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Tese de Doutorado

EFEITO DA HIPOXIA E REOXIGENAÇÃO NO MÚSCULO LOCOMOTOR DO CARANGUEJO *NEOHELICE GRANULATA* (DECAPODA, VARUNIDAE)

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II. LISTA DE ABREVIAÇÕES

SDA - Sistema de defesa antioxidante

ROS - Espécies reativas de oxigênio

ENR - Espécies nitroreativas

H_2O_2 - Peróxido de hidrogênio

HO• - Radical hidroxila

$O_2^{\cdot -}$ - Radical ânion superóxido

CHP - Hidroperóxido de cumene

H_2DCF-DA -Diacetato de 2´, 7´ diclorodihidrofluoresceína

NDA - Dicarboxaldeído de 2',3' naftaleno

ACAP - Competência antioxidante contra radicais peróxil

GSH - Glutationa reduzida

GSSG - Glutationa oxidada

CAT - Catalase

SOD - Superoxido dismutase

GPx -Se- Glutationa peroxidase

GCL - Glumatato cisteína ligase

GR - Glutationa redutase

GS - Glutationa sintase

PMSF - Fluoreto de fenilmetilsulfonil

LPO - Peroxidação lipídica

JC1- Iodeto de 5,5,6,6-tetracloro-1,1,3,3-tetraetilbenzimidazolcarbocianina

ATP- Trifosfato de adenosina

CL_{50} - Concentração letal que mata 50% dos animais

TL_{50} - Tempo letal que mata 50% dos animais

TL_{10} - Tempo letal que mata 10% dos animais

HEPES - Ácido 1-piperazinoetanosulfônico 4-2-hidroxietil

EDTA - Ácido etilenodiamino tetra-acético

EGTA - Ácido tetracético etileno-glicol

BSA – Albumina de soro bovino

TTC – Cloreto 2,3,5 de trifenil tetrazólio

III. LIST OF ABREVIATIONS

ADS- Antioxidant defense system

ROS - Reactive oxygen species

RNS - reactive nitrogen species

H_2O_2 - hidrogen peroxide

HO• - hidroxil radical

$O_2^{\cdot -}$ - superoxide anion radical

CHP - Cumene hidroperoxide

H_2DCF-DA - 2´, 7´ dichlorofluorescein diacetate

NDA- Naphthalene 2,3 dicarboxialdehyde

ACAP - Antioxidant competency against peroxides

GSH - Glutathione reduced

GSSG - Glutathione oxidized

CAT - Catalase

SOD - Superoxide dismutase

GPx-Se - Glutathione peroxidase

GCL - Glumatate systeine ligase

GR - Glutathione reductase

GS - Glutathione sinthase

PMSF - phenylmethylsulfonyl fluoride

LPO - Lipid peroxidation

JC1- 5,5,6,6-tetrachloride-1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide

ATP- Adenosine triphosphate

CL_{50} - Lethal concentration for 50% of animals

LT_{50} - Lethal time for 50% of animals

LT_{10} - Lethal time for 10% of animals

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

EDTA - Ethylenediamine tetraacetic acid

EGTA - Ethylene glycol tetraacetic acid

BSA - Serun bovine albumine

TTC - 2,3,5- triphenyl tetrazolium chloride

IV. RESUMO

Muitos crustáceos vivem em regiões com variações constantes na concentração de oxigênio sofrendo oscilações de hipoxia e reoxigenação. Dentre estes, o caranguejo *Neohelice granulata* é uma espécie frequentemente sujeita a estas situações no seu ambiente. Desta forma, o objetivo desta tese de doutorado foi verificar como o músculo locomotor do caranguejo *Neohelice granulata* altera seu metabolismo energético, se apresenta danos oxidativos e como modula seu sistema de defesa antioxidante (SDA) em função da exposição a diferentes tempos de hipoxia severa seguida de reoxigenação. Inicialmente foi avaliada a resistência e tolerância a hipoxia bem como o tempo de recuperação em reoxigenação. Após, foram avaliados alguns constituintes do metabolismo aeróbico e anaeróbico bem como parâmetros de estresse oxidativo e atividade mitocondrial. Além disso, foi realizada a análise histológica do músculo locomotor bem como observado se a hipoxia e reoxigenação causa alterações morfológicas no tecido. Por fim, foram avaliados os constituintes do sistema de defesa antioxidante no músculo locomotor durante hipoxia e reoxigenação. Este caranguejo apresentou um LC_{50} entre 2,0 e 2,5 mgO_2/L e um LT_{50} e LT_{10} em 0,5mgO_2/L de 14 e 11h, respectivamente. Durante a hipoxia o músculo locomotor de *N. granulata* utiliza o metabolismo anaeróbico intensamente nas primeiras horas. Além disso, após 4h de hipoxia a diminuição do potencial de membrana mitocondrial observada sugere um dano oxidativo mitocondrial, pois provavelmente tanto a glutationa peroxidase (GPx-Se) bem como a glutationa (GSH) e a melatonina mitocondrial estão sendo utilizadas pelo músculo. Durante a hipoxia de 10h uma diminuição do potencial de membrana mitocondrial está associada a diminuição da área de fibras aeróbicas no músculo, provavelmente responsável pelo atraso na ativação do metabolismo aeróbico. Na reoxigenação, distintas respostas foram observadas dependendo do período de exposição à hipoxia. Além disso, alterações de volume das fibras musculares, foram observadas na região que apresenta fibras com metabolismo oxidativo, demonstrando que a capacidade de recuperação do músculo locomotor de *N. granulata* depende do tempo em que este caranguejo permanece em hipoxia. No início da reoxigenação pós hipoxia de 1h e 4h, o retorno do oxigênio levou a um aumento nos níveis de espécies reativas de oxigênio (ERO) e lipoperoxidação (LPO) e uma diminuição do potencial de membrana mitocondrial que foi proporcional ao tempo de exposição à hipoxia. Porém, estes parâmetros foram rapidamente recuperados ao final da reoxigenação, provavelmente pela utilização da enzima catalase bem como GSH e melatonina citosólica possivelmente contribuindo para a rápida ativação do metabolismo aeróbico. Estes resultados sugerem que o músculo locomotor deste animal não apresenta depressão metabólica nas primeiras horas de hipoxia utilizando os componentes do seu SDA a fim de reparar e evitar os danos quando exposto por muito tempo a hipoxia severa.

Palavras-chaves: hipoxia/reoxigenação, crustáceos, metabolismo, sistema de defesa antioxidante, melatonina, mitocôndria.

V. ABSTRACT

Many crustaceans live in regions with constant variations in oxygen concentration suffering from hypoxia and reoxygenation oscillations. Among these, the crab *Neohelice granulata* crab is a species often subject to these situations in their environment. Thus, the aim of this thesis investigated how the locomotor muscles of the crab *Neohelice granulata* changes its energetic metabolism, if present oxidative damages and modulates their antioxidant defense system (ADS) as a function of exposure at different times of severe hypoxia followed by reoxygenation. Initially the resistance or tolerance to hypoxia as well as the recovery time in reoxygenation was evaluated. After, we evaluated some constituents of aerobic and anaerobic metabolism, oxidative stress parameters and mitochondrial activity. Furthermore, histological analysis was performed in the locomotor muscle and observed whether hypoxia and reoxygenation cause morphological changes in this tissue. Finally, we evaluated the constituents of the antioxidant defense system in locomotor muscle during hypoxia and reoxygenation. This crab had a LC_{50} between 2.0 to 2.5 $mgO_2.L^{-1}$ and a LT_{50} and LT_{10} in 0.5$mgO_2.L^{-1}$ of 14 and 11h, respectively. During hypoxia locomotor muscles of *N. granulata* uses intensely anaerobic metabolism in the first hours. Furthermore, after 4h of hypoxia a decrease in the mitochondrial membrane potential observed suggesting a mitochondrial oxidative damage, probably because both glutathione peroxidase (GPx–Se) as well as glutathione (GSH) and mitochondrial melatonin is being used by the muscle. During 10h of hypoxia a decrease in mitochondrial membrane potential is associated with the decrease in the area of aerobic fibers in the muscle, probably responsible for the delay in the activation of aerobic metabolism. On reoxygenation, distinct responses were observed depending on the period of exposure to hypoxia. Additionally, volume alterations in the muscle fibers were observed in the region presenting fibers with oxidative metabolism, demonstrating that the ability of the locomotor muscles of *N. granulata* recovery depends on the time in which this remains in crab hypoxia. In the early of reoxygenation post hypoxia of 1h and 4h the return of oxygen lead to increased levels of reactive oxygen species (ROS) and lipoperoxidation (LPO) and a decrease in the mitochondrial membrane potential that was proportional to the time of exposure to hypoxia. However, these parameters were quickly recovered at the end of reoxygenation, presumably through the use of enzyme catalase as well as GSH and cytosolic melatonin possibly contributing to the rapid activation of aerobic metabolism. These results suggest that the locomotor muscle of this animal has no metabolic depression in the early hours of hypoxia, using the components of your SDA to repair and prevent damage when exposed for long time under severe hypoxia.

Keywords: hypoxia/reoxygenation, crustaceans, metabolism, antioxidant defense system, melatonin, mitochondria.

VI. INTRODUÇÃO GERAL

O oxigênio é necessário para sustentar a vida da maioria dos animais que vivem em nosso planeta. Porém, esta molécula não se distribui de forma igualitária em todos os ecossistemas. Enquanto os animais de respiração aérea vivem em regiões onde os níveis de oxigênio são normalmente mais altos e constantes, os animais de respiração aquática repetidamente convivem com concentrações de oxigênio dissolvidos menores e mais variáveis. No ambiente aquático, o suporte de oxigênio é oriundo da atmosfera ou produzido através de fotossíntese pelo fitoplanctôn. Uma vez dissolvido na água, uma condição normal é que este oxigênio se misture ao longo da coluna d'água. Quando por qualquer motivo o suprimento de oxigênio ao longo desta coluna d'água é cortado, como por exemplo, durante os processos de eutrofização ou quando a taxa de consumo dos animais aumenta além da reposição do oxigênio a concentração declina abaixo do ponto de sustentação da maioria dos animais, sendo esta condição chamada de hipoxia. O ponto médio onde a maioria dos animais aquáticos começam a demonstrar sintomas desta diminuição ocorre normalmente abaixo de 2,0 mgO_2/L (Diaz e Rosemberg, 1995). Apesar de episódios de hipoxia serem uma característica comum no ambiente aquático, a ocorrência de regiões com muito baixo níveis de oxigênio, também chamada de "zonas mortas" tem aumentado muito nos últimos anos, especialmente nas zonas costeiras e estuarinas (Diaz e Rosemberg, 1995; Diaz, 2001). Quando ocorrem variações na concentração de oxigênio, dois grandes problemas fisiológicos são enfrentados pelos animais. O primeiro é quando a concentração de oxigênio na água chega a níveis muito baixos (hipoxia), ficando abaixo do necessário para manutenção das atividades básicas. O

segundo problema ocorre durante o retorno do oxigênio (reoxigenação) quando sua concentração sobe rapidamente chegando a níveis normóxicos ou, em alguns casos, níveis hiperóxicos podendo levar a sérios danos oxidativos.

No ambiente aquático, como já comentado, as zonas costeiras são consideradas uma das áreas mais estressantes em se tratando da variação de oxigênio (Smith e Able, 2003). Nestes ambientes, variações recorrentes nos níveis de oxigênio obrigam os animais a possuírem estratégias para poderem se manter nestes locais. Dentre os grupos de animais que vivem nos ambientes aquáticos, tais como peixes, moluscos e crustáceos, existe uma grande variabilidade a respeito dos limites de resistência a hipoxia, sendo os moluscos e peixes considerados mais tolerantes e os crustáceos menos (Vaquer-Sunyer e Duarte, 2008). No entanto, várias espécies de crustáceos são frequentemente encontradas em regiões com oscilações de hipoxia e reoxigenação, exigindo destes animais profundas modificações comportamentais, fisiológicas e bioquímicas a fim de conseguir sobreviver nestes locais. Um exemplo de ambiente que apresenta flutuações constantes nos níveis de oxigênio são as marismas que rodeiam os estuários do litoral do Rio Grande do Sul. Nestas regiões ocorrem variações constantes de oxigênio. Por vezes os níveis de oxigênio na água se mantêm baixos por várias horas e até dias, outras vezes as oscilações são bruscas (Turcatto 1990; D`Incao *et al*., 1992). Várias espécies são residentes nesses ambientes e enfrentam estas oscilações. Um desses animais é o caranguejo *Neohelice granulata,* anteriormente conhecido como *Chasmagnathus granulata*/*granulatus* (Sakai *et al*., 2006). Este caranguejo é encontrado nas marismas no sudoeste do oceano Atlântico, apresentando grande densidade populacional (Iribarne *et al*.,1997).

Como hábitos alimentares *N. granulata* é considerada uma espécie detritívora alimentando-se principalmente de plantas encontradas nas marismas tais como *Spartinas, Sarcocornias* e *Juncus* (Botto *et al.*, 2005; Bortolus *et al*., 2006). Além disso, nas marismas esta espécie vive em tocas e galerias construídas na região mesolitoral onde água frequentemente fica retida nas tocas pela ação da maré (Iribarne *et al*.,1997). Entretanto, tanto pelo consumo de oxigênio desta espécie bem como pela decomposição da matéria orgânica juntamente com a baixa difusão entre a atmosfera e a água trapeada nas tocas, uma rápida diminuição dos níveis de oxigênio ocorrem neste habitat (Turcatto, 1990; Iribarne *et al*.,1997). Portanto, esta espécie é um bom exemplo para se compreender os efeitos da hipoxia e reoxigenação.

Várias espécies de crustáceos decápodes, quando confrontados com a diminuição dos níveis de oxigênio, podem evitar estes locais deslocando-se para áreas mais oxigenadas. No entanto, há espécies que permanecem nestes ambientes e precisam utilizar mecanismos fisiológicos e bioquímicos a fim de compensar a falta de oxigênio. Por exemplo, alguns crustáceos são capazes de obter oxigênio suficiente durante a hipoxia aumentando a ventilação respiratória (McMahon, 2001). Em crustáceos decápodes o processo ventilatório ocorre pelo batimento do escafognatito, estrutura em forma de remo responsável por movimentar a água dentro da câmara branquial (Wilkens e McMahon, 1972). Durante a hipoxia uma estratégia observada é o aumento na velocidade e na intensidade do batimento do escafognatito levando assim a um aumento no volume ventilatório (Burggren e McMahon, 1983). Além disso, os crustáceos podem aumentar a síntese e/ou afinidade da hemocianina (Taylor e Anstiss, 1999), permitindo que a quantidade de oxigênio circulante seja

mantida em níveis suficientes para produção energética aeróbica necessária para suprir suas atividades básicas. Uma variedade de agentes efetores tem sido relatada como iniciadores do processo de aumento da síntese e afinidade da hemocianina em crustáceos e seus papéis variam dentre as espécies (Truchot, 1992; Mangun, 1997). Por exemplo, um aumento na hiperventilação leva a uma maior liberação de CO_2 acarretando em uma maior afinidade da hemocianina pelo oxigênio, fenômeno conhecido como efeito Bohr negativo (McMahon, 2001). Além disso, um aumento na concentração de Ca^+ e Mg^+ oriundos da carapaça, do lactato durante ativação do metabolismo anaeróbico, bem como, do urato e de hormônios aminérgicos e dopaminérgicos levam também a um aumento na afinidade pelo oxigênio (Lallier e Truchot, 1989; Truchot, 1992; Morris e McMahon, 1992; Lutz e Storey, 1997). No entanto, abaixo de um nível crítico de oxigênio, quando a respiração não é capaz de manter a concentração de oxigênio na hemolinfa acima do mínimo necessário para suprir adequadamente as necessidades dos diferentes tecidos para realização das suas atividades básicas, uma série de outras respostas pode ser observada. Nestas situações, muitos crustáceos respondem à hipoxia diminuindo a frequência cardíaca (bradicardia) (Airriess e McMahon, 1994), alterando ou redirecionando o fluxo sanguíneo para os tecidos que requerem maiores níveis de oxigênio (Reiber 1995; Reiber e McMahon, 1998). Por exemplo, durante a hipoxia, a diminuição dos níveis de oxigênio atua diretamente sobre o metabolismo do gânglio cardíaco levando a bradicardia (Wilkens *et al*., 1996). Modificações no volume sistólico ocorrem durante a hipoxia pela liberação de substâncias aminérgicas e dopaminérgicas oriundas do sistema nervoso ganglionar em crustáceos (McMahon *et al*., 2001). Por fim,

uma redistribuição do volume sistólico ocorre no sistema arterial, provavelmente por alterações nas válvulas cardio-arterial da árvore respiratória provavelmente por estimulação neural (Wilkens *et al*., 1996).

Porém, outras estratégias também podem ser observadas. Alguns crustáceos podem sustentar elevadas reservas de glicogênio, especialmente no hepatopâncreas e músculo (Childress e Seibel, 1998) para serem utilizados no metabolismo anaeróbico. Maciel e colaboradores (2008a) observaram um aumento nos níveis de lactato hemolinfático durante a anoxia no caranguejo *N. granulata* sugerindo ser este o principal produto durante a anaerobiose neste caranguejo. Além disso, um aumento nos níveis de lactato também foi observado no caranguejo *Lithodes santolla* quando exposto a hipoxia severa (Paschke *et al*., 2010). Por fim, resultado similar também foi observado para o camarão *Litopenaeus vannamei* quando exposto a hipoxia (Matinez-cruz *et al*., 2012). Estes resultados demonstram que durante a hipoxia uma ativação do metabolismo anaeróbico pode estar ocorrendo em crustáceos. Em alguns casos uma diminuição do metabolismo como forma de economizar energia também tem sido sugerido. Quando exposto a hipoxia severa uma diminuição do gasto energético observado através do consumo de oxigênio e atividade locomotora foi observado no anfipoda *Niphargus rhenorhodanensis* (Hervant *et al*., 1995). Além disso, muitos crustáceos que vivem em regiões pelágicas onde os níveis de oxigênio são costumeiramente baixos utilizam a depressão metabólica como forma de economizar energia, visto que a baixa temperatura nestas zonas auxilia a baixa demanda metabólica (Childress e Seidel, 1998). Quando *Carcinus maenas* foi exposto a anoxia um aumento nos níveis de GABA, considerado o principal neurotransmissor inibitório em artrópodes,

sugere que esta espécie esteja diminuindo o metabolismo provavelmente como forma de prolongar sua sobrevivência em anoxia (Nilsson e Winberg, 1993).

Apesar de algumas estratégias utilizadas pelos crustáceos durante a hipoxia serem bem conhecidas, os mecanismos utilizados durante a reoxigenação ainda não são bem compreendidas. Nestas situações é de se esperar que as reservas utilizadas durante a hipoxia devam ser reestocadas e os produtos finais do metabolismo anaeróbico devam ser oxidados, excretados e/ou utilizados em vias gliconeogênicas (Hervant *et al*., 1995;. Maciel *et al*., 2008a;. Silva-Castiglioni *et al*., 2011). Além disso, durante a reoxigenação, os tecidos devem retornar ao metabolismo aeróbico, de modo a fornecer mais energia para suas atividades. Neste sentido, é de se esperar que quanto mais rápido uma função biológica seja restabelecida mais rapidamente os tecidos associados a esta função precisam reestabelecer seu metabolismo aeróbico. O músculo associado à locomoção é um dos tecidos que precisa rapidamente estar apto a realizar alto gasto energético durante a reoxigenação. Desta forma, entendemos que os músculos locomotores são um bom objeto de estudo para entendermos os mecanismos utilizados pelos crustáceos para enfrentarem os problemas da hipoxia e reoxigenação.

Durante o processo respiratório, normalmente uma pequena parte do oxigênio utilizado pelas células na cadeia de transporte de elétrons mitocondrial é convertida em espécies reativas de oxigênio (ERO) (Fridovich, 2004). Dentre estas ERO principalmente podem se formar o radical ânion superóxido ($O_2^{\bullet-}$), o peróxido de hidrogênio (H_2O_2) que juntamente com íons ferrosos, através da reação de Fênton, pode se transformar no radical hidroxila (HO•), considerada a ERO mais deletéria de todas (Halliwell e Gutteridge, 2001). Por serem

altamente reativas, estas moléculas podem causar inúmeros danos biológicos tais como oxidação de proteínas, peroxidação lipídica, quebra da fita de DNA (Halliwell e Gutteridge, 2001). Além disso, o aumento nos níveis de ERO pode acarretar em danos na cadeia de transporte de elétrons mitocondrial, levando a diminuição na produção de energia (Korge *et al*., 2008; Halestrap 2009; Halestrap e Pasdois, 2009).

Nos seres humanos, inúmeras patologias estão relacionadas ao processo de isquemia e reperfusão, principalmente as provocadas por enfarte do miocárdio, choque circulatório, apoplexia cerebral e transplante de órgãos (Robin e Theodore, 1982; McCord, 1985; Levinson *et al*.,1986). Estas patologias decorrem de danos moleculares causados pelo aumento na geração de espécies reativas de oxigênio (ERO) formadas em excesso nos sistemas biológicos durante este processo (Li e Jackson, 2002). Durante muito tempo se acreditava que somente na reperfusão, com o aumento no aporte de oxigênio nos tecidos e órgãos, danos moleculares tais como peroxidação lipídica, oxidação de proteínas, quebra da fita de DNA ocorriam pelo aumento nos níveis de ERO principalmente oriundos da mitocôndria (Pike *et al*., 1993; Fuller *et al*., 2003; Halliwell e Gutteridge, 2007). Porém, recentemente também foi observado que durante o processo de isquemia, onde os níveis de oxigênio estão reduzidos, situações específicas na célula podem desencadear aumento dos níveis de ERO podendo também conduzir a danos moleculares (Clanton *et al*., 2007).

Durante esta situação o principal alvo é a mitocôndria. Esta organela é responsável por quase a totalidade da energia produzida pela célula no processo de fosforilação oxidativa na cadeia de transporte de elétrons que

ocorre através da redução do O_2 a H_2O. Porém, mesmo sendo o centro de produção de ATP, altos níveis de ERO são formadas na mitocôndria tanto em condições normais quanto sob condições de estresse (Storey, 1996; Hermes-Lima e Zenteno-Savín, 2002). Além disso, por ser o principal local de produção de ERO, a mitocôndria é bastante susceptível a danos (Boveris *et al*., 1976; Turrens, 2003). Nesta organela, o aumento na geração de ERO pode prejudicar a cadeia de transporte de elétrons, levando a uma diminuição na produção de ATP (Korge *et al*., 2008). Isto ocorre pela combinação de altos níveis de ERO e Ca^+ intracelular que levam a um aumento da permeabilidade mitocondrial e consequente perda de potencial de membrana (Halestrap 2009; Halestrap e Pasdois, 2009). Com este aumento da permeabilidade mitocondrial, constituintes da cadeia de transporte de elétrons, tais como a citocromo c, podem ser lançados para o citosol, o que ocasionaria uma queda na produção de energia podendo levar a célula a desencadear o processo de apoptose (Powers *et al*., 2007; Hüttemann *et al*., 2011). Dentre os tecidos que sofrem com o aumento na geração de ERO durante isquemia e reperfusão, os tecidos musculares têm sido bem estudados (Clanton *et al*., 2007; Andrianjafiniony *et al*., 2010). Nestes tecidos, o aumento nos níveis de ERO leva a geração de vários danos tais como processos inflamatórios (Shoffner, 2000), atrofia muscular (Andrianjafiniony *et al*., 2010) ou aumento do volume celular (Lambert *et al*., 2008). Todos estes danos são consequência de disfunções na cadeia de transporte de elétrons ocasionadas principalmente pelo aumento nos níveis de ERO, causando uma diminuição da produção de ATP (Griffiths *et al*., 1998; Blaisdell, 2002; Marcinek *et al*., 2003).

Nos mamíferos, muitos trabalhos têm sido relatados verificando os efeitos da isquemia e reperfusão em vários parâmetros metabólicos. Entretanto, em outros grupos de animais poucos trabalhos têm sido feitos. Dentre os animais aquáticos estudados, pouca atenção tem sido dada aos crustáceos acerca dos danos teciduais oriundos da hipoxia e reoxigenação. Os poucos estudos apontam que danos teciduais ocorrem durante a reoxigenação. Por exemplo, de Oliveira e colaboradores (2005), analisando o efeito de 8h de anoxia e subsequente recuperação nas brânquias exteriores e posteriores do caranguejo *N. granulata,* observaram um aumento na lipoperoxidação somente durante o período de recuperação. Porém, neste estudo a recuperação foi avaliada em um período curto não verificando se este caranguejo consegue reparar os danos com o passar do tempo. Por outro lado, mesmo com um aumento nos níveis de ERO no início da reoxigenação pós 24h de hipoxia severa, não foi observado a geração de danos oxidativos no hepatopancreas e músculo do camarão *L. vannamei* (Zenteno-savín *et al*, 2006). Estes dois estudos não nos permite concluir se os crustáceos são um grupo susceptível a danos oxidativos oriundos da hipoxia e reoxigenação demonstrando que mais estudos devem ser conduzidos neste grupo.

Todos os organismos apresentam um sistema de defesa antioxidante (SDA) responsável por evitar o aumento nos níveis de ERO, bem como reparar os danos oriundos da interação com constituintes celulares. Este sistema de defesa é dividido em enzimático e não enzimático (Halliwell e Gutteridge, 2001). O SDA enzimático e constituído pelas enzimas superóxido dismutase (SOD) que converte o $O_2^{\cdot-}$ em H_2O_2, a catalase e glutationa peroxidase (GPx-Se) que reduzem o H_2O_2 em água a glutationa-S-transferase (GST) que

conjuga a GSH a um hidroperóxido lipídico, a glutationa redutase (GR) que transforma a glutationa oxidada (GSSG) em reduzida (GSH) e a glutamato cisteina ligase (GCL), enzima chave na síntese da glutationa juntamente com glutationa sintetase (GS). O SDA não enzimático é constituído por antioxidantes de baixo peso molecular tais como; a GSH, a vitamina C e E (Halliwell e Gutteridge, 2007). Dentre os componentes do SDA não enzimático, a GSH é considerada um das principais moléculas detoxificantes, atuando de várias formas na célula. Este tripeptídeo é encontrado em altas concentrações tanto no citosol quanto na mitocôndria (Meister e Anderson, 1983; Meister, 1988). Como antioxidante, a GSH apresenta um alto potencial redutor na célula atuando diretamente sobre as ERO formadas (Dalton *et al*., 2004), assim como, participa indiretamente como cofator de várias enzimas antioxidantes incluindo a GPx-Se e a GST (Arthur, 2000; Rinaldi *et al*., 2002).

Além disso, outro antioxidante recentemente descoberto é a amina biogênica melatonina (Reiter, 1996) (Fig.1). A melatonina já foi identificada em vários grupos de animais (Vivien-Roels e Pévet, 1993) dentre estes os crustáceos (Withyachumnarnkul *et al*., 1992; Agapito *et al*., 1995; Tilden *et al*., 1997; Maciel *et al*., 2008b). Como antioxidante tem sido observado que esta indolamina apresenta uma ação direta como limpador "*scavenger*" de ERO capaz de neutralizar o radical hidroxila ($OH^{\cdot}$) (Tan *et al.*, 2002; Reiter *et al*., 2002), bem como atua na modulação da expressão de enzimas antioxidantes (Mayo *et al*., 2002). Acrescenta-se ainda que já foi observado que esta indolamina apresenta ação antioxidante na mitocôndria atuando na diminuição da produção de ERO principalmente nos complexos I e III, na cadeia de transporte de elétrons, onde são mais produzidos (Acunã-Castroviero *et al*.,

2003). Hardeland (2008) sugere que nos vertebrados a melatonina produzida fora da glândula pineal apresenta como principal função ação antioxidante. No caranguejo *N. granulata* a melatonina já foi identificada no pedúnculo ocular (Maciel *et al.,* 2008b) e no músculo locomotor (Geihs *et al*., 2009). Além disso, neste caranguejo possivelmente esta molécula apresenta uma ação sobre o sistema de defesa antioxidante (Geihs *et al*., 2009; Vargas *et al*., 2011).

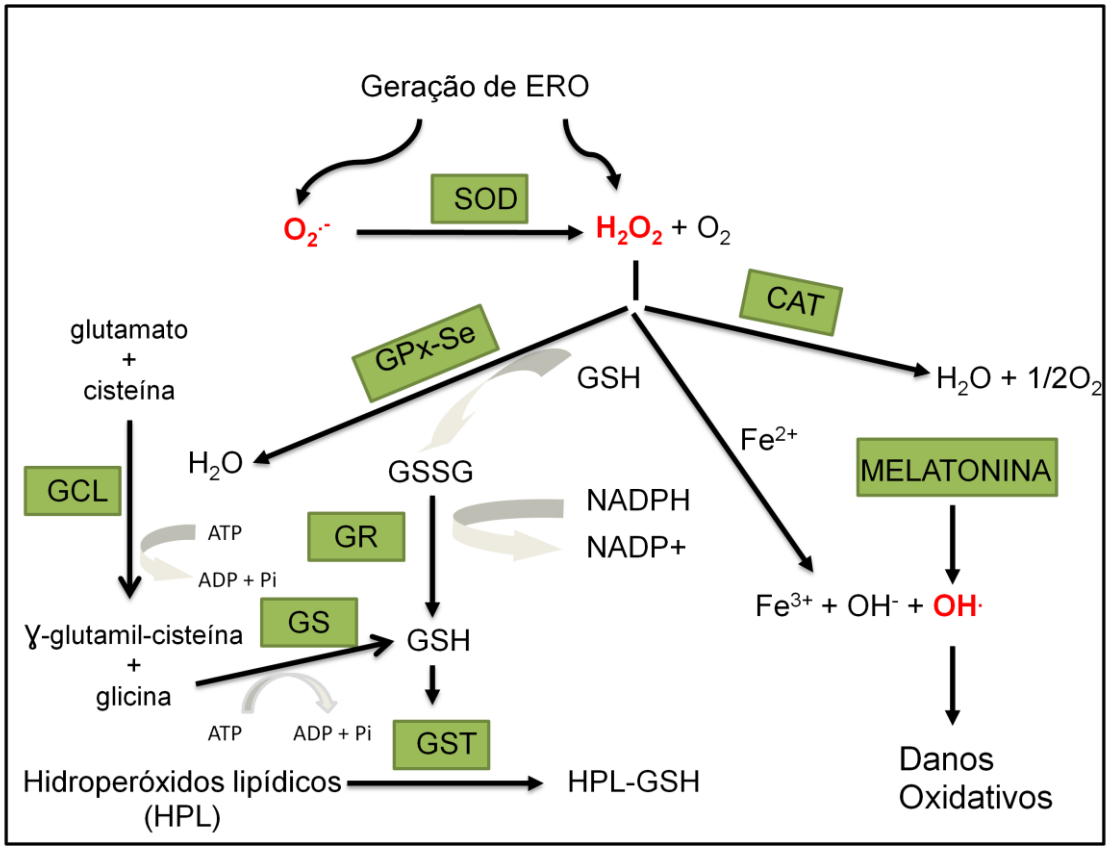

Figura 1. Desenho esquemático mostrado a ação das diversas enzimas e moléculas que compõem o sistema de defesa antioxidante (SDA). Esquema modificado a partir de Halliwell e Gutteridge, 2001.

Uma das adaptações esperadas para tolerar variações de hipoxia e reoxigenação nos animais é que apresentem um SDA bem adaptado a estas variações a fim de evitar a geração de estresse oxidativo (Sies, 1986; Jones, 2006). Neste sentido, quando submetidos à hipoxia e reoxigenação os animais de maneira geral podem manter altos os níveis basais de suas defesas antioxidantes, como já observado para algumas espécies mais tolerantes

(Hermes Lima e Zenteno-Savín, 2002; Bickler e Buck, 2007), ou modular a atividade das enzimas antioxidantes durante o momento de baixa concentração de oxigênio como uma forma de antecipar o aumento na geração de estresse oxidativo, como já observado para outras espécies (Hermes Lima e Zenteno-Savín, 2002; Gorr *et al*., 2010). Entretanto, os poucos trabalhos em crustáceos verificando as estratégias utilizadas pelo SDA durante as oscilações de hipoxia e reoxigenação dificulta a compreensão de como este grupo lida com tais situações (de Oliveira *et al*., 2005; Parrila Taylor e Zenteno-Savín, 2011). Por exemplo, de Oliveira e colegas (2005) observaram que durante 8h de anoxia um aumento na atividade das enzimas catalase e GST ocorria nas brânquias do caranguejo *N. granulata* sugerindo que possivelmente estratégias antecipatórias estariam ocorrendo neste tecido. Parrila Taylor e Zenteno-Savín (2011) observaram que variações na atividade das enzimas antioxidantes em músculo e hepatopancreas ocorriam no grupo controle no camarão *L. vannamei*, e demonstraram que durante a hipoxia e reoxigenação as enzimas SOD e GPx-se eram as que apresentavam modulação.

Portanto, como hipótese deste trabalho esperamos que o caranguejo *Neohelice granulata* seja uma espécie que apresente uma alta tolerância a hipoxia utilizando como uma importante estratégia para aumentar sua resistência a hipoxia à depressão metabólica. Além disso, a fim de evitar danos moleculares esta espécie utilize estratégias antecipatórias no SDA bem como a melatonina seja uma componente importante no SDA no músculo locomotor deste caranguejo.

VII. Objetivos

O objetivo desta tese de doutorado foi verificar se o músculo locomotor do caranguejo *Neohelice granulata* altera seu metabolismo energético, se apresenta danos e qual estratégia é utilizada pelo seu sistema de defesa antioxidante em função da exposição a diferentes tempos de hipoxia severa seguida de reoxigenação. Tendo como objetivos específicos:

1. Verificar os limites de tolerância e resistência a hipoxia;
2. Verificar o tempo necessário para recuperação da hipoxia para enfrentar nova exposição hipóxica;
3. Avaliar o efeito da variação no tempo de hipoxia severa seguida de reoxigenação sobre o metabolismo energético do músculo locomotor;
4. Verificar se diferentes tempos de hipoxia severa (0,5mgO_2/L) seguida de reoxigenação causam danos moleculares, mitocondriais e estruturais do músculo locomotor;
5. Verificar o efeito da hipoxia severa seguida de reoxigenação sobre os diferentes componentes do sistema de defesa antioxidante verificando a participação da melatonina neste sistema.

VIII. 1º ARTIGO

Efeito da hipoxia e reoxigenação no metabolismo energético do caranguejo *Neohelice granulata* (Decapoda, Varunidae)

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EFFECTS OF HYPOXIA AND REOXYGENATION ON THE ENERGETIC METABOLISM OF THE CRAB *NEOHELICE GRANULATA* (DECAPODA, VARUNIDAE)

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Abstract

Many studies have been performed concerning the effects of hypoxia in crustaceans. However, the effects of reoxygenation are not well understood. The aim of this work was to verify the effects of different times of severe hypoxia (0.5 $mgO_2.L^{-1}$) and reoxygenation on the energetic metabolism of the locomotor muscle of the crab *Neohelice granulata*. Initially the tolerance and resistance to hypoxia and the recovery time under reoxygenation were verified. Thereafter, the crabs were submitted to 1, 4 or 10 h of hypoxia (0.5 $mgO_2.L^{-1}$), 30 and 120 min of reoxygenation. Oxygen consumption (MO_2), locomotor activity, hemolymph glucose and lactate, together with glycogen, glucose, ATP content and mitochondrial complex IV activity were analyzed in the locomotor muscle. *N. granulata* showed a LC_{50} between 2.0 and 2.5 $mgO_2.L^{-1}$ and the crab presented a fast recovery after hypoxia exposure. During hypoxia, the locomotor activity did not decrease as intensely as MO_2, probably being maintained by the high levels of lactate verified after short and intermediate hypoxia exposure in the locomotor muscle. During reoxygenation, a fast activation in the aerobic metabolism was observed after short and intermediate hypoxia. However, after long exposure to hypoxia, near the resistance time of this specie, defects in the electron transport chain may be occurring. These results suggest that *N. granulata* utilize the anaerobic metabolism almost all periods of hypoxia. In the reoxygenation the capacity to rapidly activate the aerobic metabolism is dependent on the time of hypoxia exposure.

Keywords: crustaceans, hypoxia, reoxygenation, muscle, metabolism, tolerance.

1. Introduction

Although episodes of hypoxia are a common characteristic in the aquatic environment, the occurrence of regions with lack of oxygen has increased in the last years, especially in coastal zones and estuaries (Diaz and Rosemberg, 1995; Diaz, 2001). Two major physiological problems are faced in this scenario by the animals. The first is when the oxygen concentration reaches very low levels staying below what's necessary for the maintenance of aerobic metabolism. The second problem is when there is a return of oxygen to normoxic levels or, in some cases, hyperoxic levels that may cause oxidative damage. It must be also taken into account that, depending on the environmental conditions, hypoxia may be of short duration, usually called "acute hypoxia", or persist long hours, days or even weeks, defined as "chronic hypoxia". In the aquatic environment, salt marshes are considered one of the most fragile areas concerning oxygen variations (Diaz, 2001; Smith and Able, 2003). In this environment there are burrows and galleries, constructed by annelids and crustaceans, in which a rapid variation in the concentration of dissolved oxygen occurs (Turcatto, 1990).

Among the groups of animals living in aquatic environments, crustaceans are considered one of the less tolerant to hypoxia (Vaquer-Sunyer and Duarte, 2008). However, several crustacean species are frequently found in salt marshes. Many decapods, facing a decrease in oxygenation, can avoid these places and dislocate to more oxygenated areas. However, some crustaceans' species lives in these locals. To face these situations they employ physiological and biochemical mechanisms. To deal with hypoxia, for example, some crustaceans are able to maintain oxygen delivery during hypoxia by increasing

ventilation (McMahon, 2001), hemocyanin synthesis and/or hemocyanin affinity (Taylor and Anstiss, 1999). However, below the critical oxygen level, when respiration alone is not able to maintain the oxygen concentration in the hemolymph above the minimal necessary to adequately supply the needs of the different tissues for the performance of the aerobic metabolism, a series of other responses may be observed. In these situations most crustaceans respond to hypoxia by present bradycardia (Airriess and McMahon, 1994), altering or redirecting blood flow to tissues requiring high levels of oxygen (Reiber, 1995; Reiber and McMahon, 1998). Also, some crustaceans can sustain high glycogen reserves, especially in the hepatopancreas and muscle (Childress and Seibel, 1998), to utilize it in aerobic metabolism (Maciel *et al*., 2008; Paschke *et al*., 2010; Marqueze *et al*., 2011) or, in many cases, save energy by means of metabolic depression (Hervant *et al*., 1995; Childress and Seidel, 1998).

Despite the fact that some strategies used by crustaceans during hypoxia are well known, the mechanisms used during the reoxygenation process are still not well understood. In these situations it is expected that the reserves utilized in hypoxia must be restocked and the end products of anaerobic metabolism must be oxidized, excreted and/or used in gluconeogenic pathways (Hervant *et al*., 1995; Maciel *et al*., 2008; Silva-Castiglioni *et al*., 2011). In addition, during reoxygenation, tissues must return to the normal use of the aerobic metabolism in order to provide more energy for its activities. It is expected that, the faster a biological function needs to be established, more quickly the tissues associated with that function must re-establish their aerobic metabolism. The muscle associated with locomotion is one of the tissues that more quickly needs to be

able to perform a high energetic expenditure during the reoxygenation. In the locomotor muscle of crustaceans there is a predominance of glycolytic fibers (Parsons, 1982, Perry, 2008). So it is expected that in the reoxygenation the exploratory activity should increase. Thus, we understand that the locomotor muscles are a good object of study to investigate the mechanisms used to face the problems of hypoxia and reoxygenation. The crab *Neohelice granulata* that lives in the salt marshes in the estuaries along the Atlantic coast of South America is frequently subjected to variations in the oxygen concentration in the environment (D'Incao *et al.*, 1992). For this reason, it is expected that *N. granulata* is tolerant to hypoxia and that it performs a decrease of its locomotor activity and metabolic depression in its muscular tissue as a strategy to stay longer in hypoxia and during the reoxygenation the muscular tissue quickly increase its aerobic metabolism.

Intending to better understand the effects of hypoxia and reoxygenation in crustaceans, the purpose of this study was to investigate which metabolic strategies the locomotor muscle of the crab *N. granulata* exhibits when exposed to situations of hypoxia and reoxygenation in its environment. Thus, initially the hypoxia tolerance and recovery time were verified to establish the periods of hypoxia and reoxygenation to be applied. Later, we analyzed oxygen consumption (MO_2) and locomotor activity to verify the global metabolic variations of the animals. Finally, the lactate and glucose contents were measured in the hemolymph and in the locomotor muscle in order to observe the profile of the anaerobic metabolism during hypoxia and reoxygenation. Additionally, ATP content and the mitochondrial complex IV activity were

analyzed during hypoxia and reoxygenation to observe the aerobic metabolism during these two situations.

2. Material and methods

2.1. Animals maintenance

Adult male crabs of *Neohelice granulata* weighing 10.5 ± 0.2 g (mean ± S.E.M) were collected in salt marshes around Rio Grande City - Brazil and taken to the laboratory. The animals were acclimated, at least during 10 days, in tanks under constant conditions of temperature of 20°C, salinity of 20 and photoperiod of 12L:12D . The animals were fed *ad libitum* with ground beef 3 times a week.

2.2. Reagents

Citochrome c from bovine heart, *n*-Dodecyl-β-D-maltoside, JC-1 (5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide) and amyloglucosidase were purchased from Sigma-Aldrich (St Louis, MO, USA). The ATP (adenosine triphosphate) kit was purchased from Invitrogem (Oregon, USA). Lactate and glucose kits were purchased from Kovalent (São Gonçalo, RJ, Brazil).Total protein kit were purchased from Doles (Goiania, GO, Brazil).

2.3. Mortality

In the experiment of mortality the crabs were put individually into cylindrical glass chambers with base and height of 5x14cm and approximately 300ml during 96 hours. The different levels of hypoxia were obtained bubbling nitrogen gas (100%) into the aquaria containing water at constant conditions of salinity and temperature (20 and 20ºC) until the oxygen concentrations were fixed in 0.5, 1.0, 2.0, 2.5 and 3.0 mg $O_2.L^{-1}$. The oxygen concentration in the

aquaria was continuously monitored with a portable oximeter (DO- 5519, Lutron Electronic Enterprise CO). Simultaneously, the control group (6.5 $mgO_2.L^{-1}$) was maintained under the experiment. For this experiment the LC_{50}, oxygen concentration that causes mortality of 50% of the animals as well as the LT_{50} and LT_{10}, hypoxia time which cause mortality of 50% and 10% of animals were estimated, respectively. The experiment was made in triplicate being each replicate constitute for ten crabs. The results are presented as accumulated percentage.

2.4. Recovery

In order to determine the time required under control conditions for the animals recover from severe hypoxia (0.5$mgO_2.L^{-1}$), initially the crabs were individually placed in cylindrical glass chambers with base and height of 5x14cm and a volume of approximately 300ml and maintained in hypoxia for 1, 4 and 10h, comprising the times of short, intermediate and maximum survival before the beginning of mortality in 0.5 $mgO_2.L^{-1}$ exposure. After these hypoxia periods, the crabs were transferred to aquaria under control conditions (6.5 $mgO_2.L^{-1}$) and maintained in this situation for 30 min, 2, 6 or 12 h. Finally, the crabs were reintroduced to hypoxia conditions (0.5$mgO_2.L^{-1}$) and the mortality was monitored during 24h. The mortality curves obtained in the second hypoxia conditions were compared with the mortality curve of crabs submitted only to the one exposure of severe hypoxia (0.5 $mgO_2.L^{-1}$), considered the control group. The oxygen concentration was continuously monitored during the experiment. The experiment was made in triplicate being each replicate constitute for a group of ten crabs. The results are presented as accumulated percentage.

2.5. Experimental procedure of hypoxia and reoxygenation

The crabs were individually placed in cylindrical glass chambers with base and height of 5x14cm and a volume of approximately 300ml. Nitrogen gas (100%) were bubbled until the oxygen concentration reached 0.5 $mgO_2.L^{-1}$. The oxygen concentration was continuously monitored during the experiment. All analysis was made after 1, 4 and 10 h of hypoxia and subsequent 30 and 120 min of reoxygenation. The control group was maintained along the whole experiment in water of 0.5 $mgO_2.L^{-1}$ was analyzed simultaneously with the experimental group.

2.6. Oxygen consumption

The initial oxygen concentration was measured individually in the cylindrical glass chambers filled with crabs (n=5) together with a blank chamber (glass chamber without crabs) 30 minutes before the end times of hypoxia and reoxygenation and the chambers were sealed. Immediately after this period, the chambers were open and the final oxygen concentration was measured using a portable oximeter (DO- 5519, Lutron Electronic Enterprise CO). The same procedure was applied for the blank group. The final oxygen concentration in the cylindrical glass chambers under the control and hypoxic conditions never was less than 4.0 and 0.2 $mgO_2.L^{-1}$, respectively. The crabs were weighed and the cylindrical glass chambers volume were measured. The oxygen consumption (MO_2) was expressed in $mgO_2.g^{-1}.h^{-1}$.

2.7. Locomotor activity

After the experimental procedure, the crabs were transferred underwater to an arena (Fig.1) filled with water in the same oxygen concentrations used in the experimental procedure 10 minutes before the end times of hypoxia and

reoxygenation. The animals were held in the arena for 5 min before the analysis. Then, the distance walking (exploratory activity) by the crabs was recorded during 15 min under hypoxia and reoxygenation conditions. The oxygen concentration was constantly controlled in the arena during the analysis. The data were processed in the image analyzer system (EthoVision®, NOLDUS) and the distance walked was expressed in meters.

2.8. Lactate, glycogen and glucose measurements

To determine the lactate concentration in tissue, a pair of meropodites muscles (n=5) from the second pair of pereiopods were removed, weighted and homogenized in teflon (1:5 *w/v*) in cold (4ºC) buffer containing potassium phosphate monobasic and dibasic (100mM), EDTA (1mM) and phenylmethylsulfonyl fluoride (PMSF) (10uM) at pH 7.2. The samples were centrifuged at 8,000xg, 4ºC during 20 min and the supernatant was removed. To determine the lactate concentration in the hemolymph, (100µL) was collected with a sterile syringe from sinus at the base of 4^{th} or 5^{th} pair of pereiopods and immediately processed in a cold (4ºC) buffer containing EDTA (6%). The samples were centrifuged at 8,000xg, 4ºC during 20 min and the supernatant was removed. Lactate in muscle and hemolymph was measured in a spectrophotometer at 340nm using an enzymatic ultraviolet lactate kit (Kovalent – Brazil). The results are given as mg of lactate. g^{-1} for muscles and mg of lactate. ml^{-1} for hemolymph.

To determine the glucose hemolymph samples (n=5), (100µL) was collected similarly to lactate experiment and maintained on cold (4ºC). The glucose content was measured in a spectrophotometer at 490 nm using the

glucose oxidase mono-reagent kit GOD-PAD (Kovalent – Brazil). The results are given as mg of glucose. ml^{-1} of hemolymph.

The glycogen and glucose analysis were performed in the same protocol. Muscle tissues (n=5) were homogenized (1:10 *w/v*) in sodium citrate buffer (100mM) and heated at 95ºC for 5 min. In the samples were immediately added with a glycogen standard (100mg.dl^{-1}), divided into two groups and incubated at 55ºC for 150 min with or without the enzyme amyloglucosidase (1%) for glycogen and glucose, respectively. The samples were then centrifuged (7,000xg, 15ºC during 30 min) and the supernatant of both fractions were determined in a spectrophotometer at 490 nm using the glucose oxidase mono-reagent kit GOD-PAD (Kovalent – Brazil). The glycogen content was quantified in equivalents of glucose (Carr and Neff, 1984; Nery *et al*., 1993). The results are given as mg.g^{-1} for glycogen and glucose.

2.9. Measurement of mitochondrial complex IV activity

In the analysis a mitochondrial fraction was firstly prepared adapted from Parrino *et al*., (2000). Meropodites of the second pair of pereiopods (n=5) were removed, chopped and immediately placed in tubes in cold medium containing sucrose (510mM), EDTA (1mM), EGTA (200µM), HEPES (20mM) and BSA (0.5%) at pH 7.5. Muscles were immediately homogenized and centrifuged (2,000xg, 4ºC during 15 min). The supernatant was removed and centrifuged again (8,000xg, 4ºC during 15 min). The supernatant of the second centrifugation was removed and the resulting sediment was resuspended in cold medium containing sucrose (303mM), EGTA (1mM), KH_2PO_4 (4mM), KCl (90mM) and BSA (0.5%) at pH 7.5. The total protein content was determined in

muscle samples using a commercial kit (Doles - Brazil) based on biuret reagent in a spectrophotometer at 550nm.

The mitochondrial membrane potential was assessed using the cationic carbocyanine dye JC-1 according to Reers *et al*., (1995). This probe exists as a green fluorescent monomer (excitation: 485 nm; emission: 530 nm) at low concentrations (less than 300 nM). However, at high concentrations (>1mM) a very strong red-orange fluorescence occurs (excitation: 485 nm; emission: 530 nm) due to the formation of dye aggregates. Therefore, low membrane potentials will show green fluorescence while high ones will present a red-orange fluorescence, since more of the dye enters the mitochondria as is accumulated in the matrix, forming the aggregates (Reers et al., 1995). JC1 was prepared in a cold medium (4ºC) containing KCl (110mM), $MgCl_2$ (10mM), EDTA (1mM), HEPES (20mM), succinate (10mM) and ATP (10mM) at pH 7.5. Measurements were performed in aliquots of isolated mitochondria and JC-1 solution pipetted into wells of a 96well microplate. The JC-1 solution was prepared from a stock solution (40 µg/L in ethanol) by 200-fold dilution in a buffer solution containing KCl (110mM), MgCl (10mM),EGTA (1 mM), HEPES (20 mM), sodium succinate (10 mM) and ATP (10 mM). After incubation (30°C) for 30 min in the dark, fluorescence generated in the reaction mixture was read (excitation: 485 nm; emission: 590 nm) using a fluorometer (Victor 2, PerkinElmer, Waltham, MA, USA).

The mitochondrial complex IV activity was assessed according to Kirby *et al*., (2007). Cytochrome C was prepared by addition of ascorbate and separated by dialysis (Inlab Diagnostica, Brazil). The cytochrome C oxydase (E.C 1.9.3.1.) activity was performed by following the complete oxidation of cythocrome C in a

spectrophotometer at 550nm in 25ºC. A reaction buffer containing KH_2PO_4 (200mM) and n-dodecil-β-D-maltoside (30mM) at pH 7.5 was prepared. Cytochrome C (2.5mM) was added in the buffer and the baseline was registered. Thereafter, the mitochondria fraction was added and the initial rate was measured by a decrease in the wavelength, which represent the fully cythocrome C oxidation. The results are presented as µmoles. mg of protein^{-1}.min^{-1}. min.

2.10. ATP quantification

The muscles (n=5) were removed and stored at -80ºC for further use in ATP quantification assay. In the beginning of analysis, samples were frozen in liquid nitrogen (N_2) and rapidly processed in dry ice to avoid metabolic activity. The samples were sonicated (1:5 *w/v*) in pure water and centrifuged (8,000xg, 4ºC during 3 min) and the supernatant was used for ATP quantification using an ATP determination Kit (Invitrogen, USA). The results are expressed as pMol.µL^{-1}.

2.11. Statistical analysis

Statistical analysis was performed by analysis of variance (Two Way ANOVA) followed by Newman-Keuls test with α=0.05 for MO_2, locomotor activity and biochemical analysis. Normality and homogeneity of variance were verified as ANOVA assumptions and each point represents the mean ± 1 s.e.m. Mathematical transformations were performed when necessary (Zar, 1984). The nonlinear adjust of the sigmoidal curve of mortality was performed with the confidence interval α=0.05. The analysis of variance (Two Way ANOVA) followed by Bonferroni´s *post hoc* test with adjustment of α=0.05/number of comparisons was used to compare the curves of mortality.

3. Results

The hypoxia resistance analysis (Fig.2) demonstrated that there was no mortality among crabs at oxygen concentration of 3.0 $mgO_2.L^{-1}$. The onset of mortality was observed at 2.5 $mgO_2.L^{-1}$ (LT_{10} = 49.0; $CI_{0.95}$ = 46.3 - 51.9). Below this concentration, a progressive increase in mortality was observed. At 2.0 $mgO_2.L^{-1}$, the LT_{50} and LT_{10} were estimated in 43.8 ($CI_{0.95}$ = 42.6 - 45.0) and 28.7h ($CI_{0.95}$ = 24.9 - 32.5), respectively, occurring total mortality in 60h. For 1.0 and 0.5 $mgO_2.L^{-1}$ the LT_{50} was estimated in 33.3 ($CI_{0.95}$ = 25.7- 40.9) and 14h ($CI_{0.95}$ = 11.2 - 17.8) and the onset of mortality was observed at 12h ($CI_{0.95}$ = 5.9 - 18.1) and 11.4h ($CI_{0.95}$ = 9.0 - 13.8), respectively, and all crabs died in 48 and 24h, respectively. Since *N. granulata* shows a large difference in the mortality between 2.5 and 2.0 $mgO_2.L^{-1}$, after 96h the LC_{50} was not precisely estimated, being considered to be between 2.0 and 2.5 $mgO_2.L^{-1}$.

When we analyze the time required for *N. granulata* recovering from severe hypoxia it was observed that 30 min under control conditions was sufficient to recovery after 1h of severe hypoxia (0.5 $mgO_2.L^{-1}$) (Fig.3). Increasing the periods in which *N. granulata* remains in hypoxia, more time is necessary to recovery. The total recovery in animals exposed to 4 and 10 h of hypoxia was observed after 6 and 12h in control situation, respectively.

The oxygen consumption (MO_2) decreased ($p<0.05$) after exposure to 1, 4 and 10 h of hypoxia (Fig.4) when compared to the control group. When the oxygen was reintroduced, the MO_2 quickly returned to normal levels for all periods of hypoxia ($p>0.05$). The locomotor activity (Fig.5), was decreased ($p<0.05$) after exposure to 1, 4 and 10h of hypoxia when compared to control group. In the reoxygenation, a rapidly return of locomotor activity to basal levels

was verified in animals exposed to 1 and 4h of hypoxia ($p>0.05$). However, after 10h a delay in the return of the locomotor activity was observed ($p<0.05$), compared to control group.

The circulating lactate levels in the hemolymph (Fig.6) significantly increase ($p<0.05$) after 4h in hypoxia. After 10 h of hypoxia a four-fold increase ($p<0.05$) was observed in lactate compared to control group. During reoxygenation, an increase in lactate ($p<0.05$) was observed in 30 min in animals exposed to 1h of hypoxia returning to normal levels at the end of reoxygenation (120min). The lactate levels remained high during all reoxygenation period after 4 and 10h of hypoxia. A pronounced hyperglycemia ($p<0.05$) of seven and five orders of magnitude high compared to control group, was observed after 4 and 10h of hypoxia in the hemolymph, respectively (Fig.7). In animals exposed to 4h of hypoxia the glucose content remained five orders of magnitude higher significantly after 30min of reoxygenation remained higher ($p<0.05$) in 120 min. After 10h of hypoxia the glucose levels in the hemolymph returned more quickly during the first 30 min of reoxygenation compared to the same period of reoxygenation after 4h in hypoxia, but remained higher ($p<0.05$) than the control group during the reoxygenation.

Evaluation of lactate concentration in the locomotor muscle (Fig.8) showed a significant increase ($p<0.05$) after 1, 4 and 10h of hypoxia, when compared to control group. However, in the reoxygenation, the lactate content quickly returned to control levels ($p>0.05$) for all periods of hypoxia. Concerning the carbohydrates stocks in the locomotor muscle, no significant variation ($p>0.05$) in the glycogen content was observed for all periods of hypoxia and reoxygenation analyzed.

No significant variation p>0.05) was observed for any hypoxia periods in the glucose content for the locomotor muscle (Fig.9). During reoxygenation, a small but significant increase ($p<0.05$) was observed in glucose content after 120 min in the groups maintained in 4 and 10 h in hypoxia when compared to control group.

There was no significant difference ($p>0.05$) compared to control group in the mitochondrial complex IV activity (Fig.10) after 1 and 4 h of hypoxia and in the subsequent reoxygenation. However, after 10 h a significant decrease ($p<0.05$) was observed. During reoxygenation there was a quick return to activity soon at 30 min, with no significant differences from the control group. However, at 120min there was a new significant ($p<0.05$) drop in the mitochondrial complex IV activity.

During hypoxia, a significant decrease ($p<0.05$) in the ATP content (Fig.11) was observed after 1, 4 and 10h, respectively. During reoxygenation, the return in ATP content ($p<0.05$) was observed after 30 min in animals exposed to 1 and 4h of hypoxia similarly to control group. However, after 10h of hypoxia the ATP levels of the muscle remained significantly lower ($p<0.05$) than the ones of the control group.

4. **Discussion**

Concerning hypoxia tolerance, there is a significant variation among the organisms living in aquatic environments. Vaquer-Sunyer and Duarte (2008), making a comparative analysis of benthic organisms, observed that crustaceans presented the highest LC_{50} ($2.45 \pm 0.14 mgO_2.L^{-1}$), compared to other groups such as mollusks and fishes. The crab *Neohelice granulata*

demonstrated a tolerance similar to crustaceans in general (Fig. 2), with LC_{50} between 2.0 and 2.5mgO_2.L^{-1}. Although they have a low tolerance to hypoxia when compared to other groups, some species of crustacean that live in an environment with low oxygen levels show a high resistance to severe hypoxia and even anoxia surviving for many hours. For example, the LT_{50} was estimated in 60 and 43h for the mud shrimps *Calianassa jamaicense* and for *Calocaris macandreai* submitted to severe hypoxia (0.3 mgO_2.L^{-1}) (Felder *et al*., 1979; Anderson *et al*., 1994). When we analyzed the resistance to severe hypoxia (0.5 mgO_2.L^{-1}) in the crab *N. granulata* (Fig.2), a LT_{50} of 14h was observed, demonstrating a lower tolerance to hypoxia when compared to these crustaceans. Such differences are probably associated with the environment where these two groups live, since they are animals that live in more constantly low oxygen concentration environments (Zebe, 1982; Anderson *et al*., 1994) and *N. granulata* is a species that lives in salt marshes, where there are great variations of dissolved oxygen along the day, but they usually do not last long (D'incao *et al*., 1992). In these environments, maybe more important than the high capacity of resistance to hypoxia, is the capacity to quick recovery from hypoxia in order to face new hypoxia exposures. Actually, when *N. granulata* was exposed to severe hypoxia (0.5mgO_2.L^{-1}), the recovery was quickly observed. Even in the group of crabs exposed near to the limit of resistance to severe hypoxia, the animals were recovery in 12 hours under 6.5mgO_2.L^{-1} (Fig.3). In situations probably more common in the environment where the hypoxia time last less, the recovery time was far lower. When submitted to hypoxia the crab *N. granulata* did not demonstrate to be an oxyregulator species since MO_2 (Fig.4) quickly decreased. As a counterpart, during the

reoxygenation a rapid increase in the MO_2 to normal levels was observed regardless time of exposure to hypoxia (Fig.4), demonstrating that the activation of the aerobic metabolism during reoxygenation must be a strategy used by *N. granulata* and other crustaceans to survive in environments with cyclic oxygen variations (Hill *et al*., 1991; Paterson and Thorne, 1995; Valverde *et al*., 2012). With the decrease of MO_2 it is expected that activities with high metabolic costs, among them the locomotor activity, should follow the same profile as already observed in other crustaceans (Houlihan *et al*., 1984; Hill *et al*., 1991; Hervant *et al*., 1995, 1997; Childress and Seidel, 1998). However, the decrease in the locomotor activity was not as intense as observed for MO_2 (Fig.5). Thus, to maintain this expendious activity, energy must be supplied for the locomotor muscle during hypoxia. On the other hand, during reoxygenation a quick increase in the locomotor activity was observed in the animals exposed to hypoxia up to 4 hours. However, after 10 hours of hypoxia the return was not so fast when compared to MO_2, indicating that some difficulty in the energetic production may be occurring in the locomotor muscle.

Crustaceans in general exhibit important biochemical strategies to compensate for a lack of oxygen. In this sense, the utilization of anaerobic metabolism and the decrease of energy demands by metabolic depression are considered the two main biochemical routes (Hill *et al*., 1991; Hervant *et al*., 1995; Childress and Seidel, 1998; Kucharski *et al*., 2002; Spicer *et al*., 2002; Parsche *et al*., 2010). When the crab *N. granulata* was exposed to severe hypoxia, hemolymphatic lactate levels increased proportionally to hypoxia exposure, indicating that the activation of glycolytic anaerobic pathway probably is a strategy used to resist to the lack of oxygen. Simultaneously with lactate, a

hyperglycemia was also observed during hypoxia, demonstrating that carbohydrates stocks are being mobilized to provide energetic substrate for the different tissues, as have been verified in other stressing situations for this species and other crustaceans (Hervant *et al*., 1997; Marqueze *et al*., 2006; Maciel *et al*., 2008). These results demonstrate that the anaerobic metabolism is being activated intensively in the crab *N. granulata* during hypoxia. Although there is an increase in the anaerobic metabolism in general terms, there may be differences in the intensity and in the moment of activation for the different tissues. In the locomotor muscle of *N. granulata* under hypoxia a pronounced increase in the lactate levels was observed more intensively after 1 hour in severe hypoxia, decreasing along the time of hypoxia exposure. This result is in consonance with the variation in the intensity of locomotor activity, suggesting that this activity during hypoxia must have been maintained by the anaerobic metabolism. However, differently from the hemolymph, the lactate in the muscle returned quickly to normal levels during reoxygenation after all hypoxia periods. With this result, two hypotheses can be sustained: or the lactate is being quickly converted into glucose in the muscle tissue or it has been released for the hemolymph. In crustaceans there is a great variability among species regarding the lactate metabolism. For example, Maciel and colleagues (2008) observed an increase of the lactate converted into glucose in the muscle of the jaw of the crab *Neohelice granulata*, suggesting that the gluconeogenesis process must be occurring in this tissue. Morris and Adamczewska (2002) observed that gluconeogenesis occurs in the walking leg muscle of the red crab *Gecarcoidea natalis* after exercise. Besides, as a strategy to maintain the acid/base equilibrium, small quantities of lactate may also be excreted to aquatic medium

during reoxygenation (Henry *et al*., 1994; Maciel *et al*., 2008). In our work the glycogen stocks did not present significant variation ($p>0.05$) in any moment of hypoxia or reoxygenation. However, a small but significant increase in the glucose levels in the locomotor muscle was observed (Fig.9) at the same time of the decrease in the lactate levels on the muscle during reoxygenation. This fact can be indicating that the process of gluconeogenesis may be occurring in the locomotor muscle of *N. granulata*. However, the high levels of lactate in the hemolymph during all hypoxia exposure and reoxygenation are indicating that the release for the hemolymph problably is the main strategy used by the locomotor muscle to eliminate the lactate. It's also important to highlight that the gluconeogenesis must be occurring in other organs and tissues mainly in hepatopancreas, gills and muscles (Lallier and Walsh, 1991; Nery and Santos,1993; Henry *et al*., 1994; Oliveira *et al*., 2004; Schein *et al*., 2004).

As commented before, besides the activation of the anaerobic metabolism, several studies have showed that many aquatic animals exhibit metabolic depression as a strategy to find balance between the lower energy offering and a lower energetic expenditure. In several studies it have been verified also a decrease in the mitochondrial activity below the expected by the oxygen offering (Bishop *et al*., 2002; Staples and Brown, 2008; Lewis and Driedzic, 2010; Strahl *et al*., 2011). In this way, it is expected that in this moment a decrease in the activity in the mitochondrial complexes in the respiratory chain may be occurring. However, the situation of the complex IV mitochondrial activity in the locomotor muscle of the crab *N. granulata* (Fig.10) have decreased only after 10 hours of hypoxia and the ATP content (Fig.11) decreased soon after 1h of hypoxia, associated to the intense glycolytic activity

of the muscle and the lack of an intense decrease in the locomotor activity indicate that at least in the first hours of hypoxia the locomotor muscle does not present metabolic depression. However, near the resistance time to severe hypoxia the decrease in the mitochondrial complex IV activity and the decrease in the accumulation of lactate in the muscle can indicate a beginning of metabolic depression. A similar situation of delay of the induction of the metabolic depression also has been proposed for other species of crustaceans (Cowan and Storey, 2001; Dawson and Storey, 2011; Martinez-Cruz *et al.*, 2012). As a strategy to reestablish during reoxygenation, it is expected that a quick recovery of the energetic levels of the locomotor muscle occurs. Actually, in *N. granulata* a rapid increase in the ATP content during reoxygenation occurred in the animals exposed to hypoxia for 1 to 4 hours which can be a strategy of organisms that live in regions of quick and abrupt variations of oxygen. However, in the reoxygenation after 10 hours of hypoxia the increase of the locomotor activity (Fig.5) was not so quick. The mitochondrial complex IV activity increased at the beginning but decreased again at the end (Fig.10) and the ATP remained low during the entire reoxygenation (Fig.11) which may be indicating problems in the activation of the aerobic metabolism after a long exposure to hypoxia. Thus, these differences in the reoxygenation seem to be associated to the induction of the metabolism depression. Possibly during long exposure to hypoxia the delay in the activation of the aerobic metabolism may be associated to the increase in the generation of reactive oxygen species (ROS) and nitroreactive (RNS) both in hypoxia and reoxygenation. After these molecules are formed, they have the capacity to damage macromolecules such as proteins, lipids of membrane and DNA (Storey, 1996; Halliwell and

Gutteridge, 1999; Hermes-Lima and Zenteno-Savin, 2002), so they can lead to oxidative stress (Sies, 1986; Jones, 2006). Besides that, in the mitochondria, ROS and RNS combined with high levels of Ca^{+} lead to an increase of channels sensible to Ca^{+}, decreasing the mitochondrial membrane potential and releasing the electron transport chain constituents such as cytochrome c to the cellular media resulting in the activation of the programmed cell death process (Halestrap, 2009; Halestrap and Pasdois, 2009).

It seems that the main strategy of *N. granulata* to face situations of hypoxia and reoxygenation common to its environment is the activation of the anaerobic metabolism in its locomotor muscles in the first hours, with no induction of metabolic depression, which would allow a fast recovery of its energetic levels during reoxygenation. The induction of the metabolic depression in the locomotor muscles during a long permanence in hypoxia, near the resistance limit of this animal, seems to lead to greater difficulty of recovery of the energetic levels during reoxygenation.

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Captions to figures

Fig. 1. Diagram showing the running arena. The crabs were maintained in the internal arena (0.07m^2) filled with water at room of 20ºC, salinity of 20. In the external arena the oxygen concentration were controlled by bubbling oxygen or nitrogen. Water flow was permitted between the arenas. A video camera was attached above both arenas focusing only the internal arena.

Fig. 2. Tolerance and resistance analysis in the crab *Neohelice granulata* under different oxygen concentrations (3.0, 2.5, 2.0, 1.0 and 0.5 mg $O_2.L^{-1}$). The CL_{50} as well as LT_{50} and LT_{10} were estimated. The experiment was made in triplicate being each replicate constitute of crabs (n=10) and each point represents the mean ± 1 s.e.m.

Fig. 3. Recovery analysis in the crab *Neohelice granulata* exposed to hypoxia (0.5 mg$O_2.L^{-1}$) during 1(A), 4(B) or 10h (C), subsequent 30 min, 2, 6 and 12h of reoxygenation and returned to hypoxia (0.5 mg$O_2.L^{-1}$) and the mortality was monitored during 24h. The experiment was made in triplicate being each replicate constitute of crabs (n=10) and each point represents the mean ± 1 s.e.m. Significant differences between the curves compared with the control curve were verified with α= 0.01 (**) and 0.001 (***).

Fig. 4. Oxygen consumption (MO_2) in the crab *Neohelice granulata* exposed to hypoxia (0.5 $mgO_2.L^{-1}$) during 1(A), 4(B) or 10 h (C) and after 0.5 and 2 hours of reoxygenation (black columns) (H/R) and maintained in control condition (6.5 $mgO_2.L^{-1}$) (white columns). Different letters represents significant differences ($p<0.05$) between the groups. Each point represents the mean ± 1 s.e.m. (n = 5).

Fig. 5. Locomotor activity in the crab *Neohelice granulata* exposed to hypoxia (0.5 $mgO_2.L^{-1}$) during 1(A), 4(B) or 10 h (C) and after 0.5 and 2 hours of reoxygenation (black columns) (H/R) and maintained in control condition (6.5 $mgO_2.L^{-1}$) (white columns). Different letters represents significant differences ($p<0.05$) between the groups. Each point represents the mean ± 1 s.e.m. (n = 9).

Fig. 6. Lactate content in the hemolymph of the crab *Neohelice granulata* exposed to hypoxia (0.5 $mgO_2.L^{-1}$) during 1(A), 4(B) or 10 h (C) and after 0.5 and 2 hours of reoxygenation (black columns) (H/R) and maintained in control condition (6.5 $mgO_2.L^{-1}$) (white columns). Different letters represents significant differences ($p<0.05$) between the groups. Each point represents the mean ± 1 s.e.m. (n = 5).

Fig. 7. Glucose content in the hemolymph of the crab *Neohelice granulata* exposed to hypoxia (0.5 $mgO_2.L^{-1}$) during 1(A), 4(B) or 10 h (C) and after 0.5 and 2 hours of reoxygenation (black columns) (H/R) and maintained in control

condition (6.5 $mgO_2.L^{-1}$) (white columns). Different letters represents significant differences ($p<0.05$) between the groups. Each point represents the mean ± 1 s.e.m. (n = 5).

Fig. 8. Lactate content in the locomotor muscle of the crab *Neohelice granulata* exposed to hypoxia (0.5 $mgO_2.L^{-1}$) during 1(A), 4(B) or 10 h (C) and after 0.5 and 2 hours of reoxygenation (black columns) (H/R) and maintained in control condition (6.5 $mgO_2.L^{-1}$) (white columns). Different letters represents significant differences ($p<0.05$) between the groups. Each point represents the mean ± 1 s.e.m. (n = 5).

Fig. 9. Glucose content in the locomotor muscle of the crab *Neohelice granulata* exposed to hypoxia (0.5 $mgO_2.L^{-1}$) during 1(A), 4(B) or 10 h (C) and after 0.5 and 2 hours of reoxygenation (black columns) (H/R) and maintained in control condition (6.5 $mgO_2.L^{-1}$) (white columns). Different letters represents significant differences ($p<0.05$) between the groups. Each point represents the mean ± 1 s.e.m. (n = 5).

Fig. 10. Mitochondrial IV complex activity in the locomotor muscle of the crab *Neohelice granulata* exposed to hypoxia (0.5 $mgO_2.L^{-1}$) during 1(A), 4(B) or 10 h (C) and after 0.5 and 2 hours of reoxygenation (black columns) (H/R) and maintained in control condition (6.5 $mgO_2.L^{-1}$) (white columns). Different letters represents significant differences ($p<0.05$) between the groups. Each point represents the mean ± 1 s.e.m. (n = 5).

Fig.11. ATP content in the locomotor muscle of the crab *Neohelice granulata* exposed to hypoxia (0.5 $mgO_2.L^{-1}$) during 1(A), 4(B) or 10 h (C) and after 0.5 and 2 hours of reoxygenation (black columns) (H/R) and maintained in control condition (6.5 $mgO_2.L^{-1}$) (white columns). Different letters represents significant differences ($p<0.05$) between the groups. Each point represents the mean ± 1 s.e.m. (n = 5).

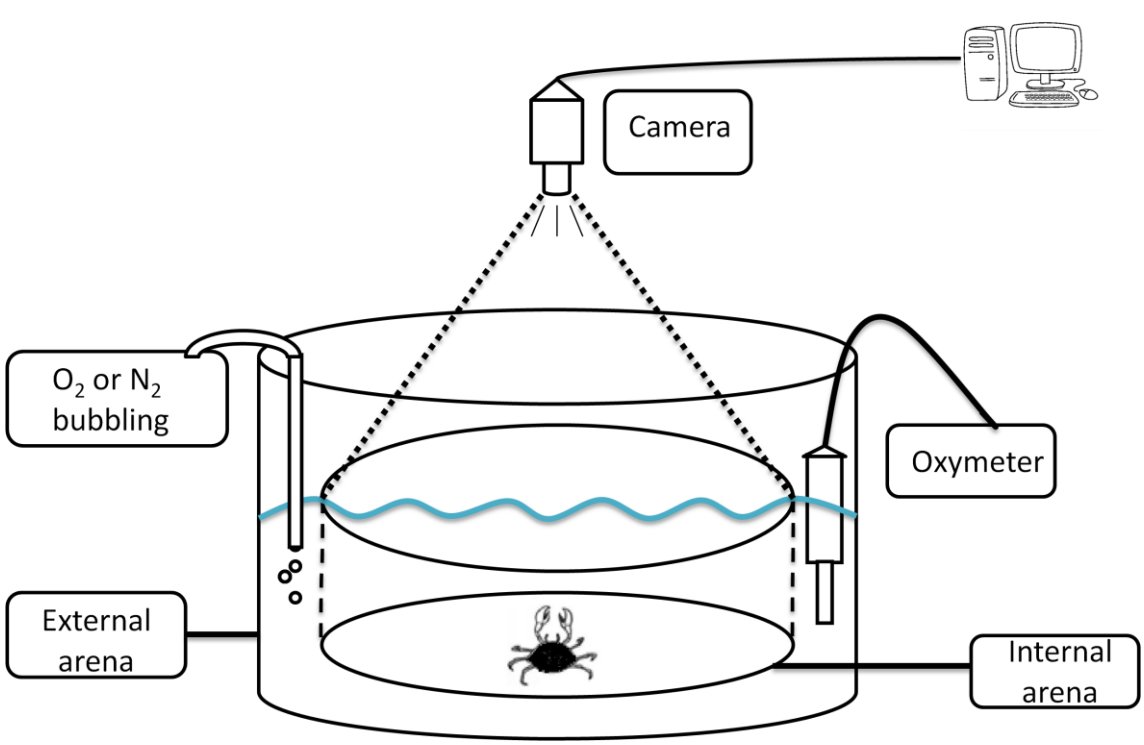
Camera
O_2 or N_2 bubbling
Oxymeter
External arena
Internal arena

Fig. 1

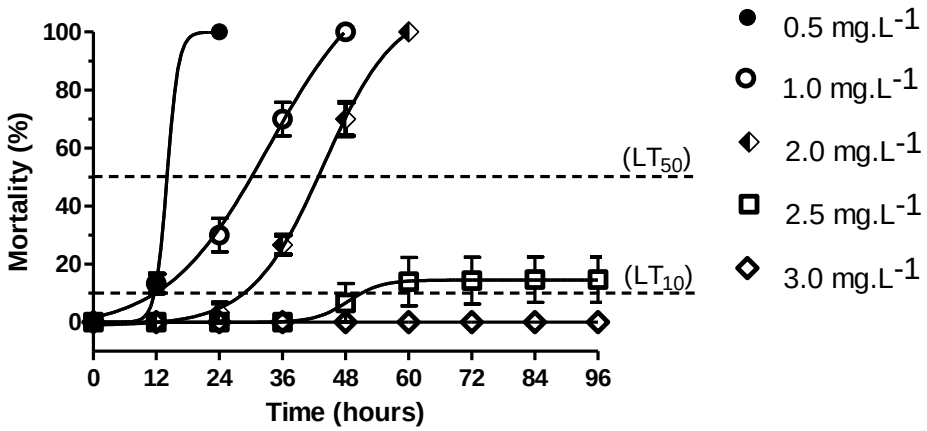
100
80
60
40
20
0
Mortality (%)
0
12
24
36
48
60
72
84
96
Time (hours)
(LT50)
(LT10)
0.5 mg.L-1
1.0 mg.L-1
2.0 mg.L-1
2.5 mg.L-1
3.0 mg.L-1

Fig. 2.

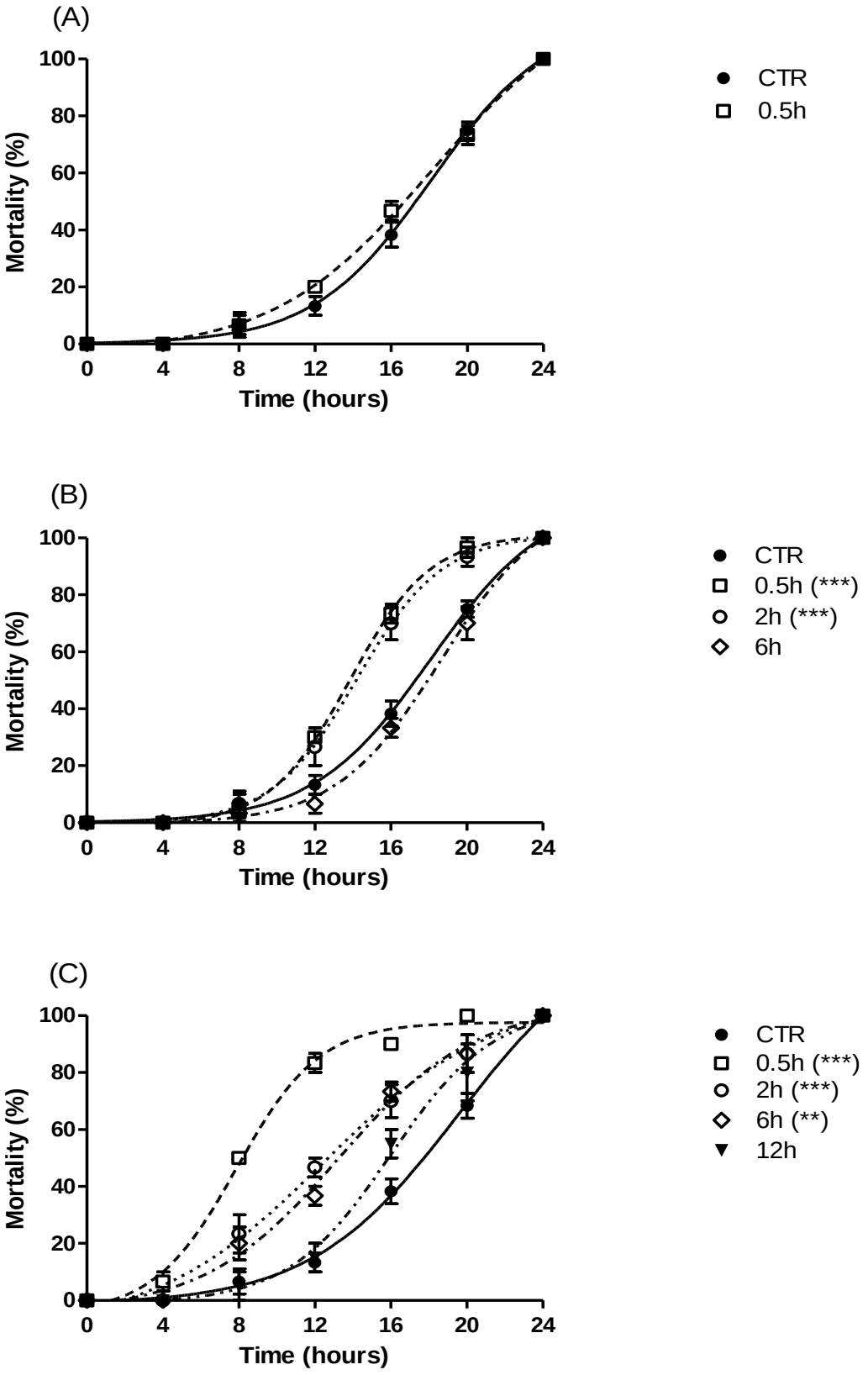
(A)
Mortality (%)
Time (hours)
CTR
0.5h
(B)
Mortality (%)
Time (hours)
CTR
0.5h (***)
2h (***)
6h
(C)
Mortality (%)
Time (hours)
CTR
0.5h (***)
2h (***)
6h (**)
12h

Fig. 3

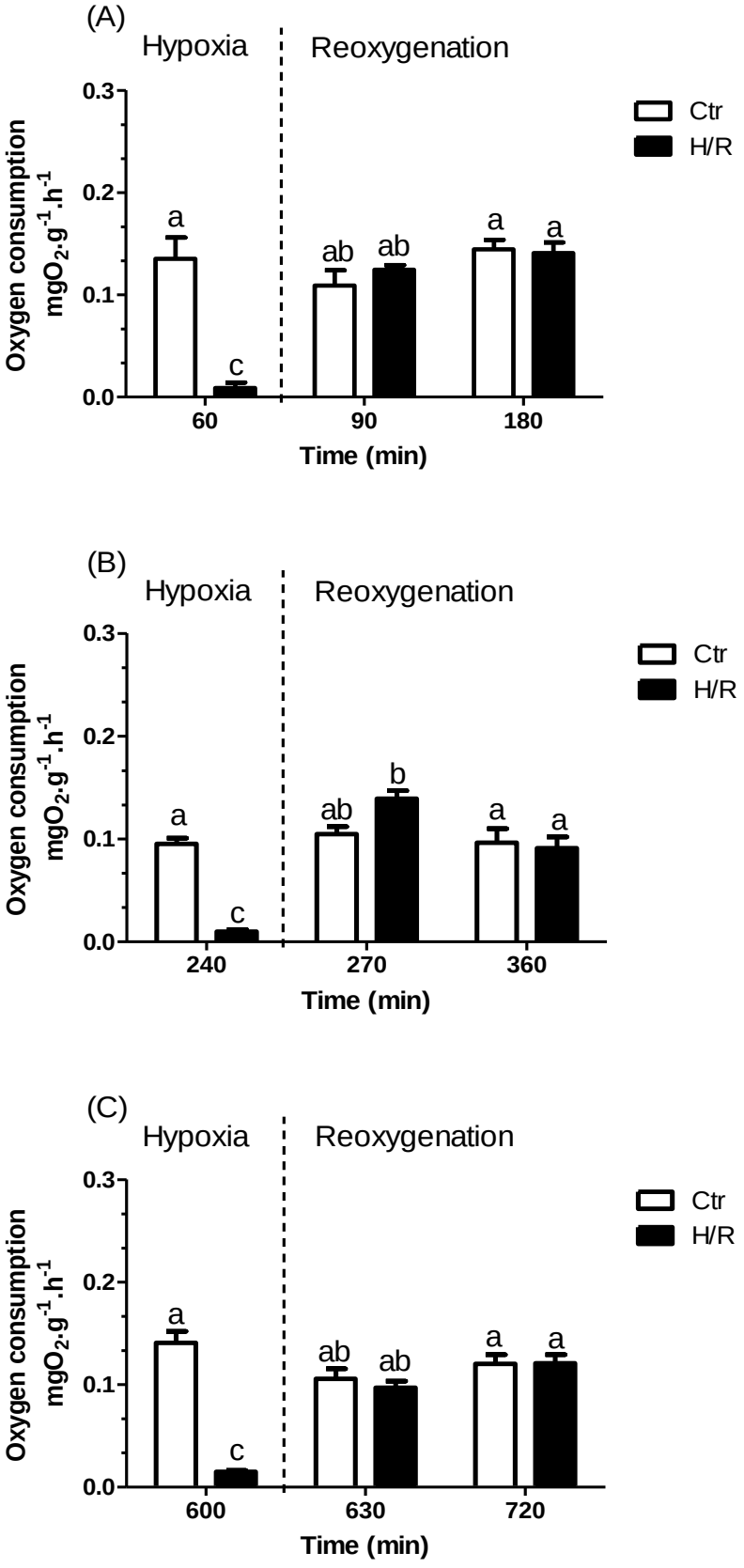
(A)
Hypoxia
Reoxygenation
Ctr
H/R
Oxygen consumption
mgO2.g-1.h-1
0.3
0.2
0.1
0.0
a
c
ab
ab
a
a
60
90
180
Time (min)
(B)
Hypoxia
Reoxygenation
Ctr
H/R
Oxygen consumption
mgO2.g-1.h-1
0.3
0.2
0.1
0.0
a
c
ab
b
a
a
240
270
360
Time (min)
(C)
Hypoxia
Reoxygenation
Ctr
H/R
Oxygen consumption
mgO2.g-1.h-1
0.3
0.2
0.1
0.0
a
c
ab
ab
a
a
600
630
720
Time (min)

Fig. 4.

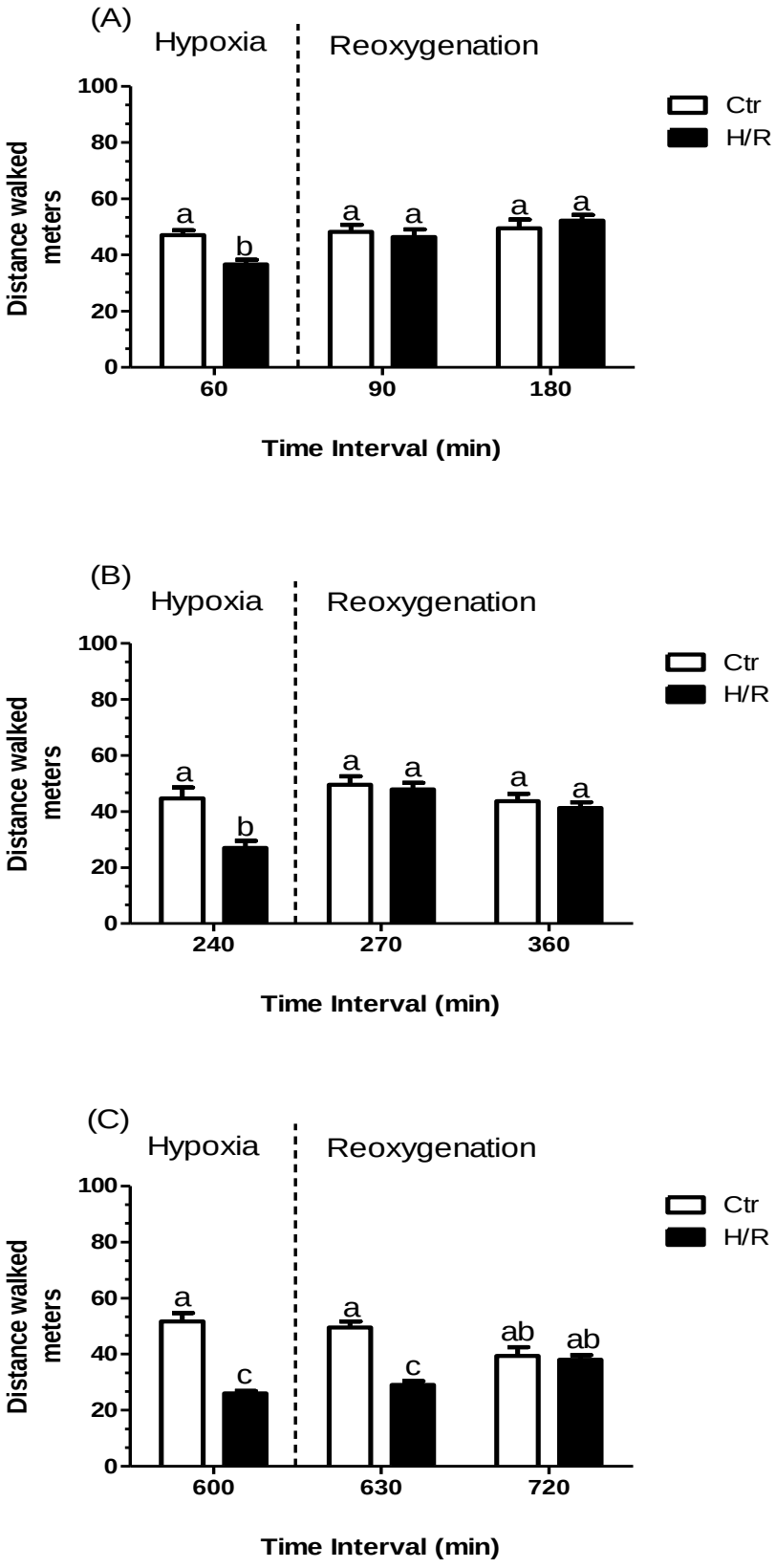
(A)
Hypoxia
Reoxygenation
Distance walked meters
100
80
60
40
20
0
a
b
a
a
a
a
Ctr
H/R
60
90
180
Time Interval (min)
(B)
Hypoxia
Reoxygenation
Distance walked meters
100
80
60
40
20
0
a
b
a
a
a
a
Ctr
H/R
240
270
360
Time Interval (min)
(C)
Hypoxia
Reoxygenation
Distance walked meters
100
80
60
40
20
0
a
c
a
c
ab
ab
Ctr
H/R
600
630
720
Time Interval (min)

Fig. 5.

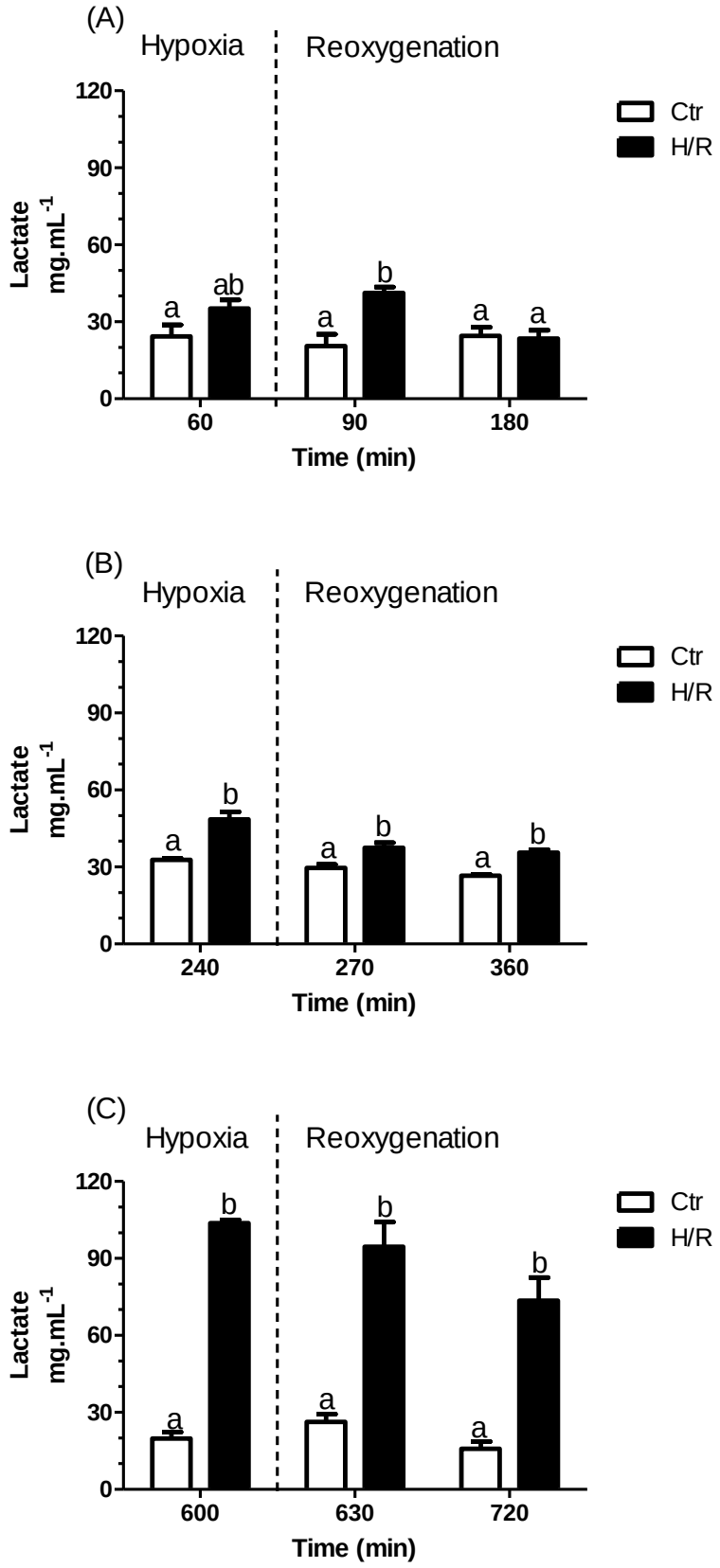
(A)
Hypoxia
Reoxygenation
Ctr
H/R
Lactate mg.mL-1
120
90
60
30
0
a
ab
a
b
a
a
60
90
180
Time (min)
(B)
Hypoxia
Reoxygenation
Ctr
H/R
Lactate mg.mL-1
120
90
60
30
0
a
b
a
b
a
b
240
270
360
Time (min)
(C)
Hypoxia
Reoxygenation
Ctr
H/R
Lactate mg.mL-1
120
90
60
30
0
a
b
a
b
a
b
600
630
720
Time (min)

Fig. 6.

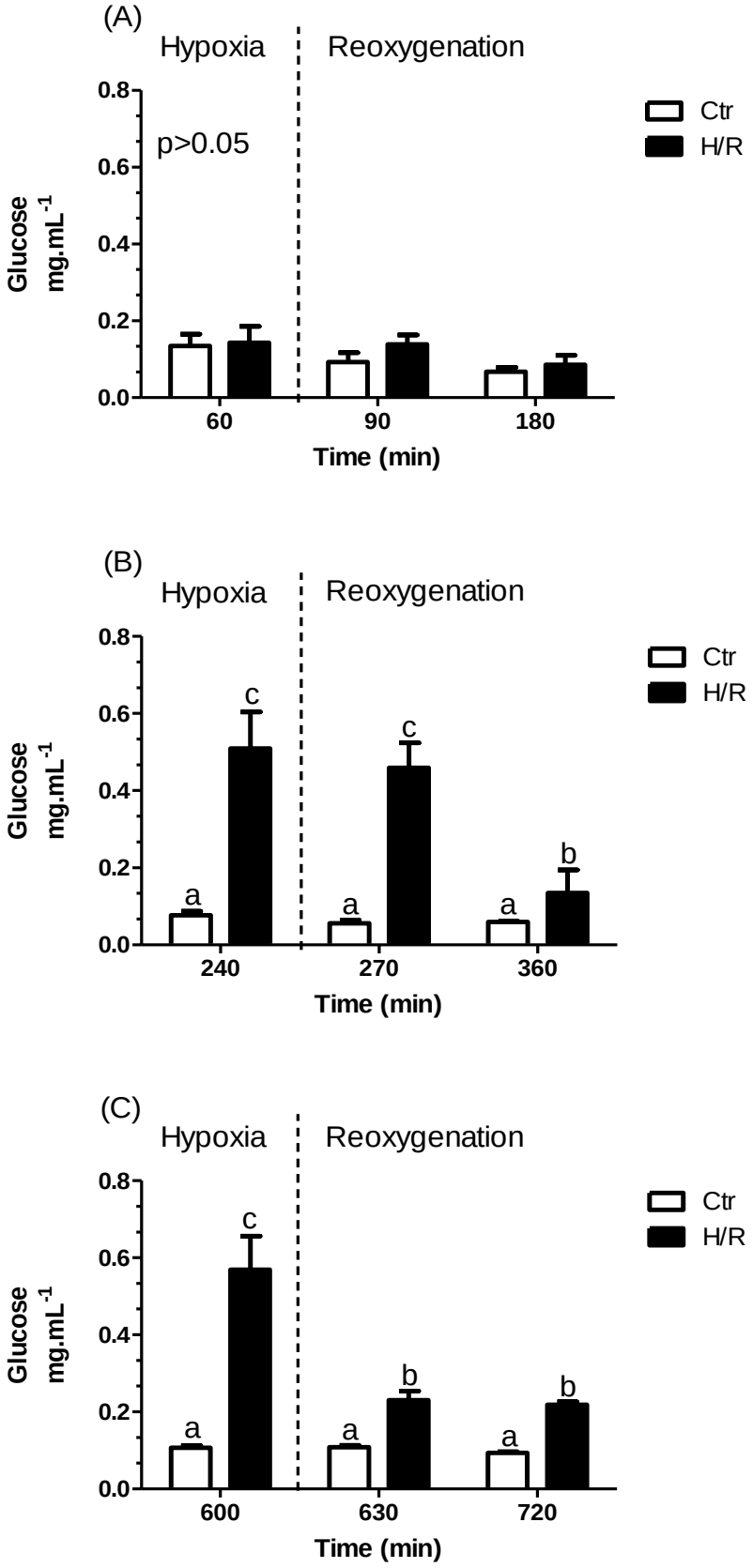
(A)
Hypoxia
Reoxygenation
p>0.05
Ctr
H/R
Glucose mg.mL-1
0.0
0.2
0.4
0.6
0.8
60
90
180
Time (min)
(B)
Hypoxia
Reoxygenation
Ctr
H/R
Glucose mg.mL-1
a
c
a
c
a
b
240
270
360
Time (min)
(C)
Hypoxia
Reoxygenation
Ctr
H/R
Glucose mg.mL-1
a
c
a
b
a
b
600
630
720
Time (min)

Fig. 7.

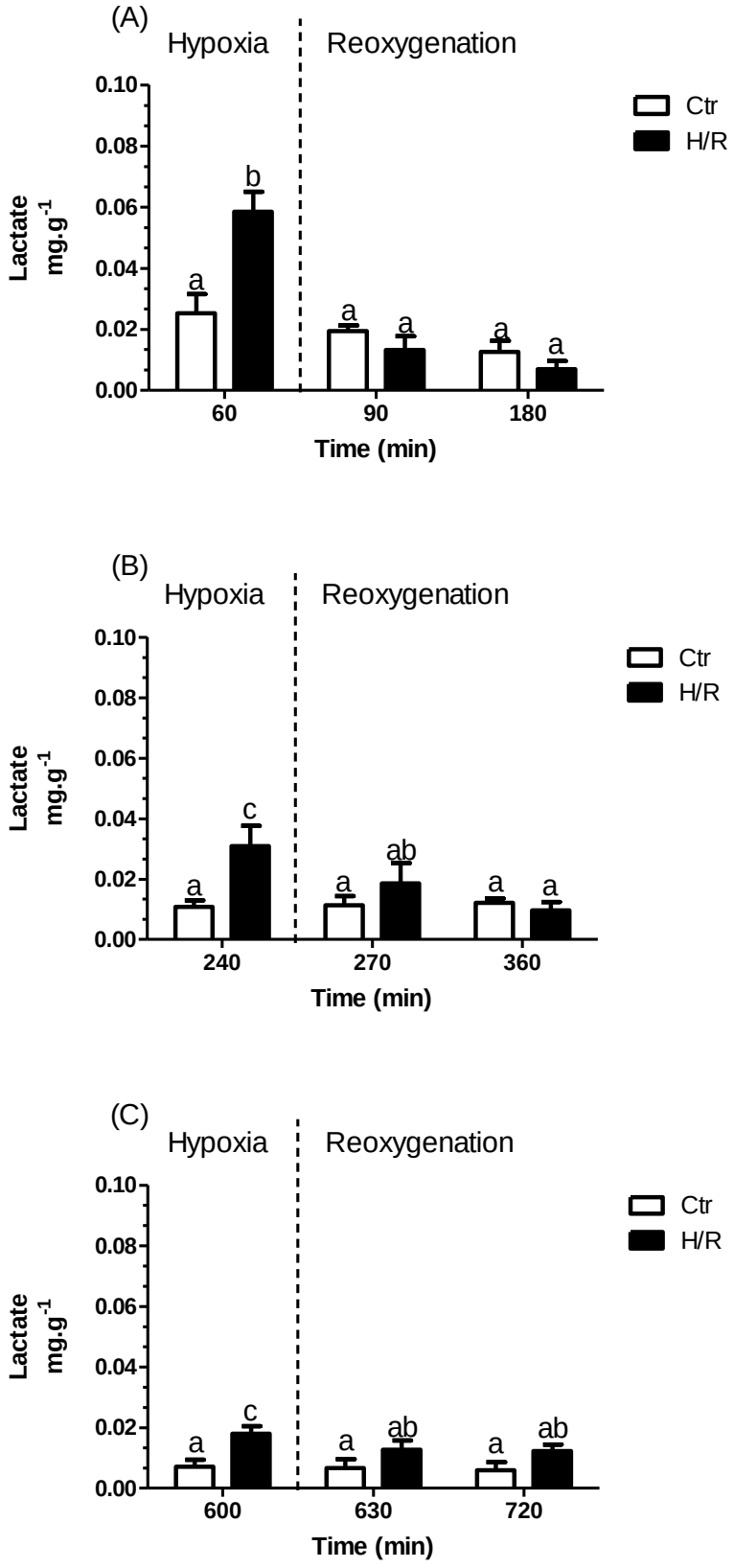
(A)
Hypoxia
Reoxygenation
Ctr
H/R
Lactate mg.g-1
0.10
0.08
0.06
0.04
0.02
0.00
a
b
a
a
a
a
60
90
180
Time (min)
(B)
Hypoxia
Reoxygenation
Ctr
H/R
Lactate mg.g-1
0.10
0.08
0.06
0.04
0.02
0.00
a
c
a
ab
a
a
240
270
360
Time (min)
(C)
Hypoxia
Reoxygenation
Ctr
H/R
Lactate mg.g-1
0.10
0.08
0.06
0.04
0.02
0.00
a
c
a
ab
a
ab
600
630
720
Time (min)

Fig. 8.

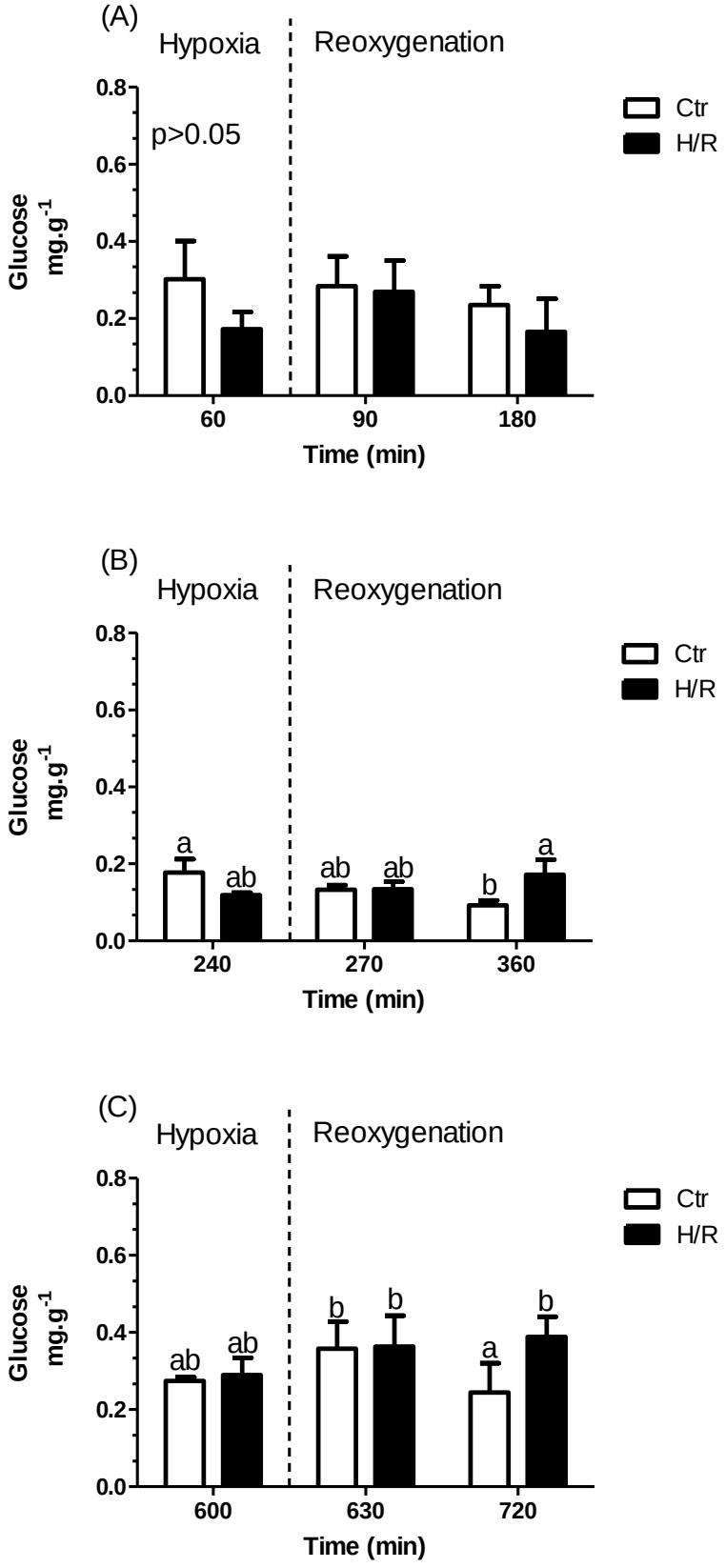
(A)
Hypoxia
Reoxygenation
p>0.05
Ctr
H/R
Glucose mg.g-1
0.0
0.2
0.4
0.6
0.8
60
90
180
Time (min)
(B)
Hypoxia
Reoxygenation
Ctr
H/R
Glucose mg.g-1
a
ab
ab
ab
b
a
240
270
360
Time (min)
(C)
Hypoxia
Reoxygenation
Ctr
H/R
Glucose mg.g-1
ab
ab
b
b
a
b
600
630
720
Time (min)

Fig. 9.

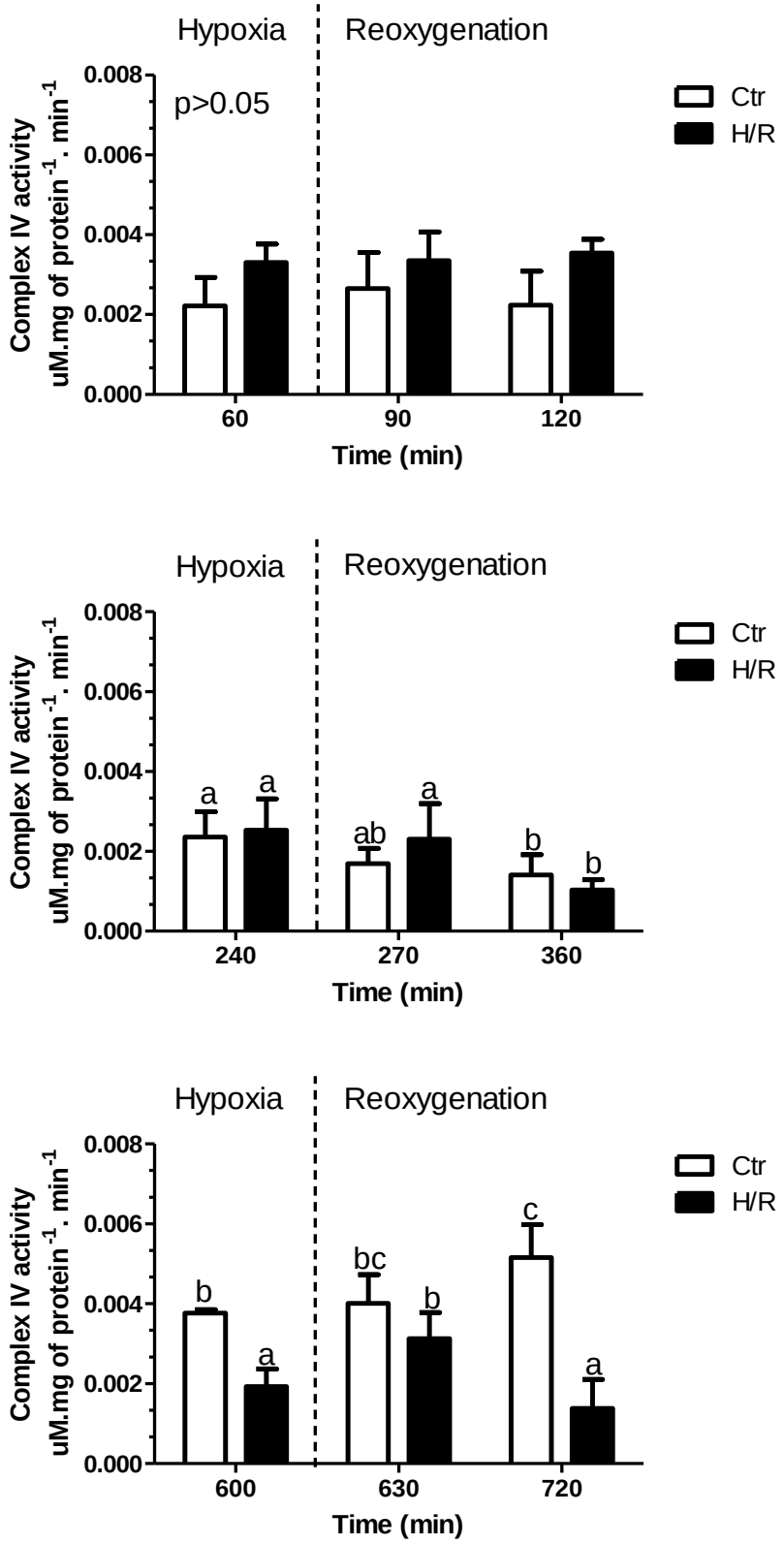
Hypoxia
Reoxygenation
p>0.05
Ctr
H/R
Complex IV activity
uM.mg of protein -1 . min-1
0.008
0.006
0.004
0.002
0.000
60
90
120
Time (min)
Hypoxia
Reoxygenation
Ctr
H/R
Complex IV activity
uM.mg of protein -1 . min-1
a
a
ab
a
b
b
240
270
360
Time (min)
Hypoxia
Reoxygenation
Ctr
H/R
Complex IV activity
uM.mg of protein -1 . min-1
b
a
bc
b
c
a
600
630
720
Time (min)

Fig. 10.

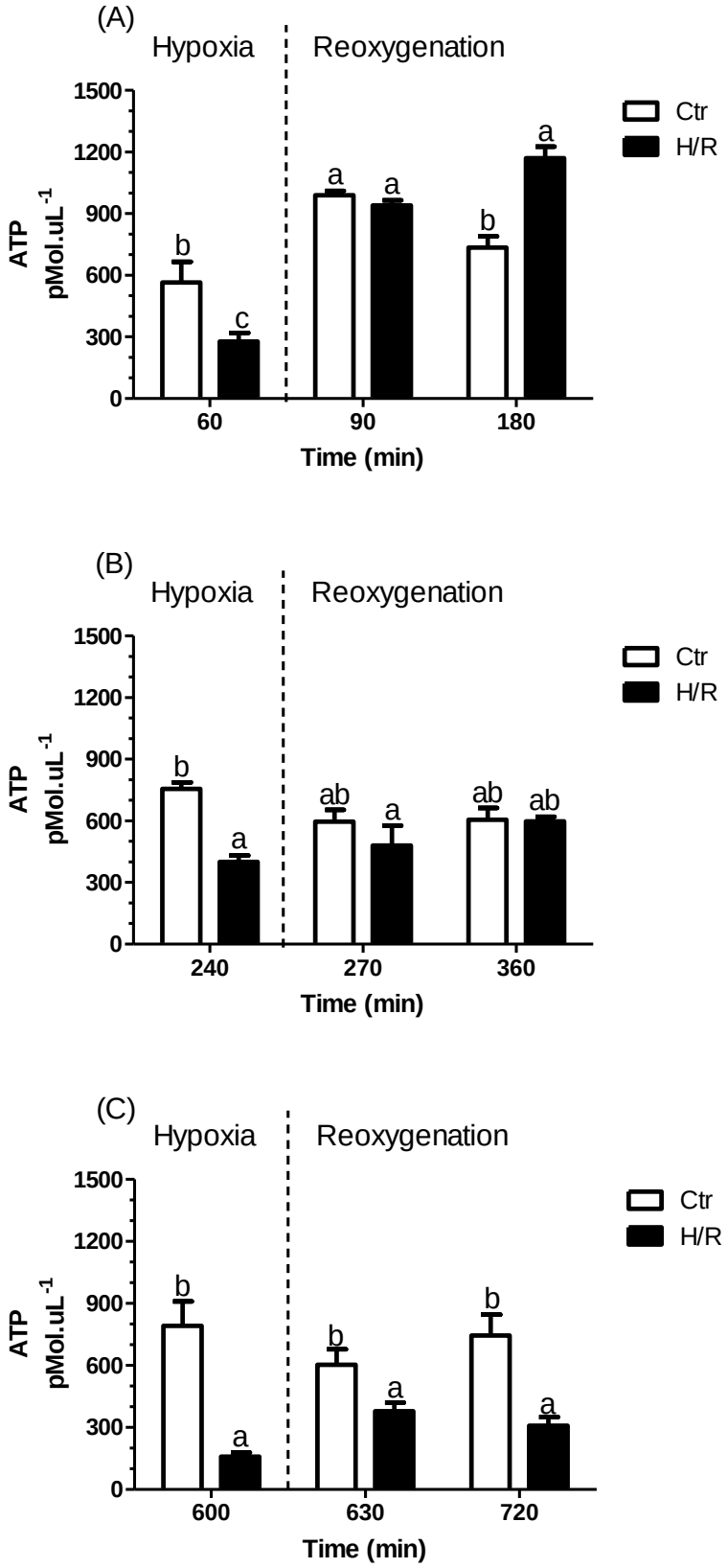
(A)
Hypoxia
Reoxygenation
ATP
pMol.uL-1
1500
1200
900
600
300
0
b
c
a
a
b
a
60
90
180
Time (min)
Ctr
H/R
(B)
Hypoxia
Reoxygenation
ATP
pMol.uL-1
1500
1200
900
600
300
0
b
a
ab
a
ab
ab
240
270
360
Time (min)
Ctr
H/R
(C)
Hypoxia
Reoxygenation
ATP
pMol.uL-1
1500
1200
900
600
300
0
b
a
b
a
b
a
600
630
720
Time (min)
Ctr
H/R

Fig. 11.

IX. 2º ARTIGO

Danos causados durante hipoxia e reoxigenação no músculo locomotor do caranguejo *Neohelice granulata* (Decapoda Varunidae)

Artigo submetido ao periódico: Free Radical Biology & Medicine.

DAMAGES CAUSED DURING HYPOXIA AND REOXYGENATION IN THE LOCOMOTOR MUSCLE OF THE CRAB *NEOHELICE GRANULATA* (DECAPODA VARUNIDAE)

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Abstract

In crustaceans few studies that verified the effects of hypoxia and reoxygenation hinder understanding if this group is susceptible to damage from the process of hypoxia and reoxygenation. For this reason, the objective of this study was to determine whether different periods of severe hypoxia (0.5 $mgO_2.L^{-1}$) followed by reoxygenation leads to damage in the locomotor muscle of the crab *Neohelice granulata.* We evaluated the levels of reactive oxygen species (ROS), lipid peroxidation (LPO), mitochondrial membrane potential and the aerobic area of muscle fibers in the end of different periods of hypoxia (1, 4 and 10h) and subsequent 30 and 120 min after the begin of reoxygenation. Changes in cell volume, mitochondrial dysfunctions and infiltration hemocytes processes were evaluated in the end of different periods of hypoxia and 24 and 48h of reoxygenation. Increasing the time of hypoxia exposure a decrease in the mitochondrial membrane potential together with a decrease in the area with aerobic fibers was observed. During reoxygenation, the increase in ROS and LPO levels and the decrease in the mitochondrial membrane potential were quickly repaired in crabs maintained in 1 and 4h of hypoxia. On the other hand, in the reoxygenation after 10h of hypoxia the mitochondrial alterations provoked morphological changes in the region of oxidative fibers on the locomotor muscle requiring much more time to recovery. Therefore, *N. granulata* suffers damage when faced with hypoxia and reoxygenation and the ability to repair is proportional to the time of exposure to hypoxia. The damages are localized in areas with predominance of aerobic cells.

Keywords: crab, hypoxia, reoxygenation, muscle, mitochondria, lipid peroxidation, reactive oxygen species.

Introduction

Numerous factors can cause variations in the oxygen offer in various organs and tissues, submitting them to situations of hypoxia and hyperoxia. Much of the knowledge about the alterations provoked by variations in oxygen tension on tissues comes from the studies of the alterations resulted from ischemia and reperfusion in mammals tissues (Robin e Theodore, 1982; McCord,1985; Levinson *et al*.,1986). Increased generation of reactive oxygen species (ROS) formed during ischemia and reperfusion, is considered the main responsible for much of the damages associated with these processes (Li and Jackson, 2002). For a long time it was believed that only during the reperfusion period, by increasing the oxygen supply to tissues and organs, molecular damages such as lipid peroxidation, oxidation of proteins and DNA strand break occurring as a result of increased ROS levels (Pike *et al*., 1993; Fuller *et al*., 2003; Halliwell and Gutteridge, 2001). However, recently, it has been observed that hypoxia per se can conduct to a raise in ROS levels (Clanton *et al*., 2007).

During the variation in oxygen levels for all tissues, mitochondrial activity plays a central role. This organelle is responsible for almost all energy produced by the cells. However, even though the center of ATP production ROS can be formed in such situations (Storey, 1996; Hermes-Lima and Zenteno-Savin, 2002), leading to mitochondrial damages (Boveris et al, 1976; Turrens, 2003). It happens due to a combination of high levels of intracellular calcium and ROS, which induces an increase in mitochondrial membrane permeability, which may lead to loss in the mitochondrial membrane potential (Halestrap 2009; Pasdois and Halestrap 2009). In mammals, this increased mitochondrial membrane permeability can result in loss of constituents of electron transport chain such as

cytochrome c, resulting in a decrease of ATP production and the activity of ion pumps such as Na^+/K^+ ATPase, which could cause a dysfunction of cellular volume (Lambert *et al*., 2,008) or even cell death (Powers *et al*, 2007; Hüttemann *et al*., 2011). Furthermore, the process of ischemia and reperfusion can similarly result in a generation of several morphological changes on the muscle tissues (Clanton *et al*., 2007; Andrianjafiniony *et al*., 2010). The increase in ROS levels is associated to many inflammatory processes (Shoffner, 2000), muscular atrophy (Andrianjafiniony *et al*., 2010) or dysfunction in osmotic cell volume (Lambert *et al*., 2008) leading to irreversible damages impairing the muscle function.

In mammals, cellular and tissue alterations caused by variations in oxygen concentration are well known. However, in other groups of animals mainly invertebrates few studies have been conducted. In the aquatic environment, variations in oxygen concentration occur more frequently when compared to terrestrial environment. Among the different aquatic environments, coastal regions are considered one of the most stressful areas concerning oxygen variation (Smith and Able, 2003). In these areas, recurring situations of hypoxia and reoxygenation require animals to adopt several strategies in order to preserve it (Lesser *et al*., 2005). In crustaceans, one of the most representative groups of the aquatic environment, the information about the molecular, cellular and morphological damages related to oxygen supply are not well known. Generally, crustaceans, when subjected to hypoxia, have a LC_{50} (lethal concentration that kills 50% of animals) higher compared to other aquatic animals such as fishes and mollusks, being considered less tolerant to hypoxia (Vaquer Sunier and Duarte, 2008). However, several species of crustaceans

live in regions with frequently variations in oxygen tension, having to avoid, tolerate or recover from the damages caused by increased ROS levels. For our knowledge, only two works have analyzed the occurrence of damages in crustaceans submitted to hypoxia and reoxygenation, on the shrimp *Litopennaeus vannamei* (Zenteno-Savín *et al*., 2006) and in the crab *Neohelice granulata* (de Oliveira *et al*., 2005). The shrimp *Litopennaeus vannamei* shows considerable tolerance to hypoxia (Seidman and Lawrence, 1985), and when exposed to severe hypoxia (1.0 $mgO_2.L^{-1}$) presented no mortality after 16 days. When exposed to severe hypoxia for 24 h followed by reoxygenation for up to 6h, the shrimp showed no lipoperoxidation in muscle, gills and hepatopancreas (Zenteno-Savín *et al*., 2006). On the other hand, the crab *N. granulata* (previously named *Chasmagnathus granulata/ granulatus)* showed tolerance to hypoxia (Geihs *et al*., 2013) similarly to other crustaceans, with a tolerance (LC_{50}) between 2.0 - 2.5$mgO_2.L^{-1}$. When exposed to anoxia and severe hypoxia (0.5 $mgO_2.L^{-1}$) the beginning of mortality was observed after 10 and 12h of exposure, respectively (Maciel, 2010; Geihs *et al*., 2013). When this crab was exposed for at least 8h under anoxic conditions followed by reoxygenation, an increase in lipid peroxidation was observed in gills during reoxygenation without any signs of recovery (de Oliveira *et al*., 2005). Both preliminary studies suggest the existence of an inverse correlation between tolerance to hypoxia and generated damages. However, it should be noted that hypoxia periods analyzed were not proportionally to the resistance time for these two species as well as the time of reoxygenation. Finally, besides the lipid peroxidation many other damages such as; mitochondrial dysfunctions and morphological changes may also be occurring in these species when subjected to hypoxia and

reoxygenation. In fact, when *N. granulata* was exposed for at least 10 h under severe hypoxia (0.5 $mgO_2.L^{-1}$) followed by reoxygenation, the activity of mitochondrial complex IV in locomotor muscle showed significant changes (Geihs *et al*., 2013). For this reason, the aim of this work is to verify whether molecular, mitochondrial and morphological damages are occurring in the locomotor muscle of the crab *N. granulata* submitted to different periods of hypoxia and reoxygenation.

Material and methods

Animals maintenance

Adult male crabs of *Neohelice granulata* weighing 11.2 ± 0.3 g (mean ± S.E.M) were collected in salt marshes around Rio Grande City - Brazil and taken to the laboratory. The crabs were acclimated, at least during 10 days, in tanks under constant conditions of temperature (20°C), salinity (20‰) and photoperiod (12L:12D). The animals were fed *ad libitum* with ground beef three times a week.

Reagents

H_2DCF-DA (diacetate of 2´,7´ dichlorofluorescein), CHP (Cumene hidroperoxide), TTC (Chloride of 2,3,5-triphenyltetrazolium) JC-1 (5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide), xylenol orange and paraplast X-TRA were purchased from Sigma-Aldrich (St Louis, MO, USA). Total protein kit were purchased from Doles (Goiania, GO, Brazil)

Experimental procedure

The crabs were individually placed in cylindrical glass chambers with base and height of 5x14cm and a volume of approximately 300ml. Nitrogen gas (100%) were bubbled until the oxygen concentration reached 0.5 $mgO_2.L^{-1}$ at

constant salinity and temperatures (20‰ and 20ºC). The oxygen concentration was continuously monitored during the experiment with a portable oximeter (DO-5519, Lutron Electronic Enterprise CO). All analysis were performed after 1, 4 and 10h of hypoxia and subsequent 30 and 120 min of reoxygenation for biochemical and 2, 24 and 48h for morphological determinations. The control group was maintained along the whole experiment in water of 6.5 $mgO_2.L^{-1}$ and was analyzed simultaneously with the experimental group.

ROS Quantification

Muscles (n=5) of the second pair of pereiopods were removed, weighted and homogenized (1:10 *w/v*) in a cold (4°C) buffer solution containing sucrose (250mM), phenylmethylsulfonyl fluoride (PMSF) (1mM), and EDTA (5mM), with pH adjusted to 7.6. The samples were centrifuged (2,000xg, 4°C for 20 min) and the supernatant were collected and centrifuged (10,000xg, 4°C for 45 min). The last supernatant was employed for reactive oxygen species (ROS) quantification (Viarengo *et al*., 1999). For ROS detection, it was used the fluorophore 2',7'-dichlrofluorescein-diacetate (H_2DCF-DA, Molecular Probes) that in the presence of ROS generates a fluorochrome that was detected using wavelengths of 488 and 525 nm for excitation and emission, respectively. The analysis were carried out in a fluorescence microplate reader (Victor 2, Perkin-Elmer, Waltham, MA, USA) with readings every 5 min for 1h. Total fluorescence area was calculated by integrating the fluorescence units (FU) along the measurement time, after adjusting FU data to a second order polynomial function. ROS concentration was referred to the total protein content present in the biological sample using a commercial kit (Doles - Brazil) based on biuret

reagent spectrophotometricaly at 550nm and the results were expressed in FU. mg of protein^{-1}.

Lipid peroxidation (LPO)

Muscles (n=5) of the second pair of pereiopods were weighted and homogenized (1:9 *w/v*) in cold methanol (4°C). The samples were centrifuged (1,000xg, 4°C for 10 min) and the supernatant was used for analysis. The protocol used was the modified FOX assay (Hermes-Lima *et al*., 1995; Monserrat *et al*., 2003), based on the oxidation of Fe (II) under acidic conditions and quantify lipid hydroperoxides, one of the main products of lipoperoxidation. For LPO measurements, $FeSO_4$ (1mM), H_2SO_4 (0.25M), xylenol orange (1mM) and MilliQ water were sequentially added. Samples (30µL) were added and incubated for 375 minutes. Thereafter, the absorbance at 550 nm was determined using a microplate reader (Victor 2, Perkin- Elmer, Waltham, MA, USA). Finally, cumene hydroperoxide (CHP) was employed as a standard. Lipid peroxidation (LPO) was express in nmoles of CHP.g^{-1} of wet tissue.

Mitochondrial membrane potential

Pereiopods muscles (n=5) were removed, chopped and immediately rinsed in tubes in ice cold (4ºC) medium containing sucrose (510mM), EDTA (1mM), EGTA (200µM), HEPES (20mM) and BSA (0.5%) at pH 7.5. Muscles were immediately homogenized and centrifuged (2,000xg, 4ºC during 15 min). The supernatant was removed and centrifuged (8,000xg, 4ºC during 15 min). The last supernatant was removed and the resulting pellet was resuspended in ice cold medium containing sucrose (303mM), EGTA (1mM), KH_2PO_4 (4mM), KCl (90mM) and BSA (0.5%) at pH 7.5. The total protein content was determined in muscle samples using a commercial kit (Doles - Brazil) based on

biuret reagent spectrophotometricaly at 550nm. The mitochondrial membrane potential was assessed using the cationic carbocyanine dye JC-1 according to Reers *et al*., (1995). This probe exists as a green fluorescent monomer (excitation: 485 nm; emission: 530 nm) at low concentrations (less than 300 nM). However, at high concentrations (>1mM) a very strong red-orange fluorescence occurs (excitation: 485 nm; emission: 530 nm) due to the formation of dye aggregates. Therefore, low membrane potentials will show green fluorescence while high ones will present a red-orange fluorescence, since more of the dye enters the mitochondria as is accumulated in the matrix, forming the aggregates (Reers *et al*., 1995). JC1 was prepared in a cold medium (4ºC) containing KCl (110mM), $MgCl_2$ (10mM), EDTA (1mM), HEPES (20mM), succinate (10mM) and ATP (10mM) at pH 7.5. Measurements were performed in aliquots of isolated mitochondria and JC-1 solution pipetted into wells of a 96 well microplate. The JC-1 solution was prepared from a stock solution (40 µg/L in ethanol) by 200-fold dilution in a buffer solution containing KCl (110mM), MgCl (10mM),EGTA (1 mM), HEPES (20 mM), sodium succinate (10 mM) and ATP (10 mM). After incubation (30°C) for 30 min in the dark, fluorescence generated in the reaction mixture was read (excitation: 485 nm; emission: 590 nm) using a microplate reader (Victor 2, Perkin-Elmer, Waltham, MA, USA). The results were expressed as the difference of fluorescence. mg of protein^{-1}.

Morphological analysis

The pereiopods muscles (n=3) were removed and instantly frozen in liquid nitrogen. Subsequently, the extensor and flexor muscles from the meropodites were carefully dissected from the carapace in order to conserve the structural

integrity. Muscles were dehydrated in crescent graded ethanol series, diaphanized in xylol and embedded in paraplast X-TRA. After this stage, serial cuts (8µm) were performed using a rotatory microtome (Leyka RM2255). The sections were stained with hematoxylin and eosin (HE) and Gomory thricrome. The morphological observations (muscle cell diameter, mitochondria alterations and hemocytes infiltration) were carried out using an Olympus BX51 microscope connected to a CCD camera (Olympus DP72), and provided with the software Image J.

Histochemistry analysis

The histochemistry analysis was performed according to modified procedure of Benedek *et al*., (2006). After the experimental procedure, the extensor and flexor muscles of the second pair of pereiopods (n=5) were carefully removed from the meropodites. Immediately the muscles were immersed in 2% of TTC (2,3,5-triphenyltetrazolium Chloride) solution for 20 min at 25ºC. TTC is enzymatically reduced to red formazan, a product of dehydrogenases, which are most abundant in viable mitochondria. Stain intensity correlates with the number and functional activity of mitochondria. The complete lack of TTC staining was defined as anaerobic area and the stained region was defined as aerobic area. The observations was made using an Olympus SZX16 stereo microscope connected to a CCD camera (Olympus DP72), and provided with commercial software for image acquisition using Image J. The aerobic area was calculated as a percentage of total muscle area and the results are expressed by means ± 1 s.e.m.

Statistical analysis

Statistical analysis were performed by analysis of variance (Two Way ANOVA) between the groups control, hypoxia and reoxygenation followed by Newman-Keuls test with $\alpha=0.05$. Normality and variance homogeneity were verified as ANOVA assumptions and each point represents the mean ± 1 s.e.m. Mathematical transformations were performed when necessary (Zar, 1984).

Results

In the locomotor muscles of *N. granulata* no significant differences ($p>0.05$) has been observed in ROS concentration after 1h of hypoxia exposure and subsequent reoxygenation (Fig.1). After 4 and 10h under hypoxic conditions, a significant increase ($p<0.05$) in ROS concentration has been observed during the first 30min of reoxygenation, returning to normal levels ($p>0.05$) after 2h.

No significant variations ($p>0.05$) were observed in the LPO levels after 1, 4 and 10h of hypoxia. During reoxygenation, after 4 and 10h under hypoxic conditions a significant increase ($p<0.05$) in the LPO levels was observed after 30 min (Fig. 2) returning to normal levels ($p>0.05$) after 2h.

After 4 and 10h under hypoxic conditions a significant decrease ($p<0.05$) in the mitochondrial membrane potential was observed in the locomotor muscle (Fig. 3). The mitochondrial membrane potential decreased ($p<0.05$) in the first 30min of reoxygenation returning to normal potential after 2h after exposure to 1 and 4 h of hypoxic conditions. On the other hand, the mitochondrial membrane potential remained lower ($p<0.05$) during all reoxygenation time after 10h in hypoxic conditions.

The meropodites is constituted by two main structures, the extensor and flexor muscles (Fig. 4A). Both of these muscles show morphologically a simple

pinnate structure and fibers with different lengths and diameters, inserted in a rigid structure (apodeme) which roams the meropodites lengths surrounded by connective tissue (Figs. 4B and C). Longitudinal sections made to characterize the locomotor muscle identified regions with cells of different lengths along the structure of extensor and flexor muscles, being classified as proximal, mid-region and distal. The proximal and distal regions comprise, each one, around 20% and the mid-region comprises about 60% of the muscle total area (Fig. 4D). The morphometric analysis along these three regions presented fibers with different diameters. The proximal and distal regions show a predominance of fibers with around 60µm. The mid-region shows a predominance of fibers with about 80µm. (Fig. 5)

The histochemistry analysis in the locomotor muscle shows regions with fibers of different metabolism. The proximal region of both muscles was the only region stained with TCC, being classified as areas with prevalence of aerobic fibers. The mid-region and distal comprises areas with prevalence of fibers with anaerobic metabolism (Fig. 6A and E). When exposed to 10h under hypoxia, a significant decrease ($p<0.05$) in the aerobic fibers area (Fig.6B e F) of 51 and 23% (Fig. 6I and J) were observed in the extensor and flexor muscles, respectively. During the 30min and 2h of reoxygenation a significant decrease ($p<0.05$) in the aerobic fibers area (Fig. 6C and D) of 43 and 45% (Fig. 6I) in extensor muscle and 87 and 59% (Fig.6J) in the flexor muscle (Fig. 6D and H) were observed.

During the different periods of hypoxia and reoxygenation, it could neither be observed mitochondrial alterations nor process of hemocytes infiltration in the locomotor muscles of *N. granulata*. However, when exposed to 10h under

hypoxic conditions a significant increase ($p<0.05$) in the muscle fiber diameter was observed after 24h of reoxygenation in the proximal region of extensor and 24 and 48h of flexor muscle, respectively (Fig. 7A and B).

Discussion

The few studies that observed the occurrence of tissue damages in crustaceans when submitted to hypoxia and reoxygenation were realized with species with different degrees of tolerance to hypoxia (de Oliveira *et al*., 2005; Zenteno-Savín *et al*., 2006). These data suggest that species with greater tolerance to hypoxia would present no damages. Conversely, less tolerant species would be more susceptible to damages during reoxygenation. However, the exposure time to anoxia/hypoxia and the recovery were not proportional to the resistant period of these species. Furthermore, the authors observed only the occurrence of lipid peroxidation. It is possible that species with different degrees of tolerance to hypoxia suffer damages when subjected to hypoxia and reoxygenation. The differences may be only on the necessary time under hypoxia to generate damages. In fact, when we submit *N. granulata* to different periods of hypoxia and reoxygenation, there was a variation in the type and intensity of damages. Although not been observed any increased ROS and LPO levels after 1, 4 and 10 h under severe hypoxia ($0.5 mgO_2.L^{-1}$), there was a decrease in the mitochondrial membrane potential on the locomotor muscle of *N. granulata* after 4 h of hypoxia, along with a decrease in the area of oxidative fibers after 10h. The decrease in the mitochondrial membrane potential observed during hypoxia may be a consequence of decreased mitochondrial complex IV activity occurred on the locomotor muscle of *N. granulata* leading to ATP decrease (Geihs *et al*., 2013). These mitochondrial alterations may be

indicating that hypoxia *per se*, is leading to increased ROS levels in the locomotor muscles, as has been observed in the skeletal muscle of rats subjected to hypoxia (Clanton *et al*., 2007).

When *N. granulata* was submitted to severe hypoxia, an increase in ROS and LPO levels and a decrease in the mitochondrial membrane potential were verified in the beginning of reoxygenation. However, at the end of this period, these parameters returned to normal conditions after 4h under hypoxia exposure. When *N. granulata* was subjected to the same periods of hypoxia and reoxygenation, a faster return of mitochondrial complex IV activity and ATP levels were observed (Geihs *et al*., 2013). These results demonstrate that oxidative damages occur on the locomotor muscle of *N. granulata* after hypoxia and reoxygenation exposure, but the damages are quickly converted at the end of reoxygenation. As this crab is an intertidal species that lives in regions with no predictable alternations of hypoxia and reoxygenation (D' incao *et al*., 1992), it's expected to have a good capacity to face the problems generated by these alternations. One of the possible strategies to confront such situations is to have a well-adapted antioxidant defense system (ADS) in order to avoid the generation of oxidative stress (Sies, 1986; Jones, 2006). In this sense, when subjected to hypoxia and reoxygenation, crustaceans, in general, can maintain high the basal levels of their ADS, as has been observed for other animals (Hermes Lima and Zenteno-Savin, 2002; Bickler and Buck, 2007), or modulate in an anticipatory way their antioxidant enzymes during the time of low oxygen concentration in order to avoid the increased ROS levels, preventing the generation of oxidative stress, as already observed for other animals (Hermes Lima and Zenteno-Savin, 2002, Gorr *et al*, 2010).

With prolonged exposure to hypoxia, mitochondrial membrane potential and the area with predominance of fibers with oxidative metabolism were not recovered. These mitochondrial alterations are possibly related to problems in the activation of aerobic metabolism during reoxygenation after 10h under severe hypoxia evidenced by the delay in the mitochondrial complex IV activity resulting in ATP decrease on the locomotor muscle (Geihs *et al*., 2013). This may be occurring due to the fact that as previously observed in mammals an increase in ROS levels during hypoxia can cause a cumulative effect during the reperfusion, taking to cellular, mitochondrial and tissue cumulative damages in the muscle (Clanton *et al*., 2007). Besides, as this period of hypoxia comprises the resistance limit of *N. granulata* probably the recovery, if happens, is occurring later.

By analyzing the effects of hypoxia and reoxygenation in the muscular structure of *N. granulata* no morphological changes such as mitochondrial dysfunctions or process of infiltrating hemocytes were observed. In crustaceans hemocytes infiltration in an important defense line against various stressors such as the exposure to heavy metals and contamination with pathogens being sometimes associated to cellular apoptosis and/or necrosis (Yang *et al*., 2007; Frías-Espericueta *et al*., 2008). However, during reoxygenation it was observed an increase in cell volume in both muscles after 10h under hypoxic exposure. Since, during this period of hypoxia mitochondrial alterations were not repaired, possibly leading to later morphological changes. In the cell the active transport of Na^+ and K^+ by the Na^+/K^+-ATPase is considered one of the main energy consumers (Gregg e Milligan, 1982; Rolfe e Brand, 1999). In crustaceans, the Na^+/K^+ ATPase are considered the main component in the osmorregulation

process (Towle, 1997; Lucu e Towle, 2003; Lovett *et al*., 2006; Tsai e Lin, 2007; Lucu *et al*., 2008). Variations in the activity of Na^+/K^+ ATPase have already been observed in gills and muscle when *N. granulata* is exposed to salinity changes (Bianchini *et al*., 2008; Pinoni *et al*., 2009). However, there are no studies that examined whether hypoxia and reoxygenation modifies its activity. Probably the observed increase in cell volume during reoxygenation is due to a decrease in ATP levels in the locomotor muscle of *N. granulata* (Geihs *et al*., 2013) related to mitochondrial alterations observed in this study. However, these damages were not as intense since at the end of reoxygenation evidences of recovery were observed in muscle tissues. The walking legs of crustaceans have been characterized by having a distribution between aerobic and anaerobic fibers in different regions of the muscle. In these crustaceans there is a prevalence of fibers with small diameter and aerobic metabolism in proximal and distal regions and fibers with large diameter and anaerobic metabolism in the mid-region (Parsons, 1982; Perry, 2008). The distribution of the fibers in the walking legs of *N. granulata* showed a slightly different distribution. Just as these crustaceans, in the mid-region a prevalence of anaerobic fibers with large diameter were observed. The distal region is composed by fibers with anaerobic metabolism and small diameter, and the proximal region present fibers with aerobic metabolism and a mixture of fibers with different diameters. These differences may be associated with locomotor characteristics of each species. Interestingly, when subjected to hypoxia and reoxygenation, changes in cell volume were observed only in regions with predominance of aerobic fibers. This observation demonstrates to have a relationship between metabolic rate and generation of molecular, biochemical

and structural changes, since, as it is an area with many viable mitochondria may be more susceptible to damage.

In conclusion, the damages occurred after short and intermediate hypoxia exposure is rapidly reversed at the end of reoxygenation. However, exposure to longer hypoxia, near the resistance limits of *N. granulata*, conducts to more intense alterations during reoxygenation, including morphological changes requiring more time for recovery. Finally, these damages are more localized in regions with predominance of aerobic cells.

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Captions to figures

Fig. 1. Reactive oxygen species (ROS) concentration of the locomotor muscle of the crab *Neohelice granulata* exposed to hypoxia (0.5 $mgO_2.L^{-1}$) during 60(A), 240(B) or 600 min (C) and after 30 and 120 min of reoxygenation (black columns) (H/E) or maintained in control condition (6.5 $mgO_2.L^{-1}$) (white columns). Different letters represents significant differences ($p<0.05$) between the groups. Each point represents the mean ± 1 s.e.m. (n = 5).

Fig. 2. Lipid peroxidation (LPO) level of the locomotor muscle of the crab *Neohelice granulata* exposed to hypoxia (0.5 $mgO_2.L^{-1}$) during 60(A), 240(B) or 600 min (C) and after 30 and 120 min of reoxygenation (black columns) (H/E) or

maintained in control condition (6.5 $mgO_2.L^{-1}$) (white columns). Different letters represents significant differences ($p<0.05$) between the groups. Each point represents the mean ± 1 s.e.m. (n = 5).

Fig. 3. Mitochondrial membrane potential of the locomotor muscle of the crab *Neohelice granulata* exposed to hypoxia (0.5 $mgO_2.L^{-1}$) during 60(A), 240(B) or 600 min (C) and after 30 and 120 min of reoxygenation (black columns) (H/E) or maintained in control condition (6.5 $mgO_2.L^{-1}$) (white columns). Different letters represents significant differences ($p<0.05$) between the groups. Each point represents the mean ± 1 s.e.m. (n = 5).

Fig. 4. Morphological characterization of the locomotor muscle of the crab *Neohelice granulata.* (A). Histological longitudinal sections of extensor muscle of *N. granulata* stained with hematoxylin and eosin. The longitudinal sections of flexor muscle are similar (B). Transversal sections of extensor muscle of the crab *N. granulata* stained with hematoxylin and eosin. The transversal sections of flexor muscle are similar (C). Histological longitudinal sections of extensor muscle of *N. granulata* stained with hematoxylin and eosin showing the regions; proximal, mid-region and distal and their estimated length. The longitudinal sections of extensor and flexor muscle are similar. Scale bar: A - 500µm; B - 500µm; C - 50µm; D - 500µm.

Fig. 5. Frequency distribution of muscle fibers diameter in the locomotor muscle of *N. granulata*, in the extensor muscle (A) and their regions; proximal (C), mid-region (E) and distal (G). Frequency distribution of muscle fiber diameter in the

flexor muscle (B) and their regions; proximal (D) mid-region (F) and distal (H). The results are expressed in percentage.

Fig. 6. Hystochemistry in the extensor and flexor muscle of *N. granulata* stained with tryfenil tetrazolium cloride (TTC) (A and E), exposed to 10 h in hypoxia (0.5 $mgO_2.L^{-1}$) (B and F) and after 30 min (C and G) and 2 h of reoxygenation (black columns) (D and H), respectively (H/E) or maintained in control condition (6.5 $mgO_2.L^{-1}$) (white columns). Effects of hypoxia and reoxygenation in the aerobic fibers area in the extensor (I) and flexor (J) muscles of *N. granulata*. Different letters represents significant differences ($p<0.05$) between the groups. Each point represents the mean ± 1 s.e.m. Scale bar: 500µm.

Fig. 7. Fiber diameter in the proximal (A), mid region (C) and distal (E) regions of extensor muscle and proximal (B), mid region (D) and distal (F) regions of flexor muscle in the locomotor muscle of *N. granulata* exposed to 10 h in hypoxia (0.5 $mgO_2.L^{-1}$) and after 2, 24 and 48 h of reoxygenation (black columns) (H/E) or maintained in control condition (6.5 $mgO_2.L^{-1}$) (white columns). Different letters represents significant differences ($p<0.05$) between the groups. Each point represents the mean ± 1 s.e.m. (n = 5).

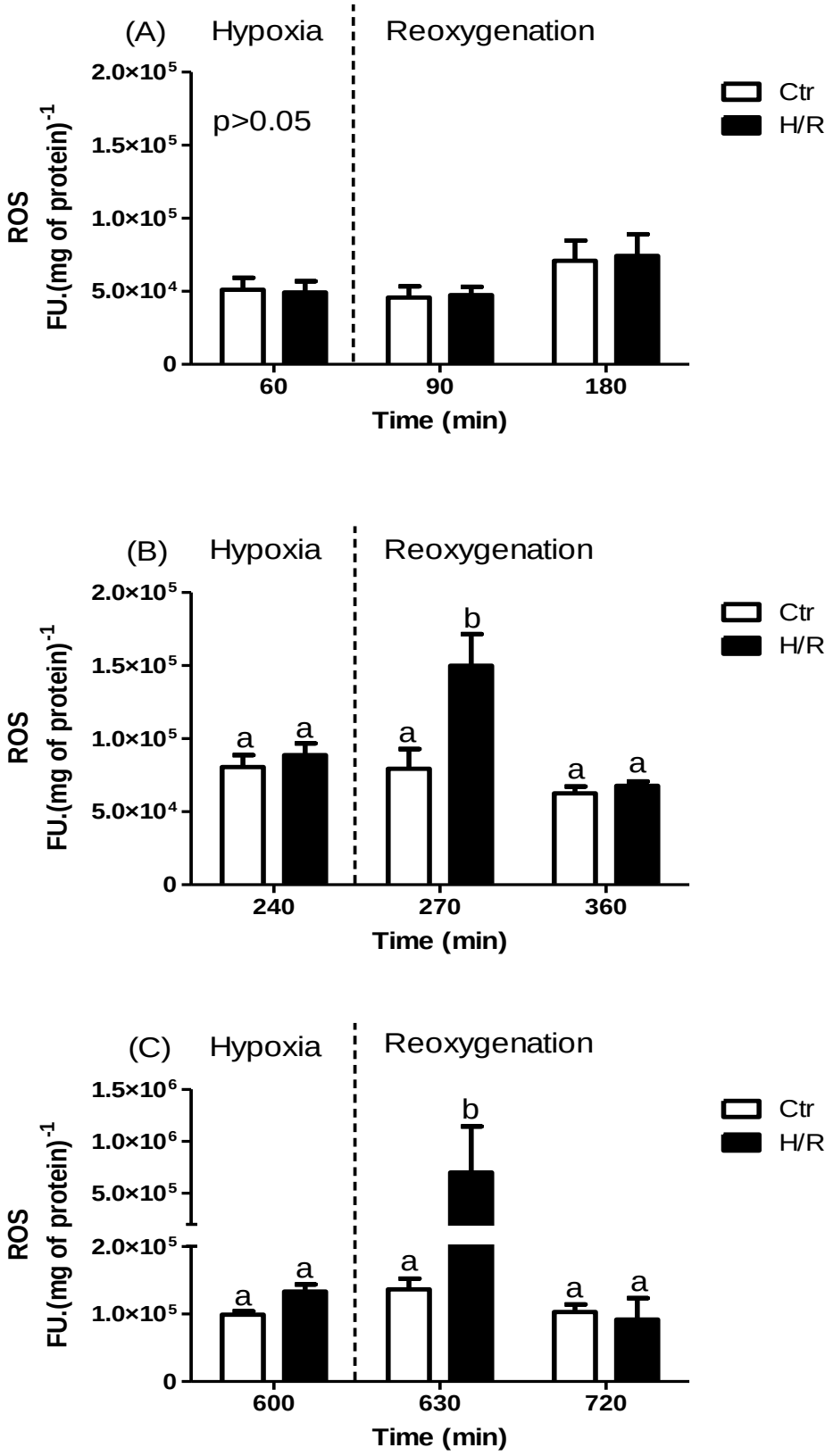
(A)
Hypoxia
Reoxygenation
p>0.05
ROS
FU.(mg of protein)-1
2.0×10⁵
1.5×10⁵
1.0×10⁵
5.0×10⁴
0
60
90
180
Time (min)
Ctr
H/R
(B)
Hypoxia
Reoxygenation
a
a
a
b
a
a
240
270
360
(C)
Hypoxia
Reoxygenation
1.5×10⁶
1.0×10⁶
5.0×10⁵
2.0×10⁵
1.0×10⁵
600
630
720

Fig. 1

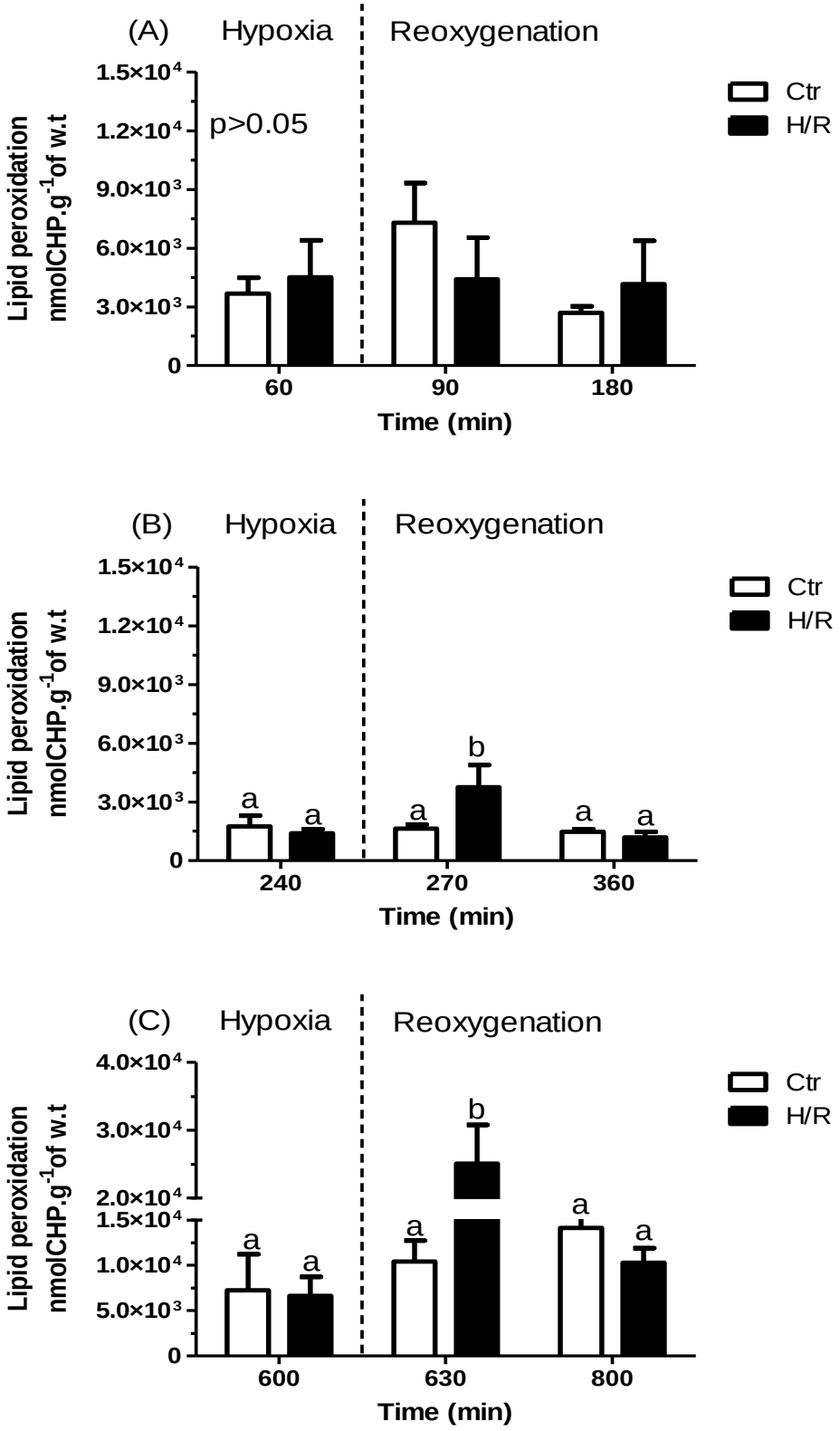
(A)
Hypoxia
Reoxygenation
p>0.05
Ctr
H/R
Lipid peroxidation nmolCHP.g-1of w.t
1.5×10⁴
1.2×10⁴
9.0×10³
6.0×10³
3.0×10³
0
60
90
180
Time (min)
(B)
Hypoxia
Reoxygenation
Ctr
H/R
Lipid peroxidation nmolCHP.g-1of w.t
1.5×10⁴
1.2×10⁴
9.0×10³
6.0×10³
3.0×10³
0
a
a
a
b
a
a
240
270
360
Time (min)
(C)
Hypoxia
Reoxygenation
Ctr
H/R
Lipid peroxidation nmolCHP.g-1of w.t
4.0×10⁴
3.0×10⁴
2.0×10⁴
1.5×10⁴
1.0×10⁴
5.0×10³
0
a
a
a
b
a
a
600
630
800
Time (min)

Fig. 2

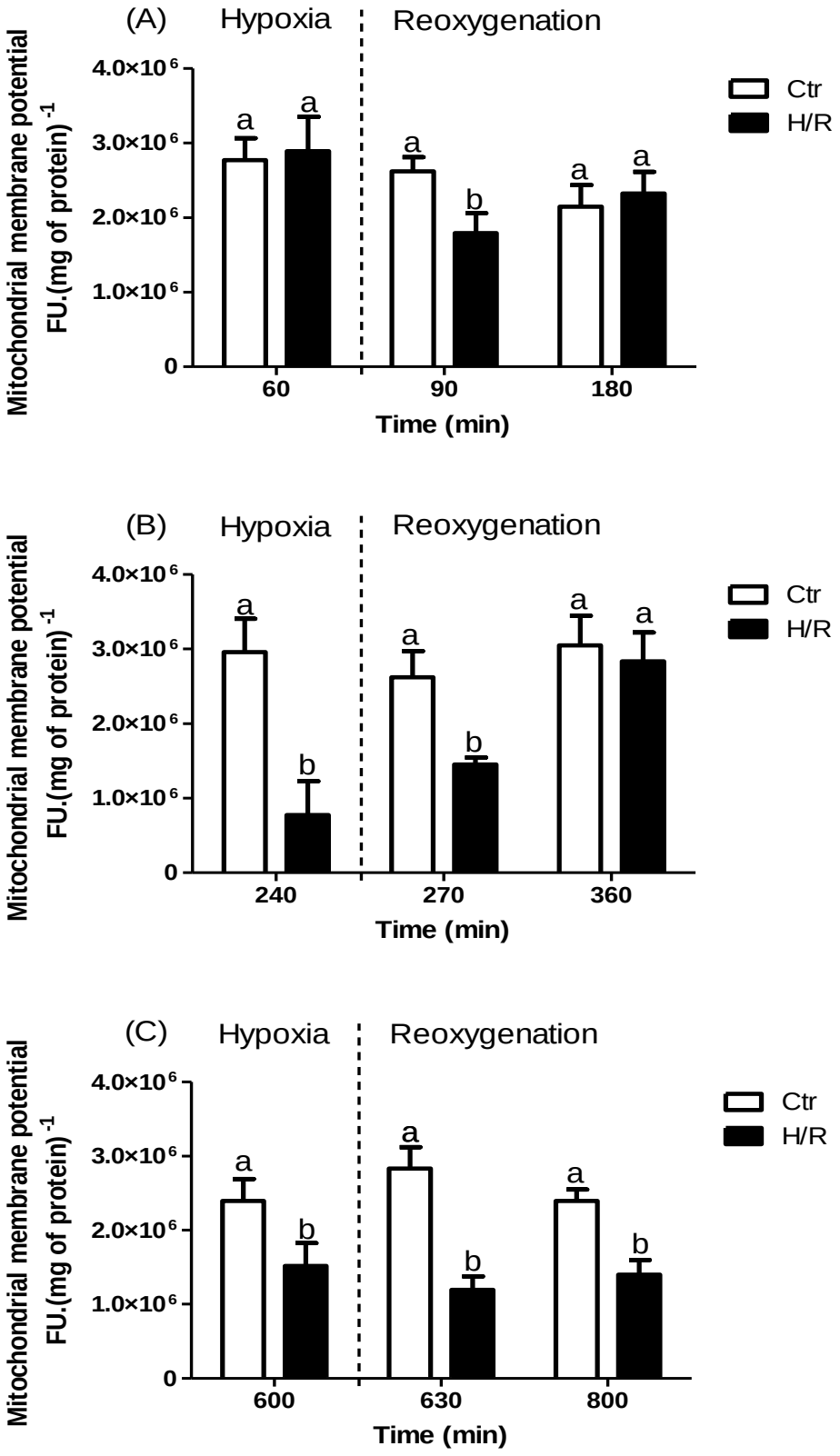
(A)
Hypoxia
Reoxygenation
Mitochondrial membrane potential
FU.(mg of protein) -1
4.0×10 6
3.0×10 6
2.0×10 6
1.0×10 6
0
a
a
a
b
a
a
60
90
180
Time (min)
Ctr
H/R
(B)
Hypoxia
Reoxygenation
Mitochondrial membrane potential
FU.(mg of protein) -1
4.0×10 6
3.0×10 6
2.0×10 6
1.0×10 6
0
a
b
a
b
a
a
240
270
360
Time (min)
Ctr
H/R
(C)
Hypoxia
Reoxygenation
Mitochondrial membrane potential
FU.(mg of protein) -1
4.0×10 6
3.0×10 6
2.0×10 6
1.0×10 6
0
a
b
a
b
a
b
600
630
800
Time (min)
Ctr
H/R

Fig. 3

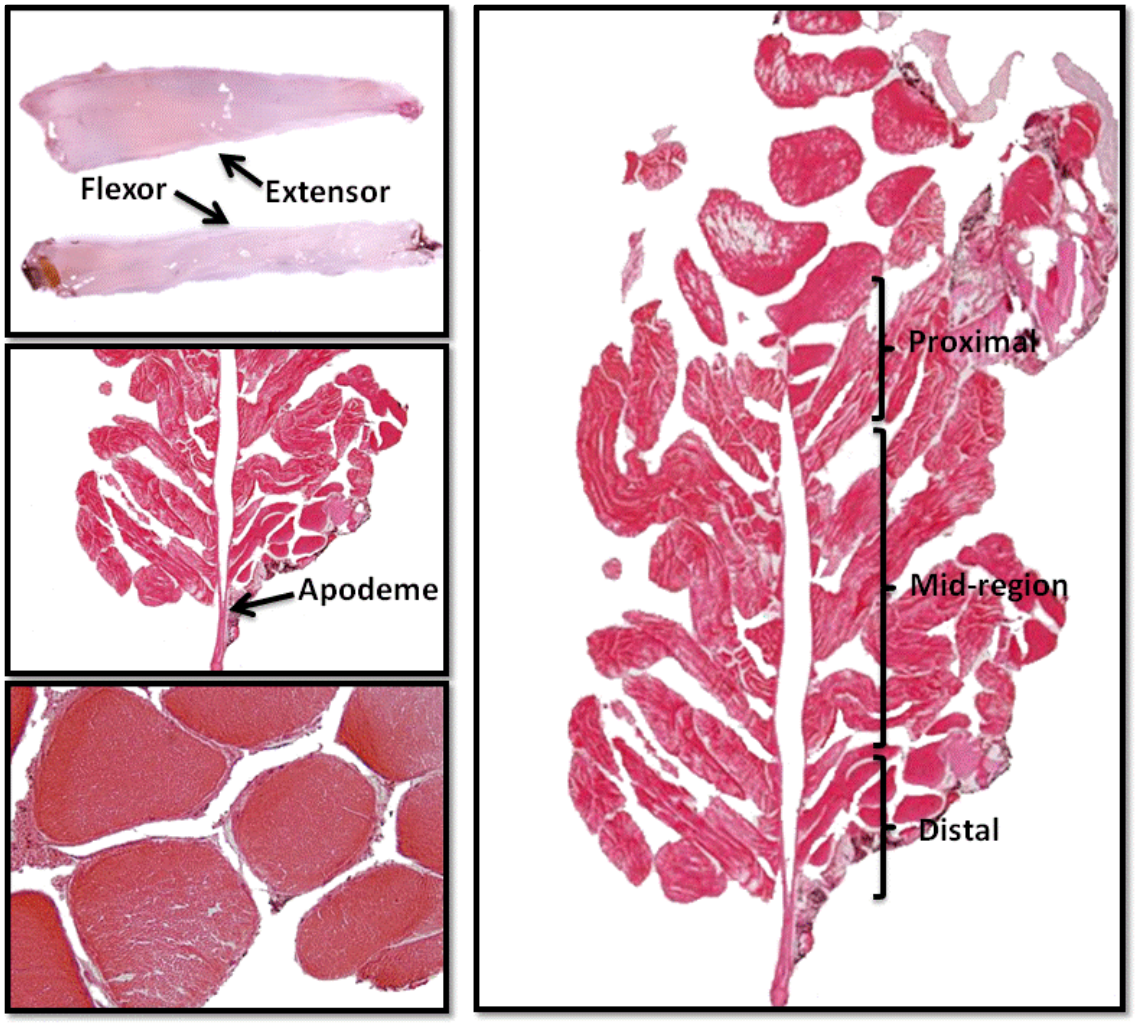

Fig. 4

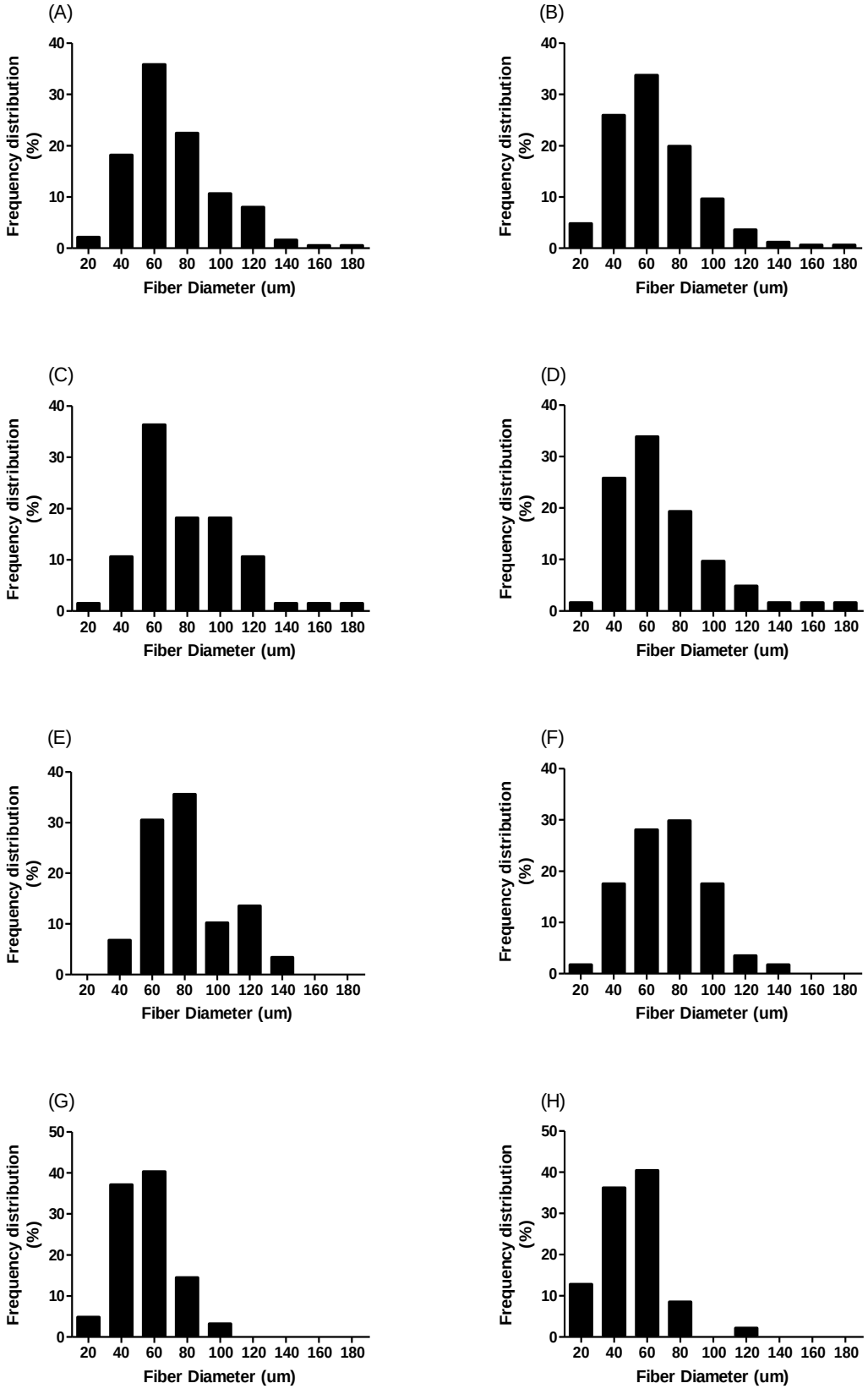
(A)
Frequency distribution (%)
0
10
20
30
40
20 40 60 80 100 120 140 160 180
Fiber Diameter (um)
(B)
Frequency distribution (%)
0
10
20
30
40
20 40 60 80 100 120 140 160 180
Fiber Diameter (um)
(C)
Frequency distribution (%)
0
10
20
30
40
20 40 60 80 100 120 140 160 180
Fiber Diameter (um)
(D)
Frequency distribution (%)
0
10
20
30
40
20 40 60 80 100 120 140 160 180
Fiber Diameter (um)
(E)
Frequency distribution (%)
0
10
20
30
40
20 40 60 80 100 120 140 160 180
Fiber Diameter (um)
(F)
Frequency distribution (%)
0
10
20
30
40
20 40 60 80 100 120 140 160 180
Fiber Diameter (um)
(G)
Frequency distribution (%)
0
10
20
30
40
50
20 40 60 80 100 120 140 160 180
Fiber Diameter (um)
(H)
Frequency distribution (%)
0
10
20
30
40
50
20 40 60 80 100 120 140 160 180
Fiber Diameter (um)

Fig. 5

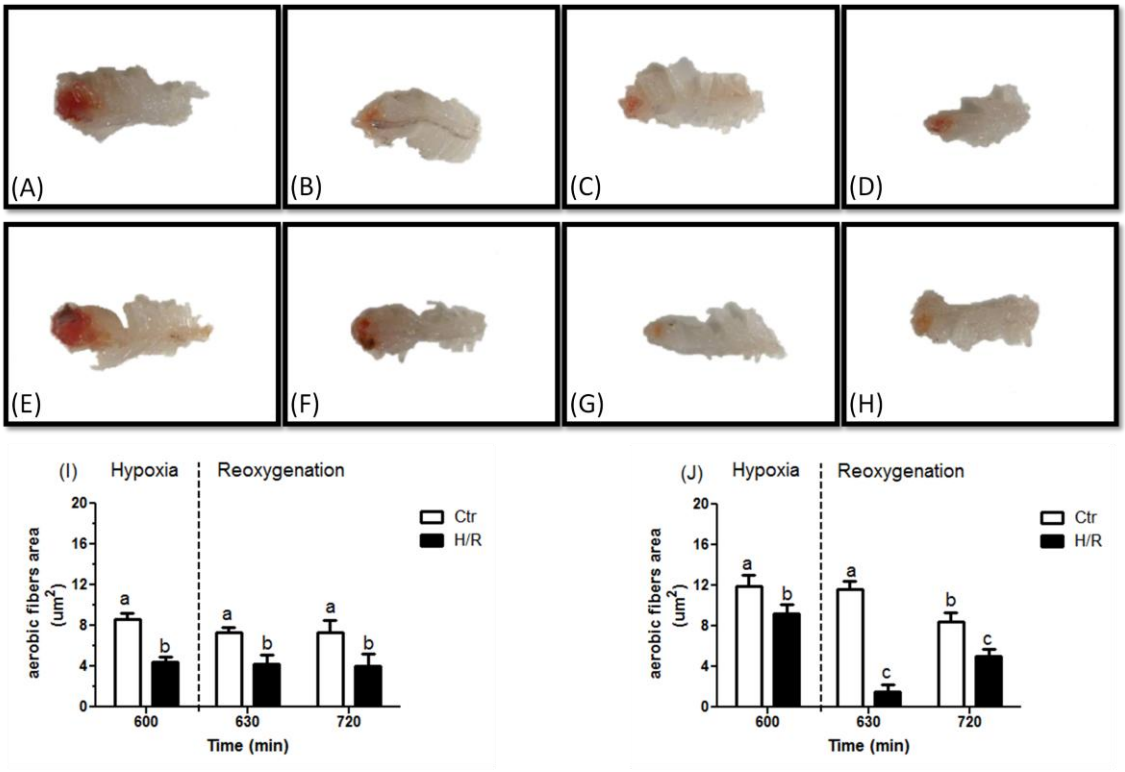
(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)
(I)
Hypoxia
Reoxygenation
aerobic fibers area (um2)
Ctr
H/R
Time (min)
600
630
720
(J)
Hypoxia
Reoxygenation
aerobic fibers area (um2)
Ctr
H/R
Time (min)
600
630
720

Fig. 6

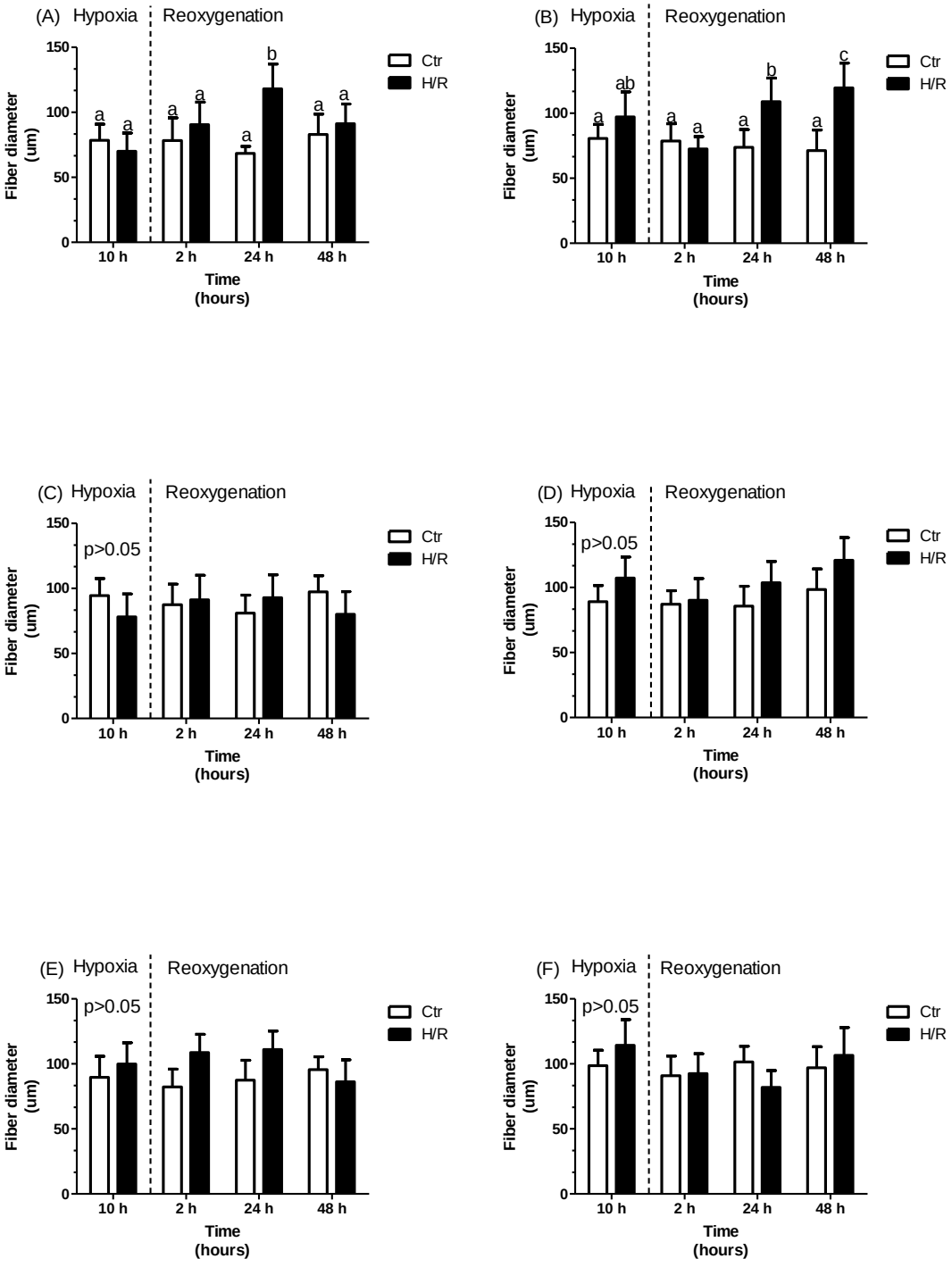
(A) Hypoxia
Reoxygenation
Fiber diameter (um)
Time (hours)
10 h
2 h
24 h
48 h
Ctr
H/R
(B) Hypoxia
Reoxygenation
(C) Hypoxia
Reoxygenation
p>0.05
(D) Hypoxia
Reoxygenation
p>0.05
(E) Hypoxia
Reoxygenation
p>0.05
(F) Hypoxia
Reoxygenation
p>0.05

Fig.7

X. 3º ARTIGO

Efeito da hipoxia e reoxigenação sobre o sistema de defesa antioxidante no músculo locomotor do caranguejo *Neohelice granulata* (Decapoda Varunidae)

Artigo a ser submetido ao periódico: Free Radical Biology & Medicine.

EFFECTS OF HYPOXIA AND REOXYGENATION ON THE ANTIOXIDANT DEFENSE SYSTEM OF THE LOCOMOTOR MUSCLE OF THE CRAB *NEOHELICE GRANULATA* (DECAPODA, VARUNIDAE)

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Abstract

Many crustaceans live in regions with oscillation of hypoxia and reoxygenation. Such oscillation results in increase in levels of ROS that may lead to oxidative damage. It is expected that these animals present an antioxidant defense system prepared to confront such situations. However, the few studies analyzing such situations hinder the understanding of adaptations used by this group when subjected to hypoxia and reoxygenation. For this reason this study analyzed the antioxidant defense system (ADS) of locomotor muscle of the crab *Neohelice granulata* when submitted to severe hypoxia ($0.5 mgO_2.L^{-1}$) and reoxygenation. We analyzed the total antioxidant capacity against peroxyl radical, and the enzymes superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx-Se), glutathione-S-transferase (GST) and glutamate cysteine ligase (GCL). Besides the glutathione levels and the concentration of melatonin in the fractions hemolymphatic, cytosolic and mitochondrial was estimated. During hypoxia an increase in the activity of GPx-Se and GCL was observed. Also, a decrease in GSH and mitochondrial melatonin level was observed. During reoxygenation it was observed an increase in the catalase activity and a decrease in cytosolic melatonin level. It appears that the ADS in the locomotor muscle of *N. granulata* modulate its components when confronting with oxidative stress.

Keywords: crustaceans, hypoxia/reoxygenation, oxidative stress, antioxidant defense system, melatonin, muscle, mitochondria.

1. Introduction

During the respiratory process, normally a short part of the oxygen taken by the cells in the oxidative phosphorylation process in the mitochondrial electron transport chain is converted in reactive oxygen species (ROS) (Fridovich, 2004). For being highly reactive, these molecules can generate many oxidative damages such as protein oxidation, lipid peroxidation, DNA strand break and damages in the mitochondrial electrons transport chain (Halliwell and Gutteridge, 2001, Halestrap 2009; Halestrap and Pasdois, 2009). All organisms presents an antioxidant defense system (ADS) which is responsible for avoiding the increase in ROS level or repair the damages caused by the interaction with the cell constituents. The enzymatic ADS is comprises by enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX-Se) and glutathione-S-transferase (GST) and the non-enzymatic ADS is made by antioxidants with low molecular weight, such as glutathione (GSH), vitamins C and E and more recently suggested melatonin (Reiter,1996; Halliwell and Gutteridge, 2001, 2007).

One of the possible consequences when animals are submitted to oscillations in the oxygen concentration on the environment, as hypoxia and reoxygenation, is the increase in ROS levels (Hermes-Lima and Zenteno-Savín, 2002; Clanton *et al*., 2007). These situations are very common in coastal regions (Smith and Able, 2003), may lead to the generation of many types of oxidative damages in the animals tissues. In order to avoid such situations, the animals, in a general way, keep high the basal levels of their antioxidant defenses, mainly observed in species considered tolerant to hypoxia/anoxia as a way to prevent the raise in the generation of oxidative stress during

reoxygenation (Hermes-Lima and Zenteno-Savín, 2002; Bickler and Buck, 2007; Gorr, 2010).

For crustaceans that as a generally group show lower tolerance to hypoxia if compared to other groups of animals as fish and molluscs (Vaquer Sunier and Duarte, 2008), the few existing studies point for an anticipatory strategy of antioxidant enzymes during anoxia/hypoxia (Hermes-Lima and Storey, 1998; Hermes-Lima and Zenteno-Savín, 2002). A study made with a specie considered most tolerant to hypoxia *Littopenaus vannamei* (Seidman and Lawrence, 1985) analyzed the muscle and hepatopancreas and observed an SOD increase during hypoxia in both tissues. However, in the reoxygenation there was a variation of different enzymatic ADS components for the different tissues avoiding the oxidative damage generation (Zenteno-Savín *et al*., 2006; Parrilla-Taylor *et al*., 2011). In experiments with the crab *Neohelice granulata,* which showed similar tolerance to other crustaceans (Geihs *et al*., 2013a), during anoxia SOD decreased and catalase and GST increased in the anterior and posterior gills. During the reoxygenation there was a variation of different components in the different tissues, however it was observed the occurrence of damages. In a more recent study, when this crab was submitted to 4h in severe hypoxia ($0.5 mgO_2.L^{-1}$), an increase in ROS levels resulted in an increase in the lipoperoxidation (LPO) and a decrease in the mitochondrial membrane potential on the locomotor muscle in the beginning of reoxygenation. However, these damages have been quickly restored because right after 2h of reoxygenation the parameters returned to normal, suggesting that the strategies in this tissue is different than observed in the gills. For this reason, the objective of this present work is to verify which strategies have been used by the crab *N.*

granulata in order to confront with the damage generation during hypoxia and reoxygenation in the locomotor muscle, as well as verify if melatonin is a functional component of these ADS in this tissue.

2. **Materials and methods**

2.1. Animals maintenance

Adult male crabs of *Neohelice granulata* weighing 10.6 ± 0.7 g (mean ± S.E.M) were collected in salt marshes around Rio Grande City - Brazil and taken to the laboratory. The crabs were acclimated, at least during 10 days, in tanks under constant conditions of temperature (20°C), salinity (20‰) and photoperiod (12L:12D). The animals were fed *ad libitum* with ground beef 3 times a week.

2.2. Experimental procedure

The crabs were individually placed in cylindrical glass chambers with base and height of 5x14cm and a volume of approximately 300ml. Nitrogen gas (100%) were bubbled until the oxygen concentration reached 0.5 $mgO_2.L^{-1}$. The oxygen concentration was continuously monitored during the experiment. All analysis was made after 4h of hypoxia and subsequent 30 and 120 min of reoxygenation. The control group was maintained along the whole experiment in water of 6.5 $mgO_2.L^{-1}$ and was analyzed simultaneously with the experimental group.

2.3. Tissue preparation

For antioxidant capacity against peroxyl radicals (ACAP), superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione-S-transferase (GST) analysis, the pair of meropodites muscles (n=5) were weighted and

homogenized (1:10*w/v*) in a cold (4°C) buffer solution containing sucrose (250mM), phenylmethylsulfonyl fluoride (PMSF) (1mM), and EDTA (5mM), with pH adjusted to 7.6. The samples were centrifuged (2,000xg, 4°C for 20 min) and the supernatant were collected and centrifuged (10,000xg, 4°C for 45 min). The supernatant of this second centrifugation was used for the analysis.

For catalase activity, muscles (n=5) were weighted and homogenized (1:10 *w/v*) in a cold (4°C) buffer solution containing Tris base (20mM), EDTA (1mM), dithiothreitol (1mM), KCl (150mM), and PMSF (0.1mM), with pH adjusted to 7.6. The samples were centrifuged (9,000xg, 4°C for 30 min) and the supernatants were then employed for the analysis.

For GCL activity and GSH content, the muscles (n=5) were weighted and homogenized(1:10 *w/v*) in a cold (4°C) buffer solution containing TRIS-HCl (100 mM), $MgCl_2$ (5mM) and EDTA (2mM) with pH adjusted to 7.6. The samples were centrifuged (2,000xg, 4°C for 20 min) and the supernatant were collected and centrifuged (10,000xg, 4°C for 45 min). The supernatant of this second centrifugation were then employed to analysis. In all enzymatic assays the reagents were supplied by Sigma-Aldrich (St Louis, MO, USA).

2.4. Total antioxidant capacity against peroxyl radical (ACAP)

ACAP was measured according to the method of Amado *et al*., (2009). Briefly, 10 µL of the supernatants prepared for enzymatic analysis were pipetted into a white 96 wells microplate, six wells per sample. The reaction buffer (127.5 µl) containing 30 mM HEPES (pH 7.2), 200 mM KCl and 1 mM $MgCl_2$ was added to the wells with samples. In three of the six wells of each sample, 7.5 µL of 2,2´-azobis 2 methylpropionamidine dihydrochloride (ABAP; 4 mM) were added while the same volume of ultrapure water was pipetted in the other three

wells. The microplate was put into a fluorescence microplate reader (Victor 2, Perkin Elmer), at a programmed temperature of 37 ^{o}C, at which peroxyl radicals were produced by thermal decomposition of ABAP. Immediately before the reading, 10µL of the fluorescent probe 2´,7´ dichlorofluorescein diacetate (H_2DCF-DA) were added to wells at a final concentration of 40µM (Ferreira-Cravo *et al*., 2007). H_2DCF-DA is cleaved by esterases present in samples and the non-fluorescent compound H_2DCF is oxidized by ROS to the fluorescent compound DCF, which is detected at wavelengths of 488 and 525 nm, for excitation and emission, respectively. The thermal decomposition of ABAP and ROS formation was monitored with readings every 5 minutes for 60 minutes. Total fluorescence production was calculated by integrating the fluorescence area along the time of the measurement, after adjusting FU data to a second order polynomial function. The results were calculated as area difference of FU × min in the same sample with and without ABAP addition and standardized to the area without ABAP. The inverse of relative difference between ROS area with and without ABAP was considered as a measure of antioxidant capacity, with high area difference meaning high antioxidant capacity. The results were expressed multiplying the inverse of relative area for 10^3. Note that calculating antioxidant competence as:

1/ [(ROS $_{área}$ ABAP – ROS $_{área}$ background)/ ROS $_{área}$ background]*10^3

2.5. Superoxide dismutase (SOD)

SOD activity was determined according to McCord and Fridovich (1969). This assay is based on the generation of superoxide anions (O^{-}_2), which reduce cytochrome C (10µM). The enzymatic activity values were expressed in SOD

units, where one unit is the amount of enzyme needed to inhibit 50% of cytochrome C reduction. min^{-1}. mg of $protein^{-1}$ at 550 nm, 25ºC, and pH 7.8.

2.6. Catalase (CAT)

Catalase activity was analyzed following Beutler, (1975), by determination of H_2O_2 (50 mM) decomposition. This procedure was performed in a digital spectrophotometer (Biomatte 3). The results were expressed in CAT units, where one unit is the amount of enzyme that hydrolyzes 1 μmol of H_2O_2. min^{-1}. mg of $protein^{-1}$ at 240 nm, 25°C and pH 8.0.

2.7. Gluthatione peroxidase (GPx-Se)

GPx-Se was measured according to Arun and Subramanian (1998). NADPH oxidation was measured in presence of excess glutathione reductase (GR), reduced glutathione (GSH), hydrogen peroxide (H_2O_2) and aliquots of the homogenate. Results were expressed in GPx units, where one unit is the amount of enzyme necessary to oxidize 1 μmol of NADPH. min^{-1}. mg of $protein^{-1}$ at 340 nm, 30 °C and pH 7.2.

2.8. Glutathione-S-transferase (GST)

GST activity was measured by monitoring the formation of a conjugate between 1mM of GSH and 1mM 1-chloro-2,4-dinitrobenzene (CDNB) (Habig *et al*., 1974; Habig and Jakoby, 1981). The results were expressed in GST units where one unit is defined as the amount of enzyme that conjugates 1 mmol of CDNB. min^{-1}. mg of $protein^{-1}$ at 340 nm, 25°C and pH 7.4.

2.9. Glutamate cysteine ligase activity (GST) and glutathione content (GSH).

GCL activity and GSH content was analyzed following White *et al*., (2003). This method is based in the reaction of naphthalene dicarboxialdehyde (NDA) with glutathione (GSH) or γ-glutamylcysteine (γ-GC) to form cyclized product

that are highly fluorescent. NDA-GSH fluorescence intensity was measured (472 ex/528 em) on fluorescence microplate reader (Victor 2, Perkin Elmer). This assay has the advantage that baseline levels of GSH can also be measured in the same tissue sample. For GCL activity, it was prepared an GCL reaction cocktail (400 mM Tris, 40 mM ATP, 20 mM glutamate, 2.0 mM EDTA, 20 mM sodium borate, 2mM serine, 40 mM $MgCl_2$) just prior to the beginning of the assay to prevent ATP degradation. The samples plate was kept on ice until were pipetted into the reaction plate (25°C) at 15-s time intervals. After 5 min of preincubation, the GCL reaction was initiated by adding 50 μl of cysteine (2 mM) to each GCL activity well (cysteine was not added to the GSH-baseline wells in this time). Soon after, the plate was incubated during 60 min and the reaction stopped by adding 50 μl of 5-sulfosalicylic acid (200mM) and then 50 μl of 2 mM cysteine was added to the GSH-baseline wells. After protein precipitation, the plate was centrifuged (750xg, 25ºC during 5 min) and then, 20 μl aliquots of supernatant from each well of the reaction plate were transferred to a 96-well plate designed for fluorescence detection (Victor 2, Perkin Elmer). The GCL activity was expressed in nM of GCL. h^{-1}. mg of $protein^{-1}$ and GSH content in nM of GSH.mg of $protein^{-1}$.

2.10. Melatonin quantification (MEL)

For melatonin quantification in the hemolymph, 100 μl were collected (n=5) with a syringe from the base of the 4th or 5th pairs of the pereiopods and stored at –80ºC. For melatonin quantification in the cytosolic and mitochondrial fractions muscles were removed (n=5), weighted and rinsed in tubes in ice cold medium containing sucrose (510mM), EDTA (1mM), EGTA (200μM), HEPES (20mM) and BSA (0.5%) at pH 7.5. Muscles were homogenized and centrifuged

(2,000xg, 4ºC during 15 min). The supernatant was removed and centrifuged again (8,000xg, 4ºC during 15 min). The supernatant and pellet constitute the cytosolic fraction and mitochondrial enriched fraction, respectively. Thereafter, methanol (500ul) were added in the three samples (hemolymphatic, cytosolic and mitochondrial) and lyophilized. The melatonin concentration was measured by radioimmunoassay according to Vakkuri *et al*., (1984a,b) and Maciel *et al.*, (2008).

2.11. Protein determinations

For all biochemical analysis the protein content was enzymatically determined using a commercial reagent kit (Doles Reagents Ltda., Goiânia, Goiás, Brazil), This method is based on the biuret reagent analyzed at 550 nm.

2.12. Statistical analysis

Statistical analysis were performed by analysis of variance (Two Way ANOVA) followed by Newman-Keuls test with $\alpha=0.05$. Normality and variance homogeneity were verified as ANOVA assumptions and each point represents the mean ± 1 s.e.m. Mathematical transformations were performed when necessary (Zar, 1984).

3. Results

During the hypoxia it was not observed significant differences ($p>0.05$) in antioxidant capacity against peroxyl radical in the locomotor muscle of the crab *N. granulata* (Fig.1). However, at the end of reoxygenation (2h) a significant reduction ($p<0.05$) was verified compared to control group.

Analyzing the enzymatic antioxidant defense system (ADS), variations were not observed ($p>0.05$) in the activity of the enzyme superoxide dismutase

(SOD) enzyme during hypoxia. However, a significant reduction ($p<0.05$) in relation to control group in the activity of this enzyme was observed after 30min and 2 h of reoxygenation (Fig. 2). The activity of the enzyme catalase enzyme had no variation ($p>0.05$) during hypoxia. However, a significant increase ($p<0.05$) compared to control group was observed after 30min of reoxygenation. On the other hand, after 2h a significant reduction in relation to the control group was observed (Fig. 3). The activity of the enzyme glutathione peroxidase (GPx-Se) increased ($p<0.05$) in relation to control group during hypoxia. However, after 30min of reoxygenation, the activity of this enzyme reduced ($p<0.05$), returning to normal levels after 2h (Fig. 4). There were no significant differences ($p>0.05$) in the activity of glutathione-S-transferase (GST) during hypoxia and reoxygenation (Fig. 5).

The activity of glutamate cysteine ligase (CGL) has demonstrated a significant increase ($p<0.05$) respect to control group during hypoxia. During reoxygenation the activity of this enzyme returned to basal levels in 30 min remaining in these situations ($p>0.05$) during all (Fig. 6). On the other hand, it was observed a significant decrease ($p>0.05$) in the glutathione (GSH) levels during hypoxia and reoxygenation in the locomotor muscle (Fig. 7).

There were no significant variations ($p>0.05$) in the content of hemolymphatic melatonin during hypoxia and reoxygenation (Fig. 8A). No significant variations were observed in the cytosolic melatonin during hypoxia. However, during reoxygenation a significant decrease in cytosolic melatonin after 30min and 2h was observed (Fig. 8B). In the mitochondrial enriched fraction, a significant decrease ($p<0.05$) in the melatonin content was observed

during hypoxia. In the reoxygenation a significant decrease ($p<0.05$) has also been observed after 2h (Fig. 8C).

4. Discussion

In crustaceans the few works that verified the effects of hypoxia/anoxia in the activity of the ADS components, verified that there was an increase of different enzymes according to the analyzed animal (de Oliveira *et al*., 2005; Parilla-Taylor *et al*., 2011), suggesting that crustaceans may use the antecipatory strategy, as it has already been observed to other groups (Hermes-Lima e Zenteno-Savín, 2002). When *N. granulata* was submitted to severe hypoxia ($0.5 mgO_2.L^{-1}$) it was not observed an increase in ROS level as well as LPO in the locomotor muscle (Geihs *et al*., 2013b). However, there was a decrease in the mitochondrial membrane potential (Geihs *et al*., 2013b) and in the mitochondrial complex IV activity leading to a decrease in ATP levels (Geihs *et al*., 2013a) demonstrating that mitochondrial alteration probably are occurring during severe hypoxia in the locomotor muscle.

By the analysis of total antioxidant capacity against peroxyl radicals during severe hypoxia, it was not observed significant variations. However, it is possible that the variations in one component mask the variations in another one. Besides, the method to quantify the antioxidant capacity is useful to detect the antioxidant competency against peroxyl radical, one of the most suitable reactive oxygen species (ROS). It's possible that others ROS or even thought reactive nitrogen species (RNS) can be formed and reacting with biological samples not be detected by this method. Indeed, it was observed an increase in GPx-Se and GCL activities during hypoxia. This result could suggest that

anticipatory responses are occurring in the locomotor muscles of *N. granulata* when exposed to hypoxia. However, the decrease in the mitochondrial membrane potential and ATP levels, in the same situation of hypoxia (Geihs *et al*., 2013a,b), may be indicating a possible increase in ROS levels in the mitochondria on the locomotor muscle as already observed in skeletal muscle of rats (Clanton *et al*., 2007). Moreover, during hypoxia, the levels of GSH have reduced, possibly due to the use as a substrate by the GPx-Se enzyme, as observed during this study and by the decrease of ATP levels (Geihs *et al*., 2013a) necessary for the increase in the synthesis of GSH through GCL enzyme. However, even with the decrease in ATP levels, it was observed an increase in the enzyme GCL activity. Because GCL is feedback inhibited by GSH (Kaplowitz *et al*., 1985) a decrease in the level of this tripeptide may cause a transient increase in GCL activity (Richman e Meister, 1975). Also, the increase in GCL activity may also be leading the transient increase in its catalytic and modulatory subunits with may be occurring by the increase in ROS levels (Griffith, 1999; Dickinson and Forman, 2002).

In the beginning of the reoxygenation an increase in ROS and LPO levels were observed in the locomotor muscle. But, at the end of reoxygenation it was observed a quick recuperation of these parameters (Geihs *et al*., 2013b) demonstrating that the locomotor muscle of *N. granulata* detains a great antioxidant capacity, to preventing or repairing the damages caused by the ROS increase after 2h of reoxygenation. By analyzing the total antioxidant capacity against peroxyl radicals no variations occurred in the beginning of reoxygenation. By analyzing the enzymes of the ADS during reoxygenation, it was possible to observe an increase in catalase activity in 30min. The increase

in catalase may be indicating that ROS produced in the mitochondria during this situation are being located in the cytosol, since the enzyme catalase is mainly concentrated in the peroxisomes (Sando *et al*.,1984; Erickson *et al*.,1992). The decrease in GPx-Se and SOD activity in the beginning of reoxygenation may be associated with the increase in ROS levels (Geihs et al.,2013b) leading to the inactivation of these antioxidants (Escobar *et al*., 1996; Cho *et al*., 2010). At the end of the reoxygenation occurred a decrease in the total antioxidant capacity against peroxyl radical probably to restore the high levels of ROS and LPO, observed in the beginning (Geihs et al.,2013b). This return is possibly due to the increase in the catalase activity and other non-enzymatic constituents in order to avoid oxidative damage.

One important component that has been suggested as antioxidant for vertebrates is the melatonin (Tan, 1998; Reiter, 2002; Hardeland, 2005). In rats it has been verified that the endogenous melatonin content tissue has an inverse relation with the levels of oxidative damages (Lardoni *et al*., 2006). In crustaceans this molecule has already been identified in the haemolymph (Agapito *et al*.,1995), nervous system (Meyer-Rochow, 2001), eyestalk (Balzer *et al*., 1997; Tilden *et al*., 1997; Pape *et al*., 2008) and muscle (Geihs *et al*., 2009). When *N. granulata* received exogenous administrations of melatonin this molecule was able to modulate the ADS components and reduce oxidative damages (Geihs *et al*., 2009; Vargas *et al*., 2011). In this present study, by our knowledge, for the first time it was verified a decrease in the levels of muscle melatonin in crustaceans in the situations of oxidative stress. During hypoxia, a decrease in the mitochondrial melatonin levels along with the increase in the GPx-Se activity are possibly reducing the generation of oxidative damages in

the locomotor muscle of this crab. Furthermore, it was not observed variations in the hemolymphatic melatonin, suggesting that the used melatonin is only muscular. During reoxygenation it was observed that the cytosolic melatonin join to the increase on the catalase activity must be contributing for the quick recovery of the locomotor muscle during reoxygenation.

In conclusion, it seems that the ADS of *N. granulata* locomotor muscle modules its components when confronting situation of oxidative stress. During hypoxia, the ADS of the locomotor muscle seems to be centered in GPx-Se activity, as well as GSH and mitochondrial melatonin. During reoxygenation, the increase in catalase activity and cytosolic melatonin appear to be responsible for reducing oxidative damage and recovery in the locomotor muscle of this species.

Acknowledgements

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Captions to Figures

Fig. 1. Antioxidant capacity against peroxyl radicals (ACAP) in the locomotor muscle of the crab *Neohelice granulata* exposed to 4 h under hypoxia (0.5 $mgO_2.L^{-1}$) and 0.5 and 2 hours of reoxygenation (black columns) (H/R) or maintained in control condition (6.5 $mgO_2.L^{-1}$) (white columns). Different letters represents significant differences ($p<0.05$) between the groups. Each point represents the mean ± 1 s.e.m. (n = 5).

Fig. 2. Superoxide dismutase (SOD) activity in the locomotor muscle of the crab *Neohelice granulata* exposed to 4 h under hypoxia (0.5 $mgO_2.L^{-1}$) and 0.5 and 2 hours of reoxygenation (black columns) (H/R) or maintained in control condition (6.5 $mgO_2.L^{-1}$) (white columns). Different letters represents significant differences ($p<0.05$) between the groups. Each point represents the mean ± 1 s.e.m. (n = 5).

Fig. 3. Catalase (CAT) activity in the locomotor muscle of the crab *Neohelice granulata* exposed to 4 h under hypoxia (0.5 $mgO_2.L^{-1}$) and 0.5 and 2 hours of reoxygenation (black columns) (H/R) or maintained in control condition (6.5 $mgO_2.L^{-1}$) (white columns). Different letters represents significant differences ($p<0.05$) between the groups. Each point represents the mean ± 1 s.e.m. (n = 5).

Fig. 4. Glutathione peroxidase (GPx-Se) activity in the locomotor muscle of the crab *Neohelice granulata* exposed to 4 h under hypoxia (0.5 $mgO_2.L^{-1}$) and 0.5 and 2 hours of reoxygenation (black columns) (H/R) or maintained in control condition (6.5 $mgO_2.L^{-1}$) (white columns). Different letters represents significant

differences ($p<0.05$) between the groups. Each point represents the mean ± 1 s.e.m. (n = 5).

Fig. 5. Glutathione-S- transferase (GST) activity in the locomotor muscle of the crab *Neohelice granulata* exposed to 4 h under hypoxia (0.5 $mgO_2.L^{-1}$) and 0.5 and 2 hours of reoxygenation (black columns) (H/R) or maintained in control condition (6.5 $mgO_2.L^{-1}$) (white columns). Different letters represents significant differences ($p<0.05$) between the groups. Each point represents the mean ± 1 s.e.m. (n = 5).

Fig. 6. Glutamate cysteine ligase (GCL) activity in the locomotor muscle of the crab *Neohelice granulata* exposed to 4 h under hypoxia (0.5 $mgO_2.L^{-1}$) and 0.5 and 2 hours of reoxygenation (black columns) (H/R) or maintained in control condition (6.5 $mgO_2.L^{-1}$) (white columns). Different letters represents significant differences ($p<0.05$) between the groups. Each point represents the mean ± 1 s.e.m. (n = 5).

Fig. 7. Glutathione content (GSH) in the locomotor muscle of the crab *Neohelice granulata* exposed to 4 h under hypoxia (0.5 $mgO_2.L^{-1}$) and 0.5 and 2 hours of reoxygenation (black columns) (H/R) or maintained in control condition (6.5 $mgO_2.L^{-1}$) (white columns). Different letters represents significant differences ($p<0.05$) between the groups. Each point represents the mean ± 1 s.e.m. (n = 5).

Fig. 8. Melatonin content (Mel) in the hemolymph (A) of the crab *Neohelice granulata* and the cytosolic (B) and mitochondrial (C) fractions of the locomotor muscle exposed to 4 h under hypoxia (0.5 $mgO_2.L^{-1}$) and 0.5 and 2 hours of reoxygenation (black columns) (H/R) or maintained in control condition (6.5$mgO_2.L^{-1}$) (white columns). Different letters represents significant differences ($p<0.05$) between the groups. Each point represents the mean ± 1 s.e.m. (n = 5).

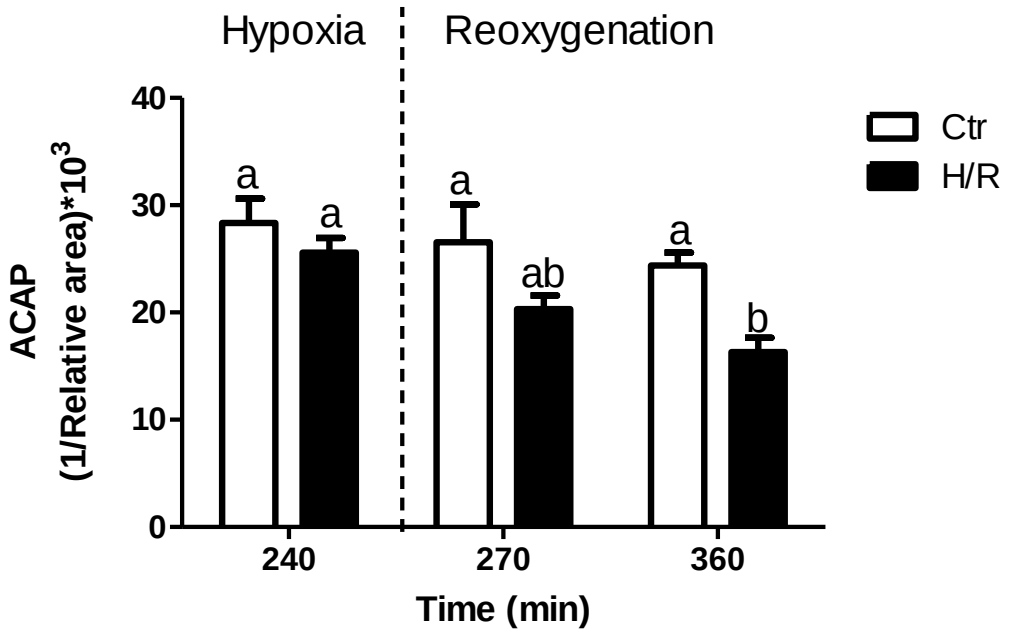
Hypoxia
Reoxygenation
Ctr
H/R
ACAP
(1/Relative area)*10^3
40
30
20
10
0
a
a
a
ab
a
b
240
270
360
Time (min)

Fig. 1

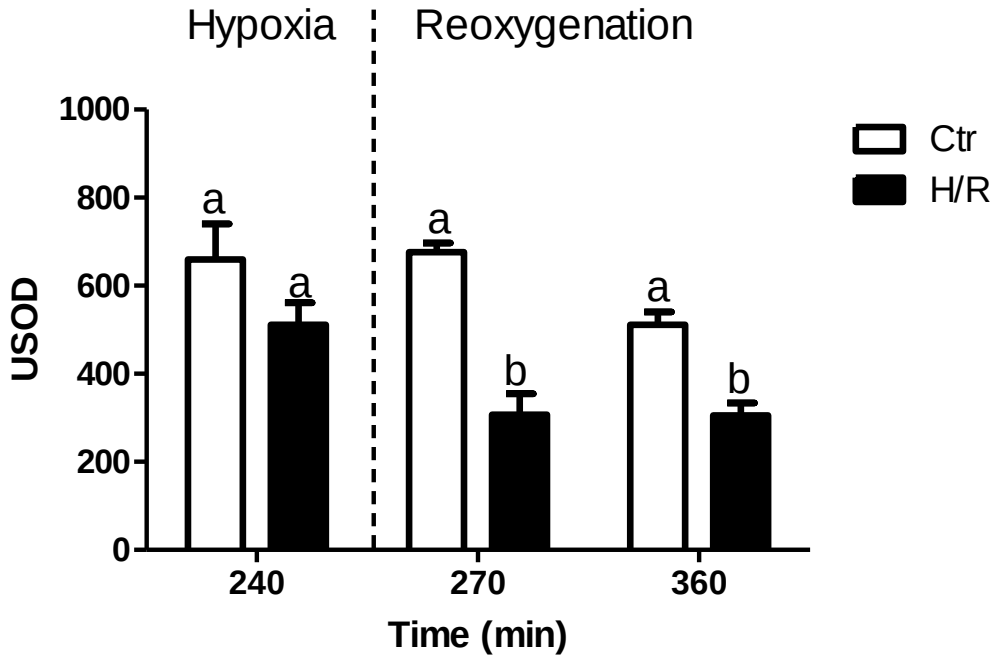
Hypoxia
Reoxygenation
1000
800
600
400
200
0
USOD
a
a
a
b
a
b
240
270
360
Time (min)
Ctr
H/R

Fig. 2

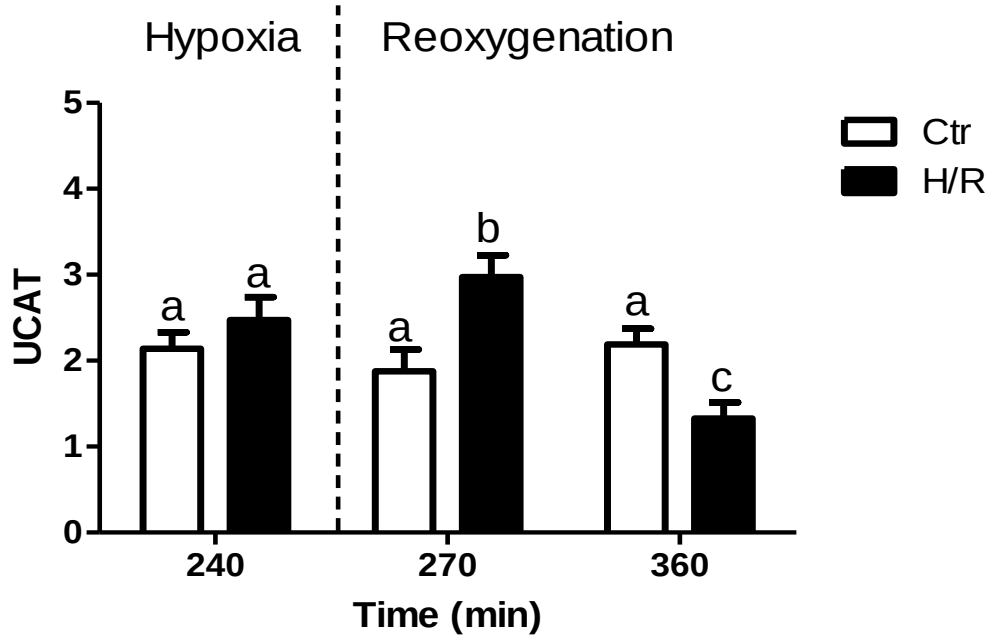
Hypoxia
Reoxygenation
Ctr
H/R
UCAT
0
1
2
3
4
5
a
a
a
b
a
c
240
270
360
Time (min)

Fig. 3

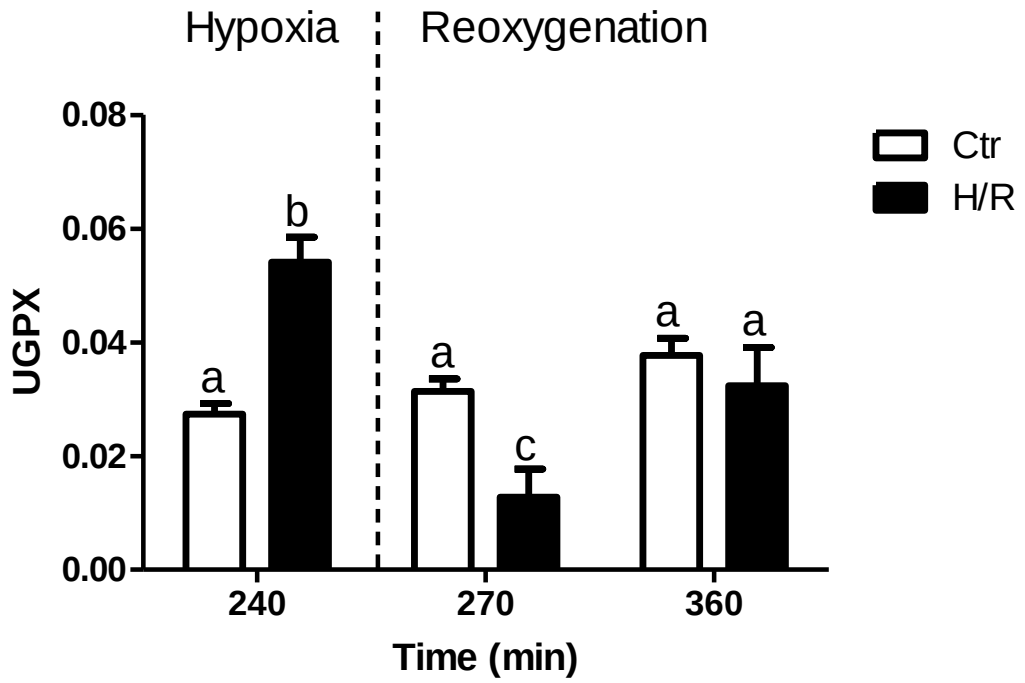
Hypoxia
Reoxygenation
Ctr
H/R
UGPX
0.08
0.06
0.04
0.02
0.00
a
b
a
c
a
a
240
270
360
Time (min)

Fig. 4

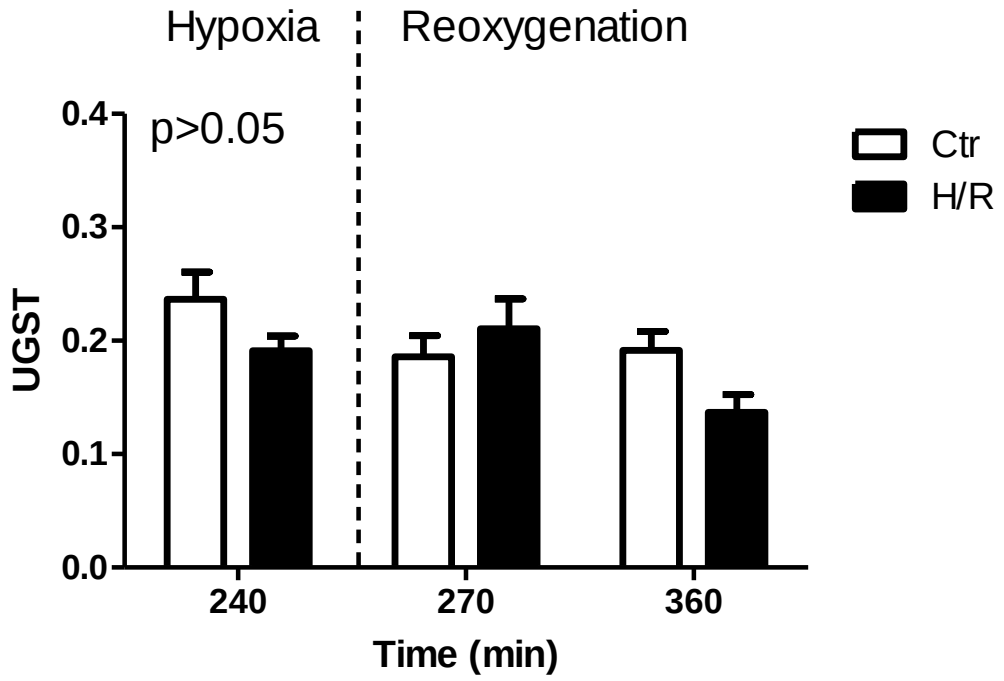
Hypoxia
Reoxygenation
p>0.05
Ctr
H/R
UGST
0.4
0.3
0.2
0.1
0.0
240
270
360
Time (min)

Fig. 5

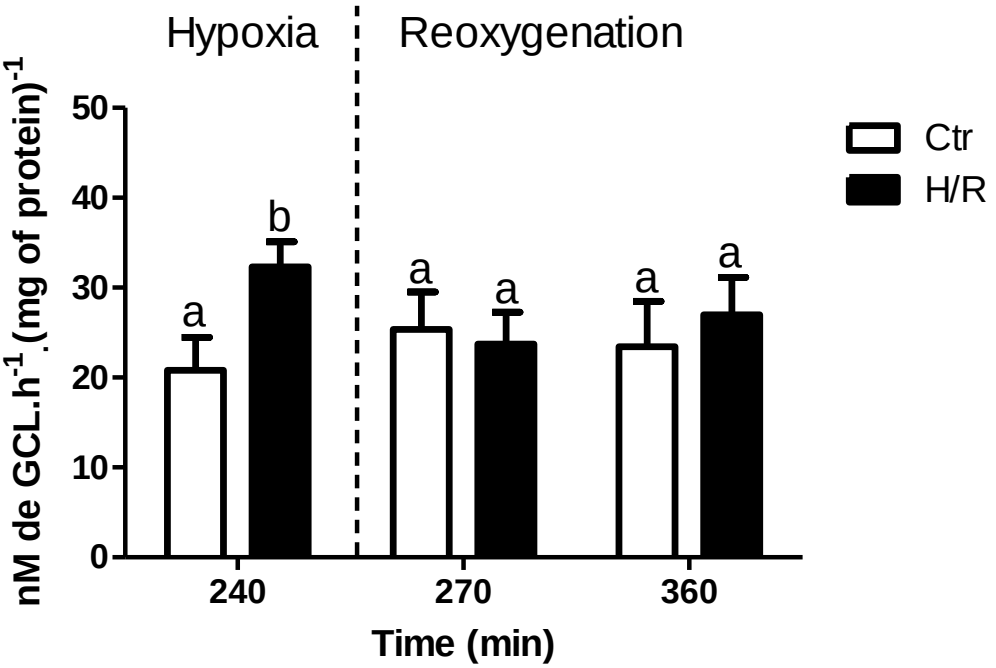
Hypoxia
Reoxygenation
nM de GCL.h-1.(mg of protein)-1
50
40
30
20
10
0
a
b
a
a
a
a
Ctr
H/R
240
270
360
Time (min)

Fig. 6

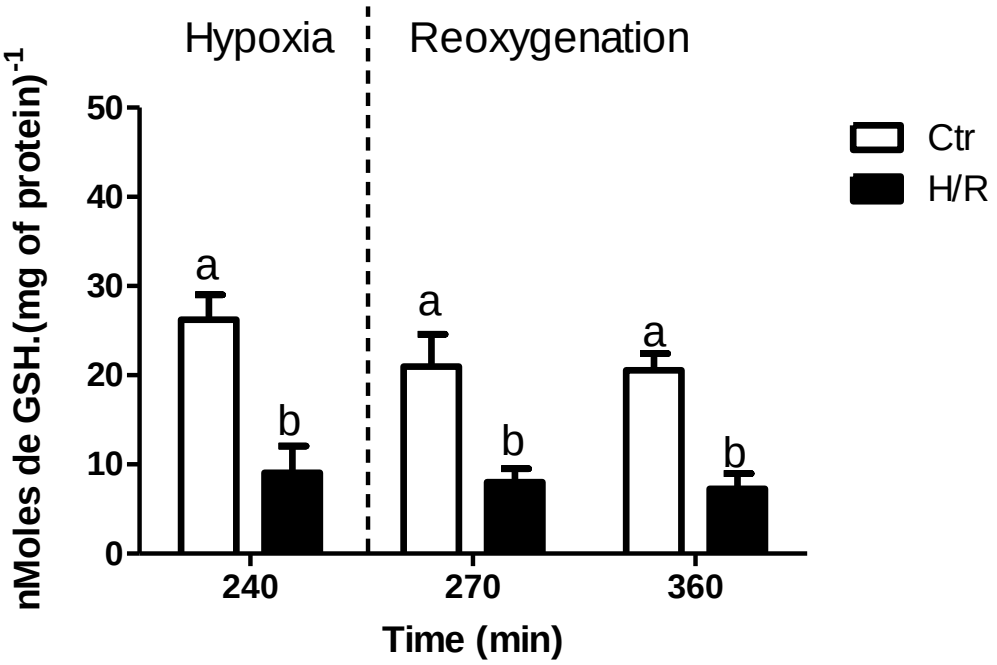
Hypoxia
Reoxygenation
nMoles de GSH.(mg of protein)-1
50
40
30
20
10
0
a
b
a
b
a
b
240
270
360
Time (min)
Ctr
H/R

Fig. 7

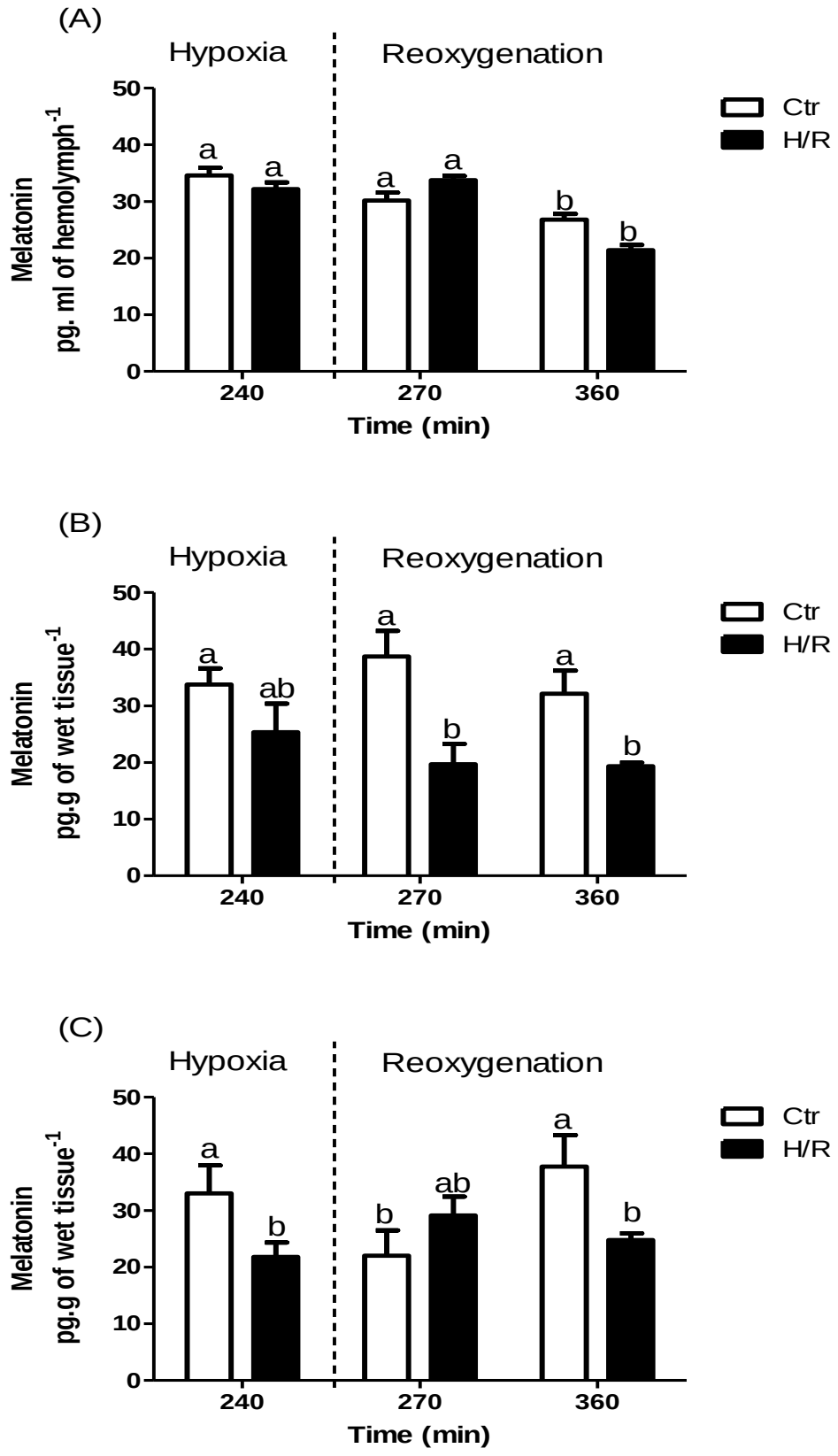
(A)
Hypoxia
Reoxygenation
Melatonin
pg. ml of hemolymph-1
50
40
30
20
10
0
a
a
a
a
b
b
240
270
360
Time (min)
Ctr
H/R
(B)
Hypoxia
Reoxygenation
Melatonin
pg.g of wet tissue-1
50
40
30
20
10
0
a
ab
a
b
a
b
240
270
360
Time (min)
Ctr
H/R
(C)
Hypoxia
Reoxygenation
Melatonin
pg.g of wet tissue-1
50
40
30
20
10
0
a
b
b
ab
a
b
240
270
360
Time (min)
Ctr
H/R

Fig. 8

IX. Conclusões

Quando o caranguejo *Neohelice granulata* foi exposto a vários níveis de hipoxia foi observado que esta espécie apresenta uma tolerância similar ao encontrado em outros crustáceos com uma CL_{50} entre 2,0 e 2,5 mgO_2/l. Além disso, quando exposto a hipoxia severa (0,5mgO_2/L) esta espécie apresentou uma TL_{50} de 14h iniciando a mortalidade (TL_{10}) a partir de 11h de exposição a hipoxia. O tempo necessário para recuperação desta espécie é proporcional ao tempo de exposição à hipoxia precisando, por exemplo, no mínimo 6h após exposição de 4h a hipoxia de 0,5mgO_2/L.

Durante hipoxia severa (0,5mgO_2/L) este caranguejo utiliza o metabolismo anaeróbico intensamente nas primeiras horas para manter o fornecimento de energia com sinais de ativar a depressão metabólica somente no seu limite de tempo de resistência. Além disso, as alterações mitocondriais observadas no músculo locomotor durante a hipoxia provavelmente estão sendo combatidas pela enzima glutationa peroxidase (GPx-Se) bem como pela GSH e melatonina mitocondrial, provavelmente responsáveis por evitar a geração de danos oxidativos neste momento.

Durante a reoxigenação observamos que não somente ocorreram alterações mitocondriais, mas também um aumento nos níveis de ERO e LPO e mais tardiamente uma hipertrofia nas fibras aeróbicas foram observados. Nesta fase de reoxigenação a catalase, GSH e melatonina citosólica são os principais constituintes do SDA evitando e recuperando os danos oxidativos. Estes resultados sugerem que este animal não apresenta depressão metabólica nas primeiras horas utilizando os componentes do seu SDA a fim de reparar e evitar os danos quando exposto por muito tempo a hipoxia severa.

X. Perspectivas

Ao final desta tese de doutorado muitas questões podem ser levantadas abrindo perspectivas que devem ser verificadas no futuro. Como foi observada neste trabalho uma diminuição da atividade mitocondrial, que esta associada a uma diminuição dos níveis de ATP durante a hipoxia, seria interessante analisar se outras vias metabólicas tais como da fosfoarginina esta sendo ativada no músculo locomotor durante a hipoxia como forma de auxiliar na produção de ATP, como já observadas em outros estudos com crustáceos.

Outra questão a ser verificada no futuro seria observar se realmente ocorre um aumento nos níveis de ERO nas mitocôndrias do músculo locomotor de *Neohelice granulata* quando exposto a hipoxia, o que corroboraria com a diminuição do potencial de membrana mitocondrial e o aumento na atividade das enzimas antioxidantes observadas neste trabalho. Além disso, se realmente observarmos um aumento nos níveis de ERO na fração mitocondrial do músculo locomotor seria interessante analisar quais os complexos enzimáticos que estão sendo responsáveis por este aumento durante a hipoxia e reoxigenação.

Na literatura alguns trabalhos realizados com espécies de crustáceos muito tolerantes a hipoxia observaram que uma estratégia para sobreviver mais tempo em hipoxia seria apresentar mitocôndrias insensíveis a Ca^{+} extracelular o que não acarretaria em um aumento na permeabilidade da membrana com diminuição do potencial de membrana mitocondrial. Como nesta tese observamos uma diminuição do potencial de membrana mitocondrial seria interessante analisar se as mitocôndrias do músculo de *N. granulata* apresentam uma sensibilidade a Ca^{+}.

A diminuição dos níveis de melatonina nas frações mitocondrial e citosólica e a não variação dos níveis de melatonina hemolinfática no músculo locomotor de *N. granulata* observadas neste trabalho possivelmente estejam indicando que a melatonina muscular apresenta uma ação antioxidante. Neste sentido, no futuro seria interessante verificar como é a metabolização desta molécula durante hipoxia e reoxigenação, e se as enzimas envolvidas na síntese de melatonina estão presentes no músculo locomotor.

Por fim, durante este trabalho observamos que a hipoxia e reoxigenação conduziu a diversas respostas tais como uma ativação do metabolismo anaeróbico durante a hipoxia, uma rápida reativação do metabolismo aeróbico na reoxigenação e um aumento na atividade de diferentes enzimas antioxidantes nas diferentes situações. Como na literatura tem sido sugerido para diversos grupos de animais que o fator induzido por hipoxia (HIF) atua como uma molécula sinalizadora de inúmeras respostas ao déficit de oxigênio seria interessante no futuro analisar se as respostas observadas no músculo de *N. granulata* são dependentes da HIF.

XI. REFERENCIAS DA INTRODUÇÃO GERAL

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XII. ANEXOS

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3. Monographs
4. Letters to the Editors
5. Book Reviews

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