Universidade Federal do Rio Grande – FURG

Instituto de Ciências Biológicas

Programa de Pós-Graduação em Ciências Fisiológicas

Fisiologia Animal Comparada

Tese de Doutorado

Papel da melatonina nas respostas metabólicas do caranguejo *Neohelice granulata* (Dana, 1851) (Decapoda; Brachyura)

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Tese defendida no âmbito do Programa de Pós Graduação em Ciências Fisiológicas-Fisiologia Animal Comparada como parte dos requisitos para obtenção do título de Doutor em Fisiologia Animal Comparada

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Rio Grande

2010

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I. Agradecimentos

Pela simples razão de nossa existência, agradeço a Deus por tudo de bom que aconteceu, acontece e estará por acontecer. Obrigado.

Bom, poderia descrever vários elogios aqui neste momento sobre meu orientador, amigo, companheiro, conselheiro, guru e etc., Prof. Dr. Luiz Eduardo Maia Nery (Carioca). Porém prefiro apenas declarar que tenho uma imensa gratidão por ti, não apenas pelos ensinamentos de FAC, mas sim de VIDA durante esta jornada (que ainda não acabou) longa, difícil, mas extremamente prazerosa. Obrigado.

Agradeço a banca examinadora desta tese (Profa. Dra. Guendalina Turcato de Oliveira, Prof. Dr. Fernando D'Incao, Prof. Dr. Elton Pinto Colares e Prof. Dr. Euclydes A. dos Santos) por ter aceitado participar e contribuir com seus conhecimentos de modo a melhorar este trabalho. Obrigado.

Também devo agradecimentos ao Prof. Dr. Horácio de La Iglesia por ter me recebido em seu laboratório na University of Washington (Seattle – EUA) e pela colaboração nesta tese. Obrigado.

Ao Prof. Dr. Ednei Primel e à Mestra Sergiane Caldas pelo apoio e empenho nas tentativas de dosagens de melatonina, ficam aqui meus agradecimentos. Obrigado.

A Profa. Dra. Silvana Allodi pelo apoio e contatos com o Prof. Dr. Olli Vakkuri, os quais estão sempre prontos a ajudar. Obrigado.

Ao Prof. Dr. Luis Fernando Marins (Luf) pelo apoio logístico, intelectual e moral no laboratório. Obrigado pela confiança.

Agradeço a CAPES pela concessão da bolsa de doutorado sanduíche que contribuiu para a conclusão desta tese.

Agradeço a paciência e tolerância do pessoal da salinha. Sei que algumas poucas vezes fui um pouquinho... assim...inconveniente (chato), mas é minha maneira de mostrar meu carinho por vocês. Obrigado.

Não poderia deixar mencionar a Bióloga Bruna Nornberg que nesta reta final me ajudou e me apoiou de maneira significativa, tanto profissional como pessoal. Obrigado pelos doces (pudim, ambrosia, arroz de leite, hummm) e salgados (lasanha, galinha descabelada, lentilha, hummm). Muito obrigado.

É óbvio que eu não esqueceria da gurizada medonha que me acompanhou e ainda acompanha nos experimentos, coletas, churrascos, X-box... Márcio (colono), Bruno e

Marcelinho, obrigado de coração pela amizade de vocês. Contem comigo sempre. Muito obrigado.

Aos meus pais (Vera Lúcia Félix Maciel e José Floriano Maciel) e meu irmão (Marcos Henrique Félix Maciel) deixo aqui meu carinho e agradeço pelos ensinamentos que irei carregar por toda a vida. Amo vocês.... Obrigado.

II. Resumo

Nos crustáceos a melatonina já foi identificada e alguns efeitos desta molécula nestes animais já foram observados tais como alteração no ritmo do eletroretinograma, regeneração de apêndices, ritmos de atividade locomotores, e mais recentemente no sistema de defesa antioxidante e metabolismo aeróbico. Estes últimos trabalhos utilizaram como modelo o caranguejo *Neohelice granulata*, que habita as marismas da região sul da América do Sul. Diante destes fatos, o primeiro objetivo desta tese de doutorado foi verificar como ocorre ajuste do metabolismo energético de *N. granulata* sob condições de hipoxia e anoxia e se estas respostas são dependente do pedúnculo ocular, bem como a identificação e expressão do gene do hormônio hiperglicemiante de crustáceos (CHH). Posteriormente, foi verificado se a melatonina exercia um efeito na expressão do CHH, consumo de oxigênio (VO_2) e concentrações de glicose e lactato circulante, além de verificar se os possíveis efeitos desta indolamina ocorrem através de receptores e também, se estes efeitos são dependentes do pedúnculo ocular. Na condição de anoxia, caranguejos ablados são significativamente menos tolerantes (TL_{50} = 8,5h) quando comparados aos intactos (TL_{50} = 14,4h). A concentração de 1 $mgO_2.L^{-1}$ significativamente (p <0,05) diminuiu o VO_2 e na hipóxia severa e anoxia (0,7 e anoxia) aumentaram os níveis de glicose (caranguejos intactos) e lactato (caranguejos intactos e eyestalkless) circulantes. Nenhuma diferença significativa na concentração de glicogênio e uma discreta alteração no teor de glicose no músculo e hepatopâncreas foram observadas entre os diferentes níveis de oxigênio dissolvido. Com relação a clonagem do CHH, foi obtido uma seqüência parcial contendo a parte final do peptídeo sinal, a seqüência completa do peptídeo relacionado ao precursor do CHH (CPRP) e cerca de 1/3 do CHH maduro, com uma similaridade de 73-82% para outros crustáceos. Em anoxia, expressão gênica do CHH foi significativamente (p <0,05) inibido. A melatonina diminuiu a expressão do CHH em animais expostos por 45 minutos a 6 (doses de 2, 200 e 20000 $\rho mol.animal^{-1}$) e a 2 (200 $\rho mol.animal^{-1}$) $mgO_2.L^{-1}$, porém este efeito não ocorre via receptores de melatonina, visto que luzindol (200 $\eta mol.animal^{-1}$) (antagonista dos receptores de melatonina) não alterou significativamente o efeito da melatonina. Já com relação a glicose e lactato a melatonina (200 $\rho mol.animal^{-1}$) aumentou os níveis destes compostos nos caranguejos expostos a 6 $mgO^2.L^{-1}$ e o luzindol (200 $\eta mol.animal^{-1}$) diminuiu este efeito, indicando o envolvimento de receptores de melatonina na hiperglicemia e lactemia em função da melatonina. Nenhum efeito da melatonina foi verificado no VO_2. Em conclusão, estes resultados sugerem que os

níveis de oxigênio dissolvido afetam o metabolismo energético em *N. granulata* e é dependente do CHH peduncular. Além disso, a melatonina mostrou ter um efeito sobre o metabolismo de *N. granulata*. Esta molécula inibiu a expressão do gene do CHH e promoveu uma hiperglicemia dependente do pedúnculo ocular e de receptores, o que sugere que a melatonina possa ter uma função sinalizadora na regulação do metabolismo deste caranguejo.

III. Abstract

In crustaceans, melatonin has already been identified and its effects have been observed on eletroretinogram rhythm changes, limb regeneration, locomotor activity rhythm, and recently in the antioxidant defense system and aerobic metabolism. These recent works used the crab *Neohelice granulata*, which inhabit salt marshes in southern Latin America. In this sense, the first objective of this doctorate thesis was to verify how is the energetic metabolism adjustment in the crab N. granulata under hypoxia/anoxia and whether these responses are eyestalk dependent, as well as the crustacean hyperglycemic hormone (CHH) cloning and gene expression. Next, we verified whether melatonin had an effect on CHH expression, VO_2 and hemolyphatic lactate and glucose concentrations, and whether these possible melatonin responses are eyestalk mediated and melatonin receptor dependent. In anoxia condition, eyestalkless crabs are significantly less tolerant (LT_{50} = 8.5h) compared to intact ones (LT_{50} = 14.4h). The concentration of 1 $mgO_2.L^{-1}$ significantly ($p<0.05$) decreased VO_2 and severe hypoxia/anoxia (0.7 and 0.0 $mgO_2.L^{-1}$) increased circulating glucose (intact crabs) and lactate (intact and eyestalkless crabs) levels. No significant differences in glycogen concentration and weakly alteration in glucose content in both muscle and hepatopancreas tissues were observed among the different dissolved oxygen concentration. A partial CHH gene encoding the final portion of signal peptide, complete CHH precursor related peptide and around 1/3 of mature CHH was cloned, with a similarity of 73-82% to other crustaceans. In anoxia, CHH gene expression was significantly ($p<0.05$) down regulated. Melatonin decreased the expression of CHH in animals exposed for 45 minutes to 6 (2, 200 and 20000 ρmol.animal^{-}1) and 2 (200 ρmol.animal^{-1}) $mgO_2.L^{-1}$, but this effect is not mediated through melatonin receptors, since luzindol (200 ηmol.animal^{-1}) (a melatonin receptor antagonist) did not significantly alter the effect of melatonin. Concerning glucose and lactate, melatonin (200 ρmol.animal^{-1}) increased levels of these compounds in crabs exposed to 6 $mgO_2.L^{-1}$ and luzindol (200 ηmol.animal^{-1}) decreased this effect, indicating the involvement of melatonin receptors in hyperglycemia. No effect of melatonin was observed on VO_2. In conclusion, these results suggest that dissolved oxygen levels affect energetic metabolism in *N. granulata* and it is dependent from the eyestalk. Moreover, melatonin showed an effect on the metabolism of *N. granulata*. This molecule inhibited CHH gene expression and promoted a hyperglycemia- eyestalk and receptors dependent, what suggest that melatonin may have a signaling function in the metabolic regulation in this crab.

IV. Introdução

A mais de 300 anos atrás, o filósofo francês René Descartes descreveu a glândula pineal como “o assento do espírito”. Contudo, apenas a partir da década de 50 a identidade química e a biossíntese da melatonina, o principal hormônio secretado pela glândula pineal de vertebrados, foi revelada. Melatonina (do grego *melano* = preto e *tonos* = cor) é uma amina biogênica com similaridades estruturais à serotonina. Como característica química, a melatonina (Figura 1) é uma molécula pequena (derivada do aminoácido triptofano), tendo uma alta lipofilicidade.

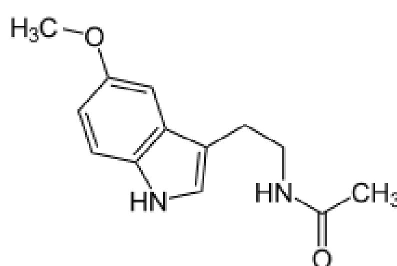

Figura 1: Representação esquemática da estrutura química da melatonina.

Esta molécula já foi identificada em vários organismos, desde vertebrados até organismos unicelulares. Contudo foi nos vertebrados, mais especificamente nos mamíferos, que sua síntese e função foram bem estudadas. Nos mamíferos, a melatonina, como descrito anteriormente, é produzida nas células da glândula pineal, o pinealócito, tendo uma síntese rítmica com um período circadiano (período que pode variar entre 20 e 28 horas). Nestas células o triptofano proveniente da corrente sanguínea é hidroxilado pela triptofano hidroxilase convertendo-o em 5-hidroxitriptofano (5-HTP) (Figura 2). O 5-HTP é então descarboxilado pela enzima 5-HTP descarboxilase em 5-hidroxitriptamina (5-HT) ou serotonina. Nesta etapa da síntese, entra em cena a serotonina-N-acetiltransferase (NAT), a qual converte a 5-HT em N-acetilserotonina. Estudos verificaram que a NAT é a principal enzima que modula a produção da melatonina, sendo caracterizada como a enzima chave do processo. Por fim, a N-acetilserotonina, pela ação da hidroxindol-O-metiltransferase (HIOMT), é convertida em N-acetil-5-metoxitriptamina ou melatonina (Marques e Menna-Barreto, 1997).

Nos vertebrados, além dos mamíferos, está bem estabelecido que a produção da melatonina ocorre de forma rítmica com um período circadiano, tendo uma maior produção durante a noite. Nestes animais a informação da variação da luminosidade externa, é captada pelos pigmentos fotorreceptores da retina, sendo transmitido para o núcleo supraquiasmático (NSQ) via trato retinoipotalâmico. O NSQ é o oscilador circadiano da produção da melatonina, visto que quando retirado ou lesado este componente, a ritmicidade da melatonina é abolida (van Esseveldt *et al.,* 2000). O NSQ também modula outros ritmos circadianos como atividade locomotora, ingestão de alimentos, temperatura corporal, atividade sexual, entre outros (Marques e Menna-Barreto, 1997; Hardeland 2008). Do NSQ a informação chega à glândula pineal via sistema nervoso simpático, estimulando a síntese da melatonina. No período noturno, como dito anteriormente, envolve a liberação de norepinefrina (NE) aumentando a atividade da NAT e conseqüentemente aumentando a produção e liberação da melatonina. A NE é liberada no espaço perivascular e se difunde para a superfície do pinealócito onde se liga e ativa receptores adrenérgicos α_1 e β_1. Estes receptores, em última instância, aumentam os níveis de AMPc dentro da célula, ativando a proteína quinase A (PKA). Em roedores, os níveis da NAT podem se elevar a partir de um aumento de sua transcrição pela atuação da PKA na fosforilação do fator de transcrição CREB. Contudo, na maioria dos mamíferos, o papel da PKA no pinealócito é ativar a NAT pré-existente dando seguimento ao processo de síntese da melatonina. Quando a NAT é fosforilada, além de ser ativada, esta enzima também adquire a capacidade de se ligar a proteínas do tipo 14-3-3, formando um complexo reversível evitando assim sua degradação por proteossomos (Ganguly *et al.*, 2002).

Apesar de a glândula pineal ser o órgão responsável pela maior parte da melatonina circulante nos fluídos corpóreos de mamíferos, outros tecidos e órgãos como a retina, glândula Harderiana, trato gastrintestinal, ovários e testículos são capazes de sintetizar esta indolamina, tendo uma função parácrina (Tan *et al.*, 1999). Em alguns destes locais os níveis desta molécula excedem várias vezes os níveis encontrados na glândula pineal podendo, ou não, apresentar um ritmo de produção circadiana (Messner *et al.*, 1998; Djeridane e Touitou, 2001).

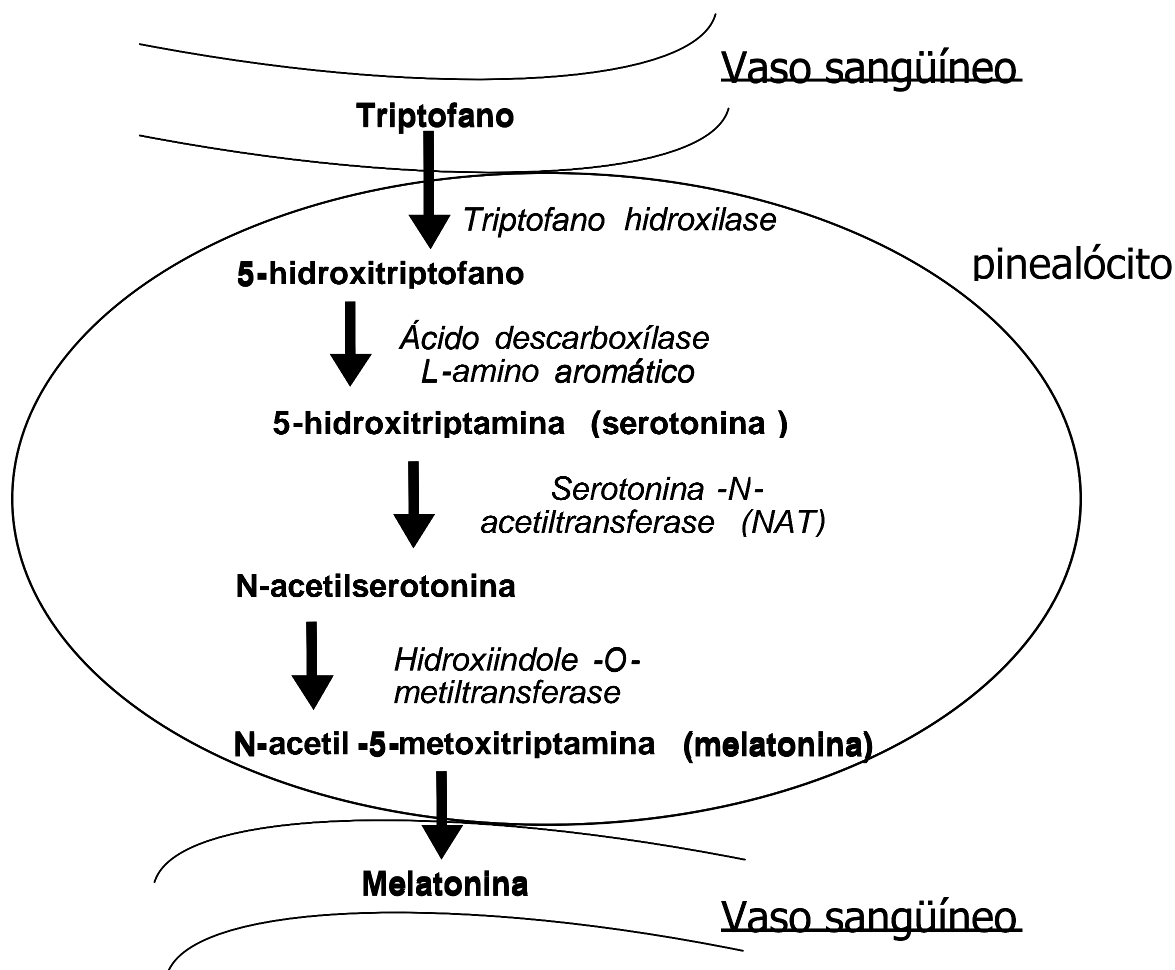

Figura 2. Representação esquemática da via de síntese da melatonina no pinealócito de mamíferos. Veja o texto para maiores explicações.

Os receptores de membrana já identificados para melatonina são classificados em MT1, MT2 e MT3 (Dubocovich, 1995; Boutin *et al*., 2005), sendo que os dois primeiros são conhecidos por serem acoplados à proteína G e o MT3 um receptor enzimático pertencente à família das quinonas redutases, porém sua via de sinalização ainda é pouco conhecida. MT1 e MT2 diferem cinética e farmacologicamente entre si. MT1 apresenta uma alta afinidade, normalmente na ordem de picomolar, e o MT2 apresenta uma menor afinidade, na ordem de nanomolar (Reppert *et al.,* 1996). MT1 apresenta ainda 3 sub-tipos, denominados Mel_{1a}, Mel_{1b}, Mel_{1c}, sendo que todos estão geralmente associados com a diminuição dos níveis de AMPc, enquanto MT2 está acoplado geralmente à hidrólise de fosfolipídeos de membrana (Reppert *et al*., 1996). Além da melatonina se ligar aos receptores de membrana, sua elevada lipofilicidade leva a sugestão de que suas ações sobre as células-alvo não sejam

necessariamente dependentes desses receptores. Acúmulos consideráveis de melatonina no núcleo de algumas células sugerem fortemente que esta indolamina possa dispor também de sítios nucleares (Menendez-Pelaez e Reiter, 1993). Ainda, a melatonina liga-se à calmodulina com alta afinidade (Benitez-King *et al.*, 1993), inibindo as atividades de fosfodiesterases e cálcio-ATPases, dependentes do complexo cálcio-calmodulina, alterando o conteúdo total dessa proteína reguladora nas células (Benitez-King *et al.*, 1991) e modificando sua distribuição sub-celular (Anton-Tay *et al.*, 1998). Desta forma, Huerto-Delgadillo *et al.* (1994) propõem que a modulação deste complexo possa ser um dos mecanismos de ação da melatonina.

Estudos demonstraram que os receptores MT1 e MT2 podem sofrer o processo de desensibilização em resposta a variações na concentração e ao tempo de exposição a melatonina. O processo de desensibilização ocorre quando um ligante específico se liga ao receptor podendo desensibilizá-lo, tornando o receptor menos responsivo ao ligante. Este fenômeno pode levar o receptor a entrar na célula, no fenômeno chamado de internalização, podendo retornar a superfície da membrana após certo tempo, ou ser degradado por proteases (Ferguson *et al.,* 1998).

Em vertebrados, em termos gerais, tem-se verificado que uma importante função da melatonina é a sinalização do comprimento da noite, e conseqüentemente do dia, preparando e regulando as funções fisiológicas do animal, de modo que este possa melhor enfrentar as variações ambientais que ocorrem sazonalmente. Segundo Reiter (1991), a melatonina pode ser considerada "a expressão química da noite", pois quanto maior o período da noite, maior o tempo de máxima produção desta indolamina. Para melhor ilustrar este fato, tomamos como exemplo a chegada do inverno. À medida que esta estação se aproxima, o comprimento da noite vai aumentando. Isto faz com que a produção da melatonina perdure por mais tempo, informando ao animal a se preparar (modificações na pelagem/plumagem, aumento na concentração de gordura) para as adversidades que estão por vir (Tamarkin *et al.*, 1985). Vários outros estudos têm evidenciado que a melatonina atua em diversas outras funções fisiológicas como na termorregulação, reprodução e atividades alimentar e locomotora (Lutterschmidt *et al.*, 2003; Mayer *et al.*, 1997; Lumineau *et al.*, 2002; Underwood, 1981, respectivamente).

A melatonina também apresenta uma marcada atuação na migração pigmentar de anfíbios e peixes, sendo esta a primeira função à ela atribuída, daí seu nome de melatonina (Lerner *et al.*, 1958). Em anfíbios este hormônio promove o clareamento da pele

provavelmente pela inibição da adenilil ciclase e uma diminuição da resposta a agonistas pigmentares, como o α-MSH (Filadelfi e Castrucci, 1996). Na maioria dos estudos realizados em peixes a melatonina tem acarretado a agregação dos pigmentos dentro das células pigmentares (Fujii, 2000). Contudo, Visconti e Castrucci (1993) verificaram que esta indolamina não tem nenhum efeito na agregação pigmentar nos melanóforos do elasmobrânquio *Potamotrygon reticulata*.

Inúmeros estudos têm também sido feitos sobre a capacidade antioxidante da melatonina. Barlow-Walden *et al.* (1995) demonstraram que administração de melatonina (500 μg/Kg) em ratos, aumenta em duas vezes a atividade da glutationa peroxidase (GPx) no cérebro destes animais. Adicionalmente, este trabalho mostrou que a atividade desta enzima neste tecido é maior na fase noturna, coincidindo com o período de maior produção de melatonina, sugerindo então que esta indolamina esteja modulando a atividade de enzimas antioxidantes como a GPx. Outros estudos indicam que este hormônio pode atuar como um “scavenger” (limpador) de espécies reativas de oxigênio (EROs), principalmente de radicais hidroxila ($HO^{\cdot}$) (Reiter *et al.*, 1996). Tan *et al.* (2000) reportaram que esta indolamina também atua como um limpador de peróxido de hidrogênio (H_2O_2). Tendo em vista que o radical $HO^{\cdot}$ é a espécie reativa mais danosa à lipídios, proteínas e DNA (Storey, 1996) e que seu precursor é o H_2O_2, a melatonina é considerada um importante antioxidante endógeno (Tan *et al.*, 2000). Adicionalmente, esta molécula não tem apenas a capacidade de modular a atividade de enzimas antioxidantes, mas também de modular a expressão gênica de enzimas antioxidantes como a superóxido dismutase (SOD) e a GPx (Mayo et al., 2002).

Como já comentado, a melatonina não é exclusiva de vertebrados, sendo encontrada em vários grupos de invertebrados e organismos unicelulares (Bittman, 1993), indicando uma origem filogenética antiga. A primeira evidência da presença desta indolamina nos invertebrados foi reportada nos olhos compostos de um inseto, o gafanhoto *Locusta migratória,* apresentando maiores valores na fase noturna (Vivien-Roels *et al.*, 1984). Para outro inseto, a mosca *Musca autumnalis*, foi reportado a presença da melatonina no cérebro com flutuações rítmicas, com um pico durante a noite (Wetterberg *et al.*, 1987). Até mesmo em organismos primitivos, como a planária *Dugesia dorotocephala* (Morita *et al.*, 1987) e a alga unicelular *Gonyaulax polyedra* (Poeggeler *et al.*, 1991), já foi verificada a presença desta indolamina. Esta molécula tem também sido encontrada nos pedúnculos oculares, cabeça, músculo e hemolinfa de crustáceos (Tabela 1), contudo o horário do dia de maior produção

não segue um mesmo padrão como é o caso nos vertebrados. Para uma mesma espécie, o lagostim *Procambarus clarkii*, em um estudo foi encontrado um pico de melatonina nos pedúnculos oculares durante o dia (Agapito *et al.,* 1995) e em outro estudo foi encontrado durante a noite (Balzer *et al*., 1997). No camarão de água doce *Macrobrachium rosenbergii* e no caranguejo *Uca pugilator* foram encontrados maiores valores de melatonina nos pedúnculos oculares na fase clara (Withyachumnarnkul *et al*., 1992a, 1992b; Tilden *et al*., 1997, 2001a). Contudo nestes mesmos tecidos em *Carcinus maenas* foram encontradas concentrações constantes durante o dia e noite, mas com diferenças entre os meses de Maio e Novembro (Vivien-Roels e Pévet, 1986). No camarão tigre *Penaeus monodon*, a melatonina foi identificada no lobo óptico, mas variações diárias não foram estudadas (Withyachumnarnkul *et al*., 1995). Meyer-Rochow (2001) observou que nos olhos do lagostim *Astacus fluviatilis* e na cabeça do isópode *Saduria entomon*, a concentração de melatonina não se diferenciou significativamente entre o dia e a noite, mas aparentemente apresentou uma variação sazonal. A ausência de variação diária na concentração de melatonina também foi verificada na hemolinfa do eufausiáceo *Euphausia superba* (Pape *et al*., 2008) e na lagosta *Nephrops norvegicus* (Aguzzi *et al*., 2009). Maciel *et al*. (2008) verificaram a presença da melatonina nos lobos ópticos do caranguejo *Neohelice granulata* com uma pequena variação diária nos níveis desta substância. Em animais mantidos por 10 dias em fotoperíodos de 12h claro e 12h escuro (12C:12E) e escuro constante (EE), foi observado um perfil bifásico na concentração de melatonina com um intervalo de 12 horas entre os picos. Já animais mantidos em claro constante (CC), a variação diária desta indolamina não foi observada (Maciel *et al*., 2008). Adicionalmente, Geihs *et al*. (2010) e Maciel *et al*. (2010) verificaram a presença desta molécula no músculo locomotor e na hemolinfa de *N. granulata*, respectivamente, porém variações diárias não foram estudadas.

Apesar de alguns estudos terem identificado a presença da melatonina nos crustáceos, sua função nestes animais ainda não foi bem elucidada. Balzer *et al*. (1997) verificaram que uma única aplicação de melatonina (10^{-4}M) aumenta o período e a amplitude do ritmo do eletroretinograma de *P. clarkii*. Tilden *et al*. (1997) também verificaram que a melatonina aumenta a taxa de regeneração de apêndices locomotores do caranguejo *U. pugilator.* Este efeito ocorreu tanto em animais intactos como apedunculados, demonstrando que o hormônio inibidor da muda (MIH), proveniente do complexo órgão-X-glândula do seio (OX-GS), não foi o alvo da atividade da melatonina. Nery *et al.* (1999) verificaram em eritróforos do camarão de água-doce *Macrobrachium potiuna*, que apesar da melatonina não

ter induzido agregação ou dispersão pigmentar *per se*, diminuiu significativamente a resposta ao hormônio concentrador de pigmentos vermelhos (RPCH). Mais recentemente foi observado que no caranguejo *N. granulata*, a melatonina não influenciou na migração dos pigmentos pretos tanto *in vitro* (10^{-10} – 10^{-7}mol.animal^{-1}) como *in vivo* (2.10^{-9}mol.animal^{-1}) (Maciel *et al.*, 2009). Tilden et al. (2003) reportaram para *P. clarkii* um aumento na transmissão sináptica nas junções neuromusculares quando injetados 10 μM de melatonina, indicando uma função moduladora na liberação de vesículas pré-sinápticas.

Tabela 1. Descrição das espécies de crustáceos nas quais já foi identificada a presença da melatonina. Adicionalmente são informados os tecidos analisados, se há diferenças entre o dia e a noite, as concentrações de melatonina encontradas, a técnica utilizada e a referência bibliográfica.

Espécie	Tecido	Variações Diárias	Concentrações	Técnica	Referência
C. maenas	Pedúnculo ocular	Sem variação	2,700 – 3,650 ρg.olho^{-1}	RIA	1
M. rosenbergii	Lobos ópticos	Dia	0.5 - 5.5 ρg.μg de proteína^{-1}	RIA	2
P. monodon	Lobos ópticos	Não estudado	30 – 35 ρg.lobos ópticos^{-1}	RIA	3
P. clarkii	Pedúnculo ocular e hemolinfa	Dia	50 – 656 ρg.olho^{-1} e 20 – 172 ρg.ml^{-1}	RIA	4
P. clarkii	Pedúnculo ocular	Noite	30 – 1,813 ρg. pedúnculo ocular^{-1}	RIA	5
U. pugilator	Pedúnculo ocular	Dia	110 – 230 ρg. pedúnculo ocular^{-1}	RIA	6, 7
A. fluviatilis	Olho	Sem variação	Não informado	RIA	8
S. entomon	Cabeça	Sem variação	Não informado	RIA	8
N. granulata	Lobos ópticos, hemolinfa e músculo	Dia e noite	7 – 20 ρg.lobos ópticos^{-1}; 102.8 ρg.ml^{-1} e 1.26 ng.mg de músculo^{-1}	RIA; ESI-LC-MS/MS	9, 10, 11
E. superba	Pedúnculo ocular e hemolinfa	Sem variação	6.3 ρg.pedúnculo ocular^{-1} e 2.9 ρg.ml^{-1}	HPLC/ELISA	12

N. norvegicus	Hemolinfa	Sem variação	40 ρg.ml^{-1}	LC-MS/MS	13

1. Vivien-Roels e Pévet (1993); 2. Withyachumnarnkul *et al*., 1992a; 3. Withyachumnarnkul *et al*., 1995; 4. Agapito et al. (1995); 5. Balzer et al. (1997); 6. Tilden et al. 1997; 7. Tilden et al. (2001); 8. Meyer-Rochow 2001; 9. Geihs et al. (2010); 10. Maciel et al. (2010); 11. Maciel et al. (2008); 12. Pape et al. (2008); 13. Aguzzi et al. (2009).

A melatonina, como reportado anteriormente, tem apresentado um importante papel no sistema de defesa de mamíferos atuando sobre as espécies reativas de oxigênio (EROs). As EROs, como por exemplo o radical ânion superóxido ($O_2^{\bullet-}$), o peróxido de hidrogênio (H_2O_2) e radical hidroxila ($HO^{\cdot}$), são capazes de promover sérios danos à célula, tais como peroxidação lipídica, oxidação de proteínas e danos no DNA (Storey, 1996). Para combater estas EROs os organismos desenvolveram um sistema de defesa antioxidante (SDA) capaz de prevenir, interceptar ou reparar as ações das EROs. Este sistema é dividido em antioxidantes não-enzimáticos (exemplos: ascorbato, α-tocoferol, β-carotenos e glutationa) e enzimáticos como a catalase (CAT), a glutationa peroxidase (GPx), a superóxido dismutase (SOD), a glutationa redutase (GR) e a glutationa-*S*-transferase (GST) (Storey, 1996; Dröge, 2002). Recentes trabalhos têm reportado a existência de variações diárias no SDA e danos oxidativos em vertebrados (Diaz-Muños *et al*. 1985; Pablos *et al*. 1998; Baydas *et al*. 2002). Com referência a crustáceos, apenas em três espécies (*Procambarus clarkii*, *Procambarus digueti* e *Neohelice granulata*) foram estudadas variações diárias nos SDA e danos oxidativos (Durán-Lizarraga *et al*., 2001; Fanjul-Moles *et al*., 2003; Maciel *et al*., 2004).

Como mencionado anteriormente, a melatonina tem sido alvo de inúmeros estudos sobre a capacidade antioxidante e regulação das enzimas antioxidantes nos vertebrados (Tan et al., 2000; Mayo et al., 2002). Adicionalmente, foi visto que esta molécula tem um efeito sobre a mitocôndria. Esta organela é responsável pela produção de energia, mas a geração de EROs é inevitável devido a fuga de elétrons na cadeia transportadora. Se a melatonina apresenta capacidades antioxidante e regulatória sobre o SDA, podemos inferir que esta molécula possa atuar sobre a mitocôndria. De fato, esta indolamina é capaz de atravessar membrana celular e mitocôndrial e de se ligar a receptores específicos (Yuan e Pang, 1991; Poon e Pang, 1992). Alguns trabalhos reportaram que a melatonina é capaz de aumentar a atividade respiratória dos complexos I e IV, permitindo uma melhor geração de ATP (Martin et al., 2000; Acuña-Castroviejo et al., 2001; 2003), e assim um melhor estado metabólico do animal. Nos crustáceos, poucos trabalhos relacionaram a melatonina com o metabolismo destes animais. Maciel et al. (2010) verificaram que a melatonina aboliu a

variação diária na capacidade antioxidante total (TOSC) e diminuiu o consumo de oxigênio (VO_2) *in vitro* nas brânquias de *N. granulata* quando injetados (2.10^{-12}mol.animal^{-1}) ou incubados (2.10^{-8}M) com melatonina. Estes foram os primeiros resultados apontando o envolvimento da melatonina no sistema de defesa antioxidante em crustáceos e tendo um possível papel depressor no metabolismo aeróbico neste caranguejo. Em outro estudo, Geihs *et al*. (2010) observaram que em caranguejos apedunculados a melatonina a curto prazo e em doses baixas induziram um aumento no VO_2 e na atividade da γ-GCL (enzima chave para a síntese de GSH) e da concentração de GSH, o que não aconteceu com altas doses de melatonina. Já a longo prazo, doses baixas diminuíram o TOSC e a atividade da catalase e em altas doses a melatonina apresentou outros efeitos diminuindo o VO_2 e aumentando o TOSC, sugerindo que a melatonina apresenta característica antioxidante dependentes da dose e do tempo.

Outros dois trabalhos verificaram efeitos da melatonina na regulação da glicose hemolinfática nos crustáceos. Tilden *et al*. (2001b) verificaram que no caranguejo *Uca pugilator* quando injetados com melatonina ($2,6.10^{-9}$mol.animal^{-1}) ocorria um deslocamento do ciclo circadiano de glicose e lactato na hemolinfa, com um único pico no meio da fase clara. Sainath e Reddy (2010) demonstraram um efeito dose-dependente da melatonina sobre a hiperglicemia no caranguejo *Oziotelphusa senex senex*.

Nos crustáceos, a hemolinfa, pelo fato de estar em contato com todos os órgãos e tecidos do animal, pode fornecer informações valiosas do estado metabólico geral. A concentração de glicose hemolinfática nestes animais permanece em uma faixa estrita, sendo finamente regulada (Verri et al., 2001). Enquanto que nos mamíferos, as concentrações mínimas de glicose circulante são ao redor de 5mM, nos crustáceos os níveis de glicose são geralmente mais baixos, variando de 0,03mM a 1,4mM dependendo da espécie (Morgulis, 1923; Keller e Orth, 1990; Garcia et al., 1993; Lüschen et al., 1993; Webster, 1996; Glowik et al., 1997; Hall e Van Ham, 1998). Esta glicose hemolinfática pode ter duas origens, uma proveniente da absorção da dieta no hepatopâncreas ou nas células epiteliais intestinais, a outra oriunda de estoques teciduais de glicogênio como, por exemplo, o hepatopâncreas (Gibson e Barker, 1979; Loret, 1993) e músculo (Nery et al., 1993) ou mesmo sintetizada através de vias gliconeogênicas (Verri et al., 2001).

Em vários estudos, a concentração de glicose hemolinfática tem sido analisada frente a diversas situações imposta ao animal tais como temperatura (Spicer et al., 1990; Morris e Olivier, 1999; Durand et al., 2000; Speed et al., 2001; Ridgway et al., 2006), salinidade

(Spaargaren e Haefner, 1987), manuseio (Paterson et al.,1997; Bergmann et al., 2001), poluentes (Lorenzon et al., 2000), imersão/emersão (Morris et al., 1986; Santos e Colares, 1986; Spicer et al., 1990; Morris e Olivier, 1999; Speed et al., 2001), hipoxia (Zou et al., 1996; Maciel et al., 2008; Silva-Castiglioni et al., 2010) ou mesmo a diferentes horários do dia (Hall e Van Ham, 1998; Aguzzi et al., 2004). Na maioria dos casos o efeito observado é uma hiperglicemia hemolinfática a fim de suprir todos os órgãos, ou pelo menos os mais vitais, frente a estas diferentes situações de estresse (Lorezon, 2004).

O primeiro relato de regulação endócrina da glicemia em crustáceos foi feito por Abromowitz et al. (1944), quando injetou extrato de pedúnculo ocular do caranguejo *Uca pugilator* no siri azul *Callinectes sapidus*, indicando a existência de um fator diabetogênico no pedúnculo ocular de crustáceos. Em estudos subseqüentes, a denominação de hormônio hiperglicemiante de crustáceos (CHH – crustacen hyperglycemic hormone) para este fator foi mais bem aceito (Kleinholtz et al., 1967; Kleinholtz, 1976; Keller e Andrew, 1973). Porém, apenas em 1989, Kegel e colaboradores isolaram e caracterizaram o CHH do caranguejo *Carcinus maenas*. Posteriormente, vários outros estudos também isolaram e caracterizaram este neuropeptídeo de várias espécies de crustáceos (para revisão veja Keller, 1992; Chang, 2001; Chan et al., 2003), verificando que se trata de um polipeptídeo de 71 – 81 aminoácidos, variando de 8000 a 9000 Da e possuindo 6 resíduos de cisteína conservados. Em uma variedade de crustáceos, moléculas estruturalmente similares ao CHH, porém com algumas diferenças na seqüência de aminoácidos e com outras funções mais marcantes além da hiperglicemia, também têm sido identificadas como o hormônio inibidor da muda (MIH- moult-inhibiting hormone), hormônio inibidor da vitelogênese (VIH - vitellogenisis inhibiting hormone) e hormônio inibidor do órgão mandibular (MOIH – mandibular-organ inhibiting hormone) (Webster, 1991; Wainwright et al., 1996; Yang et al., 1996; Chang, 2001), formando então uma família de neuropeptídeos.

Apesar dos vários estudos realizados através de ensaios de imunocitoquímica e hibridização *in situ* demonstrando que o principal sítio de produção do CHH é o pedúnculo ocular, mais especificamente o complexo OX-GS (Dircksen et al. 1988; Marco et al., 2003; Tsai et al., 2008), outros sítios de produção de CHH além do OX-GS foram observados, como a retina de *Procambarus clarkii* (Escamilla-Chimal et al., 2001), o protocérebro de *Metapenaeus ensis* (Gu et al., 2001) e *Armadillidium vulgare* (Azzouna et al., 2003), os gânglios torácico e protorácico de *Homarus americanus* (Chang et al., 1999) e *Metapenaeus ensis* (Gu et al., 2001), o órgão pericárdio, os intestinos anterior e médio e neurônios

abdominais periféricos de *Carcinus maenas* (Keller et al., 1985; Chung et al., 1999; Webster et al., 2000; Dircksen et al., 2001; Chung e Webster, 2004) além do coração e brânquias de *Penaeus monodon* (Udomkit et al., 2004).

Como mencionado anteriormente, a mobilização de glicose dos estoques teciduais é fundamental para a realização dos processos fisiológicos quando uma maior demanda energética é necessária. Vários estudos verificaram que a glicose hemolinfática, dependendo da espécie, apresenta uma variação diária (Aréchiga et al., 1985; Kallen et al., 1990; Tilden et al., 2001a; Aguzzi et al., 2004; Sathyanandam et al., 2008; Fanjul-Moles et al., 2010), geralmente correlacionada com a atividade locomotora, ou seja, uma maior concentração de glicose circulante quando a locomoção é mais intensa e concentrações basais de glicose no período de menor atividade (Hamann, 1974; Kallen et al., 1988, 1990).

O neurohormônio CHH tem como principal efeito aumentar a glicose circulante em crustáceos. Portanto, se vários estudos verificaram uma variação diária na concentração de glicose hemolinfática nestes animais, é sugestivo pensar que o CHH seja a causa desta variação e também apresente uma oscilação diária em sua concentração. De fato, alguns estudos verificaram variações diárias na concentração hemolinfática ou na liberação de CHH de células neurossecretoras do pedúnculo ocular de crustáceos. Gorgels-Kallen e Voorter (1985) verificaram através de imunocitoquímica que células secretoras de CHH no OX-GS do lagostim *Astacus leptodactylus* apresenta uma atividade rítmica, relacionada com o aumento da glicose hemolinfática 2-4 horas mais tarde, acoplada ao ciclo claro/escuro (Kallen et al 1988). Já no lagostim *Orconectes limosus*, Kallen et al. (1990) observaram um aumento na concentração de CHH hemolinfática por imunoquímica (ELISA) concomitante ao aumento de glicose ao longo do ciclo claro/escuro. Escamilla-Chimal et al. (2001) e Fanjul-Moles (2010) reportaram para o lagostim *Procambarus clarkii* também uma variação diária na concentração de CHH (imunocitoquímica e *western blot*) na retina e pedúnculo ocular. Nestes trabalhos foram observados que o mecanismo de regulação diária da síntese de CHH pode ser diferenciada nestes dois tecidos devido a diferença de fase no ritmo de produção deste neurohormônio. Adicionalmente, estes autores postulam que o CHH retinal tenha uma ação mais local, enquanto que o CHH proveniente do OX-GS atue de maneira mais ampla sobre o organismo.

Podemos observar que o CHH tem um importante papel na regulação de mecanismos adaptativos não apenas a condições de estresse, mas também na própria regulação metabólica diária nos crustáceos. Alguns trabalhos verificaram (tanto *in vitro* como *in vivo*)

que a liberação/atuação do CHH se dá, principalmente por um duplo sistema de retroalimentação (Glowik et al., 1997; Santos e Keller, 1993a; 1993b; Santos et al., 2001). Experimentos *in vitro* verificaram que neurônios produtores de CHH do complexo OX-GS são inibidos em função de glicose hemolinfática e a glicose, inibindo, portanto a liberação do CHH e conseqüentemente, a glicogenólise dos estoques de glicogênio. Já experimentos *in vivo*, revelaram uma retroalimintação positiva através do lactato. Devido a um aumento no fluxo glicolítico, o incremento na formação de lactato poderia estimular a liberação de CHH promovendo um aumento da glicogenólise. No tecido alvo, foi verificado que este peptídeo ativa a síntese de GMP através da ativação da adenilil ciclase (Goy, 1990), resultando na inibição da enzima glicogênio sintase e ativação da enzima glicogênio fosforilase e, portanto, promovendo a glicogenólise (Sedlmeier, 1982, 1988; Keller e Orth, 1990).

Condições de hipoxia e/ou anoxia em vários ambientes aquáticos são relativamente comuns, o que implica em importantes adaptações bioquímicas aos organismos que ali habitam, tais como depressão da taxa metabólica, manutenção de altos níveis de combustíveis energéticos, como glicogênio, para produção de ATP por vias fermentativas (Storey e Storey, 1990; Hervant et al., 1995; Lutz e Storey, 1997; Childress e Seidel, 1998; Hochachka e Lutz, 2001). De fato, espécies de crustáceos decápodes que habitam em tocas são geralmente mais tolerantes a hipoxia/anoxia em relação a outros crustáceos, justamente por manterem grandes quantidades de fontes de energia, como glicogênio e fosfoarginina, e pela redução da taxa metabólica, como a diminuição do consumo de oxigênio (Hervant et al., 1999). Adicionalmente, estes animais devem de alguma forma regular a produção de metabólitos oriundos da via anaeróbica como o lactato. Durante a respiração anaeróbica, vários estudos verificaram que o principal produto em crustáceos é o lactato (Gäde, 1984; Henry et al., 1994) que pode ter, a princípio, três destinos: completa oxidação, excreção e/ou reconversão à produtos energéticos como o glicogênio (Ellington, 1983). No caranguejo *Neohelice granulata*, em condições de hipoxia/anoxia o principal metabólito é o lactato, sendo encontrado em grandes concentrações. No hepatopâncreas e no músculo, foi visto que o lactato é convertido a glicose (gliconeogênese) durante períodos de hipoxia/anoxia, provavelmente para regular o balanço ácido-base e o aporte energético (Oliveira et al., 2004; Maciel et al., 2008b). Ainda nestas condições de hipoxia/anoxia ou expostos ao ar atmosférico, o aumento da glicose hemolinfática já foi descrito para esta espécie, o que sugere o envolvimento do CHH (Santos e Colares, 1986; Santos e Keller, 1993; Oliveira et al., 2001; Maciel et al., 2008).

Alguns estudos verificaram a variação dos níveis de CHH em crustáceos frente a condições de hipoxia/anoxia. No lagostim *Orconectes limosus* submetidos a hipoxia, a concentração de CHH hemolinfática aumentou em 15 minutos (Keller e Orth, 1990). Em *Cancer pagurus*, um aumento de CHH na hemolinfa foi observado após 4 horas de emersão (Webster, 1996). Chung e Zmora (2008) também verificaram um aumento na concentração deste peptídeo na hemolinfa de *Callinectes sapidus* após 1 hora de hipoxia. Adicionalmente, estes autores observaram uma diminuição na expressão gênica do CHH do pedúnculo ocular frente a hipoxia, talvez pelos altos níveis de glicose circulante causados pela maior liberação deste neurohormônio, inibindo assim a síntese deste peptídeo.

Como dito anteriormente, variações diárias de glicose e lactato hemolinfática em crustáceos já foram verificadas (Aréchiga et al., 1985; Kallen et al., 1990; Tilden et al., 2001a; Aguzzi et al., 2004; Sathyanandam et al., 2008; Escamilla-Chimal et al., 2010), sendo correlacionadas a momentos de maior atividade do animal. Interessantemente, em algumas espécies de crustáceos a melatonina varia conforme a atividade locomotora ou metabolismo aeróbico da espécie estudada (Balzer et al., 1997; Maciel et al., 2008). Portanto, é plausível inferirmos que a melatonina possa ter algum efeito sobre os níveis de carboidratos e seus produtos metabólicos na hemolinfa destes animais. De fato, apenas dois trabalhos exploraram este assunto. Tilden et al. (2001a) observou que a melatonina altera o ciclo de glicose e lactato na hemolinfa de *Uca pugilator* e que este efeito não é exclusivamente dependente do pedúnculo ocular. Já Sainath e Reddy (2010) verificaram um efeito dose-dependente da melatonina na hiperglicemia do caranguejo *Oziotelphusa senex senex*, tanto em animais íntegros como ablados. Este fato sugere que a melatonina possa estar atuando não apenas no pedúnculo ocular, mas também em outros sítios de produção e liberação de CHH.

Além das diversas funções anteriormente descritas para a melatonina, foi visto que esta molécula tem a capacidade, nos mamíferos, de influenciar a secreção de insulina, e na regulação do metabolismo de lipídeos e glicose (Hoyos et al., 2000; Nishida et al., 2003; Peschke, 2008; Robeva et al., 2008; Zanquetta et al., 2003). Adicionalmente, alguns trabalhos observaram que esta indolamina pode modular a liberação de substâncias, com funções hormonais, em insetos. Richter et al. (1999) verificou que a melatonina ocasiona uma liberação do hormônio protoracicotrópico no cérebro da barata *Periplaneta americana*, e este efeito foi inibido pelo luzindol, um antagonista do receptor pré-sináptico da melatonina. Em outro estudo, foi visto que a melatonina também promove, *in vitro*, a liberação de

peptídeos da *corpora cardíaca* do gafanhoto *Locusta migratoria*, tais como peptídeo relacionado ao precursor do hormônio adipocinético (APRP), neuroparsinas e peptídeo diurético (Huybrechts et al., 2005). Já nos crustáceos, o único trabalho semelhante foi realizado por Tilden et al. (2003), o qual observou um aumento na transmissão sináptica nas junções neuromusculares quando injetados com melatonina em *P. clarkii*. Contudo, já é sabido que a serotonina (um precursor da melatonina) tem um efeito hiperglicemiante nestes animais (Bauchau e Mengeot, 1966; Keller e Beyer 1968; Lüschen et al., 1993; Santos et al., 2001), e pelo menos em *Palaemon elegans*, este efeito é via liberação de CHH, como demonstrado por Lorezon et al. (2005), o qual verificou um aumento na concentração de CHH hemolinfática 30 minutos após tratamento com serotonina.

Diante dos fatos apresentados, esta tese de doutorado está constituída de 3 artigos relacionados com efeitos da hipoxia/anoxia e melatonina no metabolismo de crustáceos e a dependência do pedúnculo ocular nestas respostas. O primeiro artigo é uma revisão sobre as oscilações do sistema de defesa antioxidante nos crustáceos e o possível efeito da melatonina. Já o segundo artigo mostra a dependênia do pedúnculo ocular na sobrevivência e nos ajustes metabólicos de *N. granulata* sob condições de hipoxia e anoxia, bem como a clonagem do gene do CHH e sua expressão. Por fim, o terceiro artigo mostra o efeito da melatonina na expressão do CHH, VO_2, glicose e lactato circulantes frente a hipoxia e a dependência destes efeitas de receptores de melatonina.

V. Objetivos

O objetivo desta tese de doutorado foi verificar se a melatonina possui um papel na regulação do metabolismo aeróbico e anaeróbico no caranguejo *Neohelice granulata*, bem como na expressão gênica do CHH. Como objetivos específicos temos:

1. Verificar se a mortalidade, consumo de oxigênio, glicose e lactato hemolinfáticos, conteúdo de glicogênio e glucose no músculo e hepatopâncreas variam em função da hipoxia e anoxia;

2. Clonar o gene do CHH dos pedúnculos oculares de N. granulata e verificar se este gene apresenta uma variação na expressão em função da hipoxia e anoxia;

3. Verificar o efeito da melatonina na expressão gênica do CHH e se este efeito é mediado por receptores de melatonina;

4. Verificar o efeito da melatonina no consumo de oxigênio, glicose e lactato hemolinfáticos e também se este efeito é mediado por receptores de melatonina.

VI. Artigo 1

Ritmos no sistema de defesa antioxidante em crustáceos e possíveis papeis para a melatonina

Artigo publicado no periódico Frontiers in Bioscience (E2, 1448-1459)

Antioxidant defense system rhythms in crustaceans and possible roles for melatonin

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1. ABSTRACT

Animals in their habitats are subject to many cyclical patterns for different environmental parameters, resulting in selective pressure to develop biological rhythms for metabolism. To avoid oxidative stress, a rhythmic variation in the antioxidant defense system (ADS) should be associated with aerobic metabolic rhythms. In this review, we summarize and discuss the latest findings on rhythmic variations of the ADS in different tissues of crustaceans, as well as possible mechanisms for their regulation. In vertebrates, melatonin has been shown to be an important molecule in the regulation of the ADS and to be a high-capacity scavenger of reactive oxygen species. Given that this indoleamine has been identified in crustaceans, we also discuss the possible implications of this molecule in crustacean ADS regulation.

2. INTRODUCTION

The radical transformation of the primitive atmosphere, from an extremely reducing to an oxidizing environment, is considered to be a landmark in the evolution of different types of organisms. The simple act of fixing CO_2 in the presence of light to produce glucose and O_2 allowed the emergence of new organisms capable of oxidizing organic compounds to produce energy for their development and evolution. Although these aerobic organisms benefited from such an efficient method for the acquisition of energy from oxidized organic

compounds, they were (and are) also affected by molecules generated during these oxidative processes that are extremely reactive and have the potential to cause damage (1). In fact, these so-called reactive oxygen species (ROS), e.g., hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot -}$) and hydroxyl radical (HO·), can react with protein, lipids and DNA and/or disrupt the redox state of the cell (1-3). To avoid this drawback of oxidative metabolism, aerobic organisms must have a specialized antioxidant defense system (ADS) that counteracts the activities of ROS. The ADS is composed of enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-*S*-transferase (GST), as well as non-enzymatic molecules, such as α-tocopherol, ascorbate, β-carotenes and the tripeptide glutathione (GSH) (4, 5). GSH is particularly important for the redox state of the cell (3-5). Therefore, any disruption of redox signaling and/or any oxidative damage to biomolecules will cause oxidative stress, potentially affecting the survival of the animal in its habitat (1, 3, 4).

In association with the development of ADS, aerobic organisms developed mechanisms to anticipate changes in environmental stressors over time (hours, days, seasons) to avoid oxidative stress. Biological rhythms were developed due to the predictability of natural and cyclical changes in environmental factors such as photoperiod, temperature and salinity. These biological rhythms occur in almost all groups of animals and, in many cases, are linked with metabolic adjustment, involving signaling molecules, key enzymes and hormones (6-10).

Melatonin is an important molecule that is virtually ubiquitous in living animals and is involved in the modulation of many biological rhythms in vertebrates (11). This indoleamine was first isolated in the 1950s by Lerner and colleagues from thousands of bovine pineal glands (12). At least in vertebrates, melatonin production from the pineal gland peaks during the dark phase and is modulated by the key enzyme arylalkylamine N-acetyltransferase (aaNAT). Although melatonin synthesis also occurs in other tissues, such as the gonads, the harderian gland and the intestines, melatonin from these tissues may not have the same temporal profile. In these cases, melatonin may have a paracrine function instead of being released into the bloodstream to control the biological rhythms of different tissues (13-15). Along its evolutionary history, melatonin has become a multifunctional molecule, with actions varying from pigment migration (the first verified effect, hence its name) to serving as an internal signal for photoperiod length (12, 13). In the last decades, melatonin has been studied not only due to its ROS scavenging capacity but also due to its ability to modulate some components of the ADS (6, 8, 16). Given that melatonin has antioxidant capabilities and modulates a variety of biological rhythms, it may have an important role in the modulation of ADS rhythms in animals.

The Subphylum Crustacea comprises more than 50,000 described species, with an unknown number of species yet to be discovered (17). All crustaceans must inhabit water during the early stages of their development, but can subsequently inhabit every possible habitat (terrestrial, semi-terrestrial and fresh and marine aquatic environments). Studies are performed on these animals because they have multiple important roles: as significant members within the food chain (e.g., krill and copepods), as good indicators of contaminated

and polluted environments (e.g., barnacles), as part of commercial fishing and aquaculture (e.g., lobster, shrimp and crabs), and as good models for evolution and adaptation studies (all kinds of crustaceans) (17-20).

In this review, we analyze the latest findings on ADS rhythms in the different tissues of crustaceans and discuss how this system is regulated. In addition, the variation of melatonin content in crustaceans and its effects will be correlated with findings in vertebrate animal models and discussed.

3. RHYTHMIC VARIATIONS OF THE ANTIOXIDANT DEFENSE SYSTEM (ADS) IN DIFFERENT TISSUES OF CRUSTACEANS

The ADS system of any organism has a close relationship with aerobic metabolism. Any variation in aerobic metabolism function should generate a corresponding variation in ROS production, which should, in turn, induce an alteration of the ADS system to avoid cell disturbance. Due to the natural photoperiod, animals generally possess locomotor activity fluctuation along the day/night cycle, which also affects aerobic metabolism. Locomotor activity rhythms and oxygen consumption (VO_2) have been studied in crustaceans for a long time (21-29). In the portunid crabs *Callinectes similis* and *Portunus spinicarpus* and in the shrimp *Farfantepenaeus aztecus*, for example, higher VO_2 was shown to occur in the same period as higher locomotor activity (26). In contrast, few studies have addressed the daily variations in the content or activity levels of components of the ADS system and their relationships with aerobic metabolism. Prieto-Sagredo (30) observed that different photoperiods and light intensity affect the hemolymph redox state in the crayfish *Procambarus clarkii* and *Procambarus digueti*. When both species were exposed to a 20:4 photoperiod, the level of GSSG (oxidized glutathione, a pro-oxidant molecule) increased, indicating photo-oxidative stress due to the decrease in the GSH/GSSG ratio. In fact, the 20:4 light-darkness (LD) cycle produced a small, but noticeable, increase in the rate of crayfish mortality. One year later, Durán-Lizarraga (31) reported the first known rhythmic variation of the crustacean ADS, in *P. clarkii*. When the crayfish was submitted to 12:12 and constant darkness (DD) photoperiods, the GSH/GSSG ratio and GR activity in the midgut and the GSH/GSSG ratio in hemolymph showed characteristics of a circadian rhythm due to their free running in DD. A particularly interesting finding of this study was the correlation of GSH status (GSH and GSSG content and GSH/GSSG ratio) argetwith scotophase, given that *P. clarkii* is a nocturnal species (32) and its aerobic metabolism may be high during this period. Along a similar vein, Fanjul-Moles and colleagues (33) studied the same components of the ADS in the midgut gland and hemolymph of *P. digueti*, in addition to measuring GPx activity in the midgut gland of both *P. digueti* and *P. clarkii*. Some differences between the ADS of the two both species were observed in this study. As previously observed, the 20:4 LD cycle and increased light irradiance altered the GSH system in *P. digueti*, increasing GSH in the midgut and GSSG in hemolymph. The activities of GR and GPx in *P. digueti* and of GPx in *P. clarkii* were higher at scotophase, which again fits with the period of maximal locomotor activity for these species. Given that the activities of GPx in *P. digueti* and GR in *P. clarkii* varied in a circadian fashion, the midgut gland could potentially be coupled to the circadian clock, regardless of clock location (including within its own digestive system), whereas GSH status in the hemolymph may be affected by the oxidative balance of many tissues. Recently, the same group (34) published new data about the ADS in *P. clarkii*, focusing on the nervous

tissue, complex optic lobe-brain (OL-B) and retina, which are putative pacemaker sites for circadian rhythms in this species (35). Again, some components of the ADS from both tissues displayed characteristics of circadian rhythms under the DD condition but not as clearly under the 12:12 LD condition. In fact, an increase in the GSH/GSSG ratio was reported under LD 12:12 photoperiod treatment, indicating that the ADS was up-regulated to avoid photo-oxidative stress. This study also examined daily variations in the levels of lipoperoxidation (LPO), which is a measure of damage index. Under the 12:12 LD cycle, the maximum values in the OL-B were found at scotophase, whereas the maximum values in the retina were found at photophase. These data are consistent with the expected results due to the effect of light. In contrast, a paradoxical result was observed in the retina under the DD condition, in which the LPO was higher in the total absence of light than in the 12:12 LD. A possible explanation is that the electrical activity of these sensorial cells is augmented to favor ROS generation and thus results in increased damage. With respect to the ADS, an interesting observation that emerged from these studies is that the midgut gland and the complex optic lob-brain seem to be coupled by two self-sustained oscillators, whereas the retina appears to be a passive oscillator coupled with the zeitgeber light/dark cycle.

Crustacean ADS rhythms have also been investigated in our laboratory in the estuarine crab *Neohelice granulata* (previously known as *Chasmagnathus granulata/granulatus* – see ref. 36), which is a brachyuran species. This species is a semi-terrestrial crab with nocturnal locomotor activity (37). Under a 12:12 LD condition, Maciel and colleagues (29) verified two peaks in oxygen consumption in the gills and hepatopancreas of these animals that were separated by 12-h intervals. This observation leads to an interesting question: does the ADS in the gills and hepatopancreas follow a circadian rhythm, as locomotor activity does, or a tidal rhythm, as tissue VO_2 does? Given that the observed phenomenon depends on which specific antioxidant defense and which specific tissue is considered, this question will not be easy to answer. In fact, Maciel and colleagues (29) found differences in enzymatic activities (e.g., CAT and GST) and non-enzymatic content (e.g., non-proteic sulfhydryl groups, -NP-SH), as well as LPO levels, between these two tissues. Different CAT activity patterns were observed between the two tissues, with high but stable levels during the entire light/dark cycle in the gills and an activity peak at night in the hepatopancreas. The opposite situation was observed for NP-SH levels, with peak activity levels occurring at night in the gills and no variations in observed in the hepatopancreas. LPO levels were higher at photophase in the gills and at scotophase in the hepatopancreas, an observation that reinforces the tissue specificity of different antioxidant components. In a recent publication, Maciel and colleagues (10) verified that H_2O_2 induces an *in vitro* increase in NP-SH levels in the gills of *N. granulata* to the same level previously observed during the night period (29), suggesting that H_2O_2 may be the main signaling compound for activation of the GSH system. Furthermore, the same group showed a daily bimodal profile for the total peroxyl radical scavenging capacity, which serves as a general measurement of the activity of the entire ADS against peroxyl radicals generated *in vitro*, in the gills of intact and eyestalkless crabs, suggesting that gills are driven by a biological clock not located in the eyestalks. This bimodal variability may be due to antioxidants that were not studied, including enzymes, such as SOD and GPx, and non-enzymes. These observations suggest that the gill ADS follows a tidal rhythm. These studies in crayfish and crabs can be used to construct a putative flux model of the influence of photoperiod on the GSH status of crustaceans (Figure 1), in which cell damage can be initiated directly by the photoperiod or indirectly through an information flux that is initialized in

the photoreceptors. This flux is then transmitted to the biological clock that adjusts metabolic and locomotor activities, in turn leading to rhythmic variation in the levels of GSH and LPO in peripheral tissues.

Over the last ten years, other studies have focused on seasonal variations in the levels of LPO and enzymatic antioxidants in crustaceans. In addition to photoperiod, other environmental aspects, including temperature, salinity and rainfall level, and others can be extremely variable throughout the year in different ecosystems. This variability is apparent in South Asia, where monsoons significantly alter many physical and chemical aspects of the environment, leading to seasonal metabolic adjustment in the local organisms. In the digestive gland of the barnacle *Balanus balanoides*, Niyoge and colleagues (38) observed higher activity of CAT and SOD during the pre-monsoon (March-June) period with a gradual decrease during the monsoon (July-October) and post-monsoon (November-February) periods, whereas an opposite profile was seen for LPO. Temperature decreases and a possible reduction in food availability during the post-monsoon (winter) period may lead to classical oxidative stress in *B. balonoides* due to the injured metabolism. Two studies showed seasonal LPO variations in males and females of three amphipod species, i.e., *Hyalella pleoacuta*, *H. castroi* and *H. curvispina* (39, 40). In females of *H. pleacuta* and H*. castroi*, higher LPO levels were found in autumn, whereas in females of *H. curvispina*, higher LPO levels were observed in summer. These periods are correlated with reproduction in each species, when females carry juveniles in the marsupium after the eggs have hatched. In addition, the highest LPO levels for males occurred in autumn and winter for *H. pleoculata*, in summer and winter for *H. curvispina* and in autumn for *H. castroi.* Again, these variations are probably linked to copulation activity, which occurs in autumn, and to an increase in light irradiance during the summer, which may be an additional environmental stressor for these species. Another factor to consider is the variability of food antioxidant properties from season to season, which might affect the overall antioxidant capacity of these animals. Kong and colleagues (41) verified seasonal variations in CAT, SOD and GPx activities as well as LPO levels in the gills of the mud crab *Scylla serrata*. Although activities of all these enzymes were higher in summer, none were sufficient to prevent a peak of LPO during the same season. A second peak of GPx activity was observed in winter, but LPO levels also peaked during this period due to low activity of CAT and SOD. Summer appears to be the most stressful season, despite the higher activity of enzymatic antioxidants, probably due to food availability and type as well as other factors not considered in this study, such as light irradiance and/or temperature.

4. POSSIBLE MECHANISMS OF REGULATION FOR ADS

As described above, ROS are routinely generated during aerobic metabolism. These molecules cannot just be regarded as harmful by-products of aerobic metabolism because they also serve fundamentally important roles in intracellular signaling pathways. However, all current knowledge regarding the role of ROS signaling in ADS regulation is based upon mammalian studies, implying that no studies on this topic have been performed in invertebrates, including crustaceans. Given that some signaling pathway cascades are phylogenetically preserved and that ROS have been present since the formation of the aerobic atmosphere (1, 3, 4), ADS regulation by ROS signaling can plausibly be assumed to be similar in mammals and crustaceans despite their

phylogenetic distance. Therefore, a very brief overview of ADS regulation in mammals will be presented here for comparison with putative mechanisms in crustaceans.

Many reports have indicated that the GSH/GSSG ratio is an important “sensor” for ADS regulation (42). Increased GSSG levels, resulting in a decreased GSH/GSSG ratio, occur in parallel to incremental increases in ROS generation (mainly for H_2O_2). These changes promote the oxidation of protein cysteinyl thiols, which activate or deactivate specific enzymes in the signaling cascades (42-44). In fact, this oxidant-dependent mechanism can stimulate the gene expression of antioxidant enzymes through the regulation of nuclear factor (NF) κB, mitogen-activated protein kinase (MAPK), activating protein-1 (AP-1), the phosphoinositide 3-kinase (PI3K)/Akt pathway, p53 activation and the heat shock response (45). The transcription factors Nrf2 (erythroid-derived 2) and class O of forkhead box (FoxO) are also involved in the ADS signaling pathway (46, 47). It is interesting that so many signaling pathways are involved in ADS stimulation, although the likely explanation involves the role of ADS as a general response to a variety of stressors. H_2O_2 is one of the most important messengers in these signaling pathways because of its constant generation within mitochondria, its relative stability and its ability to cross a variety of membrane barriers due to its small size (48). As previously mentioned, H_2O_2 can induce an increase in the NP-SH levels in the gills of *N. granulata* (10), a similar signaling action reported in mammals (42-44).

In addition to its well-known variation following the circadian rhythm, melatonin also acts as scavenger of ROS or enzyme activity, or as an expression modulator (for review see ref. 8 and 15) in the ADS. Several studies have demonstrated the effects of melatonin on antioxidant enzymes in mammals as well as other groups of animals. Melatonin can also regulate the gene expression of antioxidant enzymes, such as CuZnSOD, MnSOD and GPx, but the specific signaling pathways involved have not yet been fully described (16). A few reports have suggested that the regulation of antioxidant enzymes by melatonin occurs through its activation of secondary messengers (e.g., cAMP, phospholipase C or intracellular calcium) via specific membrane receptors (e.g., MT1 and MT2). As a result, several transcription factors, including antioxidant enzymes, could be activated through the stimulation of MAP kinase cascades by melatonin-bound membrane receptors (16, 49). Although there are no published reports about melatonin signaling pathways in crustaceans, the effects of melatonin on the ADS in crustaceans may be similar to those reported for mammals.

5. EFFECTS OF MELATONIN ON VERTEBRATE ADS

In the early 1990s, several studies reported that, in addition to its function as a messenger that conveys information about the length of the internal photoperiod, melatonin showed higher and more direct scavenging of ROS than other, more traditional antioxidants, such as GSH (50-52). At the same time, Barlow-Walden and colleagues (53) and Pablos and colleagues (54) showed that melatonin indirectly affected ROS, through the increased activity of GPx in rat brain and several chicken tissues. Liu and Ng (55) and Ozturk and colleagues (56) also showed enhanced SOD activity in livers of rats treated with melatonin. Additional evidence supporting the regulation of ADS by melatonin was provided by Pablos and colleagues (57) and Albarran and colleagues

(58), who showed that inhibition of melatonin production by light inhibits the increased activities of SOD and GPx in several chicken tissues and in rodents (59, 60). As mentioned above, the pineal gland is the main site of melatonin production and secretion in vertebrates. Accordingly, circulating melatonin levels are dramatically decreased when this gland is extirpated. This removal, in turn, may affect the whole ADS of the animal. In fact, Baydas and colleagues (61) observed decreased GPx activity in some tissues of pinealectomized rats. As the aging process is characterized by increased ROS generation and a decline in many physiological functions, melatonin might prevent or decrease the damages associated with senescent processes (62). Along those lines, Mauriz and colleagues (8) observed a decrease in LPO levels in the livers of aged rats treated with melatonin as compared to aged control rats. Antioxidant enzymes, such as cytosolic CuZnSOD as well as mitochondrial GPx and CAT, presented higher activities in melatonin-treated rats than in aged controls, reinforcing the importance of melatonin in ADS regulation. The antioxidant activity of melatonin may not be as clear in fish as in mammals and birds, although only a few reports have been published to date. López-Olmeda and colleagues (63) observed a slight decrease in muscle LPO levels in goldfish submitted to hypoxia/re-oxygenation and treated with melatonin ($3 mg.kg^{-1}$) during re-oxygenation, but no effect was seen in liver. In the same study, melatonin was not capable of suppressing LPO production in the muscle and liver of fishes exposed to H_2O_2 for 1 h. Sreejith and colleagues (64) verified the influence of this indoleamine on the ADS *in vitro* in the teleost *Anabas testudineus*. Cell culture revealed higher LPO levels in the livers of fish kept under constant light (LL) compared to livers of control fish held under the standard 12:12 LD cycle. In contrast, cells treated with melatonin (15-min incubation) had decreased levels of LPO. In addition, melatonin increased GPx and GR activities as well as GSH levels, suggesting its involvement in fish glutathione metabolism.

As previously mentioned, melatonin is capable of stimulating not only the activity of antioxidant enzymes but also their gene expression both *in vivo* and *in vitro*. Antolin and colleagues (65) observed an increase in the mRNA levels of CuZnSOD and MnSOD in the Harderian gland of Syrian hamsters after melatonin treatment. Similar results were verified in the brain cortex of rats injected with melatonin (66). Moreover, Mayo and colleagues (16) also verified increased mRNA levels of antioxidant enzymes *in vitro* when melatonin was added to the growth medium of PC12 cells and human neuroblastoma SK-N-SH cells. Melatonin may or may not be an effective enhancer of antioxidant gene expression, however, depending on the tissue and the experimental conditions. Mauriz and colleagues (8) studied the effect of aging on antioxidant gene expression in rats and showed that melatonin did not stimulate the activities of MnSOD and CAT in the livers of aged animals, whereas CuZnSOD and both cytosolic and mitochondrial GPx activities were higher in the livers of aged animals treated with melatonin. Recently, Jimenez-Ortega and co-workers (67) reported no daily variations in the gene expression levels of CuZnSOD, MnSOD and CAT in the rat medial basal hypothalamus. However, increased gene expression of these enzymes was seen at certain time intervals, indicating that melatonin can regulate antioxidant gene expression, at least in mammals.

The mitochondrion is the organelle responsible for energy production, but ROS generation is inevitable due to leakage in the electron transport chain. Under basal metabolic conditions, cellular ADS can counteract ROS action despite the occurrence of basal levels of damage. However, the ADS may be adjusted to avoid cell

disturbance under stress conditions or daily and seasonal variations in aerobic metabolism. Thus, melatonin can be inferred to specifically regulate mitochondria for maintenance of cell homeostasis through its roles as an ROS scavenger or an ADS modulator. In fact, melatonin is a highly lipophilic molecule capable of crossing cell membranes, including those of mitochondria (68, 69), and of binding to specific receptors on these membranes (70, 71). Several reports have shown that melatonin stabilizes mitochondrial inner membranes (72) and increases the activities of respiratory complexes I and IV (73, 74), both preventing inner calcium overload and helping maintain the membrane potential for ATP generation (75). All these functions may be general features of melatonin in aerobic organisms. Additional studies on this topic should be performed in both vertebrates and invertebrates, including crustaceans, to link every possible divergence of the evolution of melatonin as a signaling molecule.

6. MELATONIN VARIATIONS IN CRUSTACEANS

Melatonin has been identified in a wide variety of vertebrate species since its first discovery by Lerner and colleagues (12) in bovine pineal glands. By contrast, the number of studies on melatonin in invertebrate is much lower, although the increasing efforts on it during the last years. With respect to crustaceans in particular, even less is known about this indoleamine. Only ten crustacean species (including crabs, prawns, isopods, lobster, crayfish and krill – see Table 1) were examined for the presence of melatonin. Some of these species showed rhythmic variations in the regulation of melatonin similar to those seen in vertebrates. Variability among species makes it difficult to establish a general pattern for the rhythmic regulation of melatonin.

Vivien-Roels and Pévet (76) published the first known report on the presence of melatonin in crustaceans, which was identified using the radioimmunoassay (RIA) method in the eye and eyestalk of the crab *Carcinus maenas* in a concentration ranging between 2,700 and 3,650 ρg.eye^{-1}. Interestingly, although no daily variation was observed, different levels of melatonin were reported during different times of the year, suggesting the existence of a putative seasonal control over its production. In the optic lobes of the freshwater prawn *Macrobrachium rosenbergii*, Withyachumnarnkul and co-workers (77) observed an increase in melatonin content at 1500 h (5.5 ρg.µg of protein^{-1}) compared to the lowest value at 2400 h (0.5 ρg.µg of protein^{-1}), which is a temporal pattern opposite to those generally reported for vertebrates. In another study, Withyachumnarnkul and colleagues (78) showed that melatonin levels in the optic lobes of sub-adult males and females of *Penaeus monodon* collected at 0900 h did not differ (30-35 ρg.optic lobes^{-1}).

Agapito and colleagues (79) showed, for the first time, melatonin content in the circulating fluid of *Procambarus clarkii* kept in a 14:10 LD cycle. Melatonin levels in the circulating fluid peaked at photophase (1900 h; 172 ρg.ml^{-1}), with increasing levels in the eye plus eyestalk at the same time point (656 ρg.eye^{-1}). Animals kept in an artificial 8:16 LD cycle also had a peak in melatonin levels in the eye at photophase, at 1600 h (749 ρg.eye^{-1}). In contrast, Balzer and co-workers (80) observed a melatonin peak in the eyestalk of *P. clarkii* at scotophase (0300 h; 1,813 ρg.eyestalk^{-1}). In this study, the amplitude of melatonin was higher (the lowest value was 30 ρg.eyestalk^{-1} at 1500 h) than in the previous work. Differences in the temporal regulation and

concentration of melatonin could be related to the geographical localization of decapod species, with the crayfish in the former and latter studies from Spain and Mexico, respectively. Chemical interference or differing sources for the melatonin antibody used in the RIA method cannot be ruled out as additional possible explanations.

Tilden and colleagues (81, 82) observed that the pattern of daily melatonin content varied in the eyestalks of *Uca pugilator* fiddler crabs kept under LD and DD. Under LD 12:12, a single melatonin peak occurred at the photophase (230 ρg.eyestalk^{-1} at 1300 h), whereas two peaks that were 12 hours apart occurred in the DD condition (196 ρg.eyestalk^{-1} at 1600 h and 111 ρg.eyestalk^{-1} at 0400 h). The second DD peak, which was not present in the 12:12 LD condition, seemed to be influenced by light and thus displayed an exogenously controlled component drift. Interestingly, melatonin levels in the LL condition were higher at almost all time points, with the maximum value at 1300 h (431 ρg.eyestalk^{-1} at 1300 h), compared to treatments with other photoperiods. This response is the opposite of the well-known pattern in mammals, in which light suppresses melatonin production. Meyer-Rochow (83) showed an absence of daily variation of melatonin content in the eye of the freshwater crayfish *Astacus fluviatilis*, in the head of the isopod *Saduria entomon* and in the eyestalk of *Carcinus maenas*. Instead, a seasonal regulation may be involved in these cases, as seems likely for *C. maenas*. In the optic lobes of the crab *Neohelice granulata*, variations in melatonin content were observed (84) at levels similar to those reported in *Uca pugilator* (81, 82). Under the 12:12 LD and DD conditions, two peaks separated by 12-h intervals occurred, and all melatonin variation was abolished in the LL condition. This pattern was similar to that reported for vertebrates but different from that reported for *U. pugilator*. Despite the similarity in the 12:12 LD and DD melatonin profiles, the concentration range in *N. granulata* (7–20 ρg.optic lobes^{-1}) was more similar to that reported for *P. monodon* (78). Recently, Maciel and co-workers (10) verified that melatonin levels (measured only in the morning interval from 0900 to 1100 h) in the hemolymph of *N. granulata* kept under the DD condition were similar to those observed in *P. clarkii* (81.8-123.8 ρg.ml^{-1}).

Pape and colleagues (85) reported the absence of a daily variation in melatonin levels in the eyestalks and hemolymph of the Antarctic krill *Euphausia superba*. Variations in melation levels were not seen in animals collected in summer (6.1 ρg.eyestalk^{-1} and 2.4 ρg.ml^{-1}) or in winter (6.3 ρg.eyestalk^{-1} and 2.9 ρg.ml^{-1}). These values are also lower than those for the different species mentioned above. Of note, this study employed a different methodology for melatonin measurement, consisting of an ethanol–chloroform extraction followed by HPLC purification and ELISA, rather than the RIA typically used in previous reports. The Norway lobster *Nephrops norvegicus* also did not display any differences in the concentration of melatonin between day and night (86). In this study, which utilized liquid chromatography/tandem mass spectrometry (LC–MS/MS), animals kept under an 12:12 LD condition with a light intensity of 10 lx, melatonin levels in hemolymph ranged between 2 to 8 ng.ml^{-1}, whereas animals exposed to the same photoperiod regime with a light intensity of 0.1 lx had even lower melatonin levels (0.03 – 0.04 ng.ml^{-1}). Geihs and colleagues (9) detected melatonin in non-neuronal tissues for the first time in crustaceans. High levels of melatonin (1,258.6 ρg.mg^{-1}), measured in the subjective morning by electrospray interface (ESI) LC–MS/MS, were seen in the locomotor muscle of *N. granulata* kept under a DD condition. This concentration is similar to that found in the eyestalk of *P. clarkii* (80). In a pioneering study in crustaceans, N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK), the main metabolite of melatonin in

mammals, was detected in the optic lobes (53.7 ρg.mg^{-1}) and supraesophageal ganglion (85.7 ρg.mg^{-1}) but not in muscle.

Thus, different crustacean species possess different melatonin profiles and concentrations in the tissues that have been analyzed. Although the number of studies has increased, a general consensus regarding melatonin variation in crustaceans still remains difficult to establish.

7. EFFECTS OF MELATONIN IN CRUSTACEAN ADS

Some studies have shown that melatonin can be involved in a variety of crustacean physiological parameters, such as electroretinograms, pigment migration, limb regeneration, hemolymph glucose and lactate rhythms and synaptic transmission (80, 81, 87-90). Studies on ADS regulation by melatonin, however, are scarce. Although some reports have shown that photoperiod-influenced variations and light intensity alter antioxidant status as an indirect and putative effect of melatonin action, only two reports (9, 10) have actually demonstrated the regulatory role of this molecule on some components of the ADS in crustaceans.

Maciel and colleagues (10) studied daily variations in the total peroxyl radical scavenging capacity (TOSC) in the gills of *N. granulata* and showed a biphasic profile similar to that of oxygen consumption in the same tissue (29). One peak occurred at photophase and the other at scotophase, separated by a 12-h lag. Interestingly, eyestalk ablation delayed the peaks by 3 hours but did not affect the overall profile. In contrast, treatment of eyestalkless crabs with $2x10^{-12}$ mol.animal^{-1} of melatonin abolished this TOSC profile, suggesting a metabolic suppression that decreases ROS production and, consequently, decreases the general antioxidant defense against peroxyl radicals, especially when taking into account the melatonin-induced decrease in *in vitro* gill VO_2 (10). Within this framework, Geihs and co-workers (9) observed that the locomotor muscle of eyestalkless crabs kept in DD and pre-treated (short-term, 30 min) with low doses of melatonin (0.002 and 0.02 ρmol.crab^{-1}) showed increased VO_2, γ-GCL activity (the rate-limiting enzyme for GSH synthesis) and GSH content without changes in ROS concentration, antioxidant capacity or LPO levels. The effects of melatonin were reduced at higher dosages (2 and 20 ρmol.crab^{-1}), probably due to the desensitization of specific membrane receptors, which suggests an increase in aerobic metabolism (opposite to what is seen in the gills), but only under non-stressful conditions. In animals pre-treated over the long-term (570 min), low dosages of melatonin decreased antioxidant capacity and CAT activity, whereas higher dosages reduced VO_2 and increased antioxidant capacity. The variability in the response of crab muscle to melatonin suggests that melatonin is capable of affecting antioxidant status in a time- and dosage-dependent manner.

8. CONCLUSIONS AND PERSPECTIVES

The ability of animals to detect natural signals from the environment and, thus, follow or even anticipate these cyclical changes by adjusting their metabolism and antioxidant defenses is fascinating. Rhythmic variations in ADS and melatonin content are well described in mammals, and knowledge about their regulation is

advanced and constantly progressing. In crustaceans, however, only a dozen reports on ADS and melatonin rhythms have been presented, providing a basic understanding of their regulation (Figure 2). Photoperiod information is transmitted from the photoreceptor site to the nervous system, where the entrainment of the putative biological clock and melatonin rhythm production occurs. This variably produced melatonin may affect the control of tissue ADS and/or paracrine melatonin production through hemolymphatic transport. Some results in crustaceans are similar to those seen in mammals, but others possess high intra- and inter-specific variabilities. Therefore, increased experimental efforts are needed for the following issues:

1. Identification of intracellular messengers for pre-existing ADS regulation;
2. Characterization of the signaling pathway for enzymatic ADS gene expression;
3. Measurement of melatonin concentration rhythms in different tissues from different species at different times of the day and on different days in different seasons to establish a generalized profile;
4. Identification of the signaling pathway and the key enzymes for melatonin synthesis;
5. Assessment of the capability of melatonin to modulate the gene expression of ADS enzymes.

Given that crustacean species are clustered in a large subphylum, the development of a generalized rhythmic consensus pattern for ADS regulation and the role of melatonin may be difficult. With increasing numbers of reports, however, a future review on this subject may provide updates on some or all of the above issues.

9. ACKNOWLEDGEMENTS

Geihs M. A. is graduate fellow by CAPES and Monserrat J. M. and Nery L.E.M. are research fellows from CNPq.

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Key Words: Antioxidant Defense System, Crustacean, Melatonin, Glutathione, Rhythm, Review

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Table 1. Day-night variations and range concentrations of melatonin in different tissues of crustacean species, as measured by distinct techniques

Species	Tissues	Day-Night variations	Range Concentrations	Technique	Reference
C. maenas	Eye plus eyestalk	No variation	2,700 – 3,650 ρg.eye^{-1}	RIA	76
M. rosenbergii	Optic lobes	Photophase	0.5 - 5.5 ρg.µg of protein^{-1}	RIA	77
P. monodon	Optic lobes	Not studied	30 – 35 ρg.optic lobes^{-1}	RIA	78
P. clarkii	Eyestalk and hemolymph	Photophase	50 – 656 ρg.eye^{-1} and 20 – 172 ρg.ml^{-1}	RIA	79
P. clarkii	Eyestalk	Scotophase	30 – 1,813 ρg. eyestalk^{-1}	RIA	80
U. pugilator	Eyestalk	Photophase	110 – 230 ρg. eyestalk^{-1}	RIA	81, 82
A. fluviatilis	Eye	No variation	Not informed	RIA	83
S. entomon	Head	No variation	Not informed	RIA	83
N. granulata	Optic lobes, hemolymph and muscle	Photophase and scotophase	7 – 20 ρg.optic lobes^{-1}; 102.8 ρg.ml^{-1} and 1.26 ng.mg^{-1} of muscle	RIA; ESI-LC-MS/MS	9, 10, 84
E. superba	Eyestalk and hemolymph	No variation	6.3 ρg.eyestalk^{-1} and 2.9 ρg.ml^{-1}	HPLC/ELISA	85
N. norvegicus	Hemolymph	No variation	40 ρg.ml^{-1}	LC-MS/MS	86

Figure 1. Putative flux model of photoperiod influence on the GSH status of crustaceans. Light can injure cells directly through its photo-oxidative capability or indirectly through photoreception. Photoreception occurs in the retina and perhaps in extra-retinal sites, with the photoperiod/light intensity information transmitted to the putative biological clock, which may facilitate the entrainment of metabolic and locomotor activities. This transmission may subsequently cause tissue-specific rhythmic variations in the GSH/GSSG ratio as the levels of GSH change. Given that variations in LPO and GSH output have been seen in the hemolymph, cellular damage to these tissues may rhythmically vary. GSH: reduced glutathione; GSSG: oxidized glutathione; GSH/GSSG ratio: reduced glutathione and oxidized glutathione ratio; ROOH: hydroperoxide; GPx: glutathione peroxidase; GR: glutathione reductase; GST: glutathione-*S*-transferase; BLPO: byproducts of lipid peroxidation; ~: rhythmic variation.

Figure 2. Putative flux model of the effects of melatonin on crustacean ADS. Photoreception occurs in the retina and perhaps in extra-retinal sites, with the photoperiod/light intensity information transmitted to the nervous system, the putative biological clock site. This biological clock may facilitate the entrainment of melatonin synthesis, which may be a species-specific pattern, as no clear overall pattern has been seen to date in

crustaceans. Melatonin secreted in the hemolymph may act in target tissues via either specific membrane receptors or transport across the cell membrane. These target tissues may also produce melatonin that may or may not act in a corresponding fashion with circulating melatonin to induce tissue-specific ADS control by scavenging ROS and modulating the gene expression of ADS enzymes. ADS: antioxidant defense system; ROS: reactive oxygen species.
List of required items

Running title: ADS rhythms and melatonin in crustaceans

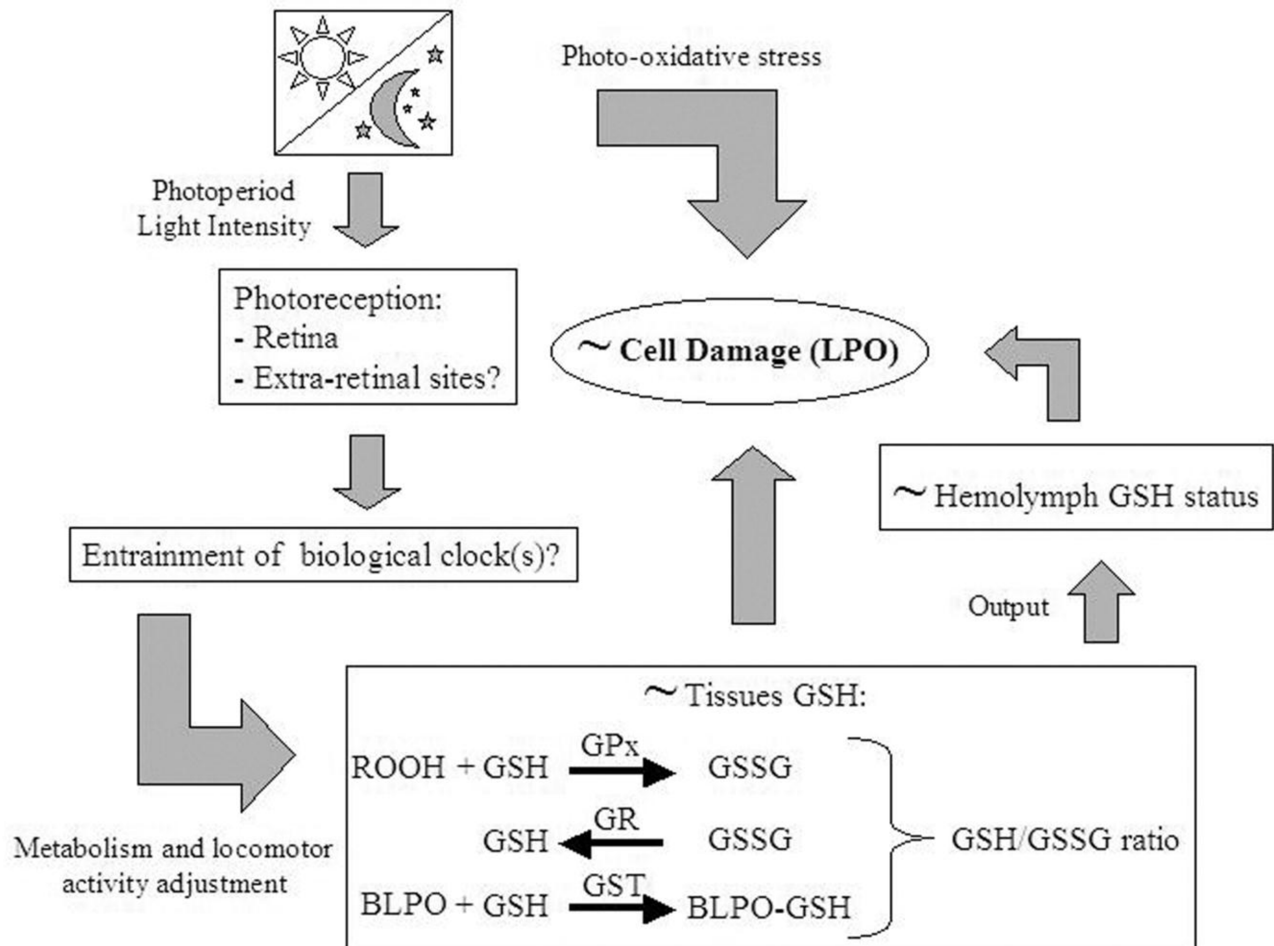

Fig. 1.

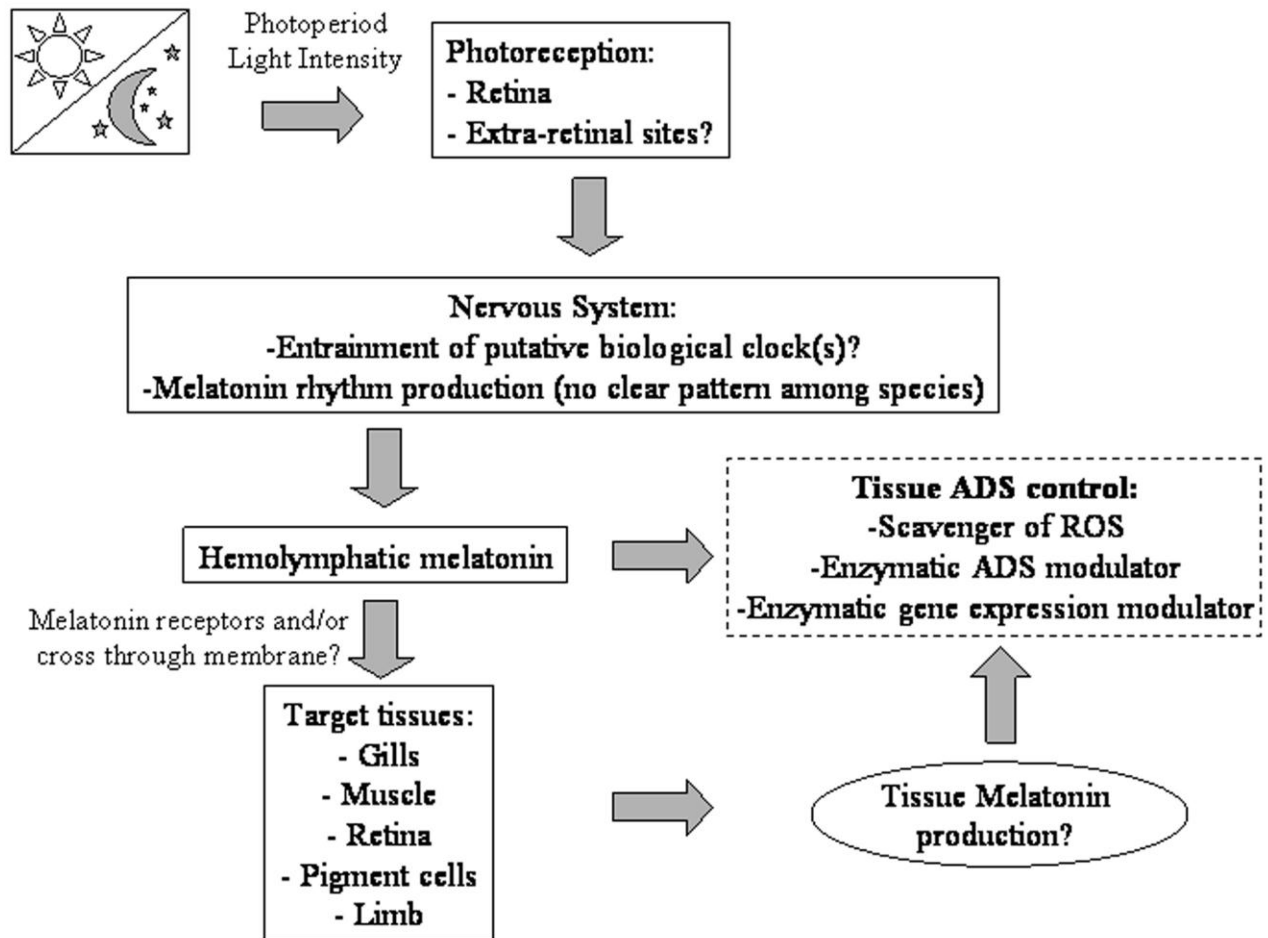

Fig. 2.

VII. Artigo 2

O ajuste do metabolismo energético é dependente do pedúnculo ocular em *Neohelice granulata* (Decapoda, Brachyura) sob hipoxia/anoxia

Submetido ao periódico Comparative Biochemistry and Physiology A

The energetic metabolism adjustment is eyestalk dependent in *Neohelice granulata* (Decapoda, Brachyura) under hypoxia/anoxia

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Abstract

Many crustacean physiological parameters are under control of the eyestalk, however its importance in the metabolic adjustment in hypoxia/anoxia conditions remains poorly investigated. In this study, we verified the tolerance, oxygen consumption (VO_2) and energetic and metabolites levels in relation to hypoxia/anoxia and eyestalk ablation in the crab *Neohelice granulata*, as well as the identification and gene expression of the crustacean hyperglycemic hormone (CHH). In anoxia condition, eyestalkless crabs are significantly less tolerant (LT_{50} = 8.5h) compared to intact ones (LT_{50} = 14.4h). The concentration of 1 $mgO_2.L^{-1}$ significantly ($p<0.05$) decreased VO_2 and severe hypoxia/anoxia (0.7 and 0.0 $mgO_2.L^{-1}$) increased circulating glucose (intact crabs) and lactate (intact and eyestalkless crabs) levels. No significant differences in glycogen concentration and weakly alteration in glucose content in both muscle and hepatopancreas tissues were observed among the different dissolved oxygen concentration. A partial CHH gene encoding the final portion of signal peptide, complete CHH precursor related peptide and around 1/3 of mature CHH was cloned, with a similarity of 73-82% to other crustaceans. In anoxia, CHH gene expression was significantly ($p<0.05$) down regulated. These results suggest that dissolved oxygen levels affect energetic metabolism in *N. granulata* and it is dependent from the eyestalk.

Keywords: Eyestalk, crustacean hyperglycemic hormone, crab, estuarine, metabolism, glucose, lactate.

1. Introduction

Hypoxia or anoxia conditions are common in several aquatic environments and are a strong selective factor in the animals survival. The estuarine crab *Neohelice granulata* (before known as *Chasmagnathus granulata*/*granulatus* – see Sakai et al., 2006) inhabiting the salt marshes in southern of South America faces cyclically and non-cyclically changes in temperature, salinity and dissolved oxygen (D´Incao et al., 1992), which can impose changes in metabolism. The main strategies adopted by crustaceans of these environments are metabolic depression, as decreasing oxygen consumption (VO_2), and maintenance of high levels of organic fuel as glycogen/glucose from different sources such as muscle, hepatopancreas, epidermis and hemocytes, to anaerobic metabolism use (Herreid II and Full, 1988; Storey and Storey, 1990; Hervant et al., 1995; Lutz and Storey, 1997; Childress and Seidel, 1998; Hochachka and Lutz, 2001). Thus, hyperglycemic responses in some crustaceans have been observed when exposed to hypoxia/anoxia (Zou et al., 1996; Maciel et al., 2008; Chung and Zmora, 2008; Silva-Castiglioni et al., 2010).

The decapod crustacean eyestalk posses a cluster of neurosecretory cells termed X-organ (Hanström, 1928), which axons are projected to the Sinus gland, an important neurohemal organ where substances are released in the circulatory system. In fact, the XO/SG complex, that is comparable to the hypothalamic-pituitary axis from vertebrates, is a source of neurohormones involved many crustacean physiological regulations and its absence influence color change, growth, reproduction and also energetic metabolism (Van Herp and Kallen, 1991; Landau et al., 1997). In this sense, glucose is one of the main energetic substrate for the general metabolic processes in crustaceans (Herreid II and Full, 1988), several studies have analyzing the circulating glucose levels against some environmental stress imposed to the animal, such as temperature (Morris and Olivier, 1999; Durand et al., 2000), salinity (Santos and Nery, 1987; Spaargaren and Haefner, 1987) and hypoxia (Zou et al., 1996; Maciel et al., 2008; Silva-Castiglione et al., 2010). The general response to these stressors is a hyperglycemia, evoked by the crustacean hyperglycemic hormone (CHH), a peptide produced mainly in the x-organ/sinus gland (XO/SG) complex identified in a great number of crustaceans species (for review see Keller, 1992; Chang, 2001; Chan et al., 2003; Fanjul-Moles, 2006). This neurohormone seems to have an important role in the adjustment of energetic metabolism in crustaceans. Interestingly, some reports have shown that CHH peptide (mature CHH cleavage from CPRP) has a double

feedback system (negative by glucose and positive by lactate) as an important releasing control of this molecule (Glowik et al., 1997; Santos and Keller, 1993a; 1993b; Santos et al., 2001; Morris et al., 2010).

Therefore, the aim of this work is to study which metabolic adjustments the crab *N. granulata* adopt against hypoxia/anoxia conditions and its dependence of the eyestalks, and also verify if this stress induce changes in CHH gene expression. This information will be of great value to understand the adaptative mechanisms of this species to tolerate hypoxia/anoxia conditions in their habitats.

2. Material and Methods

2.1. Animals

Adult male crabs of *N. granulata* (mean weight ± confidence interval: 13.5±0.9g were collected in salt marshes around Rio Grande city, Southern Brazil (32°S). Thereafter, they were acclimated to water at constant conditions of salinity (20), temperature (20°C) and photoperiod (12L:12D) for at least 7 days. During this period, the crabs were regularly fed with ground beef.

2.2. Mortality

Intact (n=16) and eyestalkless crabs (n=16, which the eyestalks were cut off with scissors and then cauterized 24 hours prior to the beginning of the experiment) were individually placed in bottles (around 300mL each) with saline water, which was purged with nitrogen gas to reduce the level of dissolved oxygen to anoxic condition. Observations for mortality (no movements of any appendices for 1 minute of mechanic stimulation) were made every hour.

2.3. Experimental design and measurements of VO_2 for different dissolved O_2 concentration

For VO_2 measurement, 6 aquaria (3L each) were purged with nitrogen gas to reduce the level of dissolved oxygen to 6, 5, 4, 3, 2 and 1 $mgO_2.L^{-1}$. Twenty four intact (n=4) and eyestalkless (n=4) crabs were exposed to the above oxygen concentrations in the respirometer chamber with saline water (salinity at 20) for 15 minutes prior to beginning of experiment. The oxygen concentration was measured at time zero and after 30 min using a

portable oxymeter (Lutron, DO-5510). Oxygen consumption was expressed in milligrams of oxygen per gram of body weight per hour. The eyestalk ablation procedure was made as previously described.

2.4. RNA isolation and cDNA synthesis

The eyestalk ganglia were used for total RNA extraction. Total RNA was isolated by TRI Reagent SolutionTM method following the manufacturer's instructions (Applied Biosystems, Brazil). Genomic DNA was degraded with DNase I (Invitrogen) and total RNA was quantified by a QubitTM fluorometer using the Quant-iTTM RNA assay kit (Invitrogen, Brazil) and calibration was carried out using a two-point standard curve. First-strand cDNA was synthesized from approximately 1 µg of total RNA and used as a template with SuperScript III Enzyme Kit (Invitrogen, Brazil) according to protocol suggested by the manufacturer.

2.5. Cloning of partial CHH gene

For CHH cloning, the first-strand cDNA was utilized to amplify a portion of the open reading frame (ORF). The polymerase chain reaction (PCR) was performed with a degenerated sense primer 5'-TCYCMRACMCRCACGCRCGC-3', and the anti-sense primer 5'-CTGTAGAGGTTGTARCAATC-3' designed based on the sequence of other Brachyura CHH nucleotide sequences from the genbank.

All reactions were realized employing Platinum Taq DNA Polymerase kit (Invitrogen, Brazil) following the manufacturer's instructions in a 12.5 µL mix. Thermal cycler program consisted of 2 min at 94°C, 40 cycles of 30 s at 94°C, 45 s at 50°C and 1 min at 72°C and a final extension step of 5 min at 72°C. PCR products were analyzed in agarose gel stained with ethidium bromide (500ng/ml). The resulting products were cloned employing TOPO 4.0 Cloning kit (Invitrogen, Brazil). Plasmids of bacterial colonies were grown overnight, purified with Ilustra^{TM} Plasmid Prep Mini Spin kit (General Electric, Brazil) and analyzed by restriction enzyme (*EcoR I,* Invitrogen, Brazil) digestion to confirm the size of the cloned cDNA fragment.

Products derived from cloning of CHH encoding genes were automated sequenced with the sequencer MegaBACE 1000 using M13 vector primers. Nucleotides sequences were aligned combining the programs ChromasPro and BLAST (directly from the website http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.6. Experimental design and measurements of CHH gene expression, hemolymph glucose and lactate levels and muscle and hepatopancreas glucose and glycogen concentrations for different dissolved O_2 concentration

Aquaria (3L each) were purged with nitrogen gas to reduce the level of dissolved oxygen to 6, 2, 0.7 $mgO_2.L^{-1}$ and anoxia. After that, the crabs (total of 84 animals) were exposed for 45 minutes in each oxygen concentration prior to eyestalk collection for CHH gene expression (n=4, pool of 4 crabs), hemolymth sampling (n=5) for glucose and lactate measurements and muscle (meropodite of the second pair of pereopods) and hepatopancreas collection for glucose and glycogen measurements. At the same conditions, an additional experiment was carried out for hemolymph, muscle and hepatopancreas sampling with eyestalkless crabs (total of 15 animals). The eyestalk ablation procedure was made as previously described.

For CHH gene expression, the eyestalks were dissected and total RNA extraction and cDNA synthesis were performed as described before. Quantitative analysis of CHH mRNA expression was carried out for 10min at 95°C and 40 cycles of 15s at 95°C and 1min at 60°C by real-time RT-PCR (7300 Real-Time System, Applied Biosystems – Brazil), using SYBR GREEN PCR Master Mix™ (Applied Biosystems), sense and anti-sense gene specific primers, Ultra Pure Distilled Water (Invitrogen, Brazil) and 1.0 μL cDNA (dilution 1:19). β–actin expression (sense 5'-CCAGATCATGTTTGAGGTGTTCA-3 and anti-sense 5'-GGGACAGCACAGCCTGGAT-3' primers) was used as an internal control to normalize the data.

The hemolymph (around 100μl) was collected with sterile syringe from the base of the 4th or 5th pairs of the walking legs and stored at –80°C for further analysis. Glucose and Lactate measurement was made using the GOD-PAD monoreagent kit (Kovalent – Brazil) at 490 nm and enzymatic ultra-violet Lactate kit (Kovalent – Brazil) at 340 nm, respectively. Glucose and lactate are expressed in mg per dl of hemolymph.

Muscles and hepatopancreas were weighted and homogenized (1:10 w/v) in sodium citrate (100mM) and heated at 100°C for 5 min. After, the samples plus a glycogen standard ($100mg.dl^{-1}$) were divided in two and incubated at 55° for 150 min with or without amyloglucosidase (1%). Next, the samples were centrifuged at 7000 rpm for 30 min and the supernatant were used for glycogen measurements as equivalent of glucose (Carr and Neff, 1984; Nery et al., 1993).

2.7. Statistical analysis

In the CHH gene expression, the REST© software was applied in the relative quantification (Pfaffl et al., 2002), which pair comparisons among groups were realized. For VO_2, glycogen, glucose and lactate, two-way ANOVA was carried out followed by an *a posteriori* means comparison (Newman–Keuls test, $\alpha = 0.05$). ANOVA assumption (normality and homogeneity variance) was verified prior to the variance analysis.

3. Results

3.1. Mortality

Intact crabs have a higher tolerance to anoxia than eyestalkless ones (Fig. 1). The first death was observed after ten hours in intact crabs whereas in eyestalkless ones the first death was observed after five hours in anoxia. Fifty percent of intact crabs have died in 14.4h of anoxia, whereas the same percentage was obtained in 8.5h of anoxia in crabs without its eyestalks. A hundred percent of death was verified after twenty one and fifteen hours in anoxia for intact and eyestalkless ones, respectively.

3.2. Dissolved oxygen concentration effects in VO_2 circulating glucose and lactate levels

Significant differences ($p<0.05$) were observed in VO_2 (Fig. 2). The lowest VO_2 was observed at $1 mgO_2.L^{-1}$ in intact and eyestalkless animals (0.016±0.001 and 0.022±0.001 $mgO_2.g^{-1}.h^{-1}$, respectively) compared to the normoxia (0.056±0.007 and 0.088±0.006 $mgO_2.g^{-1}.h^{-1}$, respectively). However, no significant differences ($p>0.05$) were observed between intact and eyestalkless crabs. Concerning glucose levels (Fig. 3a), a significant increase ($p<0.05$) was verified only in intact crabs exposed to 0.7 and anoxia (19.0±3.5 and 46.8±5.2 $mg.dl^{-1}$, respectively) compared to eyestalkless crabs in the same conditions (3.5±0.4 and 9.4±1.1 $mg.dl^{-1}$, respectively) and to the other groups. Interestingly, in both intact and eyestalkless crabs the highest lactate levels ($p<0.05$) were observed in anoxic condition (81.2±3.7 and 79.4±4.3 $mg.dl^{-1}$, respectively) compared to the other conditions (Fig. 3b), but not differed from each other ($p>0.05$). However, when crabs were exposed to 0.7 $mgO_2.L^{-1}$, a higher lactate concentration was observed in intact crabs than in eyestalkless ones (53.2±4 and 33.1±4.4 $mg.dl^{-1}$, respectively) which, in turn, is significant higher ($p<0.05$) than 2 and 6 $mgO_2.L^{-1}$ conditions.

3.3. Dissolved oxygen concentration effects in muscle and hepatopancreas glycogen and glucose concentrations

In both muscle and hepatopancreas, no significant differences ($p>0.05$) were observed in glycogen concentration in intact and eyestalkless crabs (Fig. 4a and 5a). Concerning to glucose concentrations in muscle tissue (Fig. 4b), significant differences ($p<0.05$) were verified in intact crabs with higher values at anoxia (0.31±0.02 $mg.dl^{-1}$) compared to 6 $mgO_2.L^{-1}$ condition (0.23±0.03 $mg.dl^{-1}$). In eyestalkless crabs, no differences ($p>0.05$) were observed among dissolved oxygen conditions, however, significant lower values ($p<0.05$) were observed in 6 $mgO_2.L^{-1}$ (0.08±0.01 $mg.dl^{-1}$) and anoxia (0.17±0.02 $mg.dl^{-1}$) conditions compared to intact crabs. In hepatopancreas, a significant decrease ($p<0.05$) in glucose concentration (Fig. 5b) were verified in intact crabs exposed to 2.0 $mgO_2.L^{-1}$ (1.8±0.2 $mg.dl^{-1}$) and 0.7 $mgO_2.L^{-1}$ (1.7±0.3 $mg.dl^{-1}$) conditions, compared to 6 $mgO_2.L^{-1}$ (4.1±0.6 $mg.dl^{-1}$). In eyestalkless crabs hepatopancreas, no differences ($p>0.05$) in glucose concentration were observed among dissolved oxygen conditions, however, significant higher values ($p<0.05$) were observed in 2.0 $mgO_2.L^{-1}$ (4.0±0.6 $mg.dl^{-1}$) and 0.7 $mgO_2.L^{-1}$ (3.9±0.4 $mg.dl^{-1}$) conditions compared to intact crabs.

3.4. Cloning, sequencing and eyestalk CHH gene expression

Using a PCR-based cloning strategy with sense and anti-sense degenerated primers gave rise to a fragment of approximately 250 bp that was consecutively cloned and sequenced. The PCR product from the eyestalk of *N. granulata* had 241 bp encoding a partial sequence of crustacean hyperglycemic hormone (CHH) (GenBank accession number: ADM26761). The nucleotide and deduced amino acids are shown in figure 6, which represent the final portion of signal peptide (5 deduced amino acids), the complete CHH precursor related peptide (CPRP – 42 deduced amino acids) and partial CHH mature peptide (30 deduced amino acids). Also, a dibasic cleavage site (KR) is found between CPRP and CHH. The similarity of nucleotide and amino acids sequences with other crustaceans are shown in table 1. Many CHH nucleotide sequences fitted with *N. granulata* CHH sequence, but for similarity comparisons we chosen only those which coverage 90% or higher of *N. granulata* nucleotide sequence such as *Gecarcinus lateralis* (ABF58090), *Gecarcoidea natalis* (ABL09570), *Potamon ibericum* (ABA70560) and *Pachygrapsus marmoratus* (AAO27805). *N granulata* CHH nucleotide sequence showed a similarity of 82%, 80%, 76% and 73%, respectively and the amino acids similarity were 81%, 81%, 82% and 75%, respectively. Also,

significant decrease ($p<0.05$) was verified in CHH gene expression only when the crabs were exposed to anoxia, compared to 6.0 $mgO_2.L^{-1}$ (Fig. 7).

4. Discussion

Hypoxia and/or anoxia conditions in the coastal aquatic environment are relatively common, requiring the organisms important biochemical adaptations, such as metabolic rate depression or maintaining high levels of fuel as glycogen/glucose to produce ATP by fermentative pathways (Storey and Storey, 1990; Hervant et al., 1995; Lutz and Storey, 1997; Childress and Seidel, 1998; Hochachka and Lutz, 2001). The crab *N. granulata* is poorly adapted to terrestrial life as showed by Santos et al. (1987). However, this animal has a good tolerance to air exposition (Santos and Colares, 1986; Schmitt and Santos, 1993) and up to 8h in anoxia (Oliveira et al., 2001). Thus, in order to better evaluate the tolerance to anoxia of this crab, the present work verified that *N. granulata* is less tolerant to anoxia in the absence of its eyestalk (8.5h of LT_{50} in eyestalkless crabs compared to 14.4h of LT_{50} in intact ones) (Fig. 1), suggesting that a control for metabolic adjustment against oxygen deprivation is partially dependent from this structure.

Thus, the next step was to verify what physiological and biochemical parameters are affected by eyestalk ablation in response to environment dissolved oxygen variation. Concerning to oxygen consumption (Fig.2), from 6 (normoxia) to 3 $mgO_2.L^{-1}$ this species seems to be a oxyregulator since the VO_2 did not significantly change. However, below 3 $mgO_2.L^{-1}$ the VO_2 gradually decreased, indicating a oxyconforming behavior. Interestingly, those responses are similar in both intact and eyestalkless crabs, although this later seemed to have a higher VO_2. The calculated critical point was 3.2 $mgO_2.L^{-1}$, but, is it the point where this species turns from aerobic to anaerobic metabolism? Probably no, because hemolymph glucose and lactate levels of crabs exposed at 6.0 and 2.0 $mgO_2.L^{-1}$ are similar, whereas in 0.7 $mgO_2.L^{-1}$ and anoxia a hyperglycemia and increase of circulating lactate were observed in intact crabs, which are known responses of crustaceans during anaerobic pathway activation under hypoxic/anoxia stress (Zou et al., 1996; Maciel et al., 2008; Silva-Castiglioni et al., 2010). In eyestalkless crabs, severe hypoxia and anoxia did not induced hyperglycemia, indicating that this response is dependent from eyestalk. Here, intact crabs exposed to the different dissolved oxygen concentrations evoked the similar effect of glucose in lactate concentration, *i.e.*, in 0.7 $mgO_2.L^{-1}$ and anoxia an increase of hemolymph lactate compared to the other situations. Moreover, a lower but also significant rising of lactate level was observed

in eyestalkless crabs in 0.7 $mgO_2.L^{-1}$ and similar values to intact crabs in anoxia condition, indicating that the anaerobic metabolism activation is not dependent from the eyestalk.

In order to verify the origin from increase glucose in hypoxia/anoxia conditions, measurements of glycogen and glucose in muscle and hepatopancreas were carried out. As no significant differences in glycogen concentration and weakly alteration in glucose content in both tissues were observed among the different dissolved oxygen concentration, probably the source of glucose during hyperglycemia may be a general glucose mobilization of all tissues. In fact, other sites such as gills, hemocytes and epidermis were also observed glycogen content, even higher than hepatopancreas and muscle tissues (Johnston et al., 1973; Hohnke and Sheer, 1970; Nery and Santos, 1993; Kucharski et al., 2002).

Since hypoxic/anoxia conditions induce hyperglycemia in *N. granulata*, and its effect is eyestalk dependent, it is plausible to assume that CHH is involved in this response. Some studies have investigated variations in CHH circulating levels in crustaceans under hypoxia/anoxia. In the crayfish *Orconectes limosus* subjected to hypoxia, the concentration of hemolymph CHH increased in 15 minutes (Keller and Orth, 1990). In *Cancer pagurus*, an increase of CHH in the hemolymph was observed after 4 hours of emersion (Webster, 1996). Chung and Zmora (2008) also found an increase in the concentration of this peptide in the hemolymph of *Callinectes sapidus* after 1 hour of hypoxia. Therefore, the hyperglycemic response under hypoxia/anoxia in *N. granulata* is probably CHH eyestalk dependent. At this point, the question arises is: how is the CHH gene expression in these responses? Thus, a partial cDNA sequence of CHH was isolated from the eyestalk of the estuarine crab *N. granulata*. This sequence revealed the final portion of a signal peptide, the complete portion of CPRP and around 1/3 of the mature CHH (Fig. 6). The alignment of deduced amino acid showed that the mature CHH is conserved among crustacean species. However, the CPRP sequences vary among them. Interestingly, the phylogenetic analysis grouped the CHH deduced amino acids of *N. granulata* in the brachyuran branch (data not shown), which was expected, since other reports have been shown that, at least in CHH analysis, the similarity is higher with species belonging the same infraorder (Chan et al., 2003; Marco et al., 2003; Mettulio et al., 2004). Moreover, the effects of different CHHs may differ, as shown by Nery et al. (1993). In fact, CHH from the brachyuran *C. maenas* injected in *N. granulata* was more efficient to cause hyperglycemia than CHH from the crayfish *O. limosus*.

After, the CHH gene expression was analyzed in crabs exposed for 45 minutes to different dissolved oxygen concentrations, but only in anoxia condition the CHH gene

expression was changed, with a down regulation (Fig.7). So far, only Chung and Zmora (2008) have observed a decrease in CHH gene expression in the eyestalk during hypoxia. Perhaps a high hemolymph glucose levels caused by increased release of this neurohormone, may inhibit its own synthesis (Santos and Keller, 1993a; 1993b; Glowik et al., 1997; Santos et al., 2001). In fact, higher levels of circulating glucose were observed in anoxia condition in *N. granulata,* probably by eyestalk CHH release, which its gene expression is down regulated by hyperglicemia. Moreover, others substances may be involved in the regulation of this response. Biogenic amines have been considered as neuroregulators in crustacean neurohormone releasing. Some studies verified that serotonin has a hyperglycemic effect (Fingerman et al., 1981; Santos et al., 2001). Recently, Sainath and Reddy (2010) verified in the crab *Oziotelphusa senex senex* a hyperglycemia in a dose dependent manner when injected with melatonin. It is speculated that these substances could act as neurotransmitters in the control of energetic balance in this group of animals.

In conclusion, intact *N. granulata* has a good tolerance to anoxia, probably due to reduction in VO_2 and use of anaerobic metabolism, as well as, eyestalk CHH involvement. However, in the absence of eyestalk, all these adjustments are injured, since energetic fuels are not available (hyperglycemia) through eyestalk CHH.

Acknowledgements

Maciel FE was fellow by CAPES and Nery LEM is a research fellow by CNPq.

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Table legend

Table 1. Similarity percentage of nucleotide and deduced amino acids of the crustacean hyperglycemic hormone (CHH) cDNA from the eyestalks of *Neohelice granulata* (ADM26761) compared to other crustacean sequences (*G. lateralis* - ABF58090; *G. natalis* - ABL09570; *P. ibericum* - ABA70560 and *P. marmoratus* - AAO27805), which coverage >90% of the analyzed sequence.

Captions to figures

Fig 1. Cumulative percentage mortality in intact (empty circle) and eyestalkless (full circle) crabs *Neohelice granulata* in anoxia condition (n=16).

Fig. 2. Oxygen consumption (VO_2) of intact (empty circle and dashed line) and eyestalkless (full circle and solid line) crabs *Neohelice granulata* exposed 45 minutes into six different dissolved oxygen concentration (1, 2, 3, 4, 5 and 6 $mgO_2.L^{-1}$). Each point represents the mean±SE (n=4-7). Different letters represent significant differences ($p<0.05$) between groups.

Fig. 3. Glucose (a) and lactate (b) concentration in hemolymph of intact (white columns) and eyestalkless (black columns) crabs *Neohelice granulata* exposed 45 minutes into four different dissolved oxygen concentration (anoxia, 0.7, 2.0 and 6.0 $mgO_2.L^{-1}$). Each point represents the mean±SE (n=4-5). Different letters represent significant differences ($p<0.05$) between groups.

Fig. 4. Glycogen (a) and glucose (b) concentration in muscle tissue of intact (white columns) and eyestalkless (black columns) crabs *Neohelice granulata* exposed 45 minutes into four different dissolved oxygen concentration (anoxia, 0.7, 2.0 and 6.0 $mgO_2.L^{-1}$). Each point represents the mean±SE (n=4-5). Different letters represent significant differences ($p<0.05$) between groups. Glycogen is expressed in equivalent of glucose.

Fig. 5. Glycogen (a) and glucose (b) concentration in hepatopancreas tissue of intact (white columns) and eyestalkless (black columns) crabs *Neohelice granulata* exposed 45 minutes into four different dissolved oxygen concentration (anoxia, 0.7, 2.0 and 6.0 $mgO_2.L^{-1}$). Each

point represents the mean±SE (n=4-5). Different letters represent significant differences ($p<0.05$) between groups. Glycogen is expressed in equivalent of glucose.

Fig. 6. Sequence of nucleotide and deduced amino acids of the crustacean hyperglycemic hormone (CHH) cDNA from the eyestalks of *Neohelice granulata*. The underlined nucleotides are the degenerated (external) and specific (internal) primers binding region. The dibasic cleavage site is boxed. Vertical dashed lines limit the Signal Peptide (SP), CHH Precursor Related Peptide (CPRP) and Crustacean Hyperglycemic Hormone (CHH) regions. The left and right numbers refer to nucleotide and amino acid sequence position, respectively.

Fig. 7. Crustacean Hyperglycemic Hormone (CHH) gene expression in the eyestalks of *Neohelice granulata* exposed 45 minutes into four different dissolved oxygen concentration (anoxia, 0.7, 2 and 6 $mgO_2.L^{-1}$). The bars represent the mean±SE (n=3-4). * represents significant differences ($p<0.05$) compared to 6 $mgO_2.L^{-1}$ (control group, dashed line). The values are normalized by β-actin gene expression.

Species	Nucleotide similatity (%)	Amino acids similarity (%)
Gecarcinus lateralis	82	81
Gecarcoidea natalis	80	81
Potamon ibericum	76	82
Pachygrapsus marmoratus	73	75

Table 1.

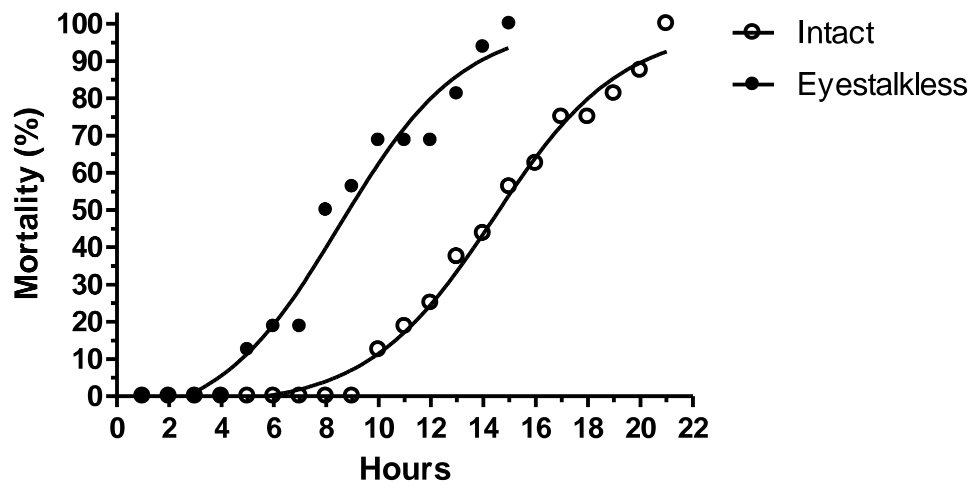


Fig. 1.

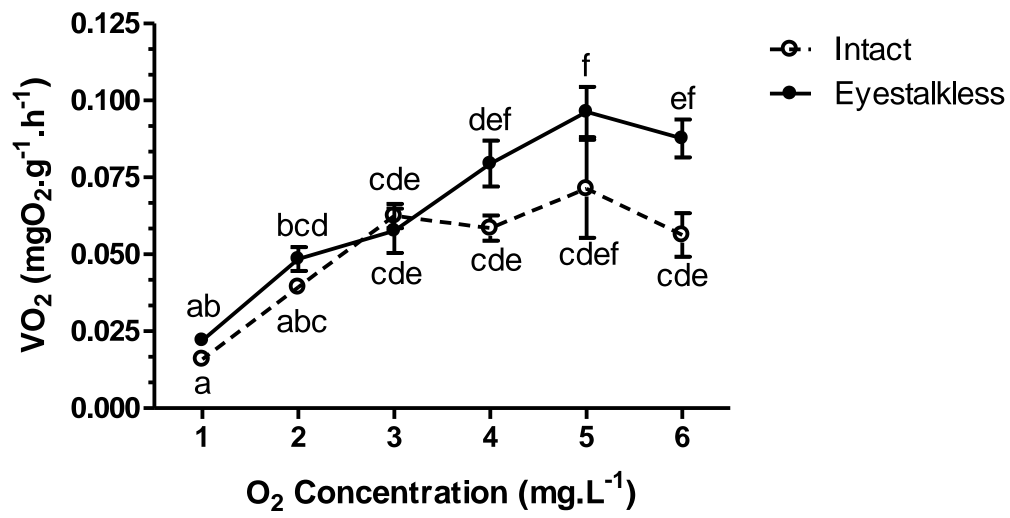
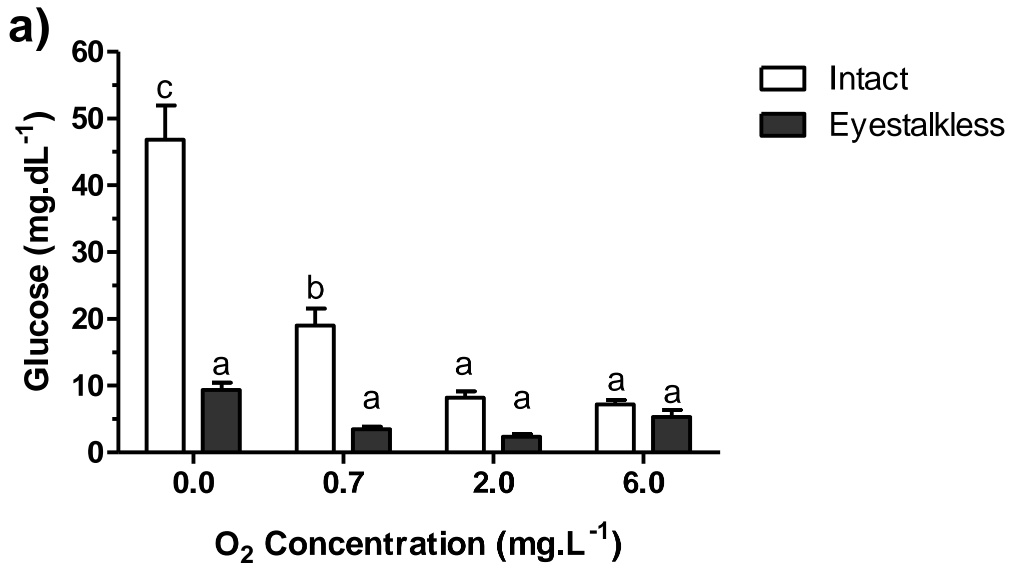


Fig. 2



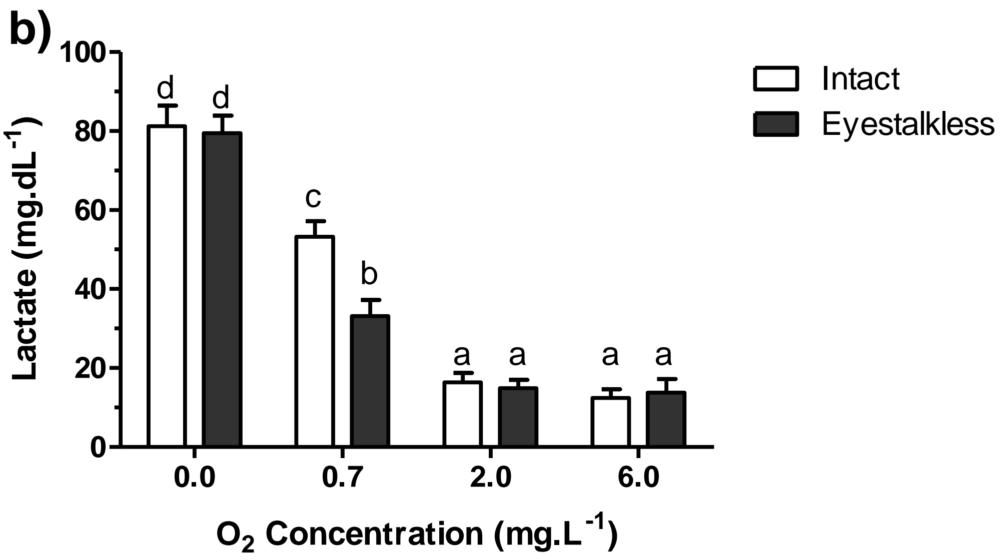
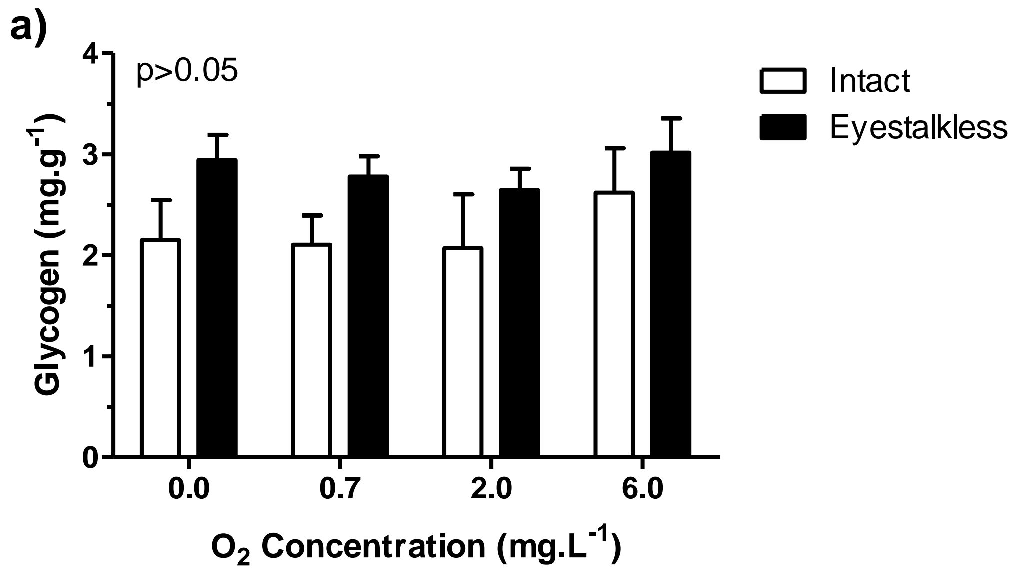


Fig. 3.



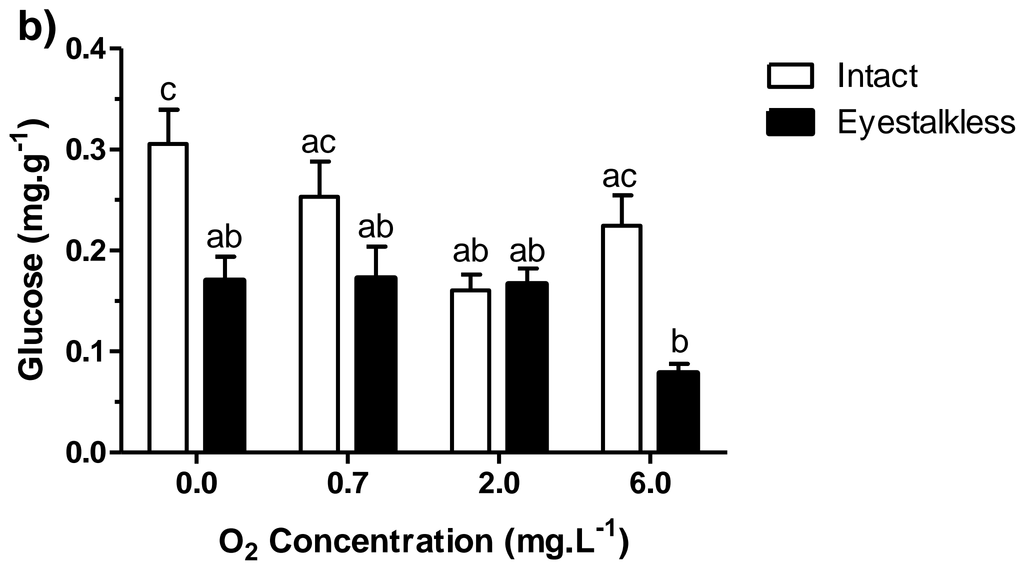
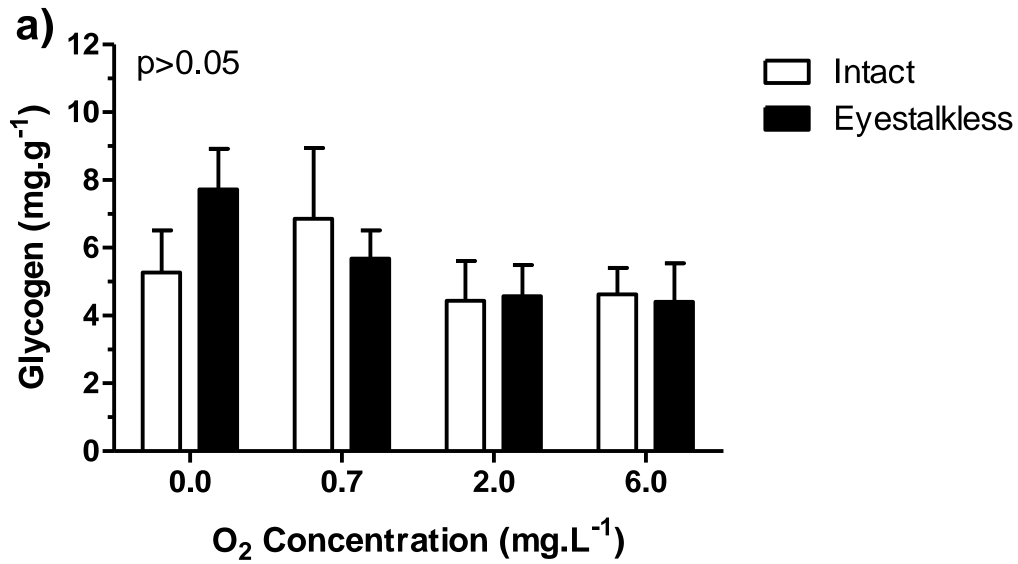


Fig. 4



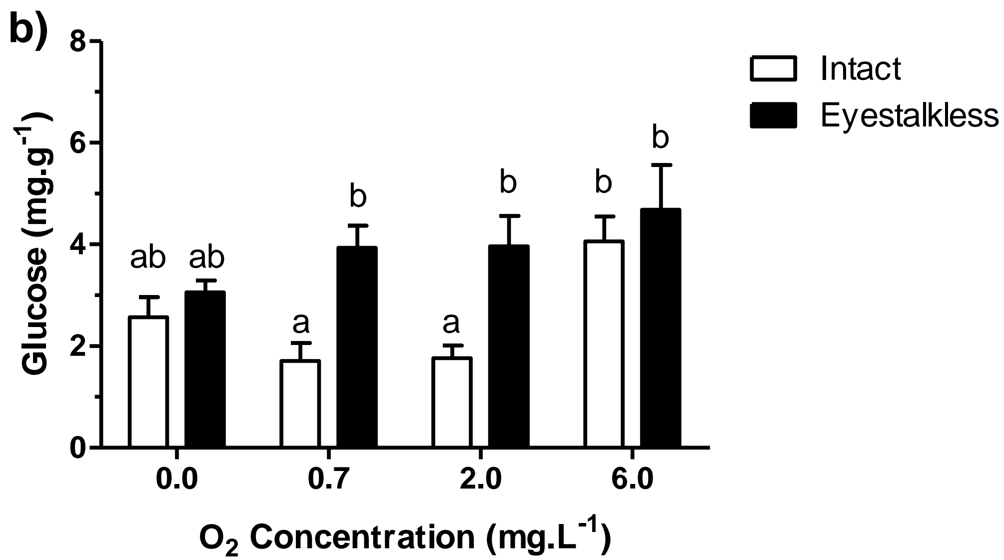


Fig. 5

```
                SP                              CPRP
1   tccccgacacgcacgcacgctcagcagaagggttcgggcgcatggagaggctgctgagtcaa
      P  D  T  H  A  R  S  A  E  G  F  G  R  M  E  R  L  L  S  Q   20

63   ctgagaggcggttcggactcctcagcagcgttgggagagatgagggtagccggggaggga
      L  R  G  G  S  D  S  S  A  A  L  G  E  M  R  V  A  G  E  G   40
                                                  CHH
124  ccagctggccaccctctggagaaacgccagatctacgaccgttcctgcaagggcatctac
      P  A  G  H  P  L  E  K  R  Q  I  Y  D  R  S  C  K  G  I  Y   80

184  gacaggtccctcttcagcaaactggaacacgtgtgcgacgattgctacaacctctacag
      D  R  S  L  F  S  K  L  E  H  V  C  D  D  C  Y  N  L  Y      79
```

Fig. 6

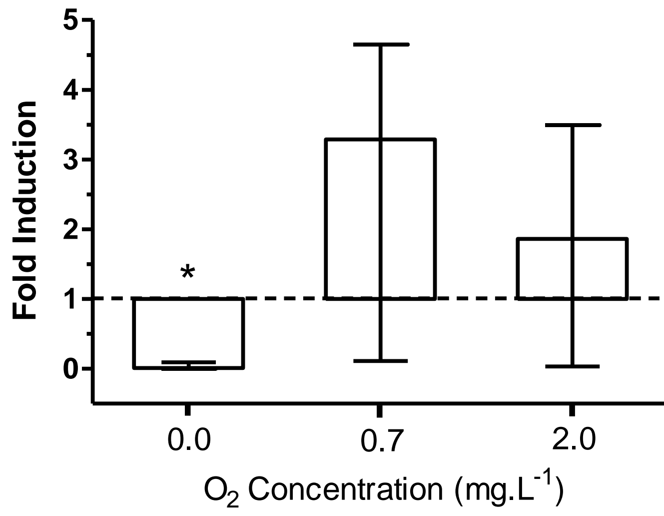


Fig. 7.

VIII. Artigo 3

Melatonina como uma molécula sinalizadora para a regulação do metabolismo do caranguejo *Neohelice granulata*

A ser submetido ao periódico Journal of Pineal Research

Melatonin as a signaling molecule for metabolism regulation in the crab *Neohelice granulata*

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Abstract

Melatonin was identified in variety of crustaceans species, but its function is not well established as in vertebrates. Recent reports have shown that this molecule affect crustacean metabolism. Thus, the aim of this work was to verify if melatonin has an effect on CHH expression, oxygen consumption (VO_2) and circulating glucose and lactate levels in response to different oxygen dissolved concentration in the crab *Neohelice granulata*, as well as if these possible effects are eyestalk and receptors dependent. Melatonin decreased CHH expression in animals exposed for 45 minutes to 6 (2, 200 and 20000 ρmol.animal^{-1}) and 2 (200 ρmol.animal-1) $mgO_2.L^{-1}$, but this was not through melatonin receptors, since luzindole (200 ηmol.animal^{-1}) did not significantly altered the melatonin effect. Concerning to circulating glucose and lactate, melatonin (200 ρmol.animal^{-1}) increased levels of these compounds in crabs exposed to 6 $mgO_2.L^{-1}$ and luzindole (200 ηmol.animal^{-1}) decreased this effect, indicating the involvement of melatonin receptors in hyperglycemia and lactemia in function to melatonin. No effect of melatonin was observed in VO_2. Therefore, melatonin seems to have an effect on the metabolism of *N. granulata*. This molecule inhibited the gene expression of CHH and promoted a hyperglycemia-dependent from eyestalk and receptors, which suggest that melatonin may have a signaling role in the metabolism regulation of this crab.

Keywords: melatonin, decapoda, hypoxia, metabolism, glucose, lactate, CHH.

Introduction

A half-century ago, Lerner et al. [1, 2] isolated from thousands bovine pineal glands the N-acetyl-5-methoxytryptamine or melatonin. Its name comes from the Greek *melano* = black and *tonos* = color, due to its action on lighting frog skin. However, the main action of this molecule, at least in vertebrates, is to inform the environment photoperiod length to the organism, which allows the animal to better adapt and also to anticipate, to the periodic environmental changes [3-5]. This indoleamine is present in a variety of organisms from mammals to unicellular algae, being produced in a circadian rhythm manner [6, 7]. In crustacean, melatonin has also been identified but its production and function is not so clear as in vertebrates [8]. Maciel and colleagues [9] verified that when the gills of the crab *Neohelice granulata* were incubated with melatonin (20.10^{-9}M), the oxygen consumption was reduced. Tilden et al. [10] observed a shift of the circadian rhythm of glucose and lactate in the hemolymph of the crab *Uca pugilator* when injected with melatonin (3.10^{-9}mol.animal^{-1}), with one peak in the middle of light phase. In another study, Sainath and Reddy [11], showed a dose-dependent effect of melatonin on hyperglycemia in the crab *Oziotelphusa senex senex*. Thus, these reports suggest that this indoleamine may have a role in the metabolism in crustaceans.

Since glucose is one of the main energetic substrate for the general metabolic processes in crustaceans [12], several studies have analyzing, the circulating glucose levels against several environmental stress imposed to the animal such as temperature [13-17], salinity [18, 19] and hypoxia [20-23]. The general response to these stressors is a hyperglycemia, evoked by the crustacean hyperglycemic hormone (CHH), a known peptide identified, as well as its nucleotide gene sequence, in a variety of crustacean species.

The estuarine crab *Neohelice granulata* is known to have a great tolerance to hypoxia/anoxia conditions imposed to oxygen decline in the water or to air exposition [24, 25], since these situations are common in their natural habitat in salt marshes [26]. Specific metabolic adjustment such as decreasing the locomotory activity and oxygen consumption (VO_2), and increase of glucose and lactate in hemolymph allow this crab to tolerate such stressors [21, 24, 25, 27-29]. In a recent report, Maciel et al. [8] verified that severe hypoxia (45 minutes at $0.7mgO_2.L^{-1}$) induced an intense decreasing of VO_2 and an increase of circulating lactate in both, intact and eyestalkless crabs, but a hyperglycemia in intact but not in eyestalkless crabs, indicating a dependence of CHH from eyestalk for this last response. In this sense, as some evidences, previously mentioned, indicate the involvement of melatonin

in the metabolism of crustaceans, the present work has the purpose to verify if melatonin is capable to induce metabolic alterations as it occurs in hypoxia and if this response is dependent from eyestalk and melatonin receptor.

Material and Methods

Animals

Adult male crabs of *N. granulata* (mean weight ± confidence interval: 11.2±1.1g) were collected in salt marshes around Rio Grande city, Southern Brazil. Thereafter, they were acclimated to water at constant conditions of salinity (20), temperature (20°C) and photoperiod (12L:12D) for at least 7 days. During this period, the crabs were regularly fed with ground beef.

Reagents

Melatonin (MEL) and luzindole (LUZ) were purchased from Sigma-Aldrich (Brazil). Stock solutions were made with ethanol and dimethyl sulfoxide (DMSO), respectively. Work solutions were made with crustacean physiological solution (PS), which contained: 1.10^{-2}M $MgCl_2$, 355.10^{-3}M NaCl, $16.6.10^{-3}$M $CaCl_2$, 5.10^{-3}M H_3BO_3, 1.10^{-2}M $KHCO_3$, 8.10^{-3}M sodium citrate ($Na_3C_6H_5O_7.2H_2O$); pH adjusted to 7.6.

RNA isolation and cDNA synthesis

The eyestalk ganglia were collected and dissected used for total RNA extraction. Total RNA was isolated by TRI Reagent SolutionTM method following the manufacturer's instructions (Applied Biosystems, Brazil). Genomic DNA was degraded with DNase I (Invitrogen) and total RNA was quantified by a QubitTM fluorometer using the Quant-iTTM RNA assay kit (Invitrogen, Brazil) and calibration was carried out using a two-point standard curve. First-strand cDNA was synthesized from approximately 1 µg of total RNA and used as a template with SuperScript III enzyme kit (Invitrogen, Brazil) according to protocol suggested by the manufacturer.

Experimental design and measurements of CHH gene expression, VO_2, glucose and lactate for different O_2 concentration and melatonin doses effects

Nine aquaria (3L each) were purged with nitrogen gas to reduce the level of dissolved oxygen to 6, 2 and 0.7 $mgO_2.L^{-1}$ (3 aquaria each). After that, the crabs (total of 144 animals)

were injected with 100µl of PS (control) or MEL (20.10^{-9} and 20.10^{-7}) and exposed for 45 minutes in each oxygen concentration prior to eyestalk collection for CHH gene expression (n=4, pool of 4 crabs) and hemolymth sampling (n=5). An additional experiment was carried out with eyestalkless crabs (total of 45 animals) at the same conditions for hemolymph sampling, which the animals had their eyestalks ablated (the eyestalks were cut off with scissors and then the crabs were cauterized) 24 hours prior to the beginning of the experiment. The eyestalks dissection, total RNA and cDNA synthesis were done as described above. Quantitative analysis of CHH mRNA expression was carried out for 10 min at 95°C and 40 cycles of 15 s at 95°C and 1min at 60° by real-time RT-PCR (7300 Real-Time System, Applied Biosystems – Brazil), using SYBR GREEN PCR Master Mix™ (Applied Biosystems), sense (5'-CGCACACGTGTTCCAGTTTG-3') and anti-sense (5'-AGATGCCCTTGCAGGAACG-3') gene specific primers, Ultra Pure Distilled Water (Invitrogen, Brazil) and 1.0 µL cDNA (dilution 1:19). β–actin expression (sense 5'-CCAGATCATGTTTGAGGTGTTCA-3' and anti-sense 5'-GGGACAGCACAGCCTGGAT-3' primers) was used as an internal control to normalize the data.

Glucose and lactate measurement was made using the GOD-PAD monoreagent kit (Kovalent – Brazil) and lactate kit (Kovalent – Brazil), respectively. Glucose and lactate are expressed in mg per dl of hemolymph.

For VO_2 measurement, 9 aquaria (3L each) were purged with nitrogen gas to reduce the level of dissolved oxygen to 6, 2 and 0.7 $mgO_2.L^{-1}$ (3 aquaria each). Thirty six intact (n=4) and 36 eyestalkless (n=4) crabs were injected with 100µl of PS (control) or MEL (20.10^{-9} and 20.10^{-7}) and exposed to the above oxygen concentrations in the respirometer chamber with saline water (salinity at 20) for 15 minutes prior to beginning of experiment. Oxygen concentration was measured at time zero and after 30 min using a portable oxymeter. Oxygen consumption was expressed in milligrams of oxygen per gram of body weight per hour. The eyestalk ablation procedure was made as describe previously.

<u>Experimental design and measurements of CHH gene expression, VO_2, glucose and lactate for different O_2 concentration and melatonin and luzindole doses effects</u>

Twelve aquaria (3L each) were purged with nitrogen gas to reduce the level of dissolved oxygen to 6, 2 and 0.7 $mgO_2.L^{-1}$ (4 aquaria each). After that, the crabs (total of 192 animals) received two injections of 100µl with 15 minutes of interval between them. One group received 2 injection of PS (control), the second group was injected first with LUZ (10^{-4})

and second with PS, the third group was injected with PS and after with MEL (20.10^{-7}), and the fourth group was injected first with LUZ (10^{-4}) and second with MEL (20.10^{-7}). Thereafter, the crabs were exposed for 45 minutes in each oxygen concentration prior to eyestalk collection for CHH gene expression (n=4, pool of 4 crabs) and hemolymth sampling (n=5). An additional experiment was carried out with eyestalkless crabs (total of 45 animals) at the same conditions for hemolymph sampling. The eyestalks dissection, total RNA, cDNA synthesis and CHH gene expression were done as described above, as well as for glucose and lactate measurements.

For VO_2 measurement, 12 aquaria (3L each) were purged with nitrogen gas to reduce the level of dissolved oxygen to 6 $mgO_2.L^{-1}$, only. Thirty six intact (n=4) and 36 eyestalkless (n=4) crabs received two injections of 100µl with 15 minutes of interval between them. The treatments were the same as described previously: PS plus PS, LUZ (10^{-4}) plus PS, PS plus MEL (20.10^{-7}) and LUZ (10^{-4}) plus MEL (20.10^{-7}). After that, the animals were exposed to the above oxygen concentration in the respirometer chamber with saline water (salinity at 20) for 15 minutes prior to beginning of experiment. Oxygen concentration was measured at time zero and after 30 min using a portable oxymeter. Oxygen consumption was expressed in milligrams of oxygen per gram of body weight per hour. The eyestalk ablation procedure was made as describe previously.

Statistical analysis

In the CHH gene expression, the REST© software was applied in the relative quantification [30], which pair comparisons were realized. For VO_2, glucose and lactate, two-way ANOVA was carried out followed by an a posteriori means comparison (Newman–Keuls test, $\alpha = 0.05$). ANOVA assumptions (normality and homogeneity variance) were verified prior to ANOVA analysis.

Results

All the three doses of melatonin were capable to significant ($p<0.05$) decrease CHH gene expression (Fig. 1) in the eyestalks of crabs exposed to 6 $mgO_2.L^{-1}$ (0.85±0.07, 0.94±0.04 and 0.96±0.03 relative expression for 2, 200 and 20000pmol.$crab^{-1}$ of melatonin, respectively), compared to control group (1.16±0.06 relative expression). Crabs exposed to 2 $mgO_2.L^{-1}$, the dose of 200pmol.$crab^{-1}$ of melatonin significant ($p<0.05$) decreased (0.79±0.03 relative expression) CHH gene expression compared to control group (1.03±0.05relative

expression). No significant differences ($p>0.05$) were observed between melatonin doses and control group in crabs exposed to 0.7 $mgO_2.L^{-1}$.

A significant hyperglycemia ($p<0.05$) was verified in intact crabs (Fig. 2a) exposed to 6 $mgO_2.L^{-1}$ and injected with 200ρmol.crab^{-1} of melatonin (16.2±0.87 mg.dl^{-1}) compared to control group (7.3±0.7 mg.dl^{-1}). This effect was not significant ($p>0.05$) in crabs exposed to 2 $mgO_2.L^{-1}$. Crabs exposed to 0.7 $mgO_2.L^{-1}$, a significant ($p<0.05$) hyperglycemia was observed in control group (19±2.6 mg.dl^{-1}) compared to the same group exposed to 6 (7.3±0.7 mg.dl^{-1}) and 2 (8.2±0.93 mg.dl^{-1}) $mgO_2.L^{-1}$. Interestingly, a significant increase ($p<0.05$) of glucose levels in crabs exposed to 0.7 and injected with 2ρmol.crab^{-1} of melatonin (33.9±4.72 mg.dl^{-1}) was observed. In eyestalkless crabs (Fig. 2b), no significant differences ($p>0.05$) were verified in all dissolved oxygen concentration and melatonin doses injections.

In intact crabs exposed to 6 $mgO_2.L^{-1}$ and injected with 20000 ρmol.crab^{-1} were verified a significant ($p<0.05$) increase of lactate (Fig. 3a) concentration (39.3±4.18 mg.dl^{-1}) compared to control group (12.5±2.1 mg.dl^{-1}). No significant effects ($p>0.05$) were observed in crabs exposed to 2 $mgO_2.L^{-1}$ and injected with the doses of melatonin. However, when the crabs were exposed to 0.7 $mgO_2.L^{-1}$, in all groups a significant increase ($p<0.05$) of lactate concentration was observed compared to the other dissolved oxygen concentration points. In eyestalkless crabs (Fig 3b) only the exposition to 0.7 $mgO_2.L^{-1}$ significant increased ($p<0.05$) lactate levels in all treatment groups.

No significant differences ($p>0.05$) were observed in VO_2 of intact and eyestalkless (Fig. 4) crabs exposed to 6 $mgO_2.L^{-1}$ and treated with melatonin.

Melatonin (200 ρmol.crab^{-1}) significant decreased ($p<0.05$) CHH gene expression in crabs exposed to 6 (0.94±0.02 1.relative expression^{-1}) and 2 (0.8±0.05 1.relative expression^{-1}) $mgO_2.L^{-1}$ independently if injected with luzindole (200ηmol.crab^{-1}), compared to control group (1.21±0.04 and 1.19±0.04 1.relative expression^{-1}, respectively) (Fig. 5). No significant differences were observed in animal exposed to 0.7 $mgO_2.L^{-1}$.

Again, injections of melatonin (200 ρmol.crab^{-1}) significantly increased ($p<0.05$) glucose levels in intact crabs (Fig. 6) exposed to 6 $mgO_2.L^{-1}$ (36.4±3.5 mg.dl^{-1}) but animals also injected with luzindole (200ηmol.crab^{-1}) (21.5±3.5) did not differ ($p>0.05$) from control group (13.4±1.3 mg.dl^{-1}). No significant effects ($p>0.05$) of melatonin or luzindole were verified in crabs exposed to 2 $mgO_2.L^{-1}$. In 0.7 $mgO_2.L^{-1}$ a general increase of glucose levels were observed in treatment groups (34.7±9.5 mg.dl^{-1}, CTR as reference; $p<0.05$ compared to CTR of 6 $mgO_2.L^{-1}$).

Similar effects were verified in lactate measurements (Fig. 7). Melatonin (200 ρmol.crab^{-1}) significant increased ($p<0.05$) lactate concentration in crabs exposed to 6 $mgO_2.L^{-1}$ (44.5±4.2 $mg.dl^{-1}$), but crabs also injected with luzindole (200ηmol.crab^{-1}) (31.5±4.8 $mg.dl^{-1}$) did not differ ($p>0.05$) from control group (21.8±2.9 $mg.dl^{-1}$). At 2 $mgO_2.L^{-1}$ luzindole significantly decreased ($p<0.05$) lactate levels (23.1±5.2 $mg.dl^{-1}$) compared with control group (45.5±6.1 $mg.dl^{-1}$) but not significant affected melatonin group. Crabs exposed to 0.7 a general lactemia were verified in all treatment groups (64.5±6.1, CTR as reference; $p<0.05$ compared to CTR of 6 $mgO_2.L^{-1}$).

In VO_2 measurements, no significant differences ($p>0.05$) were observed between the treatment groups in intact and eyestalkless (Fig. 8) crabs exposed to 6 $mgO_2.L^{-1}$.

Discussion

As mentioned before, melatonin is a ubiquitous molecule in live organisms. In crustacean, this molecule already was identified in a dozen of species [8], but its function is not so clear. Some reports have been shown that melatonin can be involved in electroretinogram rhythm and limb regeneration in crustacean. However, recent reports have been shown a connection of this molecule to metabolism in crabs. Maciel and co-workers [9] verified that gills of *N. granulata* decreased its VO_2 when incubated for 120 minutes with 20.10^{-9}M of melatonin, and also decreased gills VO_2 in eyestalkless crabs injected with melatonin after 390 min. In contrast, when equivalent dose of melatonin were injected in the same species, no effect was observed in muscle VO_2 [31]. In the present work, melatonin did not evoke any change in global VO_2 in both intact and eyestalkless crabs. All these data suggest that this indoleamine effect may be tissue specific to aerobic metabolism depression, but as muscle tissue is the most representative, the global VO_2 in this crab is not significantly affected by melatonin, at least in those dose tested.

Besides aerobic metabolism, melatonin seems have a role in glucose/lactate effects in crustaceans. In eyestalkless *Uca pugilator*, injections of melatonin (3.10^{-9}mol.animal^{-1}) caused an increase of glucose and lactate levels, indicating an independence of this effect of CHH from eyestalk [10]. In this case, melatonin could act as secretagogue of other CHH releasing tissue and/or act directly in the breakdown of glycogen to glucose through glycogen phosphorylase tissues. In fact, Sainath and Reddy [11] verified an increase of phosphorylase activity in hepatopancreas and muscle of intact and eyestalkless crab *Oziotelphusa senex*

senex when injected with melatonin (10^{-7} mol.crab^{-1}), and also a hyperglycemia in a dose dependent manner (10^{-10} – 10^{-5} mol.crab^{-1}), which reinforce that melatonin-induced hyperglycemia is not through CHH from eyestalk, at least in this species. Here, however, a hyperglycemia was observed in intact but not in eyestalkless *N. granulata* injected with 200 ρmol.carb^{-1}, indicating dependence of CHH from eyestalk. In addition, this effect seems to be by melatonin receptors, since the administration of luzindole (200 ηmol.crab^{-1}), glucose levels did not significant differ from control group.

The effect of melatonin in hyperglycemia in *N. granulata* is affected by hypoxia. In severe hypoxia (0.7 mg.L^{-1}), the lowest melatonin dose (2 ρmol.carb^{-1}) caused hyperglycemia, but not 200 ρmol.carb^{-1}. The hypoxic condition seems to increase sensibilization of melatonin effects, suggesting that melatonin could mobilize carbohydrates already in low doses of melatonin.

Melatonin (20000 ρmol.crab^{-1}) also increased lactate levels in *N. granulata*, but only when exposed to 6 $mgO_2.L^{-1}$. In severe hypoxia lactate concentration significant increased independently if crabs were treated with melatonin or not. In this species, some reports verified that lactate is the main metabolite of anaerobic metabolism. In addition, lactate level is augmented when the crab is exposed to hypoxic/anoxic situations [21, 23, 29]. However, this effect is not totally eyestalk dependent, since in eyestalkless crabs, a lower but also an increase of lactate levels was observed.

Interestingly, melatonin decreased CHH gene expression when *N. granulata* was exposed during 45 minutes to 6 (2, 200 and 20000 ρmol.crab^{-1}) and 2 (200 ρmol.crab^{-1}) $mgO_2.L^{-1}$, but its effect seems to be not through melatonin receptor. This down regulation of CHH gene expression by melatonin could be an indirect effect of this indoleamine and a negative feedback response to the increase of circulating glucose levels. Chung and Zmora [32] also verified an Inhibition of CHH expression in the blue crab *Callinectes sapidus* but in response to 1 hour of hypoxia condition. Again, a negative feedback could be regulating this gene in response to hyperglycemia seen in *C. sapidus* in hypoxic condition [32](Chung and Zmora, 2008). However, this effect was not observed in *N. granulata* [23], maybe because this species is more tolerate to hypoxia/anoxia than *C. sapidus* and 45 minutes was not sufficient to induce a down regulation in eyestalk CHH expression.

In addition to the various functions previously described for melatonin, it was seen that this molecule has the ability, in mammals, to influence insulin secretion, and metabolism of lipids and glucose [33-37]. Additionally, some studies have observed that this indoleamine

may modulate the release of neurohormones in insects, such as the protoracicotropic hormone in *Periplaneta americana* [38] and the adipokinetic precursor related peptide in *Locusta migratoria* [39]. Already in crustaceans, the only similar study was conducted by Tilden et al. [40] who observed an increase in synaptic transmission at the neuromuscular junctions when injected melatonin in *Procambarus clarkii*. In the present study melatonin induced hyperglycemia, increased circulating lactate and inhibit CHH gene expression. This indoleamine could be stimulating CHH releasing and the glucose rising inhibiting CHH gene expression, or even the own CHH peptide regulates its transcription, but in this moment only speculation can be done.

The membrane receptors for melatonin have been identified in vertebrates, and are classified as MT1, MT2 and MT3 [41, 42]. MT1 and MT2 are known to be G protein-coupled receptor and MT3 an enzyme belonging to the family of quinone reductases, but its signaling pathway is still unknown. In invertebrates the melatonin receptors knowledge is poor. Richter et al. [38] verified the luzindole (10 ηM) inhibited the protoracicotropic hormone from *P. americana* brain when incubated with melatonin (10 ηM). Concerning crustacean, Mendoza-Vargas [43] observed an inhibition of receptor potential amplitude of retinular photoreceptors by DH97 (1 ηM), a MT_2 receptor selective antagonist, whereas melatonin (100 ηM) increased receptor potential amplitude in the crayfish *P. clarkii*. Here, luzindole (200 $\eta mol.crab^{-1}$) inhibited the rise of glucose and lactate caused by melatonin (200 $\rho mol.crab^{-1}$), but not affected CHH gene expression in *N. granulata*. Thus, melatonin may have distinct ways of action in the regulation in carbohydrate metabolism, since the melatonin response was seen at transcriptional and physiological levels.

In conclusion melatonin seems to induce hyperglycemia and anaerobic metabolism in *N. granulata* since glucose and lactate are augmented, but these responses are eyestalk and melatonin receptors dependent. Also, melatonin down regulates CHH gene expression but not by melatonin receptor.

Acknowledgements

Maciel FE was fellow by CAPES and Nery LEM is a research fellow by CNPq.

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Figure legends

Fig 1. Crustacean Hyperglycemic Hormone (CHH) gene expression in the eyestalks of *Neohelice granulata* exposed 45 minutes into three different dissolved oxygen concentration (0.7, 2 and 6 $mgO_2.L^{-1}$) and injected with physiological saline (CTR) or melatonin (2, 200 or 20000 $\rho mol.carb^{-1}$). Each point represents the mean±SE (n=3-4). * represents significant differences ($p<0.05$) from the CTR. The values are normalized by β-actin gene expression.

Fig. 2. Circulating glucose concentration in intact (a) and eyestalkless (b) *Neohelice granulata* exposed 45 minutes into three different dissolved oxygen concentration (0.7, 2 and 6 $mgO_2.L^{-1}$) and injected with physiological saline (CTR) or melatonin (2, 200 or 20000 $\rho mol.carb^{-1}$). Each point represents the mean±SE (n=4-7). Different letters represents significant differences ($p<0.05$) between the groups of the same dissolved oxygen concentration. * represents significant differences ($p<0.05$) from the CTR group at 6 $mgO_2.L^{-1}$.

Fig. 3. Circulating lactate in intact (a) and eyestalkless (b) *Neohelice granulata* exposed 45 minutes into three different dissolved oxygen concentration (0.7, 2 and 6 $mgO_2.L^{-1}$) and injected with physiological saline (CTR) or melatonin (2, 200 or 20000 $\rho mol.carb^{-1}$). Each point represents the mean±SE (n=4-7). Different letters represents significant differences ($p<0.05$) between the groups of the same dissolved oxygen concentration. * represents significant differences ($p<0.05$) from the CTR group at 6 $mgO_2.L^{-1}$.

Fig. 4. Oxygen consumption (VO_2) of intact (black bars) and eyestalkless (open bars) *Neohelice granulata* exposed 45 minutes only into 6 $mgO_2.L^{-1}$ and injected with physiological saline (CTR) or melatonin (2, 200 or 20000 $\rho mol.carb^{-1}$). Each point represents the mean±SE (n=3-6). No significant differences ($p>0.05$) were observed.

Fig. 5. Crustacean Hyperglycemic Hormone (CHH) gene expression in the eyestalks of *Neohelice granulata* exposed 45 minutes into three different dissolved oxygen concentration (0.7, 2 and 6 $mgO_2.L^{-1}$) and injected with physiological saline only (CTR), luzindole (200 $\eta mol.crab^{-1}$) plus physiological saline, physiological saline plus melatonin (200 $\rho mol.crab^{-1}$) and luzindole (200 $\eta mol.crab^{-1}$) plus melatonin (200 $\rho mol.crab^{-1}$). Each point represents the

mean±SE (n=3-4). * represents significant differences ($p<0.05$) from the CTR. The values are normalized by β-actin gene expression.

Fig. 6. Circulating glucose concentration in intact *Neohelice granulata* exposed 45 minutes into three different dissolved oxygen concentration (0.7, 2 and 6 $mgO_2.L^{-1}$) and injected with physiological saline only (CTR), luzindole (200 ηmol.crab^{-1}) plus physiological saline, physiological saline plus melatonin (200 ρmol.crab^{-1}) and luzindole (200 ηmol.crab^{-1}) plus melatonin (200 ρmol.crab^{-1}). Each point represents the mean±SE (n=4-6). Different letters represents significant differences ($p<0.05$) between the groups of the same dissolved oxygen concentration. * represents significant differences ($p<0.05$) from the CTR group at 6 $mgO_2.L^{-1}$.

Fig. 7. Circulating lactate concentration in intact *Neohelice granulata* exposed 45 minutes into three different dissolved oxygen concentration (0.7, 2 and 6 $mgO_2.L^{-1}$) and injected with physiological saline only (CTR), luzindole (200 ηmol.crab^{-1}) plus physiological saline, physiological saline plus melatonin (200 ρmol.crab^{-1}) and luzindole (200 ηmol.crab^{-1}) plus melatonin (200 ρmol.crab^{-1}). Each point represents the mean±SE (n=4-6). Different letters represents significant differences ($p<0.05$) between the groups of the same dissolved oxygen concentration. * represents significant differences ($p<0.05$) from the CTR group at 6 $mgO_2.L^{-1}$.

Fig. 8. Oxygen consumption (VO_2) of intact (black bars) and eyestalkless (open bars) *Neohelice granulata* exposed 45 minutes only into 6 $mgO_2.L^{-1}$ and injected with physiological saline only (CTR) luzindole (200 ηmol.crab^{-1}) plus physiological saline, physiological saline plus melatonin (200 ρmol.crab^{-1}) and luzindole (200 ηmol.crab^{-1}) plus melatonin (200 ρmol.crab^{-1}). Each point represents the mean±SE (n=3-4). No significant differences ($p>0.05$) were observed.

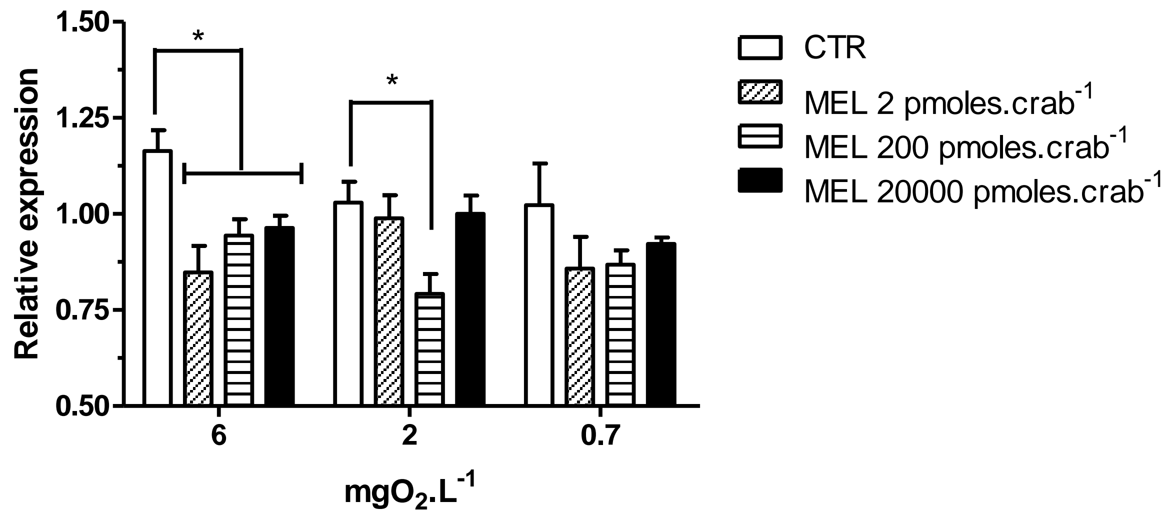
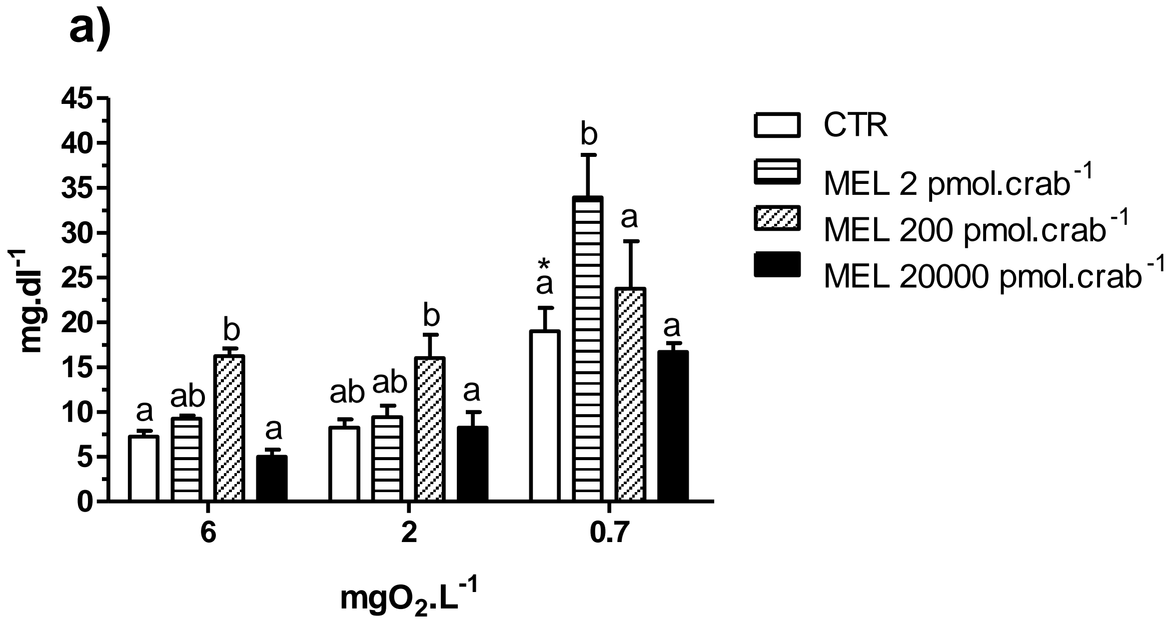
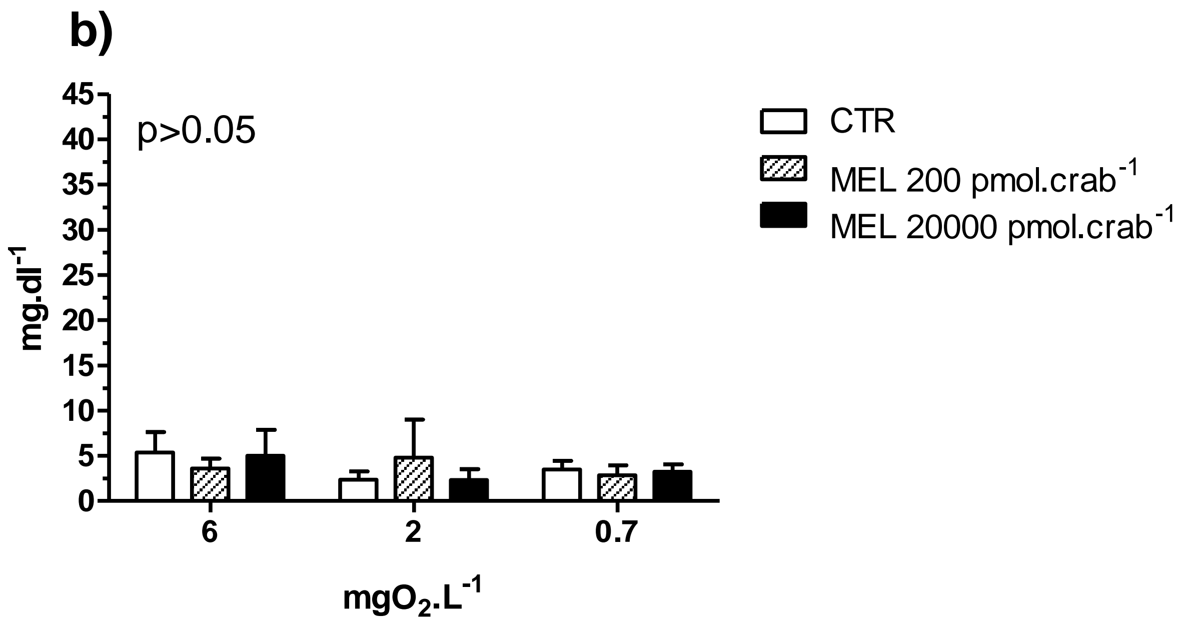


Fig. 1.





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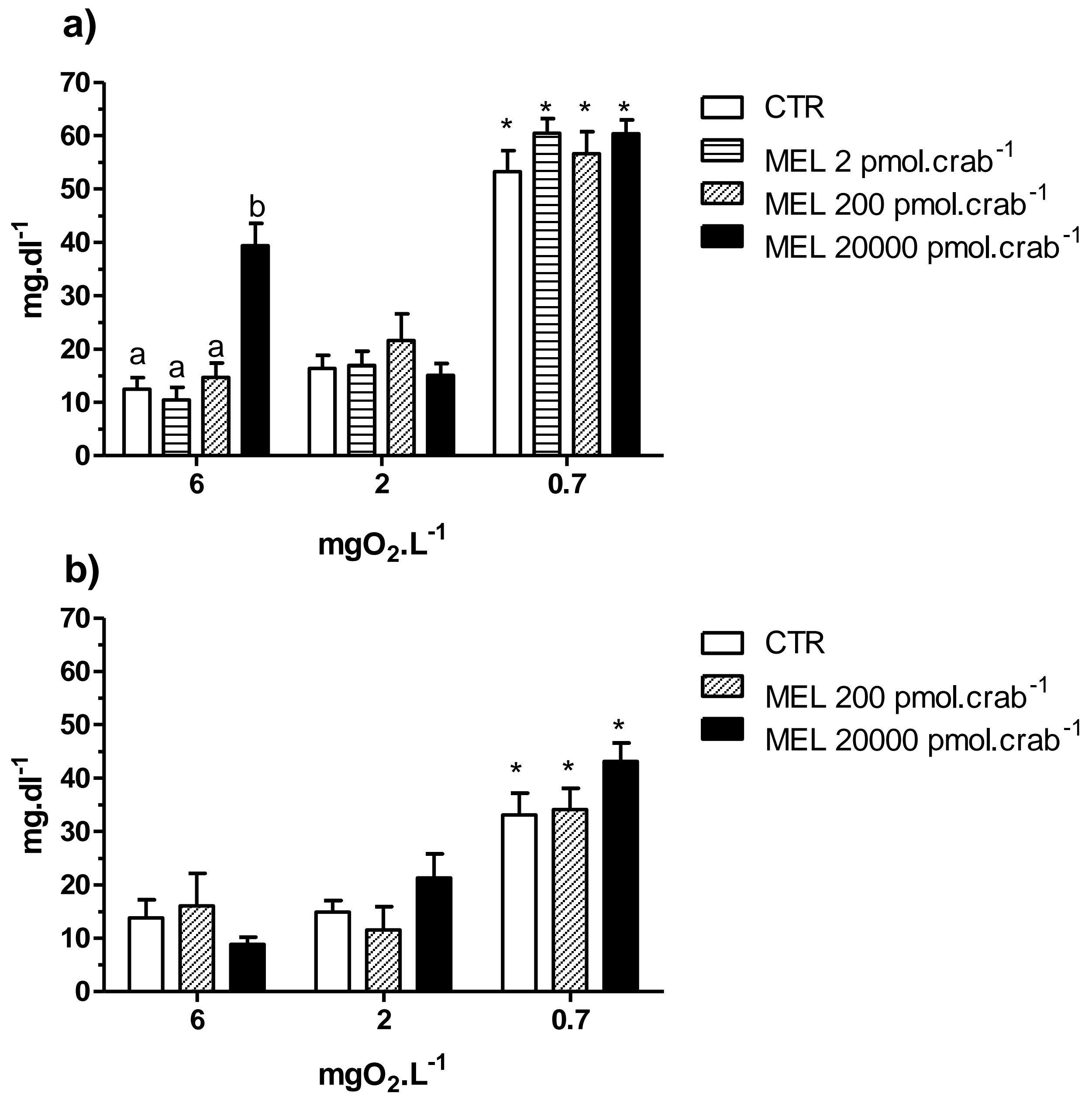

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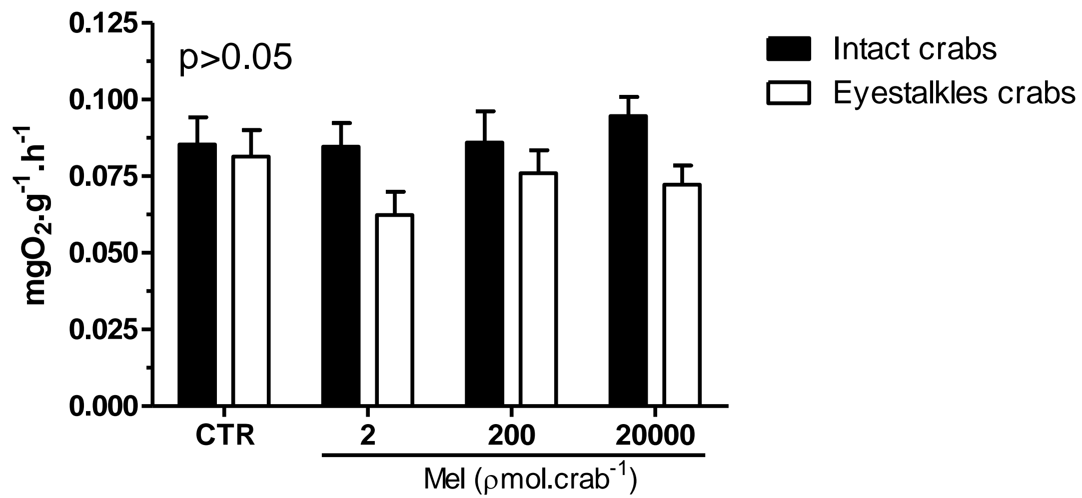
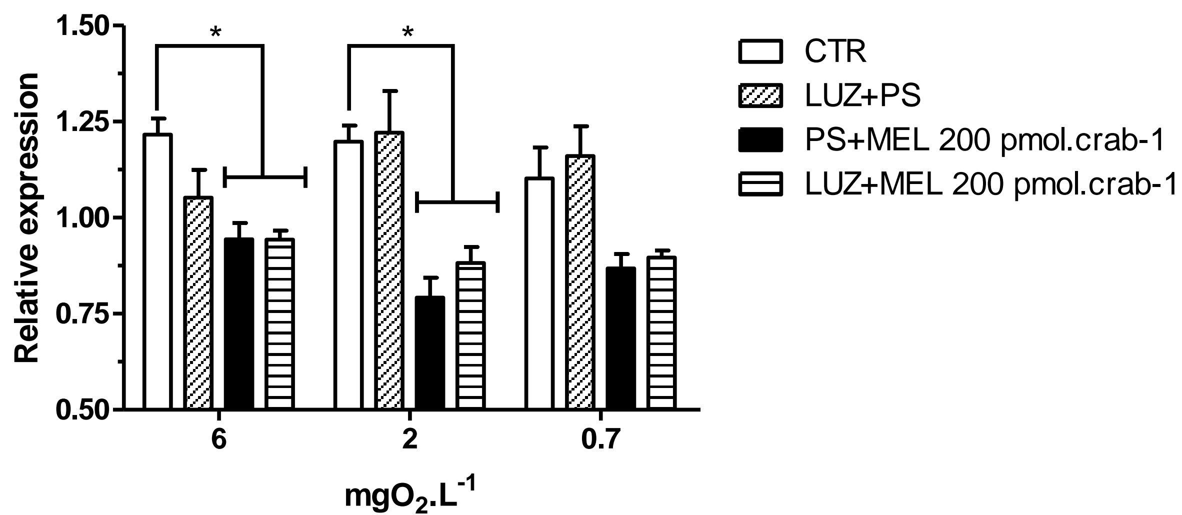


Fig. 4



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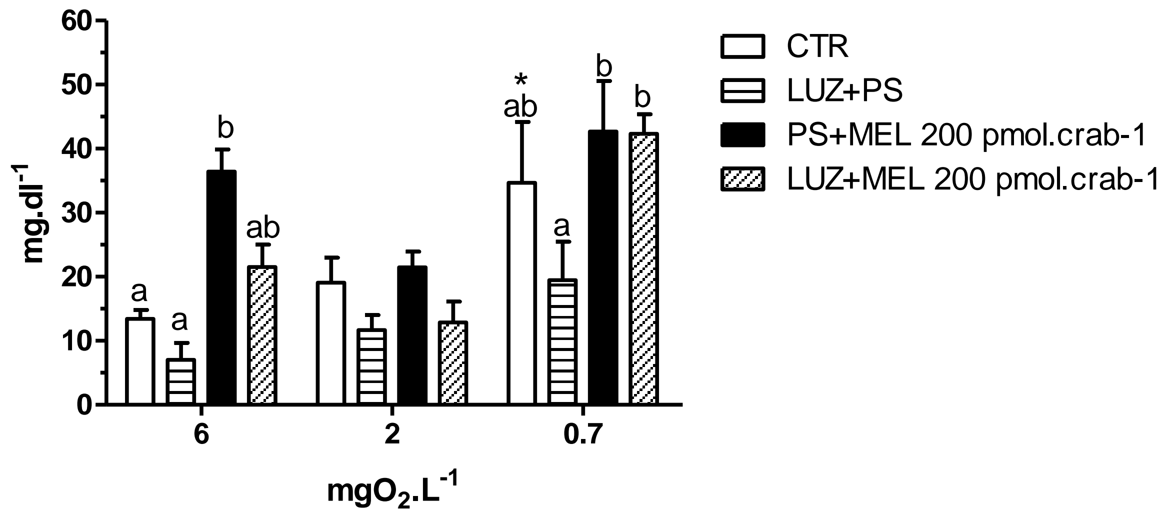

Fig. 6.

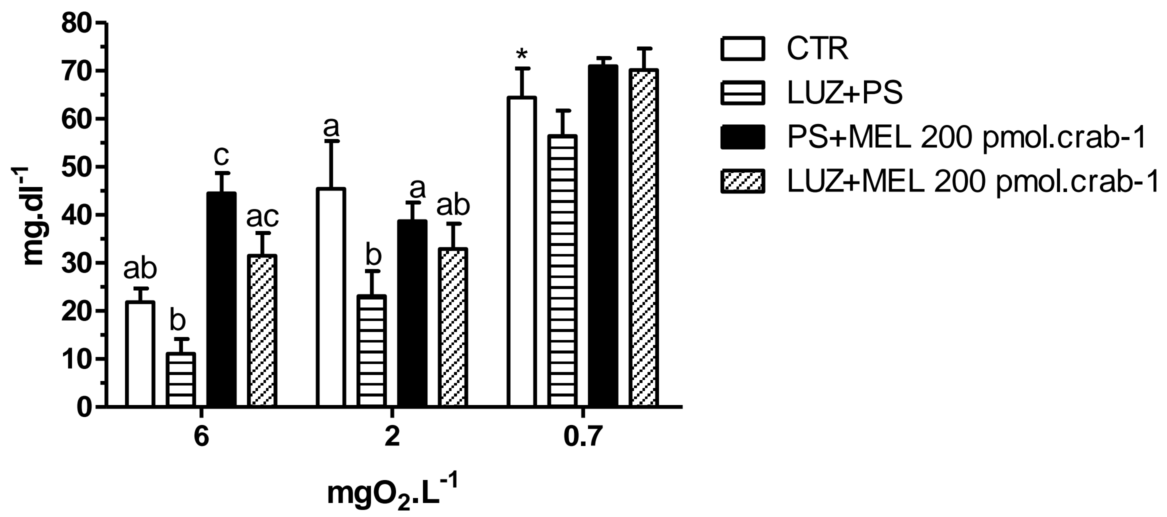

Fig. 7.

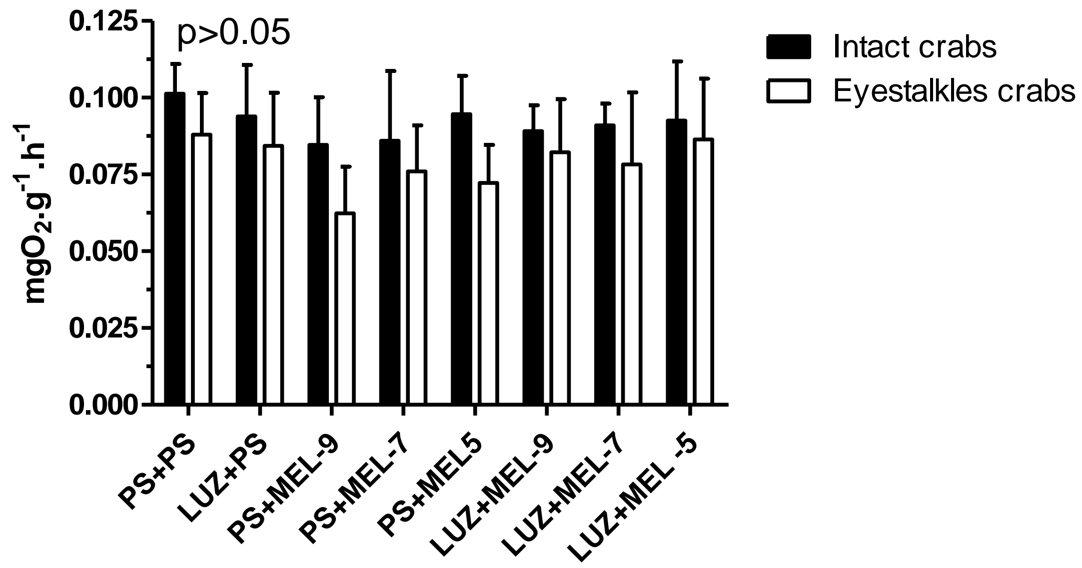

Fig. 8.

IX. Conclusões gerais

Caranguejos apedunculados são significativamente menos tolerantes a anoxia em comparação com caranguejos intactos,

A hipóxia severa diminui o consumo de oxigênio e aumenta os níveis de lactato independentemente da presença do pedúnculo ocular;

Condições severas de hipoxia e anoxia ocasionam hiperglicemia, sendo este efeito dependente do pedúnculo ocular, provavelmente da liberação do CHH proveniente deste sítio;

A blação e os diferentes níveis de oxigênio dissolvido não alteram marcadamente os níveis de glicogêni e glicose no músculo e hepatopâncreas, indicando que as reservas energéticas encontram-se distribuídas nos diferentes tecidos e não em um depósito central;

O gene do hormônio hiperglicemiante de crustáceos (CHH) clonado do pedúnculo ocular de *N. granulata* apresenta uma boa similaridade com outras sequências nucleotídicas do gene do CHH de outros crustáceos;

A sequência de aminoácidos deduzidas para o CHH se agrupa com outras sequencias do CHH de espécies de brachyura, se distinguindo de outros grupos de crustáceos;

A expressão do CHH peduncular é inibida quando os animais são expostos por 45 minutos a anoxia;

A melatonina diminui a expressão do CHH, porém este efeito não é via receptores de melatonina;

A melatonina promove hiperglicemia e lactemia, sendo estes efeitos dependentes do pedúnculo ocular e de receptores de melatonina;

O consumo de oxigênio global não é afetado pela melatonina;

A melatonina parece ter um papel de molécula sinalizadora na regulação do metabolismo de *N. granulata* similar ao efeito da hipoxia/anoxia sendo mediado, em algum grau, por receptores de melatonina e dependente do pedúnculo ocular.

X. Perspectivas

Esta tese de doutorado, a partir dos resultados obtidos, deixa como principais objetivos futuros:

1- um melhor entendimento do efeito da melatonina sobre a liberação do CHH e se este possível efeito é mediado por receptores de melatonina e quais tipos;
2- investigar se as células produtoras/liberadoras de CHH possuem receptores de melatonina;
3- verificar o efeito da melatonina na via glicolítica de crustáceos;
4- verificar o efeito da hipoxia/anoxia sobre a síntese e liberação de melatonina.

XI. Referências Bibliográficas da Introdução Geral

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