et al.,* 2001; Fernández-Galilea *et al.,* 2011). Estes resultados indicam uma ação similar ao da insulina nas células musculares, aumentado a absorção da resultando em uma maior disponibilidade energética pela biossíntese de ATP (Jacob *et al.,* 1996). Estudos avaliando a suplementação de AL na dieta de organismos aquáticos têm mostrado que esta molécula é efetiva contra situações pró-oxidativas (Monserrat *et al.,* 2008). Por exemplo, no peixe teleósteo pacu (*Piaractus mesopotamicus*), o AL mostrou restabelecer os níveis de ácido ascórbico (vitamina C) nos animais que tiveram sua dieta suplementada com o antioxidante, visto que esses animais necessitam desta vitamina para realizar alguns de seus processos fisiológicos (Terjesen *et al.,* 2004). Resultados semelhantes foram observados na truta arco-íris (*Oncorhynchus mykiss*) e a carpa-comum (*Cyprinus carpio*) (Trattner *et al.,* 2007).

Em outro estudo, a suplementação na dieta com AL, foi capaz de modular positivamente o sistema de defesa antioxidante, aumentando a atividade da enzima glutationa-S-transferase (GST) (enzima de fase II da via detoxificatória) em cérebro do peixe *Corydoras paleatus*, juntamente com o aumento da atividade de glutamato cisteína ligase (GCL) em cérebro e fígado bem como a redução dos níveis de peroxidação lipídica no fígado deste peixe (Monserrat *et al.,* 2008).

No camarão branco do pacífico *Litopenaeus vannamei,* o AL foi utilizado como suplemento administrado na ração durante quatro semanas, onde foi possível verificar a quimioprevenção do antioxidante contra os efeitos nocivos da exposição aos metais cádmio e arsênio em músculo e hepatopâncreas do camarão. Neste mesmo estudo, foi possível observar o aumento da atividade de algumas enzimas do sistema de defesa antioxidante quando comparado com grupos que não receberam a dieta suplementada com o antioxidante (Lobato *et al.,* 2013). Outro estudo também analisando o efeito quimioprotetor do AL, Martins *et al,* (2014), verificaram que a suplementação foi capaz de modular positivamente as respostas bioquímicas em brânquias do *L. vannamei* quando exposto a concentrações de oxigênio dissolvido de 3 mg O_2 L^{-1} (hipóxia moderada).

1.3 Litopenaeus vannamei

O camarão branco do Pacifico, *Litopenaeus vannamei* (Crustacea; Decapoda) (**Figura 6**) está entre as espécies mais cultivadas no mundo, sendo portanto, de grande valor econômico (Hedlund, 2007). Originalmente endêmica do México e presente até o norte do Peru, e atualmente distribuida ampalmente em diversos paises no mundo (**Figura 5**) (FAO, 2008).

Sendo uma espécie de camarão de grande valor comercial, a carcinicultura vêm se desenvolvendo exponencialmente e ganhando cada vez mais espaço dentro da aquicultura. Isso pode ser atribuído ao fato que a quantidade obtida com a pesca convencional é insuficiente para suprir a demanda do mercado pelo crustáceo (FAO, 2008).

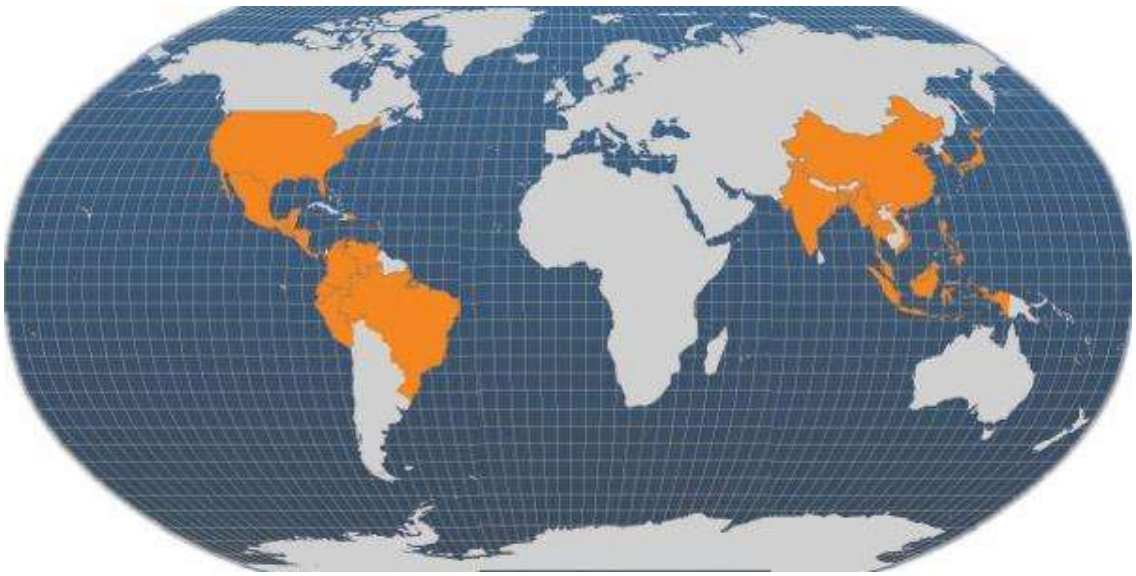

Figura 5: Os principais países produtores de *L. vannamei,* marcados na cor laranja. Fonte: FAO-Fishery Statistics, 2006.

Entretanto, justamente a produção intensificada pode trazer problemáticas à saúde em alguns aspectos na criação e cultivo de camarões, geralmente decorrentes da alta densidade populacional nos viveiros, podendo aumentar a vulnerabilidade do animal a situações estressoras, acarretando em doenças causadas por vírus, (síndrome de Taura; vírus da Mancha Branca e necrose hipodermal) e bactérias (*Vibrio spp e Vibrio parahaemolyticus*) (Zhang, 2006).

Outros fatores que estão intimamente ligados a medidas de produtividade e fortemente influenciam o bem-estar dos animais, são as condições abióticas nos viveiros como por exemplo, as técnicas de manejo adotadas pelos produtores (Sánchez *et al.,* 2001). Salinidade, temperatura e oxigênio dissolvido são os fatores mais importantes a serem estabelecidos e monitorados, pois

afetam as respostas fisiológicas dos indivíduos como, respostas imunes, crescimento, ciclo de muda e reprodução (Cheng *et al.,* 2005; Romano e Zeng, 2006).

L. vannamei é tolerante a grandes flutuação de salinidade podendo permanecer em uma ampla faixa de 2‰ até águas hipersalinas em torno de 40‰ Para o *L. vannamei* a salinidade ótima parece ser em torno de 28-32‰ (Wyban *et al*., 1995) nos viveiros, sendo esta salinidade a que mais aproxima do seu ponto isosmótico, desta forma, causando um menor gasto energético no processo de osmorregulação permitindo assim o gasto de ATP com outros processos como crescimento e reprodução, por exemplo.

A temperatura é outra variavel fundamental para o crescimento e desenvolvimento dos animais, sendo uma das mais difíceis de serem mantidas constantes, principalmente em viveiros externos que ficam dependentes das condições climaticas da região. De fato, a temperatura da água exerce efeito sobre o metabolismo dos crustáceos (Wyban *et al.,* 1995). Por exemplo, em águas mais amenas (entre 20-25 °C) a alimentação, o crescimento e a reprodução dos camarões são otimizados, entretanto, em viveiro onde a temperatura do cultivo fique acima de 30 °C ocorre uma menor concentração de oxigênio dissolvido e consequentemente uma diminuição da solubilidade de gases na água em relação a temperaturas inferiores. Assim quanto maiores as temperaturas, mais oxigênio será consumido pelo metabolismo do animal, podendo acarretar em estresse térmico diminuindo a resistência imunológica e aumentando a susceptibilidade à patogenos e infecções e causando mortalidade (Kubitza, 2003; Cheng *et al.,* 2005).

O oxigênio é outra importante e limitante variável em cultivos, geralmente refletindo de maneira geral as condições ambientais do viveiro, pois outras variaveis influenciam diretamente sobre a solibilidade deste gás (salinidade e temperarura bem como matéria orgânica). Concentrações ótimas de oxigênio dissolvido são estabelecidas entre 6 e 10 mg O_2 L^{-1} em culturas utilizando o *L. vannamei* (Brock e Main, 1994). Concentrações mínimas de oxigênio dissolvido acarretam em diversos efeitos negativos como, por exemplo, diminuição no

comportamento alimentar, reprodução, crescimento e sobrevivência dos organismos aquáticos. Desta forma é fundamental que parâmetros físicos químicos, tão importantes como temperatura, salinidade e oxigênio dissolvido tenham seus valores ótimos determinados e mantidos nos viveiros. Embora, vários estudos utilizam os parâmetros mencionados acima, até o momento não foi estudado o efeito de todas estas variáveis juntas para delinear as melhores condições para estes organismos.

Figura 6: Camarão branco do Pacífico *Litopenaeus vannamei* (Boone, 1931).

Objetivos

2.1 Objetivo Geral

Determinar valores de $CL_{50\text{-}96h}$ de OD para o camarão *Litopenaeus vannamei* sob diferentes salinidades e temperatura, bem como avaliar se o antioxidante ácido lipóico pode ser capaz de melhorar ou reverter danos causados pela hipóxia/reperfusão, considerando o metabolismo energético e sistema de defesas antioxidante.

2.2 Objetivos específicos

(a) estabelecer valores de $CL_{50\text{-}96h}$ para oxigênio dissolvido no camarão branco *Litopenaeus vannamei*, nas salinidades de 5, 25, e 35‰ e nas temperaturas de 20, 25 e 30 ºC, cruzados com diversas concentrações de oxigênio dissolvido (zero, 0.5, 1.5, 3.0 e 6.0 mg O_2 L^{-1}).

(b) avaliar os possíveis efeitos quimiprotetores do antioxidante ácido lipóico via dieta sobres os efeitos gerados na hipóxia/reperfusão.

- Considerando parâmetros de metabolismo energético como: níveis de glicose e lactato e atividade da enzima lactato desidrogenase;
- Considerando parâmetros de estresse oxidativo e sistema antioxidante como: peroxidação lipídica, capacidade antioxidante contra o radical peroxil e atividade da enzima glutationa-*S*-transferase.

3. Manuscrito I

Influence of salinity, temperature and oxygen on *Litopenaeus vannamei* survival

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ABSTRACT

The white shrimp *Litopenaeus vannamei* is one of the most cultivated species besides of worldwide economic importance. However, variation in physicochemical factors may cause large losses of animals in shrimp farming. In this context, the objective of this study was to evaluate the influence of salinity, temperature and oxygen oscillations in *Litopenaeus vannamei* survival. The mortalities of the shrimps were evaluated for 96 hours at salinity of 5, 25, and 35‰. Each salinity, was crossed with temperatures of 20, 25 and 30 ºC and different levels of oxygen: zero, 0.5, 1.5, 3 and 6 mg O_2 L^{-1}. The results were expressed as percentage of mortality and $LC_{50\text{-}96h}$ was calculated in relation to dissolved oxygen concentration for each situation. The $LC_{50\text{-}96h}$ showed a higher survival resistance to oxygen variation at lower temperature of 20 °C with higher salinities of 25 and 35‰. Also, a lower survival resistance at higher temperature of 30 °C and salinities of 5 and 25‰ were observed. Thus, we show in this study that the sensitivity of white shrimp to oxygen fluctuations is directly linked to temperature and salinity. Both high temperature and low salinity situations reflect the lower resistance to oxygen restrictions. These data, taken together, can contribute significantly to the production practices in order to minimize losses of animals, as well as on the welfare of the farming animals.

Keywords: *Litopenaeus vannamei*, crustacea, temperature, salinity, dissolved oxygen

1. INTRODUCTION

Shrimp farming is one of the most significant activities in worldwide aquaculture. Among the most economically important species of cultivated animals is the Pacific white shrimp (*Litopenaeus vannamei,* Crustacea; Decapoda) (Hedlund, 2007). Originally, this species is endemic from Mexico to the north of Peru, however, it is cultivated in several countries of the world and currently reaches larger tons in cultivation than those generated by conventional fishing (FAO, 2008).

Animal welfare and farming productivity are closely related to the environmental conditions, as well as, the appropriate management adopted by breeders. Physicochemical factors such as salinity, temperature and dissolved oxygen levels are some of the most important parameters to be established and monitored (Romano and Zeng, 2006). Dissolved oxygen is the most critical variable in shrimp farming, generally reflecting the nursery's environmental conditions. The ideal range of dissolved oxygen concentration for *Litopenaeus vannamei* is between 6 and 10 (mg O_2 L^{-1}) (Brock and Main, 1994). It is known that low oxygen concentrations with values below 1.5 mg O_2 L^{1} on prolonged exposure may be lethal to shrimp, as well as, moderate oxygen concentrations (3 mg O_2 L^{-1}), causing low feed rate, slow growth and low resistance to diseases (Welker *et al*., 2013). The solubility of oxygen is affected by temperature, atmospheric pressure, salinity and closely influenced by organic matter, therefore, the solubility of oxygen decreases both with increasing temperature and with increasing salinity (Spanopoulos-Hernández *et al.,* 2005).

For these reasons, one of the problems encountered by shrimp farming is precisely the control of the temperature of the nurseries, since it is exclusively dependent on the atmospheric temperature, oscillating throughout the day and the seasons (Barbieri and Ostrensky, 2002). The optimal temperature for *L. vannamei* growth is around 28 °C for adult animals (Wyban *et al*., 1995), however, the temperatures of water on farms vary from 15 to 30 °C in a short time

(Chen, 1990), causing thermal stress to the animals, reducing the immunological resistance and increasing the susceptibility to pathogens and infections (Cheng *et al*., 2005).

In addition, an ionic imbalance in the culture waters requires a higher energy expenditure to maintain osmotic homeostasis, thus impairing molt and growth (Brito *et al*., 2000; Romano and Zeng, 2006). Thus, another important factor for the good development of *L. vannamei* is salinity, even though it is an eurihalin species, because it tolerates a wide range of salinity ranging from 2‰ to 40‰ (Saoud *et al*., 2003), the abrupt change can cause harmful effects directly to the metabolism, growth, and consequently can affect the survival of this crustacean.

Besides, several studies employ *L. vannamei* as biological model to evalute effects of adverse situation about physiological, biochemical and molecular parameters (Pérez-Rostro *et al*., 2004; Jiang *et al*., 2005; Kniffin *et al.,* 2014; Felix-Portillo *et a*l., 2016; Li *et al.,* 2016). However, any study show the best condition to experiments, considering variables as temperature, salinity and oxygen levels together. So, in this study we evaluated the connection between these parameters in a tempt to stabilish ideal condition to performed the experiments using this specie as model. In this context, the present study had as objective to evaluate the mortality of this crustacean on different variables such oxygen, temperature and salinity levels.

2. MATERIAL AND METHODS

2.1 Shrimp maintenance

Adult white shrimp *Litopenaeus vannamei* of both sexes, with an average weight of 8 grams, were obtained with the collaboration of the Aquaculture Marine Station (Estação Marinha de Aquicultura (EMA) of the Federal Univeristy of Rio Grande-FURG and kept in the aquatic vivarium of the Institute Biological Sciences (ICB) in same University. Luminosity parameters (12L/12D), salinity of 30‰, pH 8, temperatures 20 ºC, constant aeration (5.5 ± 0.5 mg O_2 L^{-1}) were controlled and fed commercial (45% crude protein) shrimp feed were checked twice a day until satiety. The animals were kept under these conditions for at least two weeks preceding the experiments.

2.2 Experimental design

The experiments to evaluate the lethal concentration ($LC_{50\text{-}96h}$) were carried out in three distinct stages (n = 450; n=10 for each group). The following salinities were set for each step: 5, 25, and 35‰. Each salinity was crossed with different temperatures (20 ºC, 25 ºC and 30 ºC) and different levels of oxygen (zero, 0.5, 1.5, 3 e 6 mg O_2 L^{-1}). Mortality was assessed for 96 hours under all conditions. The experiment was conducted in aquaria containing 15 liters of salt water in the salinities of the study. Nitrogen gas (N_2) was used to reach the desired oxygen concentration. Oxygen and temperature were constantly monitored (Oximeter: DO-5519, Lutran Eletronic Entreprise Co) the shrimp were acclimatized for a week before the experiment.

2.3 Statistical analysis

To estimate the LC_{50} relative to dissolved oxygen concentration at different temperatures and salinities for 96 hours, the results were expressed as a parameter of the Lethal Medium Concentration required to kill 50% of the shrimp in 96 hours ($LC_{50\text{-}96h}$). These data were obtained from the analysis of Probit (Finney, 1971) with 95% confidence interval.

3. RESULTS

At the 5‰ salinity, deaths were recorded in the control group (6 mg O_2 L^{-1}) only at the highest temperature (Fig.1C). In the lowest oxygen concentrations (anoxia and 0.5 mg O_2 L^{-1}), the occurrence of death was recorded during the first hours of the experiment with mortality above 50%, rapidly reaching 100% of mortality (Fig. 1A, B and C). In the first hours of the experiment, at the lower temperature (20 °C), the occurrence of death in relation to the exposure time increased proportionally with the decrease of oxygen level (Fig. 1A). At 25‰, the concentration of 3 mg O_2 L^{-1} showed a greater effect on mortality than 1.5 mg O_2 L^{-1}. At 30 °C this effect was more pronounced, in which the oxygen concentration of 3 mg O_2 L^{-1} reaches 100% mortality after 18 hours of exposure (Fig. 1C). $LC_{50\text{-}96h}$ values for temperatures of 20 °C, 25 °C and 30 °C were set at 2.67 mg O_2 L^{-1}, 2.79 mg O_2 L^{-1} and 4.81 mg O_2 L^{-1}, respectively.

At 25‰ salinity, mortality in the control group (6 mg O_2 L^{-1}) occurred only at 30 ºC (Fig. 2C). At 20 °C, there was no mortality at 3 mg O_2 L^{-1} and the percentages of deaths were more pronounced at concentrations of 0.5 mg O_2 L^{-1} and anoxia, reaching 100% (Fig. 2A). At 25 °C, again shows a greater effect on 3 mg O_2 L^{-1} relative to the concentration of 1.5 mg O_2 L^{-1}. 0.5 mg O_2 L^{-1} and anoxia reached 50% mortality in few hours (Fig. 2B). In all oxygen concentrations expositions at 30 °C, with the exception of control, reached 100% mortality in the first hours (Fig. 2C). $LC_{50\text{-}96h}$ values for temperatures of 20 °C, 25 °C and 30 °C were set at 1.92 mg O_2 L^{-1}, 2.59 mg O_2 L^{-1} and 4.21 mg O_2 L^{-1}, respectively.

At salinity 35‰, no deaths were observed in the control groups (6 mg O_2 L^{-1}) at any temperature analyzed (Fig. 3). At 20 °C, there were no deaths at the concentration of 3 mg O_2 L^{-1} and the percentage of deaths was more pronounced in anoxia (in the first hours) and 0.5 mg O_2 L^{-1} (50% in 24h) (Fig. 3A). At 25 °C, we again observed a higher mortality rate of 3 mg O_2 L^{-1} than that of 1.5 mg O_2 L^{-1}. The concentrations of 0.5 mg O_2 L^{-1} and anoxia caused total mortality in the first hours of exposure (Fig. 3B). At 30 °C, in all oxygen concentrations, with

exception of control group, caused total mortality in 30 hours of exposure. At lower concentrations of oxygen, total mortality occurred within 12 hours exposure (Fig. 3C). $LC_{50\text{-}96h}$ values for temperatures of 20 °C, 25 °C and 30 °C were set at 1.92 mg O_2 L^{-1}, 2.81 mg O_2 L^{-1} and 3.62 mg O_2 L^{-1}, respectively.

4. DISCUSSION

Actually, shrimp farming is a globally exploited activity. In Brazil, shrimp farming began in the 1970s as a source of income for the local population in the states of the North and Northeast. Later, the activity also gained space in the southern states in the late 1990s. Then the most cultivated species of shrimp in the country became the exotic species *Litopenaeus vannamei*, due to their great adaptability to different ecosystems along the hemispheres (Ostrensky *et al*., 2000).

The demand for products generated by shrimp farming has been steadily increasing in recent years (FAO, 2008), generating interest in farming techniques that minimize losses in the production. In this way, the consequent increase in production considering the final quality of the product, leads to the necessity of approaches of aspects related to sustainability, in particular, the quality of the aquatic environment in which the production is practiced. For this reason, the physicochemical parameters discussed in this study are of important relevance for the welfare of the shrimp in the nurseries, as well as for quality productions.

The intermediate temperature and salinity used in this study was 25 °C and 25‰ respectively, which resulted in a $LC_{50\text{-}96h}$ for the dissolved oxygen of 2.59 mg O_2 L^{-1}. The temperature around 28 ºC actually seems to be the most suitable to be used in cultivation (Wyban *et al.,* 1995). The salinity variation does not seem to be an interference factor in the temperature of 25 ºC, since the values obtained of $LC_{50\text{-}96h}$ in the lowest and higher salinity used in this study (5 ‰ and 35‰) are 2.79 mg O_2 L^{-1} and 2.81 mg O_2 L^{-1}, respectively. However, $LC_{50\text{-}96h}$ values are not far from what the literature describes as moderate hypoxia (3 mg O_2 L^{-}

^{1}) (Welker *et al*., 2013), which may cause mortality over a long period of time. However, in a short period of exposure, it can leave the animals vulnerable to the attack of microorganisms, causing illnesses and with that, the low rate of growth and gain of weight (Zhou *et al*., 2009).

After 96 hours of experimentation, shrimp of lower salinity (5‰), exposed to 20 °C, had their $LC_{50\text{-}96h}$ for dissolved oxygen set at 2.67 mg O_2 L^{-1}, a value higher than the other experimental salinities (25 ‰ e 35‰) at the same temperature. This data indicates that in high salinities and temperatures of 20 ºC the survival resistance of *L. vannamei* is increased freight to a condition of almost severe hypoxia. In fact, it has been reported that temperatures around 19 °C can leave *L. vannamei* in relative immobility which reduces dietary behavior and consequently weight gain and metabolism (Ponce-Palafox *et al*., 1997). However, marine shrimp when grown at low salinity obtains the minerals required for their osmoregulation present in the water by active transport, thereby leading to increased energy expenditure (Gong *et al.,* 2004). *L. vannamei* in adulthood has a decline in osmoregulatory capacity, which entails greater energy expenditure to maintain its homeostasis, thus increasing the demand for oxygen (Vargas-Albores and Ochoa 1992).

In this study, it can be observed that in the higher experimental temperature (30 ºC), the values of $LC_{50\text{-}96h}$ for the dissolved oxygen were evidently higher in all the salinity (5, 25 and 35‰, with $LC_{50\text{-}96h}$ of 4.81, 4.21 and 3.62 mg O_2 L^{-1}, respectively). This finding directly reflects increased metabolism well as higher oxygen consumption, leading to a lower resistance to restrictions of oxygen. In this context is important to emphasize that the temperature is a factor that directly influences oxygen consumption in aquatic animals, once thatcrustaceans do not have the capacity to maintain a constant body temperature, in this way, the animal is subject to the thermal variations of its habitat, in which it directly influences the metabolic processes (Martínez-Palacios *et al*., 1996). So, high temperatures to accelerate the metabolism that front

to oxygen restriction, there are a drop in ATP levels affecting the physiological and biochemical process (Boyd, 1979; Manush *et al.,* 2006) to long time.

The rate in the oxygen consumption is generally increased steadily as the temperature rises, for example, a rise in temperature at 10 °C can result in a threefold oxygen consumption rate. This elevation in the metabolic rate is called the thermal coefficient (Q10) which represents the degree of sensitivity of organisms to temperature (Schmidt- Nielsen, 1999). In fact, Sponopoulos-Hernández *et al*. (2005) found in *Litopenaeus stylirostris* the increased oxygen consumption with increasing salinity and temperature starting from 20 °C to 30 °C. This fact corroborates with the data generated in this study, in which it shows a greater sensitivity to the variation of oxygen for higher temperatures.

In *L. vannamei* on low salinities, compensatory mechanisms are added in an attempt to maintain osmotic homeostasis, such as active Na^+/K^+-ATPase ion uptake and the mobilization of amino acids such as arginine, lysine and glycine, leading to energy expenditure (Álvarez *et al*., 2004). The highest LC_{50} value for dissolved oxygen was obtained at the lowest salinity and higher temperature, being set at 4.81 mg O_2 L^{-1}. This result can be explained because the osmoregulatory and compensatory processes are energy consuming, which results in higher oxygen consumption even for eurihalin species such as *L. vannamei*, being a potent osmoregulator in a wide range of salinity.

This way, values of temperature, salinity and dissolved oxygen are important factors to determine the stocking density and mortality in nurseries. We show in this study that the sensitivity of white shrimp to the fluctuations of oxygen is directly related to temperature and salinity. Despite the great importance of these data for the carcinucultura, little studies involving these three variables are found using *L. vannamei* adducts.

In addition, a low resistence at higher temperature (30 °C) with salinities rates (5 – 25‰) underscores the importance of monitoring of dissolved oxygen as the *L. vannamei* is

increasingly beinhg cultivated in hot regions in diferente countries using low salinities, therefore the value of 1.5 mg O_2 L^{-1} used as severe hypoxia for crustaceans, is very low in the largest value found in this study that caused mortality (4.81 mg O_2 L^{-1}), indicating that specific data of severe hypoxia are needed for each species. These data, together, can contribute significantly to the practice of production, in order to minimize the losses of animals, as well as on the welfare of animals in agriculture.

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Figure legends

Figure 1. *Litopenaeus vannamei* LC_{50} after 96 hours at 5‰ salinity in different temperatures and concentrations of dissolved oxygen. (A) 20 ºC in different concentrations of dissolved oxygen (control, 3, 1.5, 0.5 mg O_2 L^{-1} and anoxia); (B) 25 ºC in different concentrations of oxygen; (C) 30 ºC in different concentrations of oxygen. Results expressed as percentage of dead animals.

Figure 2. *Litopenaeus vannamei* LC_{50} after 96 hours at 25‰ salinity in different temperatures and concentrations of dissolved oxygen. (A) 20 ºC in different concentrations of dissolved oxygen (control, 3, 1.5, 0.5 mg O_2 L^{-1} and anoxia); (B) 25 ºC in different concentrations of oxygen; (C) 30 ºC in different concentrations of oxygen. Results expressed as percentage of dead animals

Figure 3. *Litopenaeus vannamei* LC_{50} after 96 hours at 35‰ salinity in different temperatures and concentrations of dissolved oxygen. (A) 20 ºC in different concentrations of dissolved oxygen (control, 3, 1.5, 0.5 mg O_2 L^{-1} and anoxia); (B) 25 ºC in different concentrations of oxygen; (C) 30 ºC in different concentrations of oxygen. Results expressed as percentage of dead animals.

Figure 1

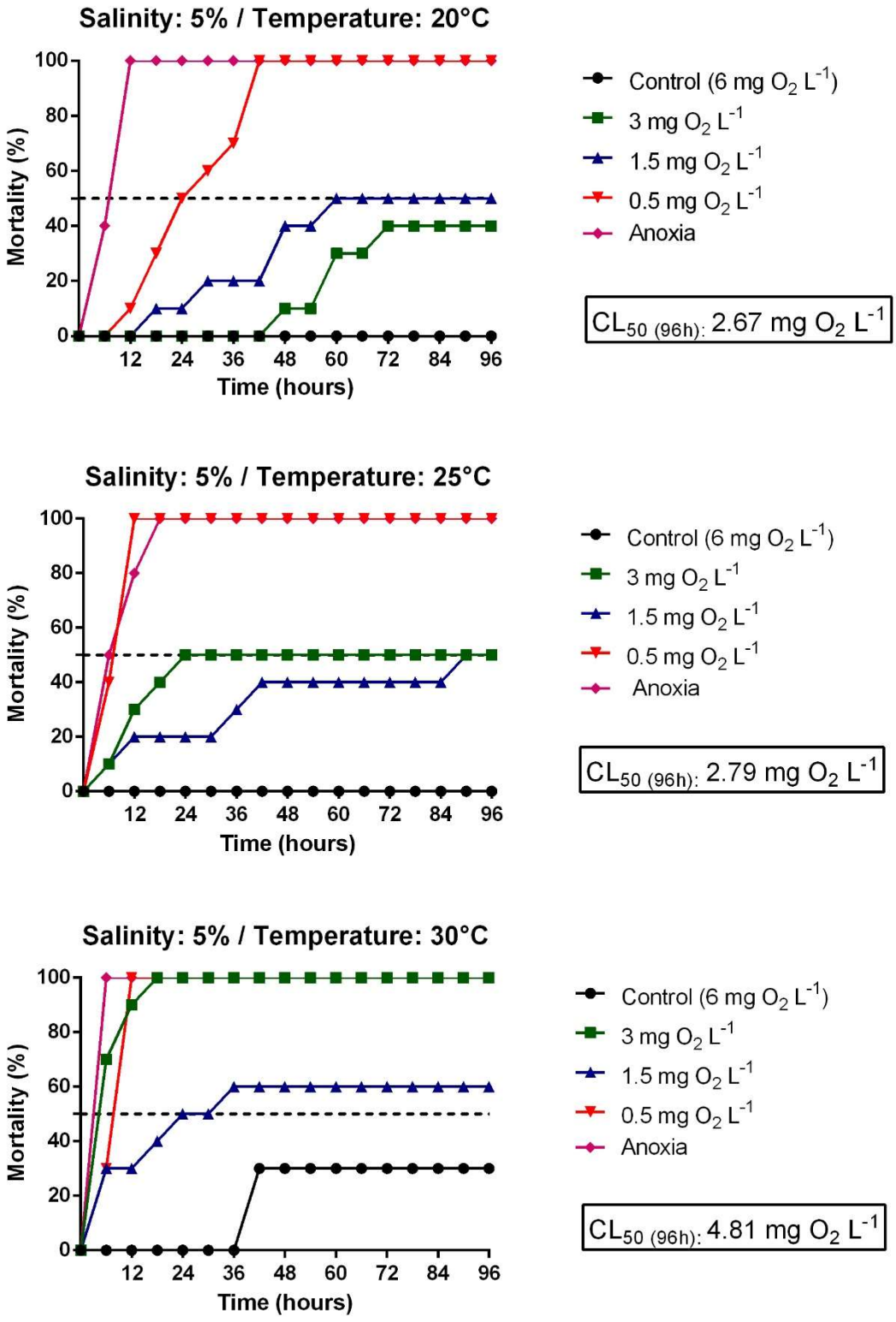

Salinity: 5% / Temperature: 20°C
Mortality (%)
Time (hours)
Control (6 mg O2 L-1)
3 mg O2 L-1
1.5 mg O2 L-1
0.5 mg O2 L-1
Anoxia
CL50 (96h): 2.67 mg O2 L-1
Salinity: 5% / Temperature: 25°C
Mortality (%)
Time (hours)
Control (6 mg O2 L-1)
3 mg O2 L-1
1.5 mg O2 L-1
0.5 mg O2 L-1
Anoxia
CL50 (96h): 2.79 mg O2 L-1
Salinity: 5% / Temperature: 30°C
Mortality (%)
Time (hours)
Control (6 mg O2 L-1)
3 mg O2 L-1
1.5 mg O2 L-1
0.5 mg O2 L-1
Anoxia
CL50 (96h): 4.81 mg O2 L-1

Figure 2

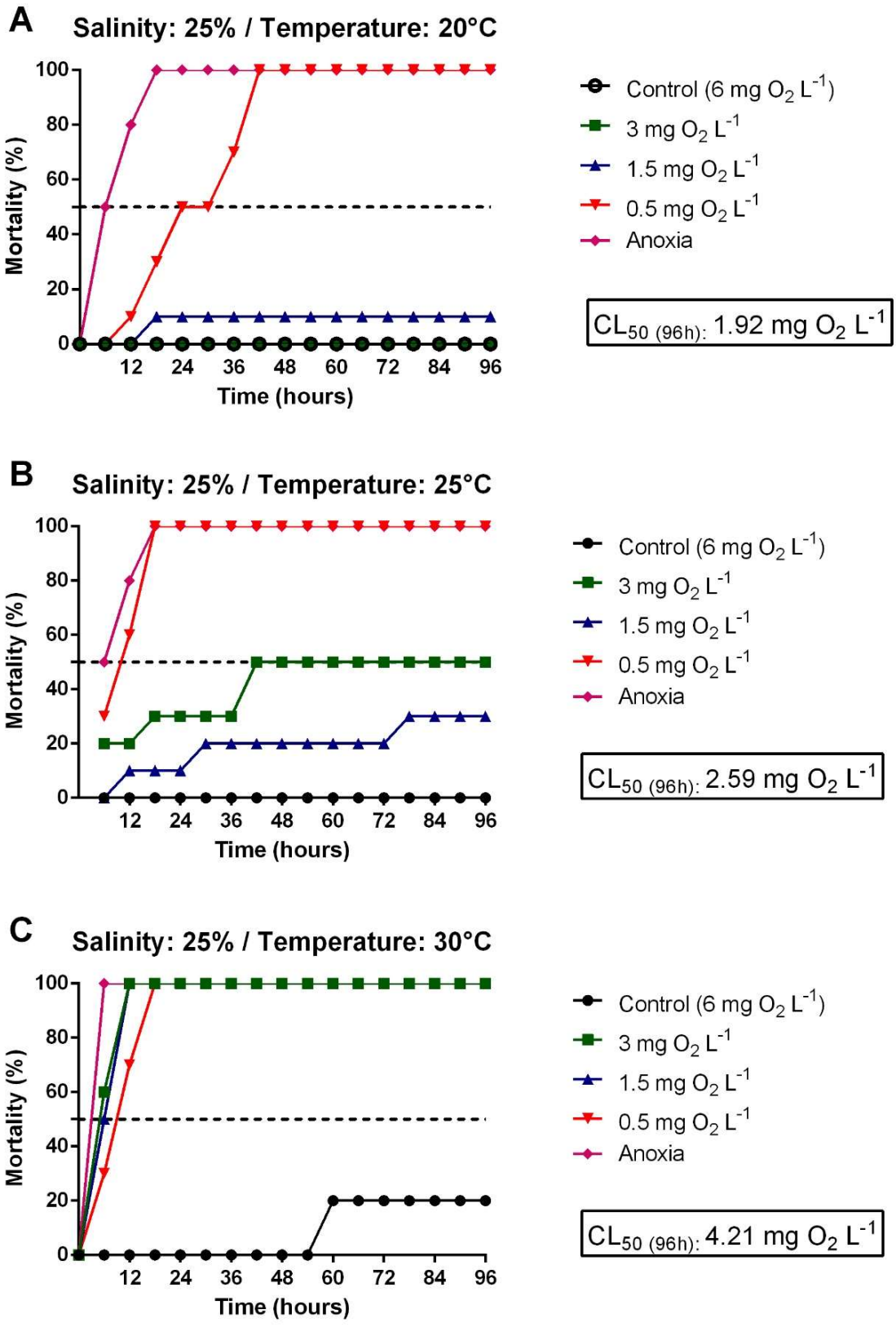
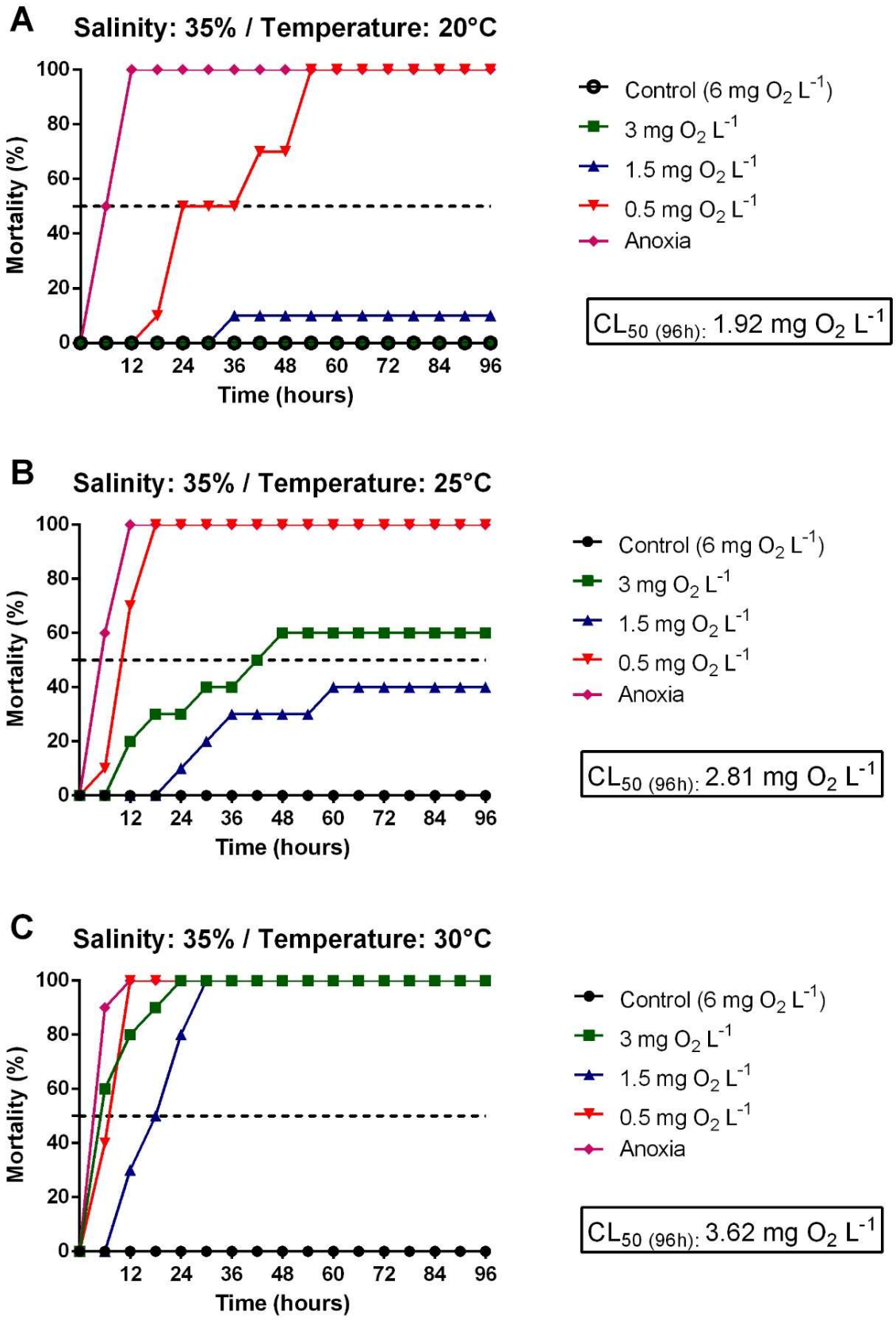

A
Salinity: 25% / Temperature: 20°C
Mortality (%)
Time (hours)
Control (6 mg O2 L-1)
3 mg O2 L-1
1.5 mg O2 L-1
0.5 mg O2 L-1
Anoxia
CL50 (96h): 1.92 mg O2 L-1
B
Salinity: 25% / Temperature: 25°C
Mortality (%)
Time (hours)
Control (6 mg O2 L-1)
3 mg O2 L-1
1.5 mg O2 L-1
0.5 mg O2 L-1
Anoxia
CL50 (96h): 2.59 mg O2 L-1
C
Salinity: 25% / Temperature: 30°C
Mortality (%)
Time (hours)
Control (6 mg O2 L-1)
3 mg O2 L-1
1.5 mg O2 L-1
0.5 mg O2 L-1
Anoxia
CL50 (96h): 4.21 mg O2 L-1

Figure 3:


A
Salinity: 35% / Temperature: 20°C
Mortality (%)
Time (hours)
Control (6 mg O2 L-1)
3 mg O2 L-1
1.5 mg O2 L-1
0.5 mg O2 L-1
Anoxia
CL50 (96h): 1.92 mg O2 L-1
B
Salinity: 35% / Temperature: 25°C
Mortality (%)
Time (hours)
Control (6 mg O2 L-1)
3 mg O2 L-1
1.5 mg O2 L-1
0.5 mg O2 L-1
Anoxia
CL50 (96h): 2.81 mg O2 L-1
C
Salinity: 35% / Temperature: 30°C
Mortality (%)
Time (hours)
Control (6 mg O2 L-1)
3 mg O2 L-1
1.5 mg O2 L-1
0.5 mg O2 L-1
Anoxia
CL50 (96h): 3.62 mg O2 L-1

4. Manuscrito II

Lipoic acid modulates energetic metabolism and antioxidant defense system in *Litopenaeus vannamei* on hypoxia/reperfusion conditions

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Juliane Ventura-Lima [1, 2, 4*].

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Lipoic acid modulates energetic metabolism and antioxidant defense system in *Litopenaeus vannamei* on hypoxia/reperfusion conditions

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ABSTRACT

Lipoic acid (LA) is a known and potent antioxidant, as well as, has shown positive effects on energy metabolism. In this study, our objective was to evaluate the protective effects of lipoic acid on metabolic and biochemical alterations on hypoxia/reperfusion injury (H/R) in shrimp (*Litopenaeus vannamei*). Two experimental groups were evaluated: non-supplemented and supplemented with LA (1% of body weight). The groups were evaluated at different times of exposure to hypoxia (6 and 24 hours) at 1.5 mg O_2 L^{-1} and different times of oxygen reperfusion (0, 1 and 3 hours). When the animals were supplemented with LA, the glucose levels were decreased in the hemolymph and increased in the muscles on H/R situation. Furthermore, no change in lactate dehydrogenase activity (LDH) activity was observed and lactate levels were either decrease or unchanged. In biochemical analyzes related to oxidative stress was showed a protective effect of LA against lipid peroxidation induced by H/R after 24 hours of exposure. LA supplementation showed to increase antioxidant capacity more effectively during reperfusion. LA differently modulated Glutathione-S-transferase (GST) activity according to exposure times to H/R and available oxygen concentration. Our results show a positive modulation induced by lipoic acid on energetic aspects and oxidative stress on H/R situation. In a context of disturbance of oxygen concentration, LA was effective as a supplement against induced damage. This study corroborates the emphasis on the unique properties of lipoic acid against the stress situation, in order to contribute positively to the problems existing in shrimp farming.

Keywords: lipoic acid, hypoxia, reperfusion, metabolic aspects, antioxidant capacity, *Litopenaeus vannamei.*

1. INTRODUCTION

Variation in oxygen levels directly influence the quality of the environment and can induce several effects in aquatic organisms. However, to cope this situation, fishes and crustaceans induce the modulation and regulation of physiological and metabolic responses (Soñanez-Organiz *et al*., 2009). ATP stores are rapidly depleted during hypoxia due to interruption of oxidative phosphorylation in mitochondria the absence of energy leads to a series of changes (Granger and Kvietys, 2015). For example, there is an increase in glycogen degradation to obtain energy, which leads to a higher production of lactate and consequently a decrease in cellular pH. These metabolic changes can result in protein changes and lead to cellular insults (Pérez-Jimenéz, 2012).

Some studies have demonstrated that when possible the aquatic organisms trend to avoid or move out of hypoxic conditions (Rosas *et al*,. 1999). However, farm animals cannot use this behavior so the development of strategies to improve the resistance of animals front hypoxia is great relevance. The Pacific white shrimp *Litopenaeus vannamei* (Crustacea; Decapoda) is one of the most cultivated species in the world. Although of *L. vannamei* has a resistance to stressful variations of their environment, such as temperature, salinity, and oxygen oscillations, these factors can trigger severe damage to the animal, mainly in a shrimp farming situation (Ballester *et al*., 2007).

During a hypoxia situation, mainly severe hypoxia (1.0-2.0 mg O_2 L^{-1}) as described by Levin *et al*. (2009), electrons are accumulated along the electron transport chain, and during the phase of reoxygenation (reperfusion) the oxygen reacts with these electrons accumulated in mitochondria, leading to a large release of reactive oxygen species (ROS) (Parrilla-Taylor and Zenteno-Savín, 2011; Welker *et al*., 2013). Although reactive species act in redox signaling and resistance against pathogens, the imbalance between these species and antioxidant capacity, can result in an oxidative stress situation (Fridovich, 2004; Zenteno-Savin *et al*., 2006). ROS are

highly reactive with biomolecules, may cause damage to lipids, proteins and DNA, resulting dysfunctions and tissues changes (Halliwell, 2007). The antioxidant defense system plays an important role in attempting to reverse or eliminate ROS, using enzymatic and non-enzymatic defense systems (Li *et al*., 2016). Among enzyme defense system are glutathione-S-transferase (GST), catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD), among others. Vitamin C (ascorbic acid), vitamin A (retinol), vitamin E (α-tocopherol) and lipoic acid (LA), reduced glutathione (GSH) among others, are part of the non-enzymatic defense system (Zhang *et al*., 2010).

Antioxidants are substances that act in the prevention or reduction of oxidation induced by reactive species (Aldini *et al*., 2010). Studies have shown that the incorporation of lipoic acid (LA) into the diet of farm animals has brought numerous benefits, as increase in its antioxidant and detoxification capacities (Lobato *et al*., 2013; Martins *et al*., 2014).

LA is derivate from fatty acid produced by the body, being essential for metabolic processes and antioxidant defense of the organisms (Packer and Cadenas, 2010). LA as an important non-enzymatic antioxidant is capable of modulating the antioxidant defense system in aquatic organisms. It can eliminate ROS, to chelate metals and play an important role in the regeneration of other antioxidants, as the GSH (Packer *et al*., 1995). In addition, it may also reduce the levels of oxidized proteins in muscle tissue; reduce in ROS levels in the brain and increase of the glutamate-cysteine ligase (GCL) activity in brain and liver of the fish *Corydoras paleatus* (Callychthyidae) as reported by Monserrat *et al*. (2008). In other studies, LA showed an increase in glutathione-S-transferase (GST) activity in carp (*Cyprinus carpio;* Cyprinidae) on microcystin-induced toxicity (Amado *et al*., 2011). Kutter *et al*. (2014) also showed an increase in the activity of this enzyme in fish brain (*Trachinotus marginatus)* when supplemented with LA-enriched ration.

Therefore, the main goal of this study was to evaluate whether the lipoic acid-supplementation diet could be able to improve or reverse the effects induced by hypoxia and reperfusion in *L. vannamei,* considering metabolic and oxidant aspects.

2. MATERIAL AND METHODS

2.1 Shrimp maintenance

The shrimps (*Litopenaeus vannamei*) were obtained from Marine Aquaculture Station, Oceanography Institute (IO), Federal University of Rio Grande-FURG and transferred to the Institute of Biological Science (ICB) of the same Institution, acclimated in tanks under controlled parameters (salinity 25‰, pH around 8.0, photoperiod 12L:12D, feeding twice at day) at least two weeks prior to the experiments.

2.2 Experimental design

Shrimp were distributed into two groups: non-supplemented (commercial ration only - Purina, 45% of crude protein) and supplemented with lipoic acid (commercial ration with lipoic acid). The food (both groups) was administrated twice daily for four weeks, a supply corresponding 1% of the body weight average. The lipoic acid (LA from SIGMA, USA) was incorporated in the commercial ration in a concentration of 70 mg/kg of ration according to Terjesen *et al*. (2004). Posteriorly, the two groups (with and without LA) were treated separately in 12 aquariums with 6 animals each (n=6) according to their respective experimental groups. The Figure 1, show the experimental designer and the distribution of the times of exposure to hypoxia and reperfusion. The objective of the choices of hypoxia exposure times it was in order of not to cause permanent damage to the animal in which it could not be remedied or prevented.

2.3 Exposure to hypoxia and reperfusion

The shrimp (n=6) were arranged in aquaria containing ten liters of seawater (salinity 25‰). Nitrogen gas was bubbled until oxygen gas concentration reached 1.5 mg O_2L^{-1} (hypoxia) normoxia was established in 6 mg O_2 L^{-1}. The oxygen concentration was continuously monitored during all experiment using an oximeter (DO-5519, Lutran Eletronic Entreprise Co). Several authors consider 1.5 mg O_2 L^{-1} as severe hypoxia for *L. vannamei* (Van Wyk and Scarpa, 1999; Vaquer-Sunyer and Duarte, 2008; Trasvinã- Arena *et al*., 2014; Li *et al.,* 2016).

2.4 Analysis of metabolic properties

Preparation of samples

Hemolymph was extracted with a hypodermic syringe (1 ml) of 13x045 mm needle, washed with anticoagulant solution [NaCl (0.45M), glucose (0.1 M), sodium citrate (0.03 M), citric acid (0.026 M), EDTA (0.01 M)] and pH 4.6. The needle was carefully inserted into at the junction between cephalothorax and abdomen of the animal and each sample obtained was stored in pre-refrigerated tubes. For analysis the hemolymph was processed in cold buffer (4 °C) with EDTA (6%). Then, muscle was removed, weighed and homogenized in a ratio of (1:5 w/v) in phosphate buffer (100 mM), EDTA (1 mM) and phenylmethylsulfonyl fluoride (PMSF) (10 μM) and pH 7.2. All samples were centrifuged at 8000 *x g*, 4 °C for 20 minutes and the supernatant was removed and stored at -80 °C for posterior analyses. Total proteins in the samples were determined following Biuret method measured by spectrophotometry at 550 nm utilizing microplate reader (Biotel ELx800).

Lactate, lactate dehydrogenase and glucose measurements.

Lactate of the muscle and hemolymph was measured spectrophotometry at 340 nm using the lactate determination kit by enzymatic method (Kovalent - Brazil). The glucose level was verified spectrophotometry at 490 nm using the glucose kit, through the enzymatic oxidation method by glucose oxidase (Kovalent - Brazil). In both tissues was used the enzymatic method (Kovalent - Brazil) to determine the lactate dehydrogenase (LDH) activity, measured by spectrophotometry at 340 nm, utilizing microplate reader (Biotel ELx800).

2.5 Analysis of antioxidant properties

Preparation of samples

After dissection, the muscle and gills were homogenized (1:4 p/v) in buffer containing Tris-HCl (100 mM), EDTA (2 mM), $MgCl_2$ (5 mM), PMSF (0.05 mM), pH adjusted in 7.75. The samples were centrifuged at 10.000 *x g* for 20 minutes at 4 ºC and the supernatant used for biochemical measurement. Total proteins in the samples were measured following Biuret method measured by spectrophotometry at 550 nm utilizing microplate reader (Biotel ELx800).

Lipid peroxides

Lipid peroxides were measured through of the thiobarbituric acid-reactive substances (TBARS) assay according to described by Oakes and Kraak, (2003). The fluorescence was measured at 520/580nm for excitation/emission wavelengths, respectively (FilterMax™ F5 microplate reader/Molecular Devices - EUA). The results were expressed as nmol of MDA/mg of protein, using tetramethoxypropane (TMP) (Sigma, USA) as standard.

Antioxidant competence against peroxyl radicals (ACAP)

This analysis was determined according to the method of Amado *et al*. (2009). Peroxyl radical was generated by thermal decomposition (37 ºC) of ABAP (2,2′-azobis (2 methylpropionamidine) dihydrochloride). Peroxyl radical reacts with H_2DCFDA (2,7

Dichlorofluorscein diacetate, Molecular Probes) and the esterases present in the sample cause deacetylation of this probe, that then is oxidized by ROS forming a fluorescent compound (DCF) detected at 485/535 nm for excitation/emission, respectively (FilterMax™ F5 microplate reader/Molecular Devices - EUA). The total antioxidant capacity against peroxyl radical was quantified by the relative area with and without ABAP, where high relative fluorescence area indicates low antioxidant competence, once high fluorescence levels were obtained after addition of ABAP meaning low capacity to neutralize peroxyl radicals.

Activity of glutathione S-transferase (GST)

GST (EC 2.5.1.18) activity was assayed on methodology described by Habig and Jakoby (1981). Basically, the assay is based on the formation of the conjugated complex of 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 1 mM reduced glutathione (GSH). The absorbance generated was monitored at 340 nm, utilizing microplate reader (Biotel ELx800).

2.6 Statistical analysis

Statistical analysis was performed by two-way ANOVA followed by Tukey's *post hoc* test. All data were expressed as mean ± standard error of mean (SEM). Differences were considered to be significant at $p<0.05$ level. Mathematical transformations were performed when necessary (Zar, 1984).

3. RESULTS

All results were statistically evaluated among the four conditions (normoxia, normoxia + LA, hypoxia and hypoxia + LA) within each time of reperfusion (0, 1 and 3 hours) on exposure times of hypoxia or normoxia (6 and 24 hours).

3.1 Glucose levels

Glucose levels in the hemolymph after 6 hours without reperfusion (0 hour) showed a significant increase in hypoxia + LA group when compared to the other groups ($p<0.05$). In the 1 hour reperfusion time after hypoxia conditions, glucose levels increased when compared to the normoxia group ($p<0.05$), whereas in LA treatment (Hypoxia + LA) glucose returned to normoxia levels. In 3 hours of reperfusion, hypoxia group had decreased in glucose levels when compared to the normoxia group ($p<0.05$), in this case, LA did not show a reversal of this effect (Figure 2A). At 24 hours in the hemolymph, all reperfusion times (0, 1 and 3 hours) were observed that the hypoxic condition induced an increase in glucose levels ($p<0.05$) when compared to animals exposed to normoxic conditions and the LA showed a reversal of this increase (Figure 2B).

In the 6 hours muscle exposure, hypoxia showed increased glucose levels after 1 hour of reperfusion, and in this case, LA did not show a reversal of this increase. While, at 0 and 3 hours reperfusion times, co-exposure to LA and hypoxia increased glucose levels compared to the other groups (Figure 2C). In 24 hours, only after 3 hours of reperfusion an increase in glucose levels ($p<0.05$) was observed in the group submitted to hypoxia and the LA did not show a reversal of this increase (Figure 2D).

3.2 Lactate levels

Lactate levels in hemolymph after 6 hours of exposure at 0, 1 and 3 hours of reperfusion showed an increase in the groups submitted to hypoxia compared to their respective normoxia groups ($p<0.05$). However, at time 0 and 1 hour of reperfusion the LA showed to decrease these levels, a contrary result was observed at 3 hours of reperfusion (Figure 3A). In all reperfusion times evaluated in the hemolymph in 24 hours to exposure, it was possible to observe that hypoxia significantly increases lactate levels when compared to their respective groups in normoxia ($p<0.05$). However, at time 0 and 3 hours of reperfusion, LA decrease lactate levels on hypoxia conditions (Figure 3B).

In muscle submitted to 6 hours of hypoxia in all reperfusion times, an increase in lactate levels was observed in groups conditioned to hypoxia compared to their respective normoxia groups ($p<0.05$). However, LA showed to restore normal lactate levels after 3 hours of reperfusion (Figure 3C). In the 24 hours in the same tissue, all groups exposed to hypoxia showed an increase in lactate levels when compared with the normoxia groups ($p<0.05$). LA treatment was shown to be effective in reversing this increase only in 1 hour of reoxygenation (Figure 3D).

3.3 LDH activity

Lactate dehydrogenase (LDH) activity in hemolymph at 6 hours, increased in the groups exposed to hypoxia at time 0 and 3 hours of reperfusion compared to their respective normoxia groups ($p<0.05$). LA did not show a protective effect in the reperfusion times evaluated (Figure 4A). In hemolymph, at 24 hours the enzyme activity showed to increase significantly in response to hypoxia, when compared to their respective normoxia groups ($p<0.05$), in all reperfusion times evaluated. Under these conditions, LA did not show a reversal of the activity increase (Figure 4B).

LDH activity in the muscle after 6 hours with 0 and 3 hours of reperfusion increased significantly in the hypoxia group compared to the normoxia group ($p<0.05$). Compared with the hypoxia + LA group, the same result was not observed (Figure 4C). LDH activity of the muscle in 24 hours was shown to be increased in all groups submitted to hypoxia when compared to their respective normoxia groups ($p<0.05$). However, LA showed to revert the enzyme activity in the reperfused groups for 1 and 3 hours (Figure 4D).

3.4 TBARS

After 6 hours of hypoxia, only was observed an increase in TBARS levels in the group exposed to normoxia +LA in muscle (Figure 5A) while in gills, no differences in lipid damage was observed (Figure 5C). However, after 24 hours, LA treatment showed a reversion in the basal levels of lipid peroxidation in muscle and gills conditioned to hypoxia followed by 3 hours of reperfusion (Figure B and D).

3.5 Antioxidant competence against peroxyl radicals (ACAP)

In the muscle after 6 hours of hypoxia, without reperfusion (0h) the total antioxidant capacity decreased in relation to their respective normoxia groups ($p<0.05$) and the LA was not able to reverse this decrease. However, LA was able to increase total antioxidant capacity in 3 hours of reperfusion when compared to the other groups (Figure 6A). In muscle, the hypoxia showed decreased total antioxidant capacity in the 24 hours at all reperfusion times when compared to their respective normoxia groups ($p<0.05$). However, LA treatment showed to increase this capacity at time without reperfusion (time 0) and the groups submitted to 3 hours of reperfusion (Figure 6B).

In 6 hours gills, the hypoxia group showed a reduction in their antioxidant capacity after 3 hours of reperfusion when compared to their respective normoxia group ($p<0.05$) and LA treatment reversed this increase (Figure 6C). Hypoxia in gills after 24 hours showed to decrease

the antioxidant capacity in the animals at times without reperfusion (time 0) and with 1 hour when compared to their respective groups in normoxia ($p<0.05$). LA treatment showed reversed this decrease at time 1 hour of reperfusion (Figure 6D).

3.6 GST activity

GST activity in the muscle was decreased after 6 hours of hypoxia followed by 1 hour of reperfusion when compared to the normoxia group ($p<0.05$), in this group the LA did not show a reestablishment of the enzyme activity (Figure 7A). In muscle after 24 hours of exposure to hypoxia, no changes in GST activity were observed regardless of the time of reperfusion or treatment with the antioxidant (Figure 7B).

In gills, after 6 hours of hypoxia only the group of 3 hours of reperfusion showed to modulate GST activity. This modulation is evidenced by the decrease in enzyme activity in the group submitted to hypoxic condition when compared to the normoxia group ($p<0.05$). In this same group, LA did not show a reestablishment of enzyme activity (Figure 7C). In the gills, after 24 hours of exposure to hypoxia, the group without reperfusion (time 0) showed to decrease enzyme activity when compared to its respective normoxia group ($p<0.05$) and LA showed to increase the activity of the enzyme in relation to all other groups. After 3 hours of reperfusion the groups submitted to hypoxia showed a positive modulus of GST activity when compared to their normoxia groups (Figure 7D).

4. DISCUSSION

The white shrimp of the Pacific *Litopenaeus vannamei* is a species found naturally from the Eastern Pacific coast to northern Peru and it is of great commercial importance. In cultivation, it is notable for being tolerant to variations in temperature, salinity and oxygen (Barnabé *et al*., 1996). However, these changes may result in greater susceptibility to infections, reduction in the growth rate and mortality (Peixoto *et al*., 2003; Ballester *et al*., 2007).

The high fluctuation in oxygen levels in shrimp farming leads to cycles of hypoxia followed by reperfusion, which prolonged periods of exposure to concentrations below 1.5 mg O_2 L^{-1} (considered severe hypoxia) can be harmful and even lethal to *L. vannamei* (Van Wyk and Scarpa 1999). Thus, due to the need to maintain adequate productivity, dietary antioxidant supplementation has been attempting to improve the health quality and growth of animals in farming (Monserrat *et al*., 2008).

One response of the organism to the effects of hypoxia is the activation of adaptation mechanisms for survival involved in both physiological and pathological functions in response to hypoxia. For example, occurs upregulation of glycolytic pathway genes, since glycolysis is the main pathway of obtaining energy when low oxygen levels do not support oxidative phosphorylation (Cruz *et al*., 2016). Some authors suggest that reactive oxygen species (ROS) play an important role in the adaptation activation (Kietzmann and Görlach, 2005).

The metabolic disorders that occur during hypoxia are very well established, however, clinical and experimental evidence demonstrates that the major events that leading to cell and tissue dysfunctions are related to subsequent reperfusion (Biddlestone *et al*., 2015). The reperfusion or reoxygenation of hypoxic tissues may induce a rapid metabolic remodeling, mitochondrial reprogramming followed by high production of ROS, reorganization of the ionic fluxes through the plasma membrane, inflammation and consequently cell death (Biddlestone *et al*., 2015; Solaini *et al*., 2010). Although a variety of molecular mechanisms have been

proposed to explain such events, the ROS production receives more attention, as a critical factor in the genesis of the reperfusion injury.

In this study, we showed changes in metabolic aspects during shorter (6 hours) and longer (24 hours) exposure to hypoxia and reperfusion (1 and 3 hours) in *Litopenaeus vannamei*, these differences were also observed between hemolymph and muscle tissue (Fig. 2, 3 and 4).

In a general analysis, we showed that supplementation with LA under exposure to hypoxia, reperfusion (H/R) decreased circulating glucose (except for 6 hours in hypoxia in hemolymph), and increased glucose levels in muscle tissue. Interestingly, although LDH activity increased in H/R, overall there was no difference between the supplemented and non-supplemented groups (except for 24 hour in reperfusion in the muscle). However, lactate production, in overall, was lower or unchanged in the supplemented-LA group when exposed to H/R (with the exception of 6 hours in hemolymph in reperfusion). The results suggest that supplementation with LA is modulating metabolic aspects of shrimp on H/R stress situation, which may be associated with an improved energetic state. However, more studies are needs to verify which pathway is being used to improve the energetic state during H/R when animals received the supplemented diet.

Lipoic acid is an essential cofactor of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes, two important enzymatic complex involved in the energy metabolism. When not covalently bound and administered exogenously to cells or supplemented in the diet, LA is a potent modulator of the redox state of cells (Packer and Cadenas, 2010). In addition to the unique antioxidant properties of LA, studies also demonstrate the protective effects of LA on cardiovascular diseases (Skibska *et al*., 2015), nervous system (Oliveira *et al*., 2017), diabetes (Golbidi *et al*., 2011) and positive effects for weight reduction (Kucukgoncu *et al*., 2017; Li *et al*., 2017).

Our results on shrimp metabolism on H/R status are similar to those already shown in a metabolic deficiency situation. Khamaisi *et al*. already in 1997 had shown that diabetic rats (type 2) supplemented with LA had a decrease of blood glucose levels, increase of the GLUT4 transporter in the muscle membrane and a non-change in the concentrations of lactic acid. In the discussion, the authors suggest that a reduced level of blood glucose may result from increased glucose utilization by peripheral tissues, such as muscle. Increased glucose in muscle tissue may reflect an increased availability of glucose to the demand for glycolysis. In this way, providing greater supply of the energetic needs of the animal in situation of restriction of oxygen.

The antioxidant properties of LA are very interesting, it is soluble in both aqueous and lipid media and able to overcome cell membranes, including the blood-brain barrier. Lipoic acid when incorporated by cells, can be reduced in part to dihydrilipoic acid (DHLA) which will act as an antioxidant in biological systems (Packer *et al*., 1998). In fact, it is known that LA can minimize oxidative damage to macromolecules such as lipids and proteins (Arivazhagan *et al*., 2002; Monserrat *et al*., 2008).

Some products are generated during lipid peroxidation and can be used as a biomarker of oxidative damage in macromolecules (Hermes-Lima, 2004). Surprisingly, in this study, the levels of TBARS (thiobarbituric acid reactives substances) in muscle of *L. vannamei* supplemented with LA in a situation of normoxia was increased (1h Rep./Fig. 5A). A similar result was also observed by Martins *et al*. (2014), which in similar conditions (normoxia) showed an increase in TBARS levels in gills of *L. vannamei* exposed to 140 mg LA/Kg, showing a pro-oxidant effect of LA. However, when the exposure time was higher (24 hours - 3h Rep./Fig. 5B, D), LA had a protective effect by decreasing levels of lipid peroxidation in muscle and gills of *L. vannamei* submitted to hypoxia.

Although the situation of hypoxia generates several metabolic and biochemical changes, the greatest oxidative damages occur during reoxygenation. When oxygen levels are restored, LA seems to be more effective after long periods of hypoxia (over 6 hours), as already reported in other studies using aquatic animals (Zenteno-Savín *et al*., 2006; Zhang *et al*., 2010; Martins *et al*., 2014). The antioxidant capacity of LA may be related to the H/R time since no protective effect of LA was observed at 6 hours in both tissues (Fig. 5A and C). In fact, Trasviña-Arena *et al*. (2014) showed that the expression of antioxidant enzymes in *L. vannamei* were only activated after 6 hours of hypoxia (1.5 mg O_2 L^{-1}) and had their highest expression after 24 hours in this condition. Therefore, this corroborates with our results, since, LA protected against increased levels of TBARS after 24 hours of H/R.

In this study the antioxidant competence against peroxyl radicals (ACAP), in overall, in both muscles and gills was increased in animals supplemented with LA (Fig. 6). This behavior underscores a critical period for aquatic animals facing to massive production of ROS after reoxygenation**.** Li *et al*. (2016) showed that *L. vannamei* exposed to 1.5 mg O_2 L^{-1} had antioxidant enzyme activity increased only in 3 hours of reperfusion when submitted to hypoxia for 12 and 24 hours. In our study, in the most critical phase during reperfusion, supplementation-LA showed to be efficient against the effects generated by oxygen recovery. It has been reported that the accumulation of electrons in the transport chain in the mitochondria are available for formation of ROS even on hypoxic condition using the available oxygen (Storey, 1996). We show in this study a decrease in the antioxidant capacity of the animals in situation of hypoxia when compared with normoxia (0h Rep./Fig. 6 A, B e D). Li *et al*. (2016) also observed this result, in similar times and tissues, showing that the antioxidant defense system in *L. vannamei* may be more effective after reperfusion.

Detoxification capacity of tissues can be evaluated through of GST activity. In muscle, only the group conditioned to 6 hours of hypoxia showed to modulate this enzyme activity after 1 hour of reperfusion and the LA not showed to reverse this decrease. However, in muscle the

LA showed to modulate the GST activity only in the group exposed to higher reperfusion period (3h), increasing its activity in normoxia (Fig. 7A). Martins *et al.* (2014), once found a different result that the supplementation-LA when exposed to 3 mg O_2 L^{-1} of hypoxia (moderate hypoxia) showed positive modulation of GST during reoxygenation.

In gills, the results in terms of GST activity were more evident than in muscle once that after 3 hours of reperfusion was observed a drop in this enzyme activity compared with normoxia group (Fig. 7C) after 6 hours of hypoxia. However, after 24 hours of hypoxia was observed a decrease in GST activity in the gills not submitted to reperfusion (0h), while an opposite result was detected after 3 hours of reoxygenation in the group under only hypoxic condition compared to normoxia group (Fig. 7D). The treatment with LA not showed to reverse changes caused by hypoxia in gills of shrimps.

A study, using hepatopancreas of *Neohelice granulata* exposure to anoxia did not cause any significant change in the enzyme GST activity, while that after aerobic recovery post anoxia enzyme showed a significant reduction in your activity (Oliveira *et al.,* 2017), In fact, GST activity is modulated in different ways by hypoxia and reoxygenation. Maciel *et al*. (2004) report that higher levels of the antioxidant defense system in gills and hepatopancreas of the crab *N. granulata* (*Chasmagnathus*) occur during the period of greater activity of the animal such as during the period of locomotion and feeding, where greater oxygen consumption and a higher production of reactive species are formed. Anyway, deprivation of oxygen affects the detoxification capacity mostly in gills of shrimps.

Independently if there was increase or decrease in GST activity, these modulation indicates insults to the system, in short time, the decrease of enzyme activity leave the organism more vulnerable to other environment conditions, while that the increase to long time is energetically expensive to cells because to synthesis of enzyme that are needs to maintain the high activity to long periods and the ATP used to increase the levels of enzyme not will be available to other physiologic functions such reproduction and growth, for example. Besides,

our results demonstrate that GST activity may be differently modulated by LA without a stress situation (normoxia), by the H/R exposure time and the available oxygen concentration.

5. CONCLUSION

In overall, shrimp supplementation-LA induced to a positive modulation in metabolic aspects and biochemical parameters, regarding lipid peroxidation and antioxidant capacity, on hypoxia and reperfusion. Our results are promising to emphasize the protective and modulatory potential of lipoic acid in stress situations, as well as to contribute significantly to improving shrimp farming.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figures

Figure 1

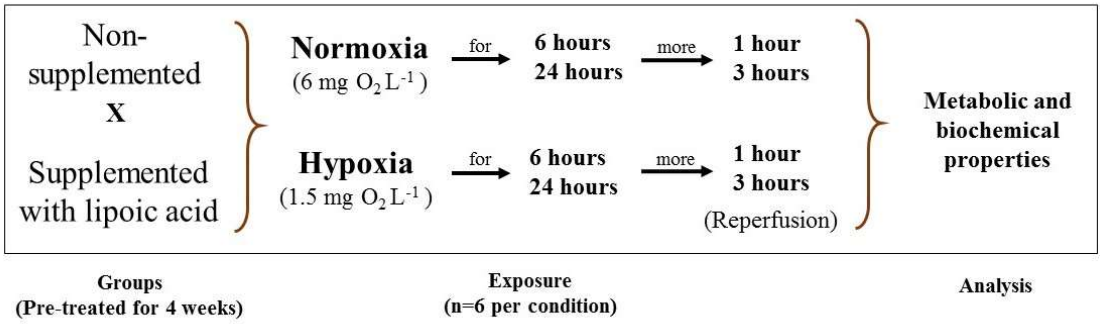

Figure 2

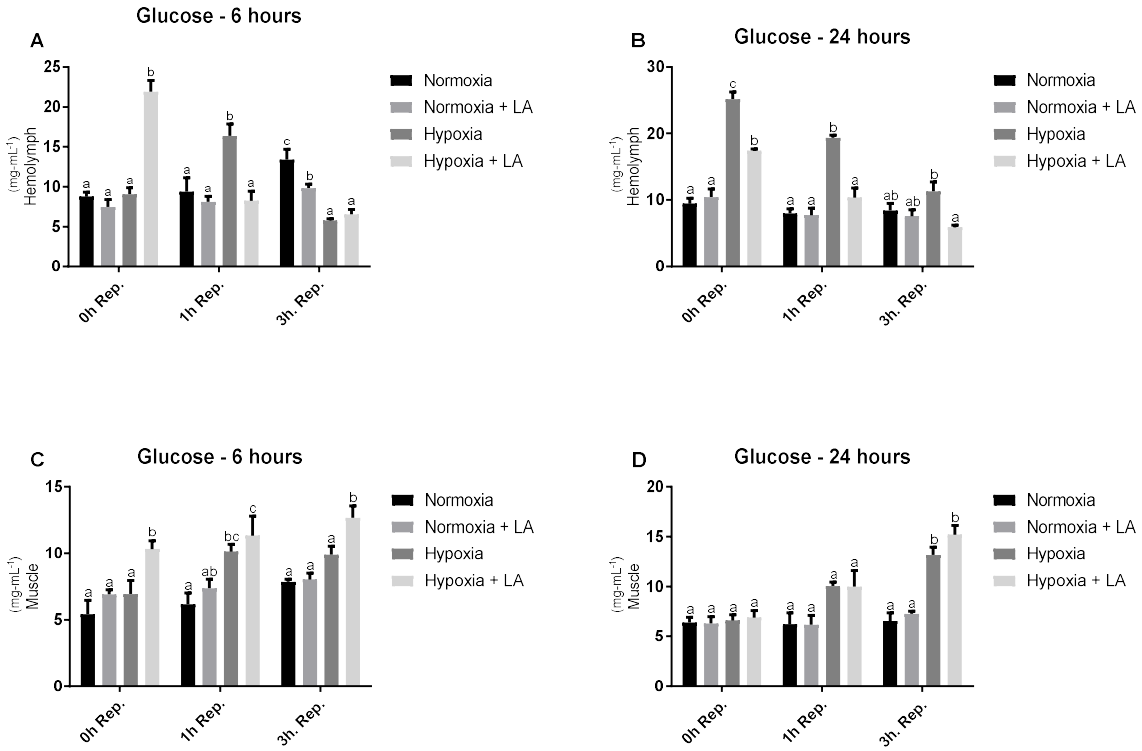
A
Glucose - 6 hours
(mg·mL⁻¹) Hemolymph
0h Rep.
1h Rep.
3h. Rep.
Normoxia
Normoxia + LA
Hypoxia
Hypoxia + LA
B
Glucose - 24 hours
C
Glucose - 6 hours
(mg·mL⁻¹) Muscle
D
Glucose - 24 hours

Figure 3

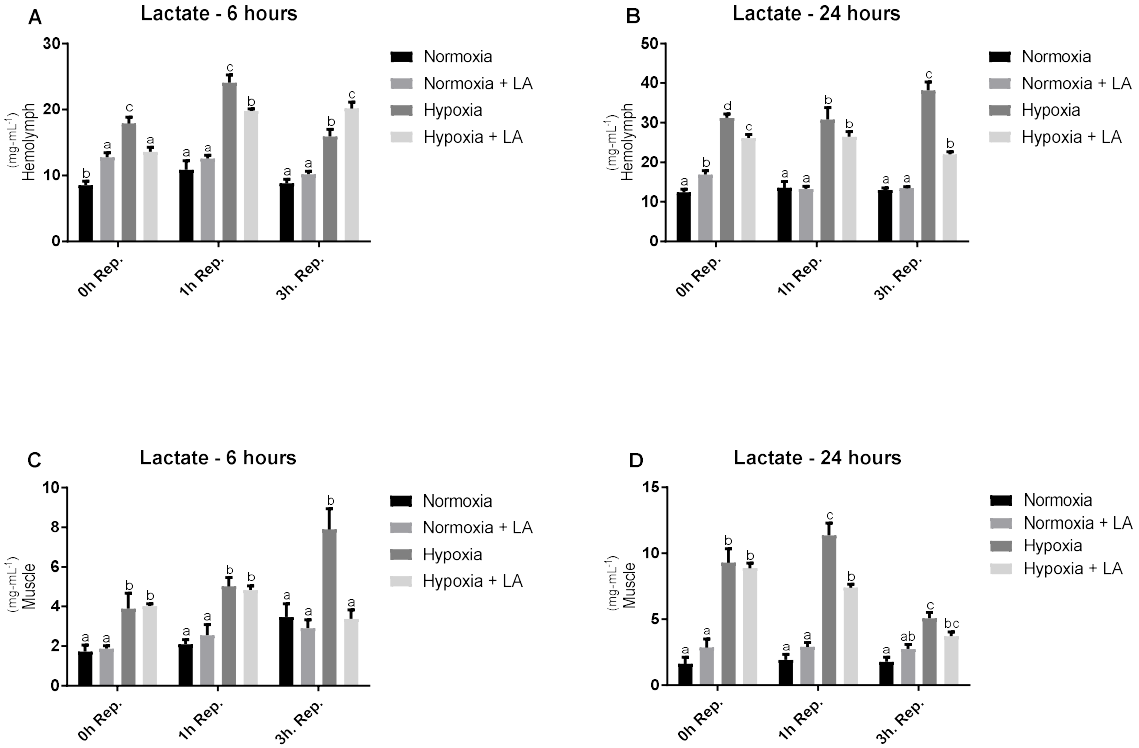
A
Lactate - 6 hours
(mg·mL⁻¹) Hemolymph
0h Rep.
1h Rep.
3h. Rep.
Normoxia
Normoxia + LA
Hypoxia
Hypoxia + LA
B
Lactate - 24 hours
(mg·mL⁻¹) Hemolymph
C
Lactate - 6 hours
(mg·mL⁻¹) Muscle
D
Lactate - 24 hours
(mg·mL⁻¹) Muscle

Figure 4

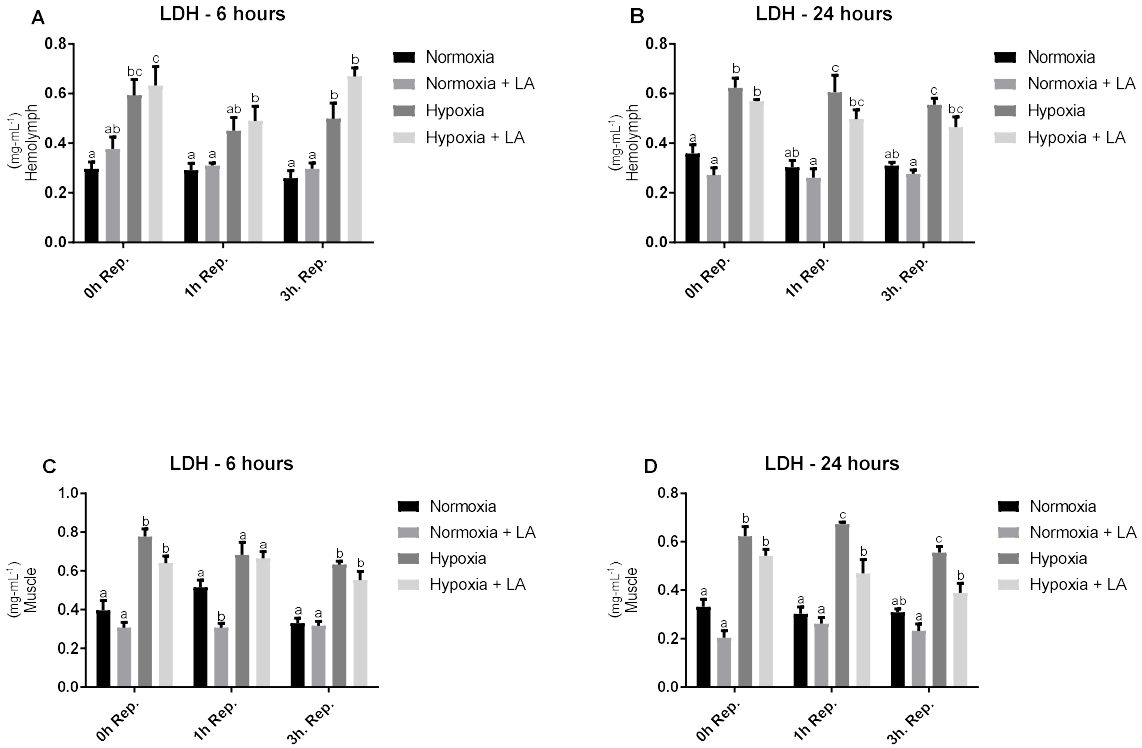
A
LDH - 6 hours
(mg-mL-1) Hemolymph
0.0
0.2
0.4
0.6
0.8
a
ab
bc
c
a
a
ab
b
a
a
b
b
0h Rep.
1h Rep.
3h. Rep.
Normoxia
Normoxia + LA
Hypoxia
Hypoxia + LA
B
LDH - 24 hours
(mg-mL-1) Hemolymph
0.0
0.2
0.4
0.6
0.8
a
a
b
b
ab
a
c
bc
ab
a
c
bc
0h Rep.
1h Rep.
3h. Rep.
Normoxia
Normoxia + LA
Hypoxia
Hypoxia + LA
C
LDH - 6 hours
(mg-mL-1) Muscle
0.0
0.2
0.4
0.6
0.8
1.0
a
a
b
b
a
b
a
a
a
a
b
b
0h Rep.
1h Rep.
3h. Rep.
Normoxia
Normoxia + LA
Hypoxia
Hypoxia + LA
D
LDH - 24 hours
(mg-mL-1) Muscle
0.0
0.2
0.4
0.6
0.8
a
a
b
b
a
a
c
b
ab
a
c
b
0h Rep.
1h Rep.
3h. Rep.
Normoxia
Normoxia + LA
Hypoxia
Hypoxia + LA

Figure 5

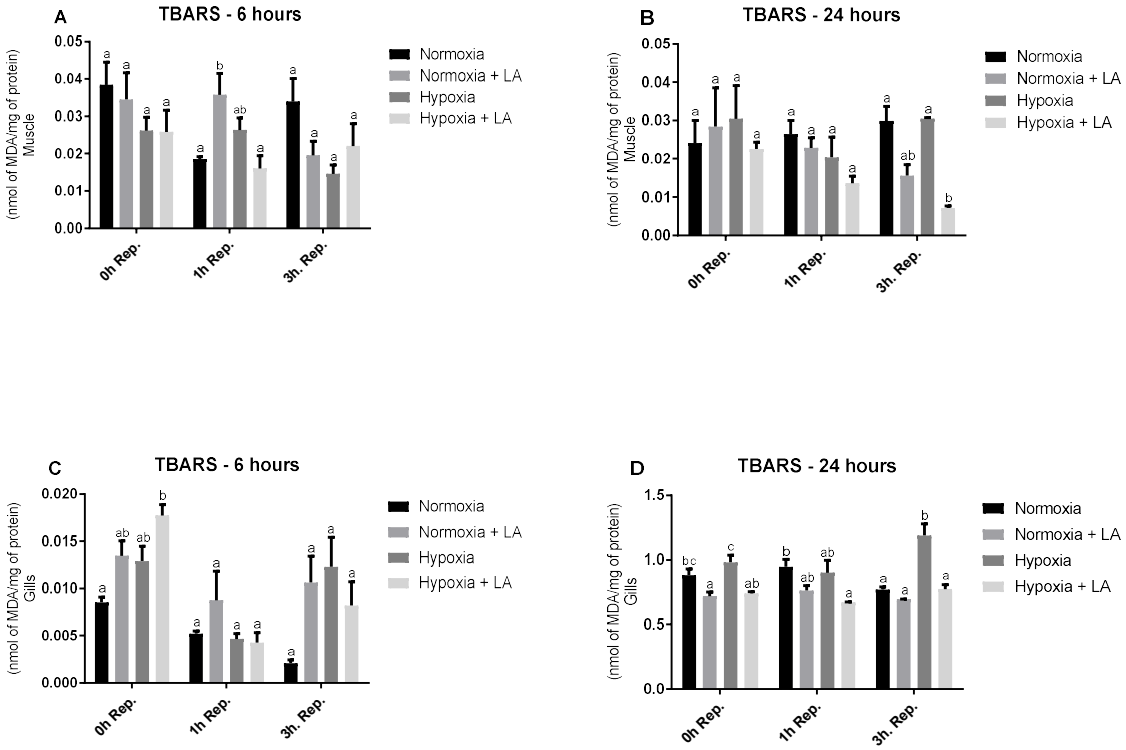
A
TBARS - 6 hours
(nmol of MDA/mg of protein)
Muscle
0.05
0.04
0.03
0.02
0.01
0.00
0h Rep.
1h Rep.
3h. Rep.
Normoxia
Normoxia + LA
Hypoxia
Hypoxia + LA
B
TBARS - 24 hours
(nmol of MDA/mg of protein)
Muscle
0.05
0.04
0.03
0.02
0.01
0.00
0h Rep.
1h Rep.
3h. Rep.
Normoxia
Normoxia + LA
Hypoxia
Hypoxia + LA
C
TBARS - 6 hours
(nmol of MDA/mg of protein)
Gills
0.020
0.015
0.010
0.005
0.000
0h Rep.
1h Rep.
3h. Rep.
Normoxia
Normoxia + LA
Hypoxia
Hypoxia + LA
D
TBARS - 24 hours
(nmol of MDA/mg of protein)
Gills
1.5
1.0
0.5
0.0
0h Rep.
1h Rep.
3h. Rep.
Normoxia
Normoxia + LA
Hypoxia
Hypoxia + LA

Figure 6

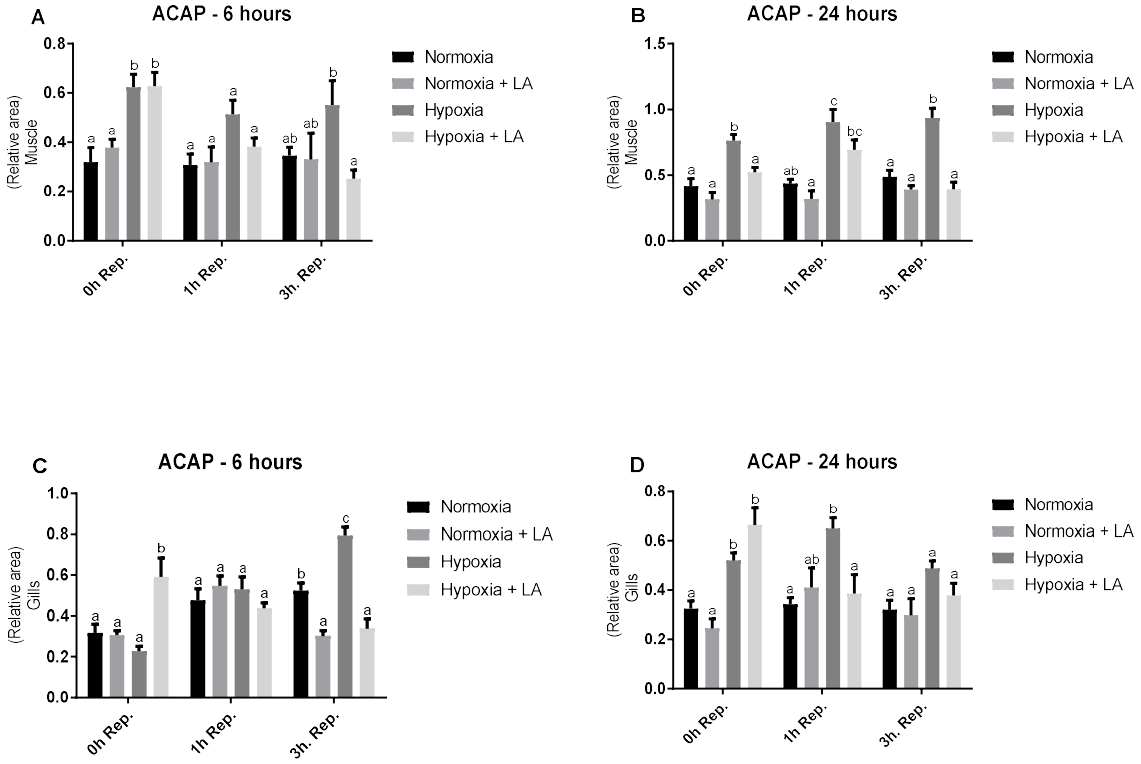
A
ACAP - 6 hours
(Relative area) Muscle
0.8
0.6
0.4
0.2
0.0
0h Rep.
1h Rep.
3h. Rep.
Normoxia
Normoxia + LA
Hypoxia
Hypoxia + LA
B
ACAP - 24 hours
(Relative area) Muscle
1.5
1.0
0.5
0.0
0h Rep.
1h Rep.
3h. Rep.
Normoxia
Normoxia + LA
Hypoxia
Hypoxia + LA
C
ACAP - 6 hours
(Relative area) Gills
1.0
0.8
0.6
0.4
0.2
0.0
0h Rep.
1h Rep.
3h. Rep.
Normoxia
Normoxia + LA
Hypoxia
Hypoxia + LA
D
ACAP - 24 hours
(Relative area) Gills
0.8
0.6
0.4
0.2
0.0
0h Rep.
1h Rep.
3h. Rep.
Normoxia
Normoxia + LA
Hypoxia
Hypoxia + LA

Figure 7

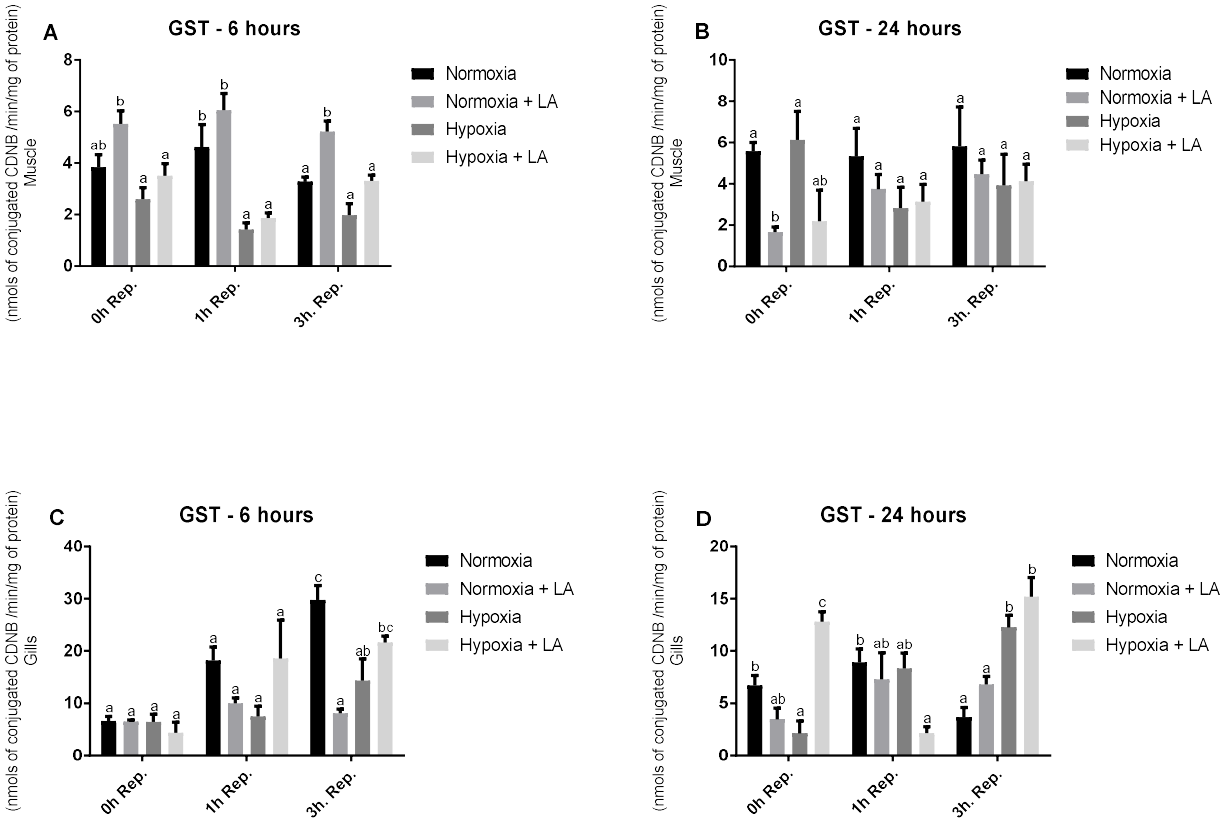
A
GST - 6 hours
(nmols of conjugated CDNB /min/mg of protein)
Muscle
Normoxia
Normoxia + LA
Hypoxia
Hypoxia + LA
0h Rep.
1h Rep.
3h. Rep.
B
GST - 24 hours
C
GST - 6 hours
Gills
D
GST - 24 hours

Figure legends

Figure 1: Schematic representation of experimental design.

Figure 2: Glucose levels. A and B: Hemolymph with 6 and 24 hours of hypoxia exposure, respectively; C and D: Muscle with 6 and 24 hours of hypoxia exposure, respectively. Different letters indicate significant differences ($p<0.05$). The statistical comparisons should be considered only within each time of reperfusion (0, 1 and 3 hours). Data are expressed as mean ± standard error (n = 6).

Figure 3: Lactate dehydrogenase (LDH) activity. A and B: Hemolymph with 6 and 24 hours of hypoxia exposure, respectively; C and D: Muscle with 6 and 24 hours of hypoxia exposure, respectively. Different letters indicate significant differences ($p<0.05$). The statistical comparisons should be considered only within each time of reperfusion (0, 1 and 3 hours). Data are expressed as mean ± standard error (n = 6).

Figure 4: Lactate levels. A and B: Hemolymph with 6 and 24 hours of hypoxia exposure, respectively; C and D: Muscle with 6 and 24 hours of hypoxia exposure, respectively. Different letters indicate significant differences ($p<0.05$). The statistical comparisons should be considered only within each time of reperfusion (0, 1 and 3 hours). Data are expressed as mean ± standard error (n = 6).

Figure 5: Thiobarbituric acid-reactive substances (TBARS) assay. A and B: Muscle with 6 and 24 hours of hypoxia exposure, respectively; C and D: Gills with 6 and 24 hours of hypoxia exposure, respectively. Different letters indicate significant differences ($p<0.05$). The statistical comparisons should be considered only within each time of reperfusion (0, 1 and 3 hours). Data are expressed as mean ± standard error (n = 6).

Figure 6: Antioxidant competence against peroxyl radicals (ACAP). A and B: Muscle with 6 and 24 hours of hypoxia exposure, respectively; C and D: Gills with 6 and 24 hours of

hypoxia exposure, respectively. Different letters indicate significant differences ($p<0.05$). The statistical comparisons should be considered only within each time of reperfusion (0, 1 and 3 hours). The results are inversely interpreted. Data are expressed as mean ± standard error (n = 6).

Figure 7: Glutathione-S-transferase (GST) activity. A and B: Muscle with 6 and 24 hours of hypoxia exposure, respectively; C and D: Gills with 6 and 24 hours of hypoxia exposure, respectively. Different letters indicate significant differences ($p<0.05$). The statistical comparisons should be considered only within each time of reperfusion (0, 1 and 3 hours). Data are expressed as mean ± sta.

5. Discussão Geral

Ao fim do presente trabalho dois manuscritos foram gerados utilizando o camarão branco do pacífico *Litopenaeus vannamei* como modelo biológico. O primeiro manuscrito teve como objetivo verificar a sobrevivência dos animais sob diferentes salinidades (35, 25 e 5‰) e temperaturas (20, 25 e 30 ºC), além desses parâmetros foram cruzadas cinco concentrações de oxigênio (6.0, 3.0, 1.5, 0.5 e zero mg O_2 L^{-1}), para estabelecer os valores de $CL_{50\text{-}96h}$ para oxigênio dissolvido em diferentes condições ambientais e de cultivo.

Sabe-se que baixas concentrações de oxigênio por determinados períodos de tempo podem vir a tornar-se letais para diversos organismos. Em crustáceos, a concentração de oxigênio dissolvido em torno de 1.5 mg O_2 L^{-1} é considerada por muitos como sendo hipoxia severa, causando inúmeras adversidades como, por exemplo, diminuição na taxa de alimentação, crescimento lento e diminuição a resistência contra diversas enfermidades (Welker *et al.,* 2013). De fato, diversos trabalhos adotam valores entre 1.0 e 2.0 mg O_2 L^{-1} de oxigênio dissolvido como sendo hipoxia severa para o *L. vannamei* (Smith *et al.,* 2003; García-Triana *et al*., 2016), entretanto, há uma grande variação nas temperaturas e salinidades experimentais utilizadas nos referidos trabalhos ficando entre 18 e 31 ºC e 28 e 37‰, respectivamente. Os valores de salinidade e temperatura interferem na disponibilidade e solubilidade do oxigênio dissolvido no ambiente aquático, o que pode vir a modificar o valor de da $CL_{50\text{-}96h}$ para o oxigênio.

No primeiro manuscrito foi possível verificar que na temperatura de 30 ºC nas salinidades de 25 e 35‰ não tiveram uma grande variação nos valores de $CL_{50\text{-}96h}$ ficando estas em torno de 4.21 e 3.62 mg O_2 L^{-1}, respectivamente. Porém, foi possível verificar que na salinidade de 5‰ o valor de $CL_{50\text{-}96h}$ para o oxigênio superior, quando comparado com o valor obtido nas maiores salinidades ($CL_{50\text{-}96h}$ de 4.81 mg O_2 L^{-1}). Isso pode ser atribuído ao fato que em salinidades mais altas os animais tendem a ter um gasto energético menor, visto que menos energia será gasta com os processos da osmorregulação, diferentemente do que ocorre em salinidades mais baixas onde

um maior consumo de oxigênio é necessário para os processos metabólicos e fisiológicos (Vargas-Albores e Ochoa, 1992).

Em animais ectotérmico, como é o caso do *L. vannamei*, a temperatura influência diretamente nas taxas metabólicas, acelerando ou retardando os processos fisiológicos. Altas temperaturas aceleram o metabolismo favorecendo o consumo de oxigênio. Geralmente acompanhando o aumento da temperatura, por exemplo, elevações em torno de 10 ºC na temperatura corpórea pode dobrar as taxas metabólicas e com isso o consumo de oxigênio, esse processo pode ser explicado pelo coeficiente termal (Q_{10}) representando o grau de sensibilidade dos organismos ao aumento da temperatura (Schmidt- Nielsen, 1999).

A salinidade mais baixa utilizada neste estudo foi de 5 ‰ obtendo valores de $CL_{50\text{-}96h}$ para oxigênio fixados em 2.67 e 2.79 mg O_2 L^{-1} nas temperaturas de 20 e 25 ºC respectivamente, e a salinidade de 25 ‰ fixando uma $CL_{50\text{-}96h}$ em 1.92 e 2.59 mg O_2 L^{-1} para as mesmas temperaturas. Excluindo o valor de $CL_{50\text{-}96h}$ na temperatura de 20 ºC na salinidade de 25 ‰ ($CL_{50\text{-}96h}$ 1.92 mg O_2 L^{-1}) os demais valores se assemelham a valores de oxigênio dissolvido descrito como sendo hipóxia moderada de 3 mg O_2 L^{-1} (Welker *et al.,* 2013).

A partir dos dados obtidos nos primeiros experimentos (relativos ao capítulo 1 da presente tese) foi originado o segundo experimento (capítulo 2) que teve com objetivo avaliar o potencial efeito quimioprotetor do ácido lipóico no camarão *L. vannamei* submetido a hipóxia/reperfusão considerando parâmetros do metabolismo energético e da capacidade antioxidante do animal. O valor de hipóxia foi fixado em 1.5 mg O_2 L^{-1} nos tempos de 6 e 24 horas, logo após ocorreu o período de reoxigenação de 1 e 3 horas com normoxia fixada em 6 mg O_2 L^{-1}.

No segundo manuscrito a salinidade e temperatura utilizadas durante os experimentos foram de 25 ‰ e 20 ºC, repectivamente, devido aos resultados obtidos a partir do primeiro manuscrito, tendo uma $CL_{50\text{-}96h}$ para oxigênio dissolvido de 1.92 mg O_2 L^{-1}, valor este próximo ao descrito na literatura como sendo hipóxia severa para crustáceos.

O AL é um antioxidante considerado universal e o mais próximo do ideal devido a algumas de suas características como: **(1)** estar presente tanto na fase aquosa como na fase lipídica, o que facilita a absorção e o transporte dessa molécula no organismo, sendo levada a todos os tecidos e facilmente atravessando as membranas biológicas (Muthuswamy *et al.,* 2006); **(2)** capacidade de quelar metais diminuindo a disponibilidade destes para exercerem efeitos tóxicos nas células; **(3)** reciclagem de diversos antioxidantes endógenos que participam do sistema de defesas antioxidante (enzimático e/ou não enzimático) como as vitaminas E e C; **(4)** interceptar espécies reativas de oxigênio (Packer *et al.,* 1995; Packer *et al.,* 1998). Os antioxidantes em geral estão sendo utilizados como uma possível ferramenta contra os efeitos gerados pelo desbalanço no sistema redox da célula. Desta forma, sendo uma alternativa a incorporação destes através da suplementação via alimentação, de fato, alguns estudos já demonstraram a melhora na saúde e capacidade antioxidante quando os animais receberam alimentação enriquecida com AL.

Neste estudo, *L. vannamei* apresentou alterações nos aspectos metabólicos, tanto em 6 como 24 horas de hipóxia seguido pela reperfusão de 1 e 3 horas no tecido muscular e no hemolinfa, atuando de forma positiva sobre a via glicolítica para geração de energia (Cruz *et al.,* 2016). O AL de forma geral mostrou ser capaz de diminuir a glicose circulante e aumentar os níveis de glicose no tecido muscular. Este resultado é de suma importância para o camarão, visto que sob o estado de privação do oxigênio o metabolismo aeróbico (responsável pela maior produção de ATP) é redirecionado para o metabolismo da glicólise anaeróbica que por sua vez, produz menos energia. A glicólise anaeróbica tem pouca capacidade de produzir energia, desta forma o aumento da glicose no tecido muscular acarreta em uma maior fonte de substrato para produção energética, visto que o saldo positivo por mol de glicose é apenas 2 moles de ATP.

Além disso, Martínez-Quintana e colaboradores, 2015 verificaram que GLUT-1 é regulado em condição de hipóxia (2 mg O_2 L^{-1}) no camarão *L. vannamei,* fator potencialmente

importante para o animal, visto que o metabolismo energético passa do aeróbico para o anaeróbico, durante a privação do oxigênio. Desta forma, podendo refletir uma maior disponibilidade de glicose para a demanda da glicólise, proporcionando assim uma fonte energética aos animais em situação de hipóxia.

Sabe-se que a reoxigenação é um período extremamente crítico quando for restabelecida a normoxia, liberando todos os produtos gerados durante a privação do oxigênio, além da retomada do metabolismo aeróbico que utiliza o oxigênio como passo final na síntese de ATP, acarretando na produção endógena de ERO (Fridovich, *et al*., 2004). Embora a hipóxia afete os animais de várias maneiras, a produção de espécies reativas merece uma especial atenção, como um fator crítico nas lesões geradas pela reperfusão. De fato, sabe-se que o AL pode minimizar o dano oxidativo a macromoléculas, como lipídios e proteínas (Arivazhagan *et al.,* 2002; Monserrat *et al.,* 2008).

Em músculo e brânquias de *L. vannamei* submetidos à hipóxia, o AL teve um efeito protetor pela diminuição dos níveis de peroxidação lipídica somente quando ocorreu a reperfusão de 3 horas após 24 horas de hipóxia. Surpreendentemente, neste estudo, em relação aos níveis de TBARS no músculo de *L. vannamei* suplementados, não houve uma melhora em uma situação de normoxia, mas sim após a retomada do oxigênio, na condição mais crítica em que o animal pode se encontrar, como já foi verificado por outros autores (Zenteno-Savín *et al*., 2006; Zhang *et al*., 2010; Martins *et al*., 2014).

A proteção dada no período de reoxigenação pelo AL é de extrema importância, principalmente sob estruturas biológicas como as membranas lipídicas, por serem alvo das reações de oxidação de espécies reativas. Os fosfolipídios e proteínas presentes nas membranas celulares quando oxidados, perde sua fluidez, e com isso sua principal característica, a seletividade e função. Esse processo pode levar a injuria da célula, uma vez que pode causar o

extravasamento do conteúdo celular e o influxo de Ca^{2+} presente no citoplasma, um grande sinalizador apoptótico (Berridge *et al.,* 2003).

Neste estudo foi observado que a suplementação com AL melhorou os parâmetros analisados quando houve a retomada nos níveis de oxigênio dissolvido (período mais crítico em termos de danos oxidativos), resultado também encontrado quando analisado à competência antioxidante contra os radicais de peroxil. De maneira geral, em ambos os tecidos (músculos e brânquias) foi evidenciado um aumento na capacidade antioxidante em animais suplementados com o antioxidante durante a reperfusão. O aumento na capacidade antioxidante pode ter colaborado para que baixos níveis de lipídios peroxidados fossem encontrados na análise de TBARS, dando um efeito protetor às membranas biológicas.

Alterações nos níveis de oxigênio dissolvido modificam o sistema redox das células, podendo alterar a capacidade detoxificatória dos animais em decorrer da formação de espécies reativa de oxigênio. A glutationa-*S*-transferase (GST) compõem a família das enzimas que participam dos processos detoxificatório nos organismos, por catalisar a conjugação de moléculas eletrolíticas à glutationa, gerando produtos menos tóxicos e mais fáceis de serem metabolizados e excretados (Halliwell e Gutteridge, 2007).

Portanto, os resultados obtidos nos dois capítulos deste trabalho, quando analisados em conjunto nos levam a concluir que efeitos causados pela hipóxia e reoxigenação, podem ser minimizados com a ação quimioprotetora do antioxidante AL. Entretanto, condições abióticas são de grande importância para determinar a concentração de OD consideradas como hipóxia severa para o camarão *L. vannamei*.

6. Conclusões da tese

Por meio dos resultados obtidos pode-se observar que na baixa salinidade de 5‰ em conjunto com uma alta temperatura (30 ºC), acarreta em um valor de $CL_{50\text{-}96h}$ para oxigênio dissolvido maior que as demais temperaturas e salinidades analisadas para *L. vannamei*. Desta forma parece ser necessário fixar valores para hipóxia severa e moderada levando-se em consideração os fatores bióticos como temperatura e salinidade que são intimamente ligados a solubilidade do oxigênio em meio aquático.

A suplementação na ração com o antioxidante AL é uma boa estratégia de quimioproteção contra os efeitos gerados pela hipóxia e reperfusão por ser capaz de modular positivamente o metabolismo energético em animais submetidos a hipóxia e reperfusão, principalmente em músculo *L. vannamei.* O melhor papel desempenhado pelo AL é durante a reperfusão, melhorando consideravelmente os parâmetros bioquímicos e do metabolismo energético analisados com exceção da atividade da GST que não mostrou ser modulada pela suplementação com AL.

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Anexo I: Normas da revista a ser submetido o manuscrito 1 e 2.

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