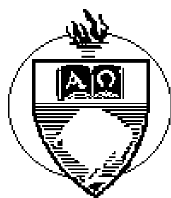

Daiana da Silva Castiglioni

# Adaptações metabólicas de *Parastacus defossus* Faxon, 1898 e *Parastacus brasiliensis* (von Martens, 1869) (Crustacea, Decapoda, Parastacidae)

Tese apresentada ao Programa de Pós-Graduação em Biologia Animal,
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Co-orientadora: Profª. Drª. Guendalina Turcato de Oliveira

**Universidade Federal do Rio Grande do Sul**

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**2010**

Adaptações metabólicas de *Parastacus defossus* Faxon, 1898 e *Parastacus brasiliensis* (von Martens, 1869) (Crustacea, Decapoda, Parastacidae)

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Tese aprovada em ________________________________________

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Prof. Dr. Luis Carlos Rios Kucharski

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**“Tudo pode ser só basta acreditar....**

**Os sonhos sempre vem pra quem sonhar....”**

(Xuxa)

**“Vivemos esperando... o dia em que seremos melhores, melhores no amor, melhores na dor, melhores em tudo...”**

(Jota Quest)

**"Se as coisas são inatingíveis... ora! Não é motivo para não querê-las. Que tristes os caminhos, se não fora a mágica presença das estrelas!"**

(Mário Quintana)

***Dedico essa tese aos meus pais, Dirlam e Tania.***

***"Pai, você é o meu orgulho, um exemplo***

***de honestidade e caráter***

***e você mãe, o meu porto seguro.***

***Amo vocês mais que o infinito..."***

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# ***Resumo***

Os lagostins são crustáceos decápodos límnicos que podem ser encontrados em água corrente, outros preferem água com pouca ou nenhuma corrente, como pequenos riachos, lagos, reservatórios e pântanos. Muitas espécies vivem em galerias subterrâneas com níveis mais baixos de oxigênio; assim, estas espécies podem mostrar adaptações metabólicas às condições hipóxicas. O objetivo desta pesquisa foi comparar o metabolismo de duas espécies de lagostins com diferentes hábitos*, Parastacus defossus* e *Parastacus brasiliensis*. *P. defossus* é uma espécie fossorial, vive em galerias com baixos níveis de oxigênio e *P. brasiliensis* é encontrado em ambientes lóticos com maiores níveis de oxigênio. Amostragens sazonais foram realizadas da primavera de 2006 ao inverno de 2007 para determinações metabólicas sazonais e posteriormente, amostragens foram realizadas durante o inverno de 2008 para análises metabólicas dos animais submetidos à hipóxia e recuperação pós-hipóxia. *P. brasiliensis* foi amostrado em Mariana Pimentel, Rio Grande do Sul (Brasil) e *P. defossus* foi amostrado no Lami, Porto Alegre, Rio Grande do Sul (Brasil). Nos experimentos de hipóxia, grupos de animais foram submetidos à hipóxia por 1, 2, 4 e 8 horas. Períodos de recuperação pós-hipóxia também foram analisados, após 4 hs de hipóxia, grupos de animais foram colocados em aquários com água aerada e foram removidos em intervalos de 1, 3, 6 e 9 hs. Após esse período foram extraídas amostras de hemolinfa e removidos o hepatopâncreas, o músculo, as brânquias e as gônadas para a determinação de glicose, lactato, glicose livre, glicogênio, proteínas totais, lipídios totais, colesterol total, arginina e arginina fosfato. Os resultados das análises sazonais mostraram diferentes respostas entre as estações do ano e entre as espécies, para todos os parâmetros metabólicos, com exceção das proteínas nas brânquias e do lactato na hemolinfa. As variações metabólicas em *P. defossus* foram principalmente relacionadas com o período

reprodutivo e os períodos de baixa concentração de oxigênio nas galerias, enquanto os resultados em *P. brasiliensis* sugerem uma alocação significativa dos nutrientes da dieta para o tecido gonadal durante o período reprodutivo, com uma menor transferência das reservas de diferentes tecidos para as gônadas. Em relação ao metabolismo dos animais submetidos à hipóxia foi observado que em ambas as espécies, os níveis de glicose e de lactato aumentaram significativamente em hipóxia. Reduções de glicogênio, lipídios e colesterol foram registradas no hepatopâncreas e no tecido muscular, especialmente de *P. defossus*. Todos os tecidos de *P. defossus* e *P. brasiliensis* mostraram reduções nos níveis de glicose livre, mas essas reduções não foram significativas. Todas as reservas das brânquias anteriores e posteriores, com exceção das reservas de glicogênio, mostraram comportamento semelhante em ambas as espécies. As duas espécies de *Parastacus* armazenaram e utilizaram arginina fosfato, principalmente *P. defossus*. Entre os resultados do metabolismo dos animais submetidos à recuperação pós-hipóxia foram observadas que a restauração dos níveis de lactato foi mais rápido em *P. defossus* quando comparado com *P. brasiliensis*. Essa espécie restabeleceu suas reservas de glicogênio do hepatopâncreas e do tecido muscular. Já os níveis de glicose livre foram rapidamente restabelecidos em todos os tecidos das duas espécies. Em relação às reservas de arginina fosfato, *P. defossus* mostrou maiores concentrações que *P. brasiliensis*. As duas espécies mostraram capacidade de restaurar os níveis de arginina fosfato, mas também utilizaram essas reservas durante períodos de recuperação. Nas espécies, as reservas de lipídios totais e colesterol parecem ser uma importante fonte de energia durante a recuperação.

**Palavras-chave**: lagostim, hipóxia, metabolismo, níveis de oxigênio, recuperação pós-hipóxia

# Abstract

Some species of crayfish live in flowing water, and others prefer water with little or no current such as small streams, lakes, reservoirs, and swamps. Many species live in subterranean burrows with lower oxygen levels, and can show metabolic adaptations to hypoxic conditions. The aim of this study was to compare the metabolism of two crayfish species with different habitats, *Parastacus defossus* and *Parastacus brasiliensis*. *P. defossus* is fossorial, living in burrows with low oxygen levels, and *P. brasiliensis* lives in lotic environments with higher oxygen levels. Seasonal sampling was conducted from spring 2006 to winter 2007 for seasonal metabolic determinations, and samples were taken during the winter of 2008 for metabolic analyses of the animals subjected to hypoxia and during the post-hypoxia recovery. *P. brasiliensis* was collected in Mariana Pimentel, Rio Grande do Sul (Brazil) and *P. defossus* at Lami, Porto Alegre, Rio Grande do Sul. In the hypoxia experiments, groups of animals were subjected to hypoxia for 1, 2, 4, and 8 h. Periods of post-hypoxia recovery were also analyzed; after 4 h of hypoxia, groups of animals were placed in tanks with oxygenated water and were then removed at intervals of 1, 3, 6, and 9 h. The hemolymph was extracted, and the hepatopancreas, muscle, gills, and gonads were removed for determination of glucose, lactate, free glucose, glycogen, total proteins, total lipids, total cholesterol, arginine, and arginine phosphate. The results of the seasonal analysis showed, for all metabolic parameters, different seasonal responses between the species, with the exception of proteins in gills, and of lactate in hemolymph. The metabolic variations in *P. defossus* were mainly related to reproductive period and periods of low oxygen concentration in the burrows. The results for *P. brasiliensis* suggested a significant allocation of dietary nutrients to gonadal tissue during the reproductive period, with a smaller transfer of reserves from different tissues to gonads. In both species, glucose and lactate levels

increased significantly in hypoxia. Reductions of glycogen, lipids, and cholesterol were recorded in hepatopancreas and muscle tissue, especially of *P. defossus*. In all tissues of *P. defossus* and *P. brasiliensis* were observed reductions in the free glucose levels, but these reductions weren't significant. All reserves in the anterior and posterior gills, except glycogen, behaved similarly in both species. Both *Parastacus* species, mainly *P. defossus,* stored and used arginine phosphate. During post-hypoxia recovery, lactate was restored more rapidly in *P. defossus* than in *P. brasiliensis*. *P. defossus* restored its glycogen reserves in the hepatopancreas and muscle tissue. Free glucose was quickly restored in all tissues of both species. In relation to the reserves of arginine phosphate, *P. defossus* showed higher concentrations than *P. brasiliensis*. The two species showed ability to restore this metabolite, but they also used this metabolite during longer periods of recovery. In both species, the reserves of total lipids and cholesterol seemed be an important source of energy during the recovery period.

**Keywords**: crayfish, hypoxia, metabolism, oxygen levels, post-hypoxia recovery

# ***Apresentação***

A presente tese está estruturada por uma “Introdução Geral” com o objetivo de proporcionar um melhor entendimento da biologia dos lagostins límnicos e em seguida uma síntese sobre o metabolismo intermediário dos crustáceos, assunto abordado nesta tese. Em seguida, a seção “Material e Métodos” que especifica as amostragens dos lagostins e as determinações metabólicas realizadas na hemolinfa e nos tecidos das duas espécies pesquisadas, *Parastacus defossus* e *Parastacus brasiliensis*. A seguir, são apresentados os três capítulos, na forma de artigos científicos. O Capítulo I caracteriza o metabolismo sazonal de *P. defossus* e *P. brasiliensis.* No Capítulo II está representado o perfil do metabolismo das duas espécies de *Parastacus* submetidas a diferentes períodos de hipóxia e o Capítulo III que apresenta o metabolismo dos animais submetidos a diferentes tempos de recuperação pós-hipóxia. Por fim, as “Considerações Finais” que contém as principais conclusões dos três artigos desenvolvidos nesta tese e, constam também algumas expectativas.

A tese está formatada em conformidade com as normas da Revista Brasileira de Zoologia. As normas para publicação desta revista e das demais revistas escolhidas para a publicação dos artigos são apresentadas nos anexos.

# Introdução

São mais de 540 as espécies de lagostins límnicos com ocorrência em todos os continentes, com exceção da África e da Antárctica (HOBBS 1988). Esses animais pertencem ao maior táxon dos crustáceos, Decapoda, e estão reunidos na infraordem Astacidea, superfamília Astacoidea De Haan, 1841, que reúnem as espécies límnicas do hemisfério norte, representadas pelas famílias Astacidae e Cambaridae e a superfamília Parastacoidea Huxley, 1879 representada pela família Parastacidae Hulex, 1879. Essa família reúne 14 gêneros e 129 espécies distribuídos pela Austrália, Tasmânia, Nova Zelândia, Madagascar e América do Sul (BUCKUP 1999).

A família Parastacidae está representada na América do Sul pelos gêneros *Parastacus* Huxley, 1879, *Samastacus* Riek, 1971 e *Virilastacus* Hobbs, 1991. No Brasil ocorrem somente espécies do gênero *Parastacus* representadas por seis espécies: *Parastacus brasiliensis* (von Martens, 1869), *Parastacus defossus* Faxon, 1898, *Parastacus varicosus* Faxon, 1898, *Parastacus pilimanus* (von Martens, 1869), *Parastacus saffordi* Faxon, 1898, *Parastacus laevigatus* Buckup and Rossi, 1980.

Os lagostins de água doce podem ser encontrados em uma grande variedade de habitats límnicos, como lagos, rios, pântanos, lagoas temporárias, represas e estuários (HOGGER 1988, NYSTROM 2002). Os parastacídeos também habitam uma grande diversidade de ambientes do limnociclo e estão entre os lagostins que exibem as maiores adaptações a condições ambientais extremas. Existem espécies que habitam águas correntes e outras que preferem águas com escassa ou nenhuma correnteza, como córregos pequenos, lagos, represas e pântanos (RICHARDSON 1983, HOGGER 1988).

No Brasil, os parastacídeos podem ser encontrados em ambientes límnicos das planícies meridionais (RS e SC), preferencialmente em áreas pantanosas e em águas lóticas de pequeno volume e correnteza fraca, faltando nos cursos d'água mais

correntosos do planalto sul brasileiro (BUCKUP & ROSSI 1980, FONTOURA & BUCKUP 1989a,b).

A maioria das espécies constrói galerias subterrâneas em forma de túneis simples ou ramificados que se ligam ao nível do lençol freático e apresentam uma ou mais aberturas na superfície do solo. As galerias abrem-se na superfície e podem ser reconhecidos por torres cônicas ou chaminés que circundam as aberturas. As chaminés são resultado do acúmulo de sedimento removido pelo lagostim durante o processo de escavação (BUCKUP & ROSSI 1993, RUDOLPH 1997, BUCKUP 1999). As espécies têm hábitos noturnos, deixando suas habitações subterrâneas em busca de alimento no interior da água ou nos ambientes mais próximos (BUCKUP 1999).

As galerias de *P. pilimanus* são formadas por vários túneis de entrada, que se unem abaixo da superfície, formando o túnel principal que desce verticalmente até a câmara habitacional, que pode estar situada a profundidade de até 2 metros, no nível da água freática. Nessas galerias coexistem indivíduos de diferentes gerações em uma mesma habitação (BUCKUP & ROSSI 1980). A espécie *P. defossus* constrói habitações semelhantes a *P. pilimanus* onde se observa um conjunto de galerias ligadas a várias aberturas na superfície do solo, com vários canais de acesso que convergem para galerias mais amplas que penetram até um metro, ou mais, no solo (NORO 2007).

Os parastacídeos são animais politróficos ou omnívoros oportunistas, com a dieta consistindo principalmente de detritos de origem vegetal (HOGGER 1988, GOODARD 1988, HOLLOWS *et al* 2002). Embora oportunistas, têm preferências por certos itens alimentares e variam sua alimentação de acordo com a idade, estação do ano e estado fisiológico (GODDARD 1988). Estudos realizados sobre o conteúdo estomacal de *P. defossus* por NORO (2007) mostraram que essa espécie alimenta-se basicamente de detritos vegetais. O mesmo foi observado em alguns espécimes de *P. varicosus* por

SILVA-CASTIGLIONI (2006).

Algumas pesquisas com as espécies de lagostins foram desenvolvidas no Brasil. BUCKUP & ROSSI (1980) realizaram uma revisão taxonômica do grupo e BUCKUP (1999) destacou aspectos biológicos e ecológicos das 4 espécies que ocorrem no Rio Grande do Sul, *P. brasiliensis*, *P. defossus*, *P. varicosus* e *P. pilimanus.* Nessa pesquisa foi incluída a espécie *P. saffordi* por ser encontrado no Uruguai e em Santa Catarina, o que permite supor que venha a ser localizada também no Rio Grande do Sul.

Aspectos da biologia de *P. brasiliensis* foram analisados por FRIES (1984) em condições de cultivo experimental em laboratório, a qual verificou que esta espécie possui características positivas para o cultivo com fins comerciais como desenvolvimento direto, cuidado da prole, baixa taxa de mortalidade e grande resistência à manipulação. A mesma espécie foi investigada por FONTOURA & BUCKUP (1989a,b) os quais analisaram o crescimento, a dinâmica populacional e a reprodução obtendo, entre outros resultados, informações sobre o período reprodutivo que se estende de setembro a fevereiro. As fêmeas atingem a maturidade sexual com, aproximadamente, 3 anos de vida e o tempo de incubação dos ovos foi estimado em 41 ± 15 dias.

CRANDALL *et al.* (2000) investigaram as relações filogenéticas dos gêneros de lagostins da América do Sul, incluindo o gênero *Parastacus*, relacionadas aos gêneros australianos. Entre os resultados, os pesquisadores observaram que os gêneros sul-americanos formam um grupo monofilético e estão relacionados com os gêneros australianos *Paranephrops e Parastacoides*.

Com relação à morfologia, estudos foram realizados sobre as setas do adulto de *P. brasiliensis* por HORN & BUCKUP (2004). A morfologia externa dos juvenis nos diferentes estágios, dessa mesma espécie, foi pesquisada por NORO *et al.* (2005) e a

morfologia externa do adulto foi pesquisada por HORN *et al.* (2008). BARCELOS (2005) descreveu a morfologia externa de *P. defossus* com ênfase nos apêndices e na distribuição dos diversos tipos de setas.

A história natural de *P. defossus* foi investigada por NORO (2007) que buscou, especialmente, informações sobre as habitações subterrâneas, comportamento escavador, dinâmica populacional, biologia reprodutiva e análise do conteúdo estomacal. Entre os resultados, observou-se que esta espécie passa a totalidade de sua existência no interior de suas galerias onde se reproduz e obtém o alimento de que necessita.

Com relação à biologia reprodutiva, as espécies de *Parastacus* apresentam em ambos os sexos o poro genital masculino no coxopodito do pereiópodo 5 e o poro genital feminino no coxopodito do pereiópodo 3. Apresentam também os dois pares de dutos genitais (ALMEIDA & BUCKUP 1999, SILVA-CASTIGLIONI *et al.* 2008). A presença de características sexuais masculinas e femininas em um mesmo indivíduo é conhecida como intersexualidade. Essas características podem estar limitadas à morfologia externa ou podem se estender à diferenciação das gônadas (SAGI *et al.* 1996, KHALAILA & SAGI 1997, SILVA-CASTIGLIONI *et al.* 2008).

A intersexualidade pode estar associada ao hermafroditismo, o qual é caracterizado como a capacidade de um conjunto de genes de proporcionar a formação de gametas de ambos os sexos. O hermafroditismo pode ser simultâneo, o qual se caracteriza pela presença de gônadas de ambos os sexos no mesmo indivíduo simultaneamente ou pode ser sequencial, caracterizado pela presença de gônadas de ambos os sexos no mesmo indivíduo em diferentes momentos de sua vida. O hermafroditismo sequencial pode ser de dois tipos: a protandria e a protoginia. Quando o primeiro sexo é o feminino a condição é chamada de protoginia e quando o primeiro

sexo é o masculino a condição denomina-se protandria (GHISELIN 1969, WARNER 1975). Nas espécies de *Parastacus*, evidências de hermafroditismo protândrico foram registradas em *P. nicoteli, P. brasiliensis, P. defossus* e *P. varicosus* pesquisadas por RUDOLPH (1995), ALMEIDA & BUCKUP (2000), NORO *et al.* (2008) e SILVA-CASTIGLIONI *et al.* (2008), respectivamente. Com exceção da pesquisa com *P. nicoleti,* as demais foram desenvolvidas no Brasil.

As espécies investigadas nessa pesquisa, *Parastacus defossus* e *Parastacus brasiliensis* (figura 1), apresentam diferentes hábitos de vida. *P. defossus* é uma espécie fossorial e escava suas galerias em terrenos baixos e alagadiços. Essas galerias subterrâneas podem apresentar 2 ou mais metros de profundidade, que ligam o nível do lençol freático com uma ou mais aberturas na superfície do solo e as concentrações de oxigênio no interior dessas galerias são muito baixas, de aproximadamente 1,4ml/L de oxigênio (BUCKUP & ROSSI 1980, NORO 2007). Enquanto *P. brasiliensis* pode ser encontrada com facilidade, principalmente sob detritos vegetais que se acumulam nos remansos e baixos dos ambientes lóticos menores; escavam suas habitações nas margens de pequenos ambientes lóticos como arroios, riachos e fontes, com concentrações elevadas de oxigênio (FONTOURA & BUCKUP 1989a, BUCKUP 1999). Portanto, uma das principais diferenças no habitat dessas duas espécies são as concentrações de oxigênio registradas.

*P. defossus* ocorre no Brasil e no Uruguai. No Rio Grande do Sul é particularmente comum em zonas pantanosas das planícies adjacentes ao estuário do Guaíba ao sul de Porto Alegre (BUCKUP & ROSSI 1980, BUCKUP, 1999). *P. brasiliensis* é uma espécie endêmica do Rio Grande do Sul e ocorre nas bacias que formam o estuário do Guaíba na depressão central do estado.

### *Metabolismo intermediário*

Os crustáceos estão expostos, em seus habitats, a variáveis ambientais que podem alterar o comportamento, a alimentação e o metabolismo. O metabolismo intermediário apresenta grande variabilidade inter e intra-específica em crustáceos. Essa variabilidade pode ser atribuída ao habitat em que vivem, ao estágio do ciclo de muda, alimentação, a sazonalidade e a reprodução (CHANG & O'CONNOR 1983, KUCHARSKI & DA SILVA 1991, ROSA & NUNES 2003, OLIVEIRA *et al*. 2003, 2007), entre outros fatores.

Nos crustáceos, as variações metabólicas estão principalmente relacionadas com o período reprodutivo das espécies, visto que diferentes órgãos podem atuar como fonte de armazenamento e transferência de reservas orgânicas para a maturação das gônadas durante o período reprodutivo (PILLAY & NAIR, 1973; ROSA & NUNES, 2003, SILVA-CASTIGLIONI *et al*. 2007). Essas reservas são armazenadas e/ou mobilizadas, principalmente do hepatopâncreas para as gônadas (PILLAY & NAIR 1973, ROSA & NUNES 2003, SILVA-CASTIGLIONI *et al.* 2006). Em decápodos, o hepatopâncreas é o principal órgão de armazenamento dos recursos energéticos (GIBSON & BARKER 1979).

O desenvolvimento gonadal está relacionado com o armazenamento de reservas orgânicas e minerais nos tecidos somáticos, que são transferidos para as gônadas durante a gametogênese (LAWRENCE 1976). Como o hepatopâncreas dos crustáceos é o maior centro de reservas energéticas, espera-se que ocorra uma mobilização dessas reservas para a maturação das gônadas. Essa mobilização pode ser observada pelos índices hepatossomático e gonadossomático como registrado em várias espécies de decápodos, onde podemos destacar as pesquisas realizadas por PILLAY & NAIR (1973), HAEFNER & SPAARGAREN (1993), CHU (1995), LÓPEZ-GRECO & RODRÍGUEZ (1999).

O período reprodutivo pode ser determinado pelo índice gonadossomático, o qual expressa a porcentagem que as gônadas representam no peso total dos indivíduos.

Esse índice é utilizado como um método qualitativo na determinação do período reprodutivo de uma espécie (GRANT & TAYLOR 1983, VAZZOLER 1996, LÓPEZ-GRECO & RODRÍGUEZ 1999). Nos lagostins de água doce esse índice foi determinado em *Procambarus clarkii* (Girard), *Cherax quadricarinatus* (von Martens), *Cherax quinquecarinatus* Gray, 1845 e *Parastacus varicosus* Faxon, 1898 pesquisados por KULKARNI *et al.* (1991), SAGI *et al.* (1996), BEATTY *et al.* (2005) e SILVA-CASTIGLIONI *et al.* (2006), respectivamente e no caranguejo de água doce *Aegla platensis* Schmitt, 1942 por SOKOLOWICS *et al.* (2006).

Mobilização das reservas do hepatopâncreas para o desenvolvimento das gônadas foi observada em *Aegla platensis* (SOKOLOWICS *et al.* 2006); contudo, no estudo desenvolvido por OLIVEIRA *et al.* (2007), com este eglideo, observaram uma transferência parcial dos nutrientes dos tecidos para a estrutura gonadal. Entretanto, em três espécies de decápodos, os camarões *Aristeus antennatus* (Risso, 1816), *Parapenaeus longirostris* (Lucas, 1846) e a lagosta *Nephrops norvegicus* (Linnaeus, 1758) pesquisadas por ROSA & NUNES (2003), não foi observada uma transferência dessas reservas para o desenvolvimento ovariano. Esses autores sugeriram que os recursos do hepatopâncreas não são esgotados e compensados diretamente pela alimentação. Em *Parastacus varicosus* pesquisado por SILVA-CASTIGLIONI *et al.* (2006) o índice hepatossomático não mostrou variação significativa durante o período amostral, mas uma redução foi registrada no verão, com o aumento do índice gonadossomático, mostrando uma transferência parcial das reservas do hepatopâncreas para as gônadas durante o período reprodutivo. Esse fato foi corroborado com análises metabólicas dessa espécie que mostraram mobilização das reservas lipídicas e proteicas (SILVA-CASTIGLIONI *et al.* 2007).

As concentrações lipídicas são bastante elevadas em crustáceos apesar de não

existir um tecido adiposo diferenciado, os principais locais de armazenamento são o músculo e o hepatopâncreas (O'CONNOR & GILBERT 1968, CHANG & O'CONNOR 1983, HERREID & FULL 1988, KUCHARSKI & DA SILVA 1991a). Mobilizações das concentrações lipídicas, principalmente as do hepatopâncreas, são registradas durante períodos de grande demanda energética, como a muda e a gametogênese. Segundo SASTRY (1983) a oogênese envolve uma intensa mobilização de lipídios para o desenvolvimento dos ovos. No caranguejo de estuário *Neohelice granulata*, foi observada uma redução dos lipídios do hepatopâncreas no período reprodutivo (KUCHARSKI E DA SILVA 1991) como também em outras espécies de crustáceos (PILLAY & NAIR 1973, CASTILLE & LAWRENCE 1989, SILVA-CASTIGLIONI *et al*. 2007).

O músculo parece ser a principal fonte de proteínas em crustáceos. Variações dessas reservas durante o desenvolvimento ovariano foram registradas em *Parastacus varicosus* por SILVA-CASTIGLIONI *et al.* (2007). Estas variações podem resultar de um aumento na biossíntese de várias proteínas, incluindo hormônios, enzimas e lipoproteínas envolvidas com a maturação das gônadas (ROSA & NUNES 2003, YEHEZKEL *et al*. 2000, OLIVEIRA *et al.* 2007).

A glicose, principal monossacarídeo na hemolinfa dos crustáceos, é armazenada na forma de glicogênio no músculo, no hepatopâncreas, no coração, nos hemócitos e nas brânquias. Sugere-se assim, que a ausência de um depósito central de glicogênio seria uma adaptação importante para animais que em seu habitat estariam submetidos a períodos de hipóxia e que possuem o sistema circulatório do tipo aberto com baixa pressão e fluxo lento, determinando uma distribuição menos efetiva de glicose para os tecidos (HOCHACHKA & SOMERO 1984).

O glicogênio é utilizado nos processos de muda, adaptação a hipóxia e/ou anoxia, osmorregulação, crescimento, diferentes estágios de reprodução e durante

períodos de jejum (CHANG & O'CONNOR 1983, KUCHARSKI & DA SILVA 1991, VINAGRE & DA SILVA 1992, ROSA & NUNES 2003). O ciclo de armazenamento/mobilização de glicogênio e os valores de glicose apresentam flutuações marcantes, dependendo, entre outros fatores, do teor de oxigênio dissolvido na água (CHANG & O'CONNOR 1983, HERREID & FULL 1988). Os principais tecidos de reserva de glicogênio em crustáceos são os músculos, o hepatopâncreas, as brânquias e os hemócitos, porém, o local de armazenamento deste polissacarídeo vária de acordo com a espécie (PARVATHY 1971, JOHNSTON & DAVIES 1972, HERREID & FULL 1988). Em *Parastacus varicosus* pesquisado por SILVA-CASTIGLIONI *et al.* (2007) altos níveis de glicogênio foram encontrados principalmente nas gônadas.

As únicas pesquisas desenvolvidas no Brasil sobre o metabolismo intermediário dos crustáceos límnicos foram realizadas com os caranguejos anomuros *Aegla ligulata* Bond-Buckup e Buckup, 1999 por OLIVEIRA *et al.* (2003) e *A. platensis*, por FERREIRA *et al.* (2005) e OLIVEIRA *et al.* (2007). O metabolismo intermediário dos lagostins *P. defossus, P. brasiliensis e P. varicosus* foi pesquisado por BUCKUP *et al.* (2008), DUTRA *et al.* (2008) e SILVA-CASTIGLIONI *et al.* (2007), respectivamente. Essas espécies límnicas, citadas anteriormente, foram analisadas somente com relação às reservas de carboidratos e gorduras. No entanto, nenhuma pesquisa foi desenvolvida no Brasil sobre o lactato e as reservas de arginina fosfato nas espécies de crustáceos límnicos, metabólitos importantes no metabolismo anaeróbico e no fornecimento de energia, respectivamente.

### *Metabolismo intermediário e hipóxia*

Os animais aquáticos estão submetidos a alterações mais frequentes e rápidas com relação aos níveis de oxigênio do que os animais terrestres, que utilizam à

respiração aérea, porque a mistura e a difusão são mais rápidas no ar do que na água. O oxigênio, em sistemas aquáticos é, aproximadamente, 25 vezes menor do que no ar atmosférico. Além disso, a alta densidade e viscosidade da água demandam maiores esforços para sua extração, por parte dos aparelhos respiratórios dos animais aquáticos, representando assim, um custo metabólico elevado. Por estas razões, o oxigênio torna-se um fator limitante para os organismos com respiração aquática (MARGALEF 1974, RANDALL *et al.* 2000).

Redução dos níveis de oxigênio (hipóxia) ou até mesmo a ausência (anoxia) podem ocorrer nos ecossistemas aquáticos. A tolerância a hipóxia/anoxia é muito variável entre os animais; muitos organismos podem tolerar longos períodos a níveis reduzidos de oxigênio, mas não conseguem sobreviver na sua completa ausência. A tolerância a hipóxia varia, consideravelmente, entre as espécies e entre os órgãos e estágios de desenvolvimento, dentro de uma determinada espécie (STOREY & STOREY 2004a).

Os animais tolerantes a hipóxia ou anoxia são encontrados entre os diferentes grupos de vertebrados e invertebrados (HOCHACHKA & SOMERO 2002, KNOLL AND CARROL 1999, LUTZ & STOREY 1997). No entanto, poucas espécies de vertebrados toleram a períodos severos de hipóxia e praticamente nenhuma resiste à anoxia crônica (LUTZ 1992). Exceções são observadas entre os peixes e os répteis; duas espécies de ciprinídeos, a carpa cruciana *Carassius carassius* (Linnaeus, 1758) e o peixe-dourado *Carassius auratus* (Linnaeus, 1758) e as tartarugas de água doce dos gêneros *Chrysemys* e *Trachemys* (HOCHACHKA & SOMERO 2002). Com relação aos invertebrados, os nematoideos, moluscos bivalvos, anelídeos e platelmintos estão entre os invertebrados que apresentam os melhores mecanismos de tolerância a hipóxia (HOCHACHKA & SOMERO 2002).

A capacidade de tolerância a hipóxia é muito variável entre os crustáceos (SCHMITT & UGLOW 1998). Deste modo, muitas espécies de decápodos são muito tolerantes a hipóxia e apresentam estratégias adaptativas que permitem a sobrevivência durante horas ou até mesmo dias (HOCHACHKA 1980, HOCHACHKA & SOMERO 1984, HERVANT *et al.* 1995, 1999a). Geralmente as espécies com hábito subterrâneo são mais adaptadas do que as espécies que vivem em águas mais oxigenadas (VERNBERG 1983, HERVANT *et al.* 1995).

Em crustáceos foram identificadas estratégias adaptativas que permitem a sobrevivência em hipóxia ou anoxia ambiental, entre estas podemos citar: 1) manutenção em todos os tecidos de altas concentrações de glicogênio e fosfato (ex. arginina fosfato); 2) utilização de vias anaeróbicas para a produção de ATP; 3) redução da atividade metabólica e 4) depressão metabólica (STOREY & STOREY 1990, HERVANT *et al.* 1995, CHILDRESS & SEIDEL 1998). A principal estratégia é a utilização de mecanismos anaeróbicos e a principal via para a produção de ATP na ausência de oxigênio é a glicólise anaeróbica (HOCHACHKA 1980, HOCHACHKA & SOMERO 1984).

Embora a glicólise anaeróbica seja considerada a principal via de produção de energia, sob baixas condições de oxigênio, existem algumas limitações dessa via no fornecimento de ATP, comparado com as vantagens do metabolismo aeróbico. A primeira limitação é que a glicólise aeróbica pode utilizar carboidratos, lipídeos e proteínas como combustíveis oxidativos enquanto em condições anaeróbicas os organismos estão restritos ao uso de carboidratos e poucos aminoácidos como combustíveis fermentáveis. A segunda limitação é com relação à produção de energia, pois a glicólise anaeróbica produz somente 2 mol de ATP por mol de glicose catabolisada a lactato comparada com 36 mol de ATP gerados pela glicólise aeróbica. Outra limitação é com relação aos produtos finais, que na glicólise anaeróbica,

geralmente o lactato, se não excretado ou neutralizado pode causar toxicidade enquanto o produto final da glicólise aeróbica, o $CO_2$ é excretado ou exalado (STOREY & STOREY 2004b). Portanto, a glicólise anaeróbica embora seja a principal via para a produção de ATP, na ausência de oxigênio, ela apresenta limitações.

Os invertebrados podem utilizar quatro principais vias anaeróbicas (BARNES *et al*. 1993): a primeira é a via do lactato, a mais conhecida de todas. A segunda via é a das opinas, um derivado de aminoácido, similar à via do lactato e também adaptada para as atividades energéticas intensas, que necessitam de uma produção rápida de ATP. A terceira via metabólica é a via do succinato, que ocorre em organismos como os moluscos bivalves que habitam substratos lodosos anóxicos e em endoparasitas que vivem em locais anaeróbicos de seus hospedeiros, como o intestino de vertebrados. Esses organismos desenvolveram vias metabólicas que não são capazes de gerar ATP de forma rápida, mas produzem mais ATP por resíduo de glicose do que as vias das opinas e do lactato. A quarta, principal, via utilizada é a via dos fosfagênios, que são importantes durante períodos de intensa atividade e anoxia.

A via do lactato é a mais utilizada pelos crustáceos, pois esse metabólito é o principal produto do metabolismo anaeróbico (ZEBE 1982, GÄDE 1984, TAYLOR & SPICER 1987, HILL *et al.* 1991, SANTOS & KELLER 1993, ANDERSON *et al.* 1994, HERVANT *et al.* 1995, 1996, 1997, SCHMITT & UGLOW 1998, SPICER *et al.* 2002, OLIVEIRA *et al.* 2004). Em várias espécies foi observado aumento nos níveis de lactato durante a hipóxia ou anoxia como no lagostim *Orconectes limosus* (Rafinesque, 1807), no caranguejo *Carcinus maenas* (Linnaeus, 1758), na lagosta *Nephrops norvegicus* (Linnaeus, 1758) pesquisados por GÄDE (1984), HILL *et al*. (1991) e SCHMITT & UGLOW (1998), respectivamente.

Os organismos mais resistentes à hipóxia ou anoxia apresentam maiores reservas

de glicogênio, principal polissacarídeo fermentável armazenado nas células animais (URICH 1994, LUTZ & STOREY 1997, HOCHACHKA & SOMERO 2002). O metabolismo de carboidratos é crucial para a vida hipóxica ou anóxica, uma vez que, esse substrato pode gerar energia através da fermentação. Como esperado, os organismos tolerantes a hipóxia ou anoxia apresentam altas concentrações de glicogênio (HOCHACHKA & SOMERO 2002, LUTZ & STOREY 1997, URICH 1994).

Além das altas concentrações de glicogênio, os crustáceos também podem manter altas reservas de fosfoarginina (arginina fosfato), composto de arginina e ácido fosfórico pertencente ao grupo de compostos denominados fosfatos de alta energia (fosfagênio) que apresenta um papel importante na manutenção dos níveis normais de ATP (tamponamento) (TJEERDEMA *et al.* 1991). A arginina fosfato é muito utilizada em crustáceos para fornecer energia durante períodos de hipóxia, períodos de intensa atividade muscular e sob estresse (ENGLAND & BALDWIN 1983, HILL *et al.* 1991, SPEED *et al.* 2001). Poucas pesquisas foram realizadas sobre as reservas de arginina fosfato nos crustáceos submetidos à hipóxia onde podemos destacar as desenvolvidas por HILL *et al.* (1991), SPEED *et al*. (2001) e HERVANT *et al.* (1995, 1996, 1997).

Além das adaptações aos ambientes hipóxicos ou anóxicos, as espécies também precisam enfrentar períodos de reoxigenação, posterior ao estresse hipóxico/anóxico, período conhecido como recuperação pós-hipóxia ou pós-anoxia. A recuperação tem importância funcional para o organismo, pois é nesse período que os produtos do metabolismo anaeróbico precisam ser oxidados, excretados ou convertidos novamente a glicose ou glicogênio pela gliconeogênese e as reservas energéticas utilizadas durante a hipóxia ou anoxia precisam ser restabelecidas (ELLINGTON 1983). Segundo HERVANT & RENAULT (2002) a rápida recuperação das reversas energéticas pode ser uma resposta adaptativa de várias espécies subterrâneas como estratégias para sobreviverem em

ambientes com restrição de oxigênio.

Dois processos básicos ocorrem durante a recuperação, que permitem o organismo ou tecido, retornar à condição metabólica anterior a hipóxia ou anoxia,: o restabelecimento das concentrações de ATP e de fosfogênios e a distribuição dos produtos finais do metabolismo anaeróbico para excreção, oxidação ou reconversão em substratos anaeróbicos como, por exemplo, o glicogênio (ELLINGTON 1983).

As únicas pesquisas no Brasil sobre o metabolismo dos crustáceos submetidos à hipóxia e recuperação pós-hipóxia foram desenvolvidas com o caranguejo de estuário *Neohelice granulata* por OLIVEIRA *et al.* (2001, 2004), MARQUEZE *et al.* (2005) que analisaram o metabolismo dessa espécie mantida com diferentes dietas e submetidas a anoxia. OLIVEIRA *et al.* (2005) analisaram o balanço oxidativo das brânquias e MACIEL *et al.* (2008) verificaram as reservas de lactato no músculo da mesma espécie durante a hipóxia. No entanto, nada é conhecido sobre o metabolismo das espécies límnicas submetidas à hipóxia e a recuperação pós-hipóxia.

## *Objetivos*

O objetivo geral desta pesquisa foi investigar e comparar as adaptações metabólicas de duas espécies de lagostins límnicos com diferentes hábitos, *Parastacus defossus* e *Parastacus brasiliensis* frente à sazonalidade, a hipóxia e a recuperação pós hipóxia, contribuindo para a ampliação dos conhecimentos sobre o metabolismo dos parastacídeos.

## *Objetivos específicos*

### *Capítulo I*

1) Conhecer o perfil do metabolismo de glicose, lactato, glicose livre, glicogênio, proteínas totais, lipídeos totais, colesterol total, arginina e arginina fosfato na hemolinfa, hepatopâncreas, músculos, brânquias e gônadas de *P. defossus* e *P. brasiliensis*, verificando se há diferença significativa entre as espécies, entre os sexos e entre as estações do ano;

### *Capítulo II*

2) Caracterizar o perfil do metabolismo de glicose, lactato, glicose livre, glicogênio, proteínas totais, lipídeos totais, colesterol total, arginina e arginina fosfato na hemolinfa, hepatopâncreas, músculos e brânquias de *P. defossus* e *P. brasiliensis* submetidos a diferentes períodos de hipóxia, verificando se as espécies apresentam

adaptações metabólicas a hipóxia e se as espécies diferem significativamente entre si;

***Capítulo III***

3) Caracterizar o perfil do metabolismo de glicose, lactato, glicose livre, glicogênio, proteínas totais, lipídeos totais, colesterol total, arginina e arginina fosfato na hemolinfa, hepatopâncreas, músculos e brânquias de *P. defossus* e *P. brasiliensis* submetidos a diferentes períodos de recuperação pós-hipóxia, verificando se as espécies apresentam adaptações metabólicas a essas condições e se as espécies diferem significativamente entre si.

# ***Materiais e Métodos***

## ***1) Amostragem***

As amostragens de *Parastacus defossus* foram realizadas na região do Lami, no município de Porto Alegre, Rio Grande do Sul, Brasil, com uma bomba de sucção visando retirar os espécimens do interior de suas galerias (figura 2A). *Parastacus brasiliensis* foi amostrado em um riacho em Mariana Pimentel, Rio Grande do Sul, Brasil com armadilhas contendo iscas de fígado. As armadilhas foram colocadas no local de coleta e retiradas após 2 dias devido ao hábito noturno da espécie, facilitando assim a captura (figura 2B,C).

Nos locais de amostragens (figura 3,4), parâmetros ambientais como temperatura, pH e oxigênio dissolvido foram registrados em todas as estações. A temperatura foi obtida com termômetro de escala interna, o pH com um medidor portátil de pH (Cole-Parner) e o oxigênio dissolvido com auxílio de um termo-oxímetro portátil (OXI 330 WTW).

## ***2) Determinações metabólicas sazonais***

Amostras sazonais de *Parastacus defossus* e *Parastacus brasiliensis* foram realizadas durante a primavera de 2006 ao inverno de 2007, na metade de cada estação. Aproximadamente 150 e 120 espécimes de *P. defossus* e *P. brasiliensis* foram amostrados, respectivamente e em seguida, transportados em recipientes com água fria para o laboratório de Carcinologia da Universidade Federal do Rio Grande do Sul. Devido às diferentes estratégias de amostragem tentamos minimizar o estresse nos animais durante o transporte.

Os espécimes foram pesados e separados por sexo. O sexo foi confirmado pela observação da gônada sob microscópio óptico, pois esses lagostins não apresentam dimorfismo sexual. Os animais mostraram uma variação de tamanho de 17.7- 47.5 em *P.*

*brasiliensis* e 16.8-31.0 em *P. defossus* (comprimento de cefalotórax em mm).

A hemolinfa foi obtida com seringas contendo oxalato de potássio a 10% (anticoagulante). Amostras do hepatopâncreas, músculo do abdômen, brânquias (anteriores e posteriores) e gônadas foram extraídas, pesadas e armazenadas, assim como a hemolinfa a uma temperatura de –80° C. As brânquias foram separadas em anteriores e posteriores devido as diferenças metabólicas já observadas entre as brânquias de *Parastacus varicosus* por Oliveira *et al.* (2010).

Os níveis de glicose, lactato, glicose livre, glicogênio, proteínas totais, lipídeos totais, colesterol total, arginina e arginina fosfato foram quantificados. As determinações metabólicas foram realizadas através de métodos espectrofotométricos padronizados para outros crustáceos no laboratório de Fisiologia da Conservação da Pontifícia Universidade Católica do Rio Grande do Sul (FERNANDES *et al.* 2003, OLIVEIRA *et al.* 2007, SILVA-CASTIGLIONI *et al.* 2007, BUCKUP *et al.* 2008 e DUTRA *et al.* 2008).

Os índices gonadossomáticos e hepatossomáticos foram determinados para verificar possíveis mobilizações metabólicas durante o período reprodutivo das espécies. Os índices foram calculados segundo GRANT & TYLER (1983) e VAZZOLER (1996): IG= PG/PA X 100 (PG = peso da gônada e PA = peso do animal); multiplicado por 100 para obter a porcentagem, e IH= PH/PA X 100 (PH = peso do hepatopâncreas).

### *2.1) Determinações metabólicas na hemolinfa*

**a.** Os níveis de glicose foram quantificadas através do método da glicose oxidase com emprego do kit da Bioclin (glicose GOD-CLIN) . Os resultados foram expressos em mg/ml.

**b.** Para a determinação do lactato as amostras foram desproteinizadas com ácido perclórico. A dosagem de lactato foi realizada através do Kit da Boehringer-Mannheim, no sentido da

formação do piruvato (L-lactato + $NAD^+$ NADH + $H^+$). Os resultados foram expressos em mmol/L.

**c.** As proteínas totais foram quantificadas segundo método descrito por LOWRY (1957) com a albumina bovina como padrão, os resultados expressos em mg/ml.

**d.** Os lipídeos totais foram quantificados através do kit do método da sulfofosfovalina, com os resultados expressos em mg/dl.

**e.** O colesterol total foi quantificado através do kit da Lab test (liquiform- Cat. 76.2), com os resultados expressos em mg/dl.

### *2.2) Determinações metabólicas no hepatopâncreas, no músculo, nas brânquias e nas gônadas*

**a.** O glicogênio nos diferentes tecidos foi extraído segundo VAN HANDEL (1965) e quantificado como glicose, após hidrólise ácida (HCl) e neutralização com CaCO3 (GEARY *et. al*, 1981), utilizando-se o kit da Bioclin (glicose GOD-CLIN) (glicose oxidase). Os resultados foram expressos em mg/g.

**b.** A glicose livre foi determinada segundo CARR & NEFF (1984). Os tecidos foram pesados e homogeinizados com Ultra-Turrax. Para separar a fração lipídica, as amostras foram fixadas em uma solução de clorofórmio-metanol e centrifugadas. A concentração de glicose livre foi determinada pelo método calorimétrico da glicose-oxidase (Kit Biodiagnóstico) em uma fração intermediária obtida depois da centrifugação. Os resultados foram expressos em mg/g.

**c.** As proteínas totais foram quantificadas segundo método descrito por LOWRY (1957) com a albumina bovina como padrão, os resultados foram expressos em mg/ml.

**d.** Os lipídeos totais foram extraídos pelo método do clorofórmio: metanol (2:1) (FOLCH *et al.,* 1957) e determinados por uso do método da sulfofosfovalina (FRING & DUNN 1970), com

os resultados expressos em mg/g.

**e.** O colesterol total foi quantificado através do kit da Lab test (liquiform- Cat. 76.2), utilizando-se o método de extração de FOLCH *et al.* (1957), com os resultados expressos em mg/g.

**f.** Arginina e arginina fosfato foram determinadas utilizando o método de BERGMEYER (1985). A arginina foi determinada pela mudança na absorbância de 339 nm na reação catalisada pela octopina desidrogenase: arginina + piruvato + NADH + $H^+$ ↔ octopina + $NAD^+$ + $H_2O$. Para hidrolisar arginina fosfato em arginina e fosfato, 100 ul de 1 mol $l^{-1}$ HCl foi adicionado a 100 ul de homogeneizado tecidual e incubados. O ensaio foi repetido e a concentração de arginina subtraída para obter a arginina fosfato. Os resultados foram expressos em mmol/g.

### *3) Determinações metabólicas dos animais submetidos a hipóxia e a recuperação pós-hipóxia*

Espécimes de *Parastacus defossus* e *Parastacus brasiliensis* foram amostrados no inverno de 2008, aproximadamente 80 espécimes de cada espécie; 40 para os experimentos de hipóxia e 40 espécimes para os experimentos de recuperação. Os animais mostraram uma variação de comprimento de cefalotórax de 18.8-35.2 mm (média: 26 mm) em *P. defossus* e 21.4-44.0 mm (média: 31.3 mm) em *P. brasiliensis*. Os lagostins foram conduzidos em recipientes com água fria ao laboratório da Carcinologia da Universidade Federal do Rio Grande do Sul. Os animais foram aclimatados a temperatura constante de 19°C e alimentados durante 10 dias com ração para peixe de composição balanceada.

Grupos de animais foram mantidos em aquários individuais em condições normóxias de aproximadamente 10mg/L de oxigênio. Gás nitrogênio foi aerado nos aquários, em um

sistema único de aeração, para reduzir a concentração de oxigênio até 2mg/L; visto que esta concentração de oxigênio foi observada no habitat de *P. defossus* por NORO (2007). Os níveis de oxigênio foram constantemente monitorados com um oxímetro.

Os animais foram removidos dos aquários nos seguintes tempos: 1, 2, 4 e 8 horas; sendo parte dos animais utilizados como controle. Amostras de hemolinfa foram coletadas com uma seringa contendo anticoagulante (oxalato de potássio 10%). O hepatopâncreas, músculo abdominal, brânquias anteriores e posteriores foram removidos e armazenados no freezer a -80°C até a determinação de glicose, lactato, glicose livre, glicogênio, proteínas totais, lipídeos totais, colesterol total, arginina e arginina fosfato.

Para a realização dos experimentos de recuperação pós-hipóxia foi escolhido o tempo de 4 horas de hipóxia a 2 mg/L de oxigênio. Esse tempo foi escolhido pela análise da curva do lactato, pois foi observado o maior aumento significativo dos níveis de lactato e também porque no período posterior (8 horas), as concentrações mostram uma diminuição. Portanto, grupos de animais, após 4 horas de hipóxia, foram colocados em aquários com água aerada e submetidos a diferentes períodos de recuperação pós-hipóxia (1, 3, 6 e 9 horas). Parte dos animais do grupo de 4 h de hipóxia foi utilizada como grupo controle também foi monitorado. Os animais foram retirados dos aquários, após hipóxia, para a extração da hemolinfa e dos diferentes tecidos. Os metabólitos analisados e métodos utilizados para as determinações metabólicas na hemolinfa e nos diferentes tecidos dos animais submetidos a hipóxia e a recuperação pós-hipóxia foram os mesmos descritos anteriormente para as análises do metabolismo sazonal.

### *4) Análises estatísticas*

Todos os parâmetros metabólicos foram analizados com o teste de Levene para

homogeinidade e normalidade com o teste de Kolmogorov–Smirnov. ANOVA de três vias foi aplicada para verificar diferenças no metabolismo entre as espécies, os sexos e as estações do ano. Para verificar diferenças sazonais, ANOVA de uma via foi utilizada, seguida pelo teste de Bonferroni. Para os índices hepatossomáticos e gonadossomáticos foi também utilizado ANOVA de uma via seguido de Tukey. Nas análises estatísticas dos diferentes tempos de hipóxia e de recuperação pós-hipóxia, um teste de ANOVA de uma via foi utilizado, seguido pelo teste de Bonferroni. Para comparação entre as espécies, ANOVA de duas vias foi utilizada. O nível de significância adotado para todas as análises foi de 5% e todos os testes foram realizados utilizando o programa SPSS (Statistical Package for the Social Sciences (SPSS- 11.5).

Cabe salientar que as análises estatísticas aqui utilizadas já foram realizadas em outras pesquisas sobre o metabolismo de crustáceos, onde podemos destacar as desenvolvidas por KUSCHARSKI & DA SILVA (1991), HERVANT *et al.* (1999b), OLIVEIRA *et al.* (2007), SILVA-CASTIGLIONI *et al.* (2007) e DUTRA *et al.* (2008).

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**A** **B**

Figura 1. Espécies de lagostins. A) *Parastacus defossus*. B) *Parastacus brasiliensis*.

A

B

C

Figura 2. Local de amostragem de *Parastacus defossus:* Região do Lami, Porto Alegre, Rio Grande do Sul, Brasil. A) Vista panorâmica da área de amostragem; B,C) Chaminés contruídas por *P. defossus*.



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Figura 3. Local de amostragem de *Parastacus brasiliensis:* Mariana Pimentel, Rio Grande do Sul, Brasil.

B

A

C

Figura 4. Material utilizado nas amostragens dos lagostins. A) Bomba de sucção utilizada nas amostragens de *Parastacus defossus*. B,C) Armadilhas utilizadas nas amostragens de *Parastacus brasiliensis*.



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# *Capítulo I*

**Comparison of the seasonal responses of the intermediary metabolism in two species of freshwater crayfish from southern Brazil**

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**Abstract.** The aim of this study was to compare seasonal variations in the metabolism of two crayfish species with different habitats, *Parastacus defossus* that lives in subterranean galleries with low oxygen concentrations and *Parastacus brasiliensis* that lives in oxygenated environments. The metabolism of the species was compared, mainly evaluating the mobilization of the reserves for the reproductive period. Seasonal sampling was conducted from spring 2006 to winter 2007. *P. brasiliensis* was collected at Mariana Pimentel, and *P. defossus* at Lami, Porto Alegre, Rio Grande do Sul. Hemolymph, hepatopancreas, muscle, gills, and gonads were removed for determination of glucose, free glucose, glycogen, lactate, total protein, total lipids, total cholesterol, arginine, and arginine phosphate. The results showed different seasonal responses between the species for all metabolic parameters, with the exception of proteins in gills, and of lactate. Higher lactate levels were observed in *P. defossus* than in *P. brasiliensis* during the period of reduced oxygen, although these differences were not statistically significant. A significant difference between sexes was recorded in only some metabolites. The variability of the metabolism appears to be related to the reproductive period, but the activity of exploration in the environment also seems to be related to these variations. However, other factors may also be influencing the metabolism, such as the availability of food, and environmental conditions such as water temperature and dissolved oxygen levels, as previously observed for other decapods.

**Keywords:** Crustacea, Parastacidae, Metabolism, Seasonality.

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**Introduction**

The energy and resources obtained from the environment in the form of nutrients are allocated among maintenance, growth, and reproduction (Tytler and Calow, ’85). The growth rate and reproductive effort are largely a result of the amount of resources that organisms are capable of allocating to these alternative metabolic pathways after basic maintenance needs are fulfilled. Normally, organisms store extra resources in specific tissues, to be utilized later when the conditions and environmental clues favor or trigger either reproduction or growth (Oliveira et al., 2007; Silva-Castiglioni et al., 2007; Buckup et al., 2008).

Crustaceans show variations in intermediary metabolism, which are mainly related to the reproductive period because of the high energy demands at this time (Rosa and Nunes, 2003a; Kucharski and Da Silva, ’91a; Silva-Castiglioni et al., 2008). In reproduction, many reserves are stored and/or used; for example, a significant mobilization of lipids may occur, especially those in the hepatopancreas to the gonads (Pillay and Nair, ’73, Rosa and Nunes, 2003b, Silva-Castiglioni et al., 2006). According to Gibson and Barker (’79), in decapods, the hepatopancreas is the main organ where the energy resources are stored, and a mobilization of energy reserves for gonad maturation is expected during the reproductive period (RP). The RP can be determined by the gonadosomatic index, which expresses the percentage of gonad weight, compared to the total body weight (Grant and Taylor, ’83; Vazzoler, ’96).

Metabolic reserves are also used and/or stored during periods of reduction of the environmental oxygen level, where, especially, there is an increase in lactate as a product of the anaerobic metabolism; in crustaceans, lactate is the main anaerobic metabolite (Hill et al., ’91; Hervant et al., ’95, ’97; Zou et al., ’96; Oliveira et al., 2004). Other metabolites important in anaerobic metabolism are glycogen and arginine phosphate, which reach high concentrations and help the animals to tolerate different periods of the hypoxia/anoxia

(Hochachka and Somero, ’84; Tjeerdema et al., ’91; Lutz and Storey, ’97).

The only investigations of the intermediary metabolism of freshwater crustaceans were carried out with the anomuran crabs *Aegla ligulata* by Oliveira et al. (2003), *Aegla platensis* by Oliveira et al. (2003), and *Aegla platensis* by Ferreira et al. (2005) and Oliveira et al. (2007); and a study with two sympatric species of Amphipoda of the genus *Hyalella* by Dutra et al. (2007). Studies were also conducted with the crayfishes *Parastacus varicosus*, *Parastacus defossus,* and *Parastacus brasiliensis* by Silva-Castiglioni et al. (2007), Buckup et al. (2008), and Dutra et al. (2008), respectively. These studies showed the importance of the carbohydrates, total proteins, and fats in the metabolism of the animals, which were related mainly to the diet or reproductive period of the species, and showed seasonal variations in reserves.

The variability of the intermediary metabolism in crustaceans can be also attributed to other factors, including the habitat; however, comparative studies of the metabolisms of crustaceans with different life habits have not been performed in Brazil. In the present investigation, two crayfish species with different habits were investigated, *Parastacus defossus* and *Parastacus brasiliensis. P. defossus* is fossorial, and constructs its burrows in low and marshy land reaching 1.5 meters deep (unpublished observations). *P. brasiliensis* lives mainly in flowing surface waters, under plant debris that accumulates in backwaters and smaller lotic environments (Buckup and Rossi, ’80; Fontoura and Buckup, ’89; Buckup, ’99).

Seasonal variations of the metabolic reserves were observed in each species and, a comparison was realized between species mainly evaluating the mobilization of their reserves for the reproductive period. In addition, we launched the hypothesis that *P. defossus* must store significant energy reserves in the tissues to transfer them to the gonads in the reproductive months and during periods of oxygen deficit, and to use them as a source of

ATP, while *P. brasiliensis* must allocate energy reserves of nutrients directly from the diet to transfer them to the gonads during the period reproductive.

## Materials and Methods

Seasonal samples of *P. brasiliensis* and *P. defossus* were taken at Mariana Pimentel and Porto Alegre, Rio Grande do Sul, respectively, from spring 2006 to winter 2007. The specimens of *P. brasiliensis* were collected with traps baited with liver and, specimens of *P. defossus* were collected from their burrows with the use of suction pumps. Both species were sampled in the intermolt period. During the sampling, the temperature, pH, and dissolved oxygen were measured.

The animals were transported in containers with cold water to the Carcinology Laboratory of the Universidade Federal do Rio Grande do Sul. Due to different sampling strategies try to minimize the stress of the animals during the transport. Approximately, 120 specimens of *P. brasiliensis* and, 150 specimens of *P. defossus* were sampled. The animals showed a variation of size (cephalothorax length in mm) of 17.7- 47.5 in *P. brasiliensis* and, 16.8-31.0 in *P. defossus*.

In the laboratory, hemolymph was obtained with syringes containing 10% potassium oxalate. The animals were weighed and separated by sex. Samples of hepatopancreas, abdominal muscle, gills (anterior and posterior), and gonads were extracted, weighed, and stored, as well as the hemolymph, at a temperature of -80 °C. The anterior and posterior gills were separated because of the differences in the metabolic analyses between the gills of *Parastacus varicosus* observed by Oliveira et al. (2010). Subsequently, the levels of glucose, lactate, arginine phosphate, free glucose, glycogen, total protein, total lipids, and cholesterol were measured. The tests were performed by standard spectrophotometric methods as used

for other crustaceans in the Laboratório de Fisiologia da Conservação, of the Pontifícia Universidade Católica do Rio Grande do Sul (Oliveira et al., 2003; Oliveira et al., 2007; Silva-Castiglioni et al., 2007; Buckup et al., 2008; Dutra et al., 2008).

The gonadosomatic and hepatosomatic indices were determined to compare with the results of the metabolic tests, to check possible links with the reproductive period of the species. The indices were calculated according to Grant and Tyler ('83) and Vazzoler ('96): IG = PG / PA X 100 (PG = weight of gonad and PA = weight of the animal), multiplied by 100 to obtain the percentage; and IH = PH / PA X 100 (PH = weight of hepatopancreas).

**Hemolymph Measurements**

The levels of glucose were quantified by the glucose oxidase method with the use of the Bioclin kit (glucose GOD-CLIN). The results were expressed as mg/ml. The proteins were quantified by the method described by Lowry ('51) with bovine albumin as standard, and the results were expressed as mg/ml.

The total lipids were quantified by the method of sulphosphovanillin (Frings and Dunn, '70), with the results expressed in mg/dl. Cholesterol was determined by the Lab test kit (Cat. liquiform-76.2), with the results expressed in mg/dl. For the determination of lactate, samples were deproteinized with perchloric acid, and the determination was carried out using the Boehringer-Mannheim kit, for the formation of pyruvate (L-lactate + NAD$^+$ NADH + H$^+$). The results were expressed in mmol/L.

**Tissue Measurements: hepatopancreas, muscle, gills, and gonads**

The free glucose was determined according to Carr and Neff ('84). Tissues were weighed and homogenized with Ultra-Turrax. To separate the lipid fraction, the samples were

mixed in a solution of chloroform-methanol and centrifuged. The concentration of free glucose was determined by the calorimetric glucose-oxidase method (Biodiagnóstico Kit) in an intermediate fraction obtained after centrifugation. The results were expressed as mg/g of tissue. The glycogen in different tissues was extracted according to Van Handel ('65), and quantified as glucose after acid hydrolysis (HCl) and neutralization with $CaCO_3$ (Geary et. al., '81), using a Bioclin kit (glucose GOD-CLIN) (glucose oxidase). The results were expressed as mg/g. The proteins were measured as described by Lowry ('51), with bovine albumin as the standard, and the results were expressed as mg/ml.

The total lipids were extracted by method of Folch et al. ('57) of chloroform: methanol (2:1), and determined by the sulphosphovanillin method (Fring and Dunnm, '70), with the results expressed in mg/g. Cholesterol was measured by the lab test kit (Cat. liquiform-76.2), using the extraction method of Folch et al. ('57), with the results expressed in mg/g.

Arginine and arginine phosphate were determined using the method of Bergmeyer ('85). The arginine was determined by the change in absorbance at 339 nm in the reaction catalyzed by octopine dehydrogenase: arginine + pyruvate + NADH + $H^+$ ↔ octopine + $NAD^+$ + $H_2O$. To hydrolyze arginine and arginine phosphate to phosphate, 100 µl of 1 mol $l^{-1}$ HCl was added to 100 µl of tissue (homogenate) and incubated in tightly capped tubes for 90 s in boiling water. The hydrolysates were then cooled and neutralized with 100 µl of 1 mol l -1 NaOH. The arginine (assay) was repeated, and the previous concentration of arginine subtracted to obtain the level of arginine phosphate. The results were expressed in mmol/g.

**Statistical Analyses:**

All the metabolic parameters were homogeneous (Levene test), and were normally distributed (Kolmogorov−Smirnov test). The three-way ANOVA test was applied to evaluate

the differences in metabolism among species, sexes, and seasons. To evaluate seasonal differences, a one-way ANOVA test was used, followed by a Bonferroni test. For the hepatosomatic and gonadosomatic indices, a one-way ANOVA test was also used. The significance level adopted was 5%. All the tests were done with the program Statistical Package for the Social Sciences (SPSS- 11.5) for Windows.

**Results:**

For the metabolic analysis, we sampled 140 and 123 specimens of *P. brasiliensis* and *P. defossus* respectively. In the determinations of the gonadal tissue, only gonads of females were used.

***Haemolymph Measurements***

**Glucose:** The glucose levels showed a different response in the two species, but did not differ significantly between the sexes, in either species. *P. defossus* showed higher reserves in summer than did *P. brasiliensis* ($p<0.05$). Seasonal variations were recorded. The glucose reserves in *P. defossus* increased in summer by approximately 3.6- and 4-fold in males and females, respectively, compared with spring ($p<0.05$). A reduction in autumn was recorded, and in winter the reserves increased approximately 1.4 times in females ($p>0.05$) and 1.7 times in males ($p<0.05$) (Fig. 1a). In relation to the *P. brasiliensis* reserves, we observed in males an increase in glucose levels in autumn ($p>0.05$), with a reduction until the lowest levels were reached in spring, approximately 3.6 times lower than in autumn ($p<0.05$). Females showed the highest concentrations in autumn, with a small reduction in winter. The lowest concentrations occurred in spring, approximately 2.8 times lower than in autumn ($p<0.05$) (Fig. 1a). We observed an interaction among the three factors of species, sex, and season.

**Lactate:** Differential responses between species and sexes were not recorded. However, differential behavior was observed between seasons, and showed a dependence on the sex. In the hemolymph of *P. defossus*, principally in males, the lowest levels of lactate were recorded in spring and winter ($p<0.05$). In summer we recorded the highest reserves, in males 2.5 times higher than those recorded in spring ($p<0.05$), and 2.8 and 1.9 times higher in males and females, compared with the winter, respectively ($p<0.05$) (Fig. 1b). In *P. brasiliensis,* we observed the lowest lactate concentration in autumn and winter, approximately two times lower than the levels recorded in spring and summer in both sexes ($p<0.05$), but males showed the highest levels in spring, and females in summer ($p<0.05$) (Fig. 1b).

**Total Proteins:** We recorded differential responses between species and seasons. The principal difference between species was the concentration of total proteins in spring, when *P. defossus* showed approximately double the levels of *P. brasiliensis*. Both species showed a similar behavior between sexes. In relation to the seasons, *P. defossus* showed the highest protein levels in spring and summer. However, in autumn and in winter the levels decreased, by approximately 30% in both sexes ($p<0.05$) (Fig. 1c). In *P. brasiliensis* we observed an increase of 38% in summer, compared with spring ($p<0.05$). In spring, males showed approximately 31% lower levels compared with autumn ($p<0.05$). Females also showed higher protein reserves in the hemolymph ($p<0.05$) (Fig.1c).

**Total Lipids:** Differential behaviors in the lipid reserves were recorded between species, seasons, and sexes ($p<0.05$). In spring and summer we observed higher concentrations in *P. defossus* than in *P. brasiliensis*, approximately 5 times higher in spring. In *P. defossus*, the highest levels occurred in summer, principally in females, when we observed an increase of 1.5 times compared with the other periods ($p>0.05$) (Fig.1d). *P. brasiliensis* males showed the lowest levels in spring, but with a significant increase to the highest levels in autumn,

approximately 8 times higher than in spring. In winter a reduction was recorded, compared with autumn ($p>0.05$). However, in spring and summer we recorded increases in the reserves, approximately 6.8 and 1.4 times, respectively ($p<0.05$) (Table I). The same pattern was observed for females, but they contained lower reserves than males. In autumn we observed an increase of 7.7 times over the lowest levels found in spring ($p<0.05$) (Fig. 1d).

**Total Cholesterol:** A differential response pattern was recorded in total cholesterol reserves between species and seasons. However, the sexes did not show a differential pattern. *P. defossus* showed approximately three times the reserves of *P. brasiliensis* during spring. In summer the reserves were also higher than those found for *P. defossus*, but in winter *P. brasiliensis* showed higher concentrations. With respect to the seasonal variations, reductions in spring were observed for *P. defossus*. However, during the summer increases of 2.4 and 3.5 times occurred in males and females, respectively ($p<0.05$). In females, reductions were recorded in autumn and winter. In males, there was an increase in autumn and a reduction in winter ($p<0.05$) (Fig. 1e). *P. brasiliensis* males showed an increase of the cholesterol levels in autumn and winter ($p<0.05$), decreasing by 6.5 times in spring ($p<0.05$). In summer, a significant increase of 5.5 times was recorded, compared with spring. In spring, we also recorded lower concentrations in females. In summer we observed an increase of 3.8 times ($p<0.05$), but the highest concentrations occurred in autumn, approximately 9 times higher than those found in spring ($p<0.05$) (Fig. 1e).

Significant interactions between the three factors of species, seasons, and sexes were recorded ($p<0.05$).

***Hepatopancreas Measurements:***

**Glycogen:** Between species and seasons, we recorded differential responses in glycogen

levels, but not between sexes ($p>0.05$). Glycogen, in *P. defossus*, was higher in summer, principally in females, when it increased to 10 times the levels found in *P. brasiliensis*. *P. defossus* showed the lowest reserves in spring, principally compared with summer when it showed levels approximately 5 and 8 times higher in males and females, respectively. In *P. brasiliensis* the levels were reduced in spring and summer, principally in males, which showed an increase of 3.3 times in autumn and 4.4 times in winter, compared with spring ($p<0.05$). The females increased their reserves by approximately 2.5 times in winter, over the lowest concentration found in summer ($p>0.05$) (Table I).

**Free Glucose:** Free glucose in the hepatopancreas showed no differential response between species ($p<0.05$). *P. defossus* contained higher reserves than *P. brasiliensis* in all periods. The highest levels were found in spring, principally in females, differing significantly from the other periods ($p<0.05$). In *P. brasiliensis*, much lower reserves were recorded in winter than in spring and autumn. In both species of *Parastacus*, we observed no significant difference between the sexes (Table I).

**Total Proteins:** Differential behavior was observed between species, seasons, and sexes ($p<0.05$). In *P. brasiliensis* we observed higher reserves in *P. defossus*, except in autumn. In *P. defossus*, principally in males, we observed the lowest levels in spring ($p<0.05$). The reserves showed no significant variation from summer until winter, with the exception of the reserves of females in summer, which differed from the autumn. In males, the highest concentration was observed in summer, differing only from the spring ($p<0.05$); whereas females showed the highest reserves in winter, with a significant difference from the spring. In *P. brasiliensis*, the concentrations were depleted in autumn, with a reduction of 23 and 29 times compared with the previous period (summer) in males and females, respectively ($p<0.05$). In winter, there were increases of 13 and 15 times in males and females,

respectively ($p<0.05$). The reserves in spring were 3 times lower than those observed in winter ($p<0.05$) (Table I).

**Total Lipids:** Lipid reserves showed differences between species, seasons, and sexes ($p<0.05$). In all periods, principally in spring, the lipid concentrations in the hepatopancreas of *P. defossus* were higher than in *P. brasiliensis*. Significant reductions were recorded in summer and winter for *P. defossus*. In spring we observed the highest levels, approximately 4 and 4.8 times higher than those found in summer, and 2.6 and 5.3 times higher than in winter, in males and females, respectively ($p<0.05$). In autumn, the lipid reserves were higher than in summer and winter, but lower than in spring, approximately 1.5 and 1.8 times in males and females, respectively ($p>0.05$). Seasonal variations were recorded for *P. brasiliensis*. In both sexes, we observed the lowest reserves in summer, with a reduction of 2.5 times from the spring levels, but this difference was significant only for males (Fig.1c). The highest levels were observed in autumn, approximately 3.5 and 2.5 times higher in males and females, respectively ($p<0.05$) (Table I).

**Total Cholesterol:** Differential responses were observed between species, seasons, and sexes, showing a dependence on the factors analyzed. In all periods except winter, cholesterol in *P. defossus* was higher than in *P. brasiliensis*. In autumn we observed a significant increase in *P. defossus*, 8 times higher than the levels found in spring, and 7 and 11 times higher than the levels in summer, in males and females, respectively. The reserves were depleted in winter, principally in the hepatopancreas in females, which showed a 45-fold reduction ($p<0.05$). Depletion was also observed in *P. brasiliensis*, but this depletion occurred in spring. The levels in summer increased 4.6 and 7.4 times in males and females, respectively ($p<0.05$), but the highest values were recorded in autumn, approximately 3.2 and 2.9 times higher than in summer ($p<0.05$). The levels decreased in the subsequent period

(winter), compared with autumn (p>0.05) (Table I).

The sexes showed different changes in total cholesterol with the seasons, and the sp ecies, sexes, and seasons all showed significant differences.

**Arginine:** Differential responses were observed between species, sexes, and seasons. In *P. defossus* the highest levels of arginine were observed in autumn (p<0.05). Reductions were recorded in winter, reaching the lowest levels in summer with 63 and 28% reductions from the previous period (spring), and 80 and 60% compared with autumn (p<0.05) in males and females, respectively (Fig. 2a). In the hepatopancreas of *P. brasiliensis*, the largest arginine reserves were observed in spring, principally in females, which contained approximately double the reserves of the subsequent period (summer) (p<0.05). The hepatopancreatic tissue in males showed no significant differences between the reserves in summer and autumn, but increased 18% in winter (p>0.05); females lost approximately 39, 55, and 48% of their reserves in summer, autumn, and winter, respectively, compared with spring (p<0.05) (Fig. 2a).

**Arginine phosphate:** Differential responses were recorded between species, sexes, and seasons. Arginine phosphate reserves in hepatopancreatic tissue in *P. defossus* were highest in winter (p<0.05). Reductions of 74 and 58% were recorded in spring, in males and females, respectively (p<0.05). Depletions, in both sexes, were also recorded in summer (p<0.05), but in autumn, the reserves increased 2.5 times in males (p>0.05) and 6 times in females (p<0.05) (Fig. 3a). In *P. brasiliensis* the highest reserves were recorded in autumn and winter (p<0.05). In spring the concentrations were depleted to approximately 94% lower than in the previous period, in both sexes (p<0.05). In summer the reserves increased 90 and 86% in males and females, respectively, compared with spring (p<0.05) (Fig. 3a).

***Muscle Measurements:***

All the metabolites of the muscle tissue except arginine and arginine phosphate showed differential responses between species and seasons. Between the sexes, no differential response was recorded.

**Glycogen:** In all periods, higher glycogen reserves were recorded in muscle tissue of *P. defossus*, principally in summer and autumn ($p<0.05$). A significant increase was observed in summer, 4.6 and 3.6 times higher than in spring, in males and females, respectively. The lowest concentrations occurred in spring and winter ($p<0.05$). *P. brasiliensis* showed low concentrations in spring, differing only from the winter ($p<0.05$). In summer, glycogen increased 2.5 times compared with the spring ($p<0.05$), but the highest concentration was recorded in winter, differing significantly from the spring and autumn ($p<0.05$) (Table II).

**Free Glucose:** A differential response between the species was recorded, with higher reserves in all seasons in *P. defossus* ($p<0.05$). Free glucose also differed significantly between the sexes. The highest concentrations in *P. defossus* were observed in spring and winter, differing from the summer and autumn ($p<0.05$); while in *P. brasiliensis* the highest reserves were found in spring and summer, with a significant difference fromof the lowest levels found in autumn. Males also showed a difference between the levels observed in winter and spring, while females showed a difference between winter and autumn ($p<0.05$) (Table II).

**Total Proteins:** With the exception of the autumn, higher protein reserves occurred in *P. brasiliensis* than in *P. defossus*. In muscle tissue of *P. defossus* we observed the lowest reserves in spring, principally in females. In males we observed an increase in autumn and the highest concentrations in winter, differing significantly from the spring and summer; while in females, muscle tissue also increased in autumn and winter, but the highest reserve

occurred in autumn ($p<0.05$). Depletions were recorded in autumn in *P. brasiliensis*, approximately 13 times lower than the previous period (summer), which showed the highest reserves, in both sexes ($p<0.05$). High concentrations were also recorded in winter ($p<0.05$) (Table II).

**Total Lipids:** The muscle tissue of *P. defossus* showed higher lipid concentrations than in *P. brasiliensis*. *P. defossus* showed higher reserves in spring, with an increase of 6.4 and 4.9 times compared with summer in males and females, respectively, but a significant difference was recorded only for the muscle of males. In autumn, lipids in males increased 5.4 times compared with summer ($p<0.05$), while females showed an increase of 3.6 times ($p>0.05$). In *P. brasiliensis* the lowest levels were observed in spring, with a significant increase of 7.2 and 4.8 times in summer, in males and females, respectively. Males reduced lipid levels by approximately 3-fold in autumn ($p<0.05$); females showed no significant variations from summer until autumn, but in winter a 2-fold reduction was observed ($p>0.05$) (Table II).

**Total Cholesterol:** *P. defossus* showed higher reserves than *P. brasiliensis*, principally in autumn ($p<0.05$). In winter the cholesterol levels decreased, but a significant difference appeared only in autumn. Low levels were also observed in spring, compared with the highest concentrations in autumn ($p<0.05$). In muscle tissue of *P. brasiliensis* males, we observed a reduction of 1.9 times in summer compared with the spring. We recorded a significant difference in autumn, and the highest levels in winter, but a significant difference was found only when compared with summer. The increase in winter was 2.3 times compared with summer, when the lowest reserves were measured. In the muscle of females, a 2.3-fold decrease was recorded in summer, compared with spring ($p<0.05$). The levels increased in autumn and winter, with the highest concentrations in spring ($p<0.05$) (Table II).

**Arginine:** The species showed different behaviors in arginine reserves of the muscle tissue.

In *P. defossus*, arginine reserves decreased significantly in summer, approximately 81 and 35% lower than those in spring, in males and females, respectively. The highest concentrations were recorded in autumn, approximately 86 and 70% higher than in summer, in males and females, respectively ($p<0.05$) (Fig. 2b). In *P. brasiliensis*, the arginine levels increased in spring, approximately 62 and 80% above the previous period (winter), and decreased in summer by 76 and 64% in males and females, respectively ($p<0.05$). Compared with summer, males showed higher values in autumn, while females showed a reduction in autumn ($p<0.05$) (Fig. 2b). Overall, arginine levels differed significantly between the sexes.

**Arginine phosphate:** Differences were recorded between species, sexes, and seasons. In muscle tissue of *P. defossus*, the highest arginine phosphate reserves were observed in spring ($p<0.05$). In other seasons the reserves declined, principally in summer when they showed decreases in both sexes ($p<0.05$). In autumn, we observed significant reductions of 90 and 73%, and in winter of 78 and 69%, compared with spring, in males and females, respectively (Fig. 3b). In *P. brasiliensis* we recorded the highest reserves in winter, with a reduction of 92 and 80% in spring ($p<0.05$). In summer and autumn, arginine phosphate concentrations were higher than in spring, but lower than in winter ($p<0.05$) (Fig. 3b).

***Measurements of the anterior gills (AG):***

**Glycogen:** Glycogen reserves in the anterior gills differed between the species ($p<0.05$). *P. defossus* showed higher concentrations than *P. brasiliensis*, except in winter. In both species, the sexes showed no significant differences. The anterior gills of *P. defossus* showed low levels in spring and winter, while in summer the highest concentrations were observed, with an increase of 15 and 13 times in males and females, respectively, compared with spring ($p<0.05$). In autumn, the levels decreased 50% compared with summer ($p<0.05$). In the

anterior gills of *P. brasiliensis*, depletions were observed in spring, differing from summer and winter ($p<0.05$). In autumn, low levels were also recorded, in both sexes. Increases of 18 and 20 times were recorded in winter compared with spring, and 11 and 18 times compared with autumn in males and females, respectively ($p<0.05$) (Table III).

**Free Glucose:** Significant differences were not recorded between the species ($p<0.05$). Similarly to the hepatopancreas and muscle tissue, *P. defossus* also showed, in all periods, the highest free glucose reserves in the anterior gills. The highest concentrations occurred in spring and summer, with a significant reduction in autumn and winter. In anterior gills of *P. brasiliensis* the highest concentrations of frees glucose occurred in spring, with a significant reduction of 46 and 40% in summer in males and females, respectively, and 72%, in both sexes, in autumn. In winter, the reserves increased, but differed significantly only from the autumn levels ($p<0.05$). Significant differences between sexes were not recorded in either species (Table III).

**Total Proteins:** A differential response was recorded only between seasons ($p<0.05$). *P. defossus* and *P. brasiliensis* showed similar patterns in their protein reserves. In the anterior gills of *P. defossus*, the proteins did not show a significant difference between seasons, except for the reserves of males in summer compared with autumn. The proteins in the anterior gills of *P. brasiliensis* increased in summer compared with spring, but this increase was not significant. In autumn, the levels were depleted in both sexes, showing a reduction of 85% from summer levels. In winter we observed the highest reserves, with an increase of 92 and 95% in males and females, respectively, compared with the autumn (Table III).

**Total Lipids:** The anterior gills showed a differential pattern between species and seasons in relation to the lipid reserves ($p<0.05$). The sexes did not differ in the lipid response. The lipid levels in *P. defossus* were higher than in *P. brasiliensis*, except in spring. *P. defossus* showed

the lowest reserves in spring ($p<0.05$). The reserves increased in summer by 70 and 73% in males and females, respectively, with higher levels in winter, principally in the anterior gills of females, which increased 84% compared with spring ($p<0.05$). In relation to the *P. brasiliensis* anterior gill reserves, a significant variation was also observed. The lowest levels occurred in summer, with a significant difference from the winter reserves. However, the highest reserves were found in winter, approximately 4 and 3 times higher in males and females, respectively, compared with summer reserves ($p<0.05$) (Table III).

**Total Cholesterol:** A differential response was observed in total cholesterol levels in the anterior gills between species and seasons, but the sexes did not show a differential behavior. *P. defossus* showed higher levels in summer and autumn than *P. brasiliensis*. However, in winter *P. brasiliensis* had higher reserves than *P. defossus*. In relation to the seasonal differences, *P. defossus* showed a significant increase in summer and autumn, in both sexes, principally in autumn when the reserves were approximately 5.5 times higher than in spring and 10 times higher than in winter, in males and females, respectively. The lowest reserves were found in winter, differing significantly from summer and autumn. In *P. brasiliensis*, cholesterol increased in the anterior gills in both sexes, approximately 2 times higher in winter compared with autumn in both sexes ($p>0.05$), and 5.5 and 9.5 times compared with the lowest levels in summer, in males and females, respectively ($p<0.05$) (Table III).

**Arginine:** Differential responses were recorded between species, sexes, and seasons. *P. defossus* showed a reduction in summer of arginine reserves in the anterior gills ($p<0.05$), approximately 85 and 63% lower in males and females, respectively, compared with spring. The highest levels were observed in autumn ($p<0.05$). In winter the arginine content in the gills was reduced approximately 26% compared with autumn, but this reduction was not significant (Fig. 2c). In the anterior gills of *P. brasiliensis,* variations in arginine levels were

recorded. Males contained the lowest reserves in summer and females in autumn, approximately 35% lower than in preceding periods (p<0.05) (Fig. 2c).

**Arginine phosphate:** Levels of arginine phosphate in the anterior gills were detectable only in the spring in *P. defossus*, probably because of the small amounts of tissue obtained in the other seasons of both species. The males of *P. defossus* showed higher reserves than females (p>0.05).

***Measurements of the posterior gills (PG):***

**Glycogen:** Similarly to the anterior gills, glycogen in the posterior gills showed a differential behavior between species and seasons. The reserves were higher in *P. defossus* than in *P. brasiliensis*, except in winter. The response did not differ between sexes. Significant increases of 5.6 and 7.3 were observed in summer, and 3.4 and 3.6 in autumn, compared with spring, in males and females, respectively. The levels recorded in summer were similar to those found in winter. In the posterior gills of *P. brasiliensis*, high glycogen levels were recorded in summer and winter (p<0.05). Therefore, in winter the posterior gills contained approximately half the level of the glycogen reserves in the anterior gills. The lowest levels were found in spring, approximately 8.3 and 7.2 times lower than those recorded in winter, in males and females, respectively (p<0.05) (Table IV).

**Free Glucose:** Between sexes and species we observed a differential response in free glucose in the posterior gills (p<0.05). Similarly to the other tissues, the posterior gills of *P. defossus* showed higher concentrations than the posterior gills of *P. brasiliensis*. The lowest levels were found in autumn, differing from the summer and winter in males (p<0.05), and in females showed significant differences in all periods (p<0.05). The posterior gills of males of *P. brasiliensis* contained the lowest reserves in winter, differing from the spring and summer

($p<0.05$); the posterior gills of females showed the lowest concentrations in autumn, differing only from the spring reserves ($p<0.05$) (Table IV).

**Total Protein:** No differential response between species was observed in relation to the protein reserves of the posterior gill tissue. Differences were found between sexes and seasons ($p<0.05$). The levels in *P. defossus* did not differ in summer and winter. In spring, we recorded a significant reduction in male posterior gills. In female posterior gills, we observed the lowest levels in spring, but this difference was not significant. In *P. brasiliensis* we observed a depletion in autumn, when compared with other periods, approximately 16 and 33 times lower in males and females, respectively, compared with the highest reserves recorded in winter ($p<0.05$) (Table IV).

The three factors analyzed, sex, seasons, and species, showed significant interactions.

**Total Lipids:** The species and seasons showed a differential response ($p>0.05$). In all periods, we recorded higher reserves in *P. defossus* than in *P. brasiliensis*. In *P. defossus* we observed an increase in lipids from summer to winter ($p<0.05$), with a higher increase in females. In posterior gills of *P. brasiliensis*, lipids were lower in summer, increased in autumn, and reached their highest point in winter, approximately 5 and 8.5 times higher in males and females, respectively, compared with summer ($p<0.05$). In males, the reserves were similar in spring and autumn; females showed a reduction of 2.4 times in autumn from the winter reserves ($p<0.05$) (Table IV).

**Total Cholesterol:** Total cholesterol in posterior gills showed a differential pattern between species and seasons ($p<0.05$), and also between the sexes we ($p>0.05$). The reserves in *P. defossus* were higher in summer and autumn. In *P. defossus* we observed a significant increase of cholesterol in summer, and principally in autumn, when males showed reserves 9.3 and 14.5 times higher than in spring and winter, respectively. Females showed an increase

of 6.7 and 15.6 times in autumn over with spring and winter, respectively ($p<0.05$). The posterior gills of *P. brasiliensis* contained the highest reserves in spring and winter. The lowest concentrations were recorded in summer, approximately 3 and 4.5 times lower than the highest reserves in winter, but this difference was statistically significant only in males (Table IV).

**Arginine:** The species showed no differential responses in arginine reserves. In posterior gills of *P. defossus*, we observed a reduction in arginine in summer, approximately 93 and 82% lower than in the previous period (spring) ($p<0.05$); the highest reserves were recorded in autumn, 95 and 90% higher than in summer in males and females, respectively ($p<0.05$) (Fig. 2d). The posterior gills of *P. brasiliensis* contained higher arginine reserves in spring, similarly to the other tissues, principally in females with 46% higher levels than those recorded in summer. Males reduced their reserves by approximately 36% in summer compared with spring ($p<0.05$). The lowest reserves, in both sexes, were found in winter ($p<0.05$) (Fig. 2d). The differences between the sexes were significant.

**Arginine phosphate:** Levels of arginine phosphate were detectable only in the spring, probably because of the small amounts of tissue obtained in the other seasons of both species. *P. brasiliensis* showed higher reserves than *P. defossus,* mainly the females ($p>0.05$)

***Gonadal Measurements:***

The metabolic analyses of the gonads were carried out only for females, because the males contained insufficient gonadal tissue to determine all the metabolic parameters.

**Glycogen:** The glycogen reserves in the gonads showed a differential response between species ($p<0.05$), with higher reserves in spring and autumn in *P. defossus*. In the different seasons, significant differences were recorded in the gonads of *P. defossus*, except in autumn

and winter. The highest glycogen concentrations were observed in spring, and the lowest in summer (Fig. 4a). With respect to the gonadal reserves in *P. brasiliensis*, we observed the highest concentration in winter, approximately 1.8 and 2.8 times higher than in summer and autumn, respectively ($p<0.05$) (Fig. 4a).

**Total Protein:** The gonads showed a differential response between species in relation to protein reserves. *P. brasiliensis* showed higherst reserves than *P. defossus* in autumn and winter ($p<0.05$). In *P. defossus* we did not record seasonal variations ($p>0.05$) (Fig. 4b). *P. brasiliensis* showed significant differences between seasons, except in spring and summer (Fig. 4b).

**Total Lipids:** The species showed no differences in lipid reserves ($p>0.05$). Lipids in gonads of *P. defossus* showed no seasonal variations, but the highest concentrations were recorded in spring and winter ($p>0.05$) (Fig. 4c). In *P. brasiliensis*, high levels were recorded in autumn, but the highest reserves were observed in winter, differing significantly from the spring and summer ($p<0.05$). The concentrations in autumn also differed from the reserve levels observed in spring and summer ($p<0.05$) (Fig. 4c).

**Total Cholesterol:** The total cholesterol reserves showed no differences between species and seasons ($p>0.05$). In *P. defossus*, the levels increased 2.7 and 2.2 times in winter and spring, respectively ($p>0.05$) compared with autumn (Fig. 4d). In *P. brasiliensis,* the highest levels were observed in autumn and winter, approximately 2 and 1.7 times higher than those found in summer ($p>0.05$) (Fig. 4d).

***Determination of the gonadosomatic index (GI) and hepatosomatic index (HI):***

*P. defos*sus showed the highest GI in the spring (2.709) ($p<0.05$) and the lowest in summer (0.295), which differed from the spring and winter ($p<0.05$). The highest value of HI

was reached in winter (6.369) ($p<0.05$) and the lowest values in autumn and spring (Fig. 5a). In *P. brasiliensis* we observed the highest GI in winter (1.919) ($p <0.05$), with a decrease in spring, reaching the lowest value in summer (0.296). The values of the indexes did not differ between autumn and winter ($p> 0.05$). However, the HI was highest in summer (6.589), differing significantly from the spring and autumn (Fig. 5b).

***Environmental parameters:***

The environmental parameters showed seasonal variations. The main difference between the species was the concentration of dissolved oxygen: the habitat of *P. defossus* showed lower concentrations in all the periods analyzed (Table V).

**Discussion**

The metabolic reserves of the two crayfishes showed seasonal variations related to the differences between their habitats and adaptation strategies, mainly with the reproductive period (RP) of the species, level of oxygen in galleries of *P. defossu*s (mean:1.6 mg/L), activity exploratory, and temperature variations, mainly in *P. brasiliensis* (11.5-21.1ºC). The RP of *P. brasiliensis* and *P. defossus* were determined by the gonadosomatic index, which expresses the percentage that gonads represent of the total body weight of individuals, and is used as a qualitative method to determine the RP of a species (Grant and Taylor, ’83; Vazzoler, ’96). In *P. defossus,* the RP begins in the winter, with the highest reproductive activity (reproductive peak) in the spring, as previously observed by Noro and Buckup (2008); whereas in *P. brasiliensis* the RP starts in autumn, reaching a peak in winter.

Knowing the RP of the species and variations in energy reserves, it is possible to assess the importance of the mobilization of these reserves by certain organs or tissues, and it

is also possible to evaluate the transfer of nutrients to gonadal tissues. In *P. defossus* the process of reserve allocation of the tissues to the gonads was more evident than in *P. brasiliensis.* This was reinforced for the hepatosomatic index, because significant mobilization of the hepatopancreas reserves was observed during reproductive period only in *P. defossus.*

Seasonal variations were observed in the levels of hemolymph glucose in *P. brasiliensis* and *P. defossus*. In *P. brasiliensis,* the highest levels of hemolymph glucose were observed in autumn and winter. These results coincide with a decrease of free glucose and glycogen in the muscle and gills during autumn, and also with a reduction of free glucose and glycogen in the hepatopancreas during winter. We also observed a decrease of lactate in the hemolymph in this period, when lactate can be used in the hepatopancreas to synthesize glucose for the gluconeogenic pathway, as observed in other decapods by Oliveira et al. ('97). In *P. defossus,* the levels of hemolymph glucose showed a relationship to the reproductive period, because a significant decrease was observed in spring, the period of the reproductive peak.

Higher levels of glucose, as expected, were reached by *P. defossus* in the summer and autumn, the period when the galleries contained the lowest concentrations of dissolved oxygen. This suggests an adaptation to the hypoxic environment, because the rapid increase of the hemolymph glucose appears to function as a physiological preparation for the high demand for fermentation substrate. Hall ('98), studying the prawn *Penaeus monodon* noted that reduction of oxygen is a stress factor leading to an increase of glucose in this shrimp, and the same response was observed by Oliveira et al. (2001), and this can be also suggested for *P. defossus*. The glucose reserves showed significant differences between the species, although the limits of variation were similar. In *P. defossus* the limit was 10.76 to 45.81,

while in *P. brasiliensis* it was 8.12 to 38.31. These glucose levels are similar to those observed in the anomuran crab *A. platensis* (Ferreira et al., 2005) and the lobster *Jasus edwardsii* (Radford et al., 2005) maintained on a carbohydrate-rich diet, witch showed increased levels of circulating glucose.

Free glucose is stored in the cells in tissues of crustaceans, and it serves as a buffer so that the animals can respond more quickly to environmental variations (Oliveira et al., 2001; 2004). The reserves of free glucose in *P. brasiliensis* seem to be related to the reproductive period, because reductions were observed in the muscle and hepatopancreas in the autumn and winter, respectively. This suggests a mobilization to the gonads during reproduction in winter, or its use for synthesis of ATP and maintenance of homeostasis. In this period (winter) we observed a reduction in exploratory activity and encountered great difficult in collecting the species. Therefore, the reduction of free glucose can be compensated by the increase of glycogen during this period as observed in other crustaceans, including the crabs *Neohelice granulata*, *Aegla ligulata,* and *Ocypode quadrata*, have a shorter activity period and decreased metabolism, as well as higher glycogen levels in the hepatopancreas during winter (Kucharski and Da Silva, ’91b; Oliveira et al., 2003; Vinagre et al., 2007). In *P. defossus,* free glucose appears not to be utilized during the reproductive period; however, probably, this carbohydrate was used to maintain the levels of hemolymph glucose. This was observed after an intense decrease of glycogen in all tissues during the summer.

Several studies have shown that lactate is the main product of anaerobic metabolism in crustaceans. However, in vertebrates the lactate is produced not only under hypoxic and anoxic conditions, but under conditions of normoxia (Philip et al. 2005). This was also observed in the estuarine crab *Neohelice granulata* by Maciel et al. (2008) and, also in the crayfish *P. brasiliensis* that produced high levels of lactate under conditions of normoxia in

its relatively well-oxygenated habitat. The production of lactate in normoxia may be related to the exploratory activities of *P. brasiliensis* during spring and summer. In *P. defossus,* the highest concentrations were recorded in summer and autumn, showing that it produces lactate via anaerobic metabolism in the low concentrations of oxygen in its galleries (summer and autumn). In these periods, *P. defossus* produced higher levels than *P. brasiliensis*, although the differences between the species were not significant, thus demonstrating a similar behavior with respect to lactate reserves.

The stored glycogen is used in the processes of molting, hypoxia or anoxia, osmoregulation, growth, different stages of reproduction, and during periods of fasting (Chang and O'Connor, ’83; Kucharski and Da Silva, ’91b; Rosa and Nunes, 2003b). In all the tissues of *P. brasiliensis*, we observed the largest reserves of glycogen in the winter and a subsequent decrease during the transition from winter to spring, suggesting an increase of the use of this polysaccharide for synthesis of ATP and increased exploratory activity, as observed after the winter; females probably need increased energy for maternal care in spring, following the peak of reproduction in winter. In spring, we observed greater availability of food in the environment and found that the animals were easier to collect; the same pattern was observed in populations of *P. brasiliensis* in another locality (Dutra et al. 2008).

According to Kucharski and Da Silva (’91b), a decrease in temperature and metabolic rate may cause a decrease in exploratory activity. In this study, the lower temperatures in autumn and winter may have resulted in a decrease in exploratory activity, because fewer specimens of *P. brasiliensis* were collected in winter. This decrease in activity may be responsible for the increase in carbohydrates. Increases in glucose were observed in the autumn and winter, together with an increase of the glycogen reserves in all tissues except the

posterior gills in females. The increase of carbohydrates caused by a decrease in temperature and in exploratory activity was also suggested for the estuarine crab *Neohelice granulata* and the marine crab *Ocypode quadrata* studied by Kucharski and Da Silva ('91b) and Vinagre et al. (2007), respectively. In the muscle tissue of females, in winter we observed higher reserves of glycogen than in males, suggesting that females may be reducing their exploratory activity to save energy for vitellogenesis and maternal care, probably in spring. A similar response was observed by Oliveira et al. (2007) in the anomuran crab *Aegla platensis*.

In *P. defossus*, a decrease of this polysaccharide was observed during the transition from summer to autumn, until the lowest levels in winter. This may be related to depletion of arginine phosphate in summer and the intense decrease of oxygen levels in summer and autumn (1.5-1.4 mg $O_2$/L), which activa the anaerobic metabolism to maintain ATP synthesis.

The glycogen of the hepatopancreas of *P. defossus* appears to be mobilized to the gonads during the reproductive period, because the lowest concentrations were observed in the spring during the peak of reproduction. No similar mobilization was observed in *P. brasiliensis*. The use of glycogen in all tissues of *P. defossus* does not seem to be related only to the reproductive period, but these reserves were stored during the beginning of the period of decreased oxygen (summer) when the animal uses all its reserve of arginine phosphate and then uses part of the reserve of this polysaccharide. According to Zebe ('91), the high concentration of glycogen is an adaptation to oxygen-deficient environments. Therefore, *P. defossus* appears to store glycogen in the tissues as an adaptation to its habitat, when the oxygen levels are decreased.

The crayfishes stored more glycogen in the gills than the other tissues examined. Similar results were observed in the crab *Uca pugilator,* investigated by Keller and Andrew

('73), and also in the crab *Neohelice granulata* by Vinagre and Da Silva ('92) and Nery and Santos ('93). The increase of glycogen in the gills of *N. granulata* is important source for osmoregulation. The same can be suggested for the species of *Parastacus,* because according to McMahon (2002), crayfish are exposed constantly to the hypo-ionic and hypo-osmotic environment, are subject to constant loss of ions, and gain of water through the body surface. Therefore, a crayfish must constantly control its internal ionic composition through regulatory mechanisms in order to survive in freshwater. Studies are necessary to improve understanding in this area, because the crustaceans of marine, estuarine, and freshwater environments face very different osmotic challenges, and have very different osmoregulatory abilities.

Between the two species, significant differences in glycogen levels were recorded in all tissues. *P. defossus* contained higher concentrations than *P. brasiliensis*, with the exception of the gills during the winter. This was expected, because *P. defossus* needs more reserves to live in underground galleries and is therefore tolerant of hypoxic conditions, which occur in all the seasons, compared with the environment of *P. brasiliensis*. According to Hervant et al. ('99), subterranean species have higher glycogen concentrations than epigean species, and maintain these high concentrations as a metabolic adaptation that contributes to their survival when subjected to anaerobiosis. Glycogen is an important substrate for anaerobic metabolism (Urich, '94; Hervant et al., '95). *P. defossus* appears to make this adjustment, because in all tissues the glycogen increased significantly in the summer, when the beginnings of low oxygen levels were observed.

Arginine phosphate is widely used in crustaceans to provide energy during periods of hypoxia, intense muscular activity, and stress (England and Baldwin, '83; Hill et al., '91; Speed et al., 2001). In *P. brasiliensis* we observed an increase in the hepatopancreas and

muscle of the levels of arginine, and a reduction of arginine phosphate reserves in spring. This reduction may be due to the intense exploration activity of *P. brasiliensis,* which are much easier to collect in spring. In all tissues of *P. defossus,* we observed a reduction of arginine and a sharp depletion of arginine phosphate in the summer. This depletion must function to maintain the ATP production due to low oxygen concentrations in the galleries of *P. defossus*, mainly in the summer (1.4 mg $O_2$/L), as suggested for the crayfish *Cherax destructor* by England and Baldwin ('83) and Baldwin et al. ('99).

In spring we observed high reserves of arginine phosphate in the posterior gills of both species, and also in the anterior gills of *P. defossus*. The levels of arginine phosphate at other seasons could not be determined ie, the gills didn't shown enzymatic activity probably because of the small amount of little gill tissue obtained for analysis. Therefore, studies about the seasonal variations of arginine phosphate in crustaceans are desirable to better understand the role of this compound**.**

Animal food, although it is the smallest part of the diet, is important to provide amino acids and organic components (Gherardi, 2002). These amino acids must be metabolized to promote growth or be used as an energy source (Goddard, '88). Noro and Buckup (2008) observed the largest animal reserve in *P. defossus* in summer, compared with other periods. Therefore it is expected that the reserves will increase in this period, due to the protein diet; this was observed for *P. defossus*, which showed an increase of proteins in the hemolymph in spring and summer.

The gills of both species showed similar behavior in the protein concentrations. According to Lydon and Houlihan ('98) the gill tissue has a high degree of plasticity in protein metabolism, even under adverse conditions, thus ensuring the maintenance of the integrity of the gills, which is of great importance for the survival of the animal. The same

pattern to seem occurs in these species of *Parastacus*. Lyndon and Houlihan ('98) showed that, in fish and crustaceans, the gill tissue is the most active in terms of protein synthesis, followed by liver, hepatopancreas, muscle, and heart.

Species of *Parastacus* showed a mobilization of tissue proteins to the gonads in the reproductive period, showing it to be related to vitellogenesis, as previously observed in the crayfish *P. varicosus* by Silva-Castiglioni et al. (2007). In *P. brasiliensis,* part of these proteins can be used in gluconeogenesis to reestablish the glycogen reserves in different tissues during the winter. The capacity for gluconeogenesis has also been observed in other crustaceans (Oliveira et al., '97). During crustacean vitellogenesis, the growing oocyte accumulates yolk proteins (vitellus) in one of the most energy-demanding processes in females (Okuno et al., 2002; Okumura et al., 2007).

According to Sastry ('83), oogenesis involves intense mobilization of proteins and lipids for the development of eggs. Spring was the only period when ovigerous females of *P. brasiliensis* were collected, but the reproductive period began in winter, as indicated by the gonadosomatic index. In *P. defossus,* the protein reserves were used during the peak of reproduction (spring), although ovigerous females were not observed in any sampling period. Therefore, for *P. defossus* we suggest that intense mobilization of protein of the hepatopancreas and muscle for the development of eggs, occurs during the transition from winter to spring. The failure to collect ovigerous females of *P. defossus* may be related to their habit of living in underground galleries. Galleries of decapods serve as a refuge from predators, a place to mate and molt, incubate the eggs and/or protect their offspring, and protection against environmental conditions (Nash et al., '84; Gherardi, 2000; Shimoda and Tamaki, 2004). Collection of ovigerous females of *P. defossus* may have been hampered by their remaining sheltered in their galleries.

The lipids of the hepatopancreas of *P. brasiliensis* appear to contribute significantly to the reproductive period, because of the observed decrease in winter compared to autumn, especially in males, where larger reserves were recorded. This may be related to increased energy expenditure by males and females in search of food. This was corroborated with the increase of lipids in the gonads in autumn and winter, thus showing that during periods of high energy demand such as gametogenesis, the lipids are mobilized, mainly from the hepatopancreas to the gonad (Pillay and Nair, ’73; Read and Caulton, ’80; Castille and Lawrence, ’89; Rosa and Nunes, 2003b; Vinagre et al., 2007; Silva-Castiglioni et al., 2007).

The development of the ovary in the RP of *P. brasiliensis* shows a dependence on lipid reserves of the muscle and hepatopancreas. However, the ovarian development also seems to depend on the dietary nutrients, because by the determination of the hepatosomatic index has not observed mobilization of reserves to the gonads during the reproductive period. Cholesterol also seems to depend more on dietary nutrients in *P. brasiliensis*. Studies of other species of decapods by Castille and Lawrence (’89), Cavalli et al. (2001), Rosa and Nunes (2003b), and Oliveira et al. (2007) also showed that the mobilization and application of lipids in the ovarian development seems to depend more on dietary nutrients than on the hepatopancreas reserves.

*P. defossus* contained larger reserves than *P. brasiliensis*, except for protein and glycogen in the gills during the winter. It was expected, because higher concentrations of metabolites is an adaptive strategy to the hypoxic and/or anoxic environment (Storey and Storey, ’90; Hervant and Mathieu, ’95; Hervant et al., ’95, ’96, ’99; Malard and Hervant, ’99). Therefore, *P. defossus*, living in underground galleries with low oxygen concentrations, has the "capacity" to store larger reserves than *P. brasiliensis,* as an adaptive strategy.

The intermediary metabolism of *P. brasiliensis* and *P. defossus* showed behavioral

differences in almost all the metabolites examined. Seasonal variations in *P. defossus* were mainly related to the reproductive period, and to periods of low oxygen concentration in the galleries. This parastacid showed metabolic adaptations to the hypoxic environment, including increases of glucose and lactate and larger reserves of arginine phosphate and glycogen. For *P. brasiliensis,* the results suggest a significant allocation of dietary nutrients for the reproductive period, with a smaller transfer of the reserves of different tissues; the metabolic parameters were also related to the activity of the animals.

The study of crustacean metabolism may contribute information about their ability to adapt to environmental variations. The more efficiently an individual captures and uses its reserves of energy, the greater will be its ability to compete with other individuals, and the greater will be the adaptive capacity of the species in an evolutionary sense.

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**Table I.** Seasonal concentrations of metabolic analysis of the hepatopancreas of *Parastacus defossus* and *Parastacus brasiliensis*. The results show the mean and standard error. The different letters indicate significant differences (capital letters for males and small letters for females) ($p < 0.05$). Gly= Glycogen; FG= Free Glucose; Pro= Protein; Lip= Lipid; Chol= Cholesterol; M= male; F= female.

| | | **Hepatopancreas** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Seasons** | | ***Parastacus defossus*** | | | | | ***Parastacus brasiliensis*** | | | | |
| | | **Gly** | **FG** | **Pro** | **Lip** | **Chol** | **Gly** | **FG** | **Pro** | **Lip** | **Chol** |
| **Spring** | **M** | 0.19 ± 0.07B | 19.47 ± 1.10B | 6.86 ± 1.54A | 113.69 ± 28.14B | 7.34 ± 1.50B | 0.06 ± 0.02A | 9.75 ± 0.48B | 12.61 ± 1.77A | 50.26 ±9.90CB | 0.370 ± 0.082A |
| | **F** | 0.16 ± 0.06b | 22.39 ± 1.26a | 10.69 ± 0.87a | 123.50 ± 32.40a | 8.72 ± 2.25b | 0.13 ± 0.03a | 12.19 ± 1.21b | 15.74 ± 1.78a | 30.81 ± 9.80a | 0.216 ± 0.054a |
| **Summer** | **M** | 0.94 ± 0.12A | 13.56 ± 1.50AB | 16.13 ± 0.86B | 29.45 ± 2.73A | 8.18 ± 1.63B | 0.10 ± 0.02AB | 6.72 ± 0.68AB | 80.22 ± 2.8B | 18.50 ± 2.65A | 1.72 ± 0.44AC |
| | **F** | 1.32 ± 0.25a | 12.55 ± 1.24b | 15.60 ± 0.93b | 25.72 ± 2.41b | 6.05 ± 1.04b | 0.11 ± 0.05ab | 8.10 ± 1.37ab | 88.83 ± 3.56b | 13.32 ± 4.01a | 1.59 ± 0.88ab |
| **Autumn** | **M** | 0.47 ± 0.07B | 8.95 ± 1.09A | 15.90 ± 1.28B | 79.94 ± 11.11BC | 64.40 ± 10.98A | 0.20 ± 0.05BC | 10.18 ± 2.14B | 3.45 ± 0.38C | 65.55 ± 11.2B | 5.49 ± 2.15AB |
| | **F** | 0.58 ± 0.07b | 10.78 ± 0.96b | 16.93 ± 2.33b | 67.73 ± 9.91ab | 67.50 ± 10.93a | 0.13 ± 0.03ab | 9.50 ± 1.77b | 3.06 ± 0.46c | 32.80 ± 11.15a | 4.55 ± 1.73b |
| Winter | M | 0.30 ± 0.02B | 13.54 ± 1.47AB | 14.35 ± 0.81B | 44.77 ± 5.96AC | 3.63 ± 0.48B | 0.28 ± 0.04C | 2.84 ± 0.44A | 45.48 ± 4.72B | 20.46 ±7.67AC | 4.45 ± 0.84C |
| | F | 0.18 ± 0.02b | 15.24 ± 2.35b | 18.99 ± 1.30b | 23.10 ± 3.06b | 1.47 ± 0.21b | 0.27 ± 0.04b | 2.90 ± 0.28a | 45.90 ± 4.78d | 17.63 ± 2.70a | 3.68 ± 0.98ab |
| | | (mg $g^{-1}$) | (mg $g^{-1}$) | (mg $ml^{-1}$) | (mg $g^{-1}$) | (mg $g^{-1}$) | (mg $g^{-1}$) | (mg $g^{-1}$) | (mg $ml^{-1}$) | (mg $g^{-1}$) | (mg $g^{-1}$) |

**Table II.** Seasonal concentrations of metabolites analyzed in the muscle tissue of *Parastacus defossus* and *Parastacus brasiliensis*. The results show the mean and standard error. The different letters indicate significant differences (capital letters for males and small letters for females) ($p<0.05$). Gly= Glycogen; FG= Free Glucose; Pro= Protein; Lip= Lipid; Chol= Cholesterol; M= male; F= female.

| Muscle | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Seasons** | | ***Parastacus defossus*** | | | | | ***Parastacus brasiliensis*** | | | | |
| | | **Gly** | **FG** | **Pro** | **Lip** | **Chol** | **Gly** | **FG** | **Pro** | **Lip** | **Chol** |
| **Spring** | **M** | 0.18 ± 0.04B | 12.27 ± 0.84A | 9.06 ± 1.74A | 46.00 ± 15.76B | 4.20 ± 1.37B | 0.03 ± 0.01B | 3.36 ± 0.23B | 24.35 ± 1.59A | 0.74 ± 0.11B | 0.58 ± 0.09B |
| | **F** | 0.27 ± 0.12b | 16.28 ± 1.65a | 12.21 ± 1.47a | 35.86 ± 11.71a | 8.39 ± 2.48b | 0.04 ± 0.01b | 2.93 ± 0.25b | 29.56 ± 1.95a | 0.77 ± 0.13a | 0.10 ± 0.12a |
| **Summer** | **M** | 0.85 ± 0.06A | 8.19 ± 0.91B | 13.62 ± 0.47A | 7.19 ± 0.92A | 6.68 ± 0.73B | 0.09 ± 0.02AB | 3.12 ±0.37BC | 89.38 ± 1.62B | 5.34 ± 0.87A | 0.30 ± 0.06A |
| | **F** | 1.01 ± 0.09a | 9.68 ± 0.77b | 13.73 ± 1.02a | 7.3 ± 1.77a | 6.19 ± 1.15b | 0.09 ± 0.04ab | 2.77 ± 0.39b | 94.92 ± 0.94b | 3.67 ± 1.01bc | 0.28 ± 0.07a |
| **Autumn** | **M** | 0.54 ± 0.08B | 5.15 ± 0.62B | 18.33 ± 1.44B | 38.50 ± 7.52B | 27.10 ± 2.98A | 0.04 ± 0.004B | 1.35 ± 0.15A | 7.04 ± 0.54C | 1.60 ± 0.59B | 0.62 ± 0.08AB |
| | **F** | 0.38 ± 0.04c | 6.65 ± 0.39b | 20.34 ± 2.21b | 26.49 ± 8.85a | 22.94 ± 4.17a | 0.04 ± 0.004b | 1.11 ± 0.14a | 7.16 ± 0.73c | 3.28 ± 1.27bc | 0.36 ± 0.10a |
| **Winter** | **M** | 0.18 ± 0.01B | 10.89 ± 1.15A | 21.31 ± 1.36B | 22.30 ± 2.78AB | 2.13 ± 0.32B | 0.15 ± 0.03A | 2.12 ± 0.29AC | 53.11 ± 3.94D | 1.44 ± 0.24B | 0.71 ± 0.59B |
| | **F** | 0.19 ± 0.01b | 16.46 ± 0.2a | 19.11 ± 1.27b | 25.77 ± 5.83a | 1.28 ± 0.43a | 0.18 ± 0.04a | 2.69 ± 0.27b | 57.33 ± 5.01d | 1.74 ± 0.46ab | 0.59 ± 0.17a |
| | | (mg $g^{-1}$) | (MG $g^{-1}$) | (mg $ml^{-1}$) | (mg $g^{-1}$) | (mg $g^{-1}$) | (mg $g^{-1}$) | (mg $g^{-1}$) | (mg $ml^{-1}$) | (mg $g^{-1}$) | (mg $g^{-1}$) |

**Table III.** Seasonal concentrations of metabolites examined in the anterior gills of *Parastacus defossus* and *Parastacus brasiliensis*. The results show the mean and standard error. The different letters indicate significant differences (capital letters for males and small letters for females) ($p < 0.05$). Gly= Glycogen; FG= Free Glucose; Pro= Protein; Lip= Lipid; Chol= Cholesterol; M= male; F= female.

| | | **Anterior Gills** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Seasons** | | ***Parastacus defossus*** | | | | | ***Parastacus brasiliensis*** | | | | |
| | | **Gly** | **FG** | **Pro** | **Lip** | **Chol** | **Gly** | **FG** | **Pro** | **Lip** | **Chol** |
| **Spring** | **M** | 0.36 ± 0.14B | 38.8 ± 3.88A | 10.64 ± 1.81A | 18.62 ± 4.84A | 12.15 ± 2.53B | 0.16 ± 0.03A | 17.70 ± 3.07A | 8.50 ± 1.67B | 20.3 ± 3.86B | 15.62 ± 4.00AB |
| | **F** | 0.40 ± 0.17b | 39.2 ± 4.72ab | 10.18 ± 1.41a | 17.20 ± 3.91a | 11.40 ± 3.81b | 0.16 ± 0.04a | 20.76 ± 2.86a | 8.50 ± 1.30b | 35.64 ± 7.10a | 21.68 ± 5.75b |
| **Summer** | **M** | 5.38 ± 0.31A | 60.24 ± 12.50A | 8.11 ± 0.42A | 63.86 ± 7.91B | 45.68 ± 4.1C | 2.05 ± 0.18B | 9.48 ±1.01BC | 12.67 ± 0.56B | 13.76 ± 2.93B | 4.71 ± 1.07A |
| | **F** | 5.28 ± 0.56a | 58.72 ± 1.87a | 7.85 ± 0.63a | 63.55 ± 5.47c | 42.81 ± 5.03c | 2.89 ± 0.42b | 11.14 ± 0.70b | 12.72 ± 0.79b | 18.37 ± 4.64a | 2.45 ± 0.91a |
| **Autumn** | **M** | 2.38 ± 0.25B | 19.13 ± 1.17A | 9.72 ± 0.72A | 71.51 ± 8.83B | 67.40 ± 4.45A | 0.27 ± 0.04A | 4.88 ± 0.64C | 1.51 ± 0.25A | 22.64 ± 5.37B | 12.00 ± 3.13AB |
| | **F** | 2.16 ± 0.62b | 29.35 ± 1.18bc | 12.52 ± 0.91b | 71.73 ± 17.38bc | 67.64 ± 8.78a | 0.19 ± 0.05a | 5.04 ± 0.50b | 1.07 ± 0.17a | 28.37 ± 11.66a | 11.44 ± 1.98ab |
| **Winter** | **M** | 0.70 ± 0.11B | 26.56 ± 2.81A | 10.40 ± 0.48A | 72.25 ± 7.06B | 6.74 ± 0.79B | 3.05 ± 0.07B | 12.48±0.57AB | 19.56 ± 2.79C | 56.19 ± 13.49A | 25.70 ± 5.93B |
| | **F** | 0.52 ± 0.06b | 21.78 ± 3.70c | 10.24 ± 0.78a | 106.56 ± 15.04b | 4.98 ± 0.40b | 3.35 ± 0.73b | 14.74 ± 1.44a | 20.81 ± 2.82c | 56.43 ± 12.76a | 23.17 ± 4.09b |
| | | ($mg\ g^{-1}$) | **($mg\ g^{-1}$)** | **($mg\ ml^{-1}$)** | **($mg\ g^{-1}$)** | **($mg\ g^{-1}$)** | **($mg\ g^{-1}$)** | **($mg\ g^{-1}$)** | **($mg\ ml^{-1}$)** | **($mg\ g^{-1}$)** | **($mg\ g^{-1}$)** |

**Table IV.** Seasonal concentrations of metabolites examined in the posterior gills of *Parastacus defossus* and *Parastacus brasiliensis*. The results show the mean and standard error. The different letters indicate significant differences (capital letters for males and small letters for females) ($p < 0.05$). Gly= Glycogen; FG= Free Glucose; Pro= Protein; Lip= Lipid; Chol= Cholesterol; M= male; F= female.

| | | **Posterior Gills** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Seasons** | | ***Parastacus defossus*** | | | | | ***Parastacus brasiliensis*** | | | | |
| | | **Gly** | **FG** | **Pro** | **Lip** | **Chol** | **Gly** | **FG** | **Pro** | **Lip** | **Chol** |
| **Spring** | **M** | 0.51 ± 0.15B | 21.71 ± 2.1AB | 7.98 ± 0.96A | 56.62 ± 5.84A | 7.02 ± 1.36B | 0.14 ± 0.023A | 11.67 ± 1.14B | 5.00 ± 0.50A | 31.29 ±0.57AB | 11.23 ± 3.74AB |
| | **F** | 0.38 ± 0.12b | 35.15 ± 1.98b | 9.94 ± 1.15a | 64.97 ± 3.78c | 10.37 ± 2.82b | 0.17 ± 0.030a | 13.81 ± 2.20b | 6.49 ± 0.49b | 29.99 ± 5.82ab | 14.27 ± 4.57a |
| **Summer** | **M** | 2.87 ± 0.17A | 37.97 ± 4.89B | 13.30 ± 1.05B | 47.82 ± 5.09AC | 39.74 ± 3.80C | 1.70 ± 0.25B | 10.16 ± 1.52B | 12.68 ± 0.53B | 12.53 ± 2.64B | 6.76 ± 1.14A |
| | **F** | 2.81 ± 0.27a | 56.92 ± 2.95b | 11.21 ± 1.08a | 43.13 ± 5.69bc | 40.47 ± 5.61c | 1.97 ± 0.47b | 9.18 ± 0.57a | 13.99 ± 0.61a | 6.98 ± 1.68b | 3.53 ± 1.00a |
| **Autumn** | **M** | 1.77 ± 0.30C | 11.31 ± 1.16A | 10.60 ± 0.78B | 73.14 ± 12.94BC | 65.27 ± 5.59A | 0.20 ± 0.03A | 17.21 ±3.52Bb | 1.21 ± 0.16C | 27.58 ±7.00AB | 8.82 ± 1.99AB |
| | **F** | 1.39 ± 0.15b | 15.34 ± 1.30a | 11.65 ± 1.12a | 74.30 ± 11.13ab | 67.30 ± 5.09a | 0.16 ± 0.04a | 23.31 ± 2.35a | 0.92 ± 0.09b | 12.54 ± 4.15b | 5.49 ± 1.03a |
| **Winter** | **M** | 0.50 ± 0.07B | 32.90 ± 6.59B | 10.18 ± 0.43B | 83.79 ± 8.35B | 4.57 ± 0.63B | 1.21 ± 0.07B | 4.40 ± 0.74A | 19.25 ± 2.07D | 61.81 ± 13.91A | 20.23 ± 3.86B |
| | **F** | 0.43 ± 0.04b | 41.50 ± 4.97b | 11.20 ± 1.15a | 111.93 ± 20.98a | 4.35 ± 0.57b | 1.20 ± 0.14b | 7.74 ± 1.13ab | 30.44 ± 4.41c | 59.50 ± 14.68a | 15.66 ± 2.55a |
| | | (mg $g^{-1}$) | (mg $g^{-1}$) | (mg $ml^{-1}$) | (mg $g^{-1}$) | (mg $g^{-1}$) | (mg $g^{-1}$) | (mg $g^{-1}$) | (mg $ml^{-1}$) | (mg $g^{-1}$) | (mg $g^{-1}$) |

**Table V.** Seasonal variations of environmental parameters in the habitats of *Parastacus defossus* and *Parastacus brasiliensis*. The results show the mean and standard error.

| **Seasons/ Environmental parameters** | ***Parastacus defossus*** | | | ***Parastacus brasiliensis*** | | |
|---|---|---|---|---|---|---|
| | **Oxygen (mg/L)** | **Temperature (ºC)** | **pH** | **Oxygen (mg/L)** | **Temperature (ºC)** | **pH** |
| **Spring** | 2.0 ± 0.44 | 23.2 ± 0.58 | 5.5 ± 0.28 | 12.1 ± 0.23 | 21.1 ± 0.64 | 7.6 ± 0.86 |
| **Summer** | 1.5 ± 0.50 | 27.0 ± 0.44 | 7.0 ± 0.22 | 10.0 ± 0.35 | 19.2 ± 0.74 | 6.6 ± 1.13 |
| **Autumn** | 1.4 ± 0.68 | 19.1 ± 0.52 | 5.6 ± 0.36 | 13.1 ± 0.43 | 20.1 ± 0.85 | 7.2 ± 0.95 |
| **Winter** | 1.7 ± 0.86 | 18.0 ± 0.48 | 5.2 ± 0.25 | 13.2 ± 0.37 | 11.5 ± 0.46 | 5.4 ± 0.30 |

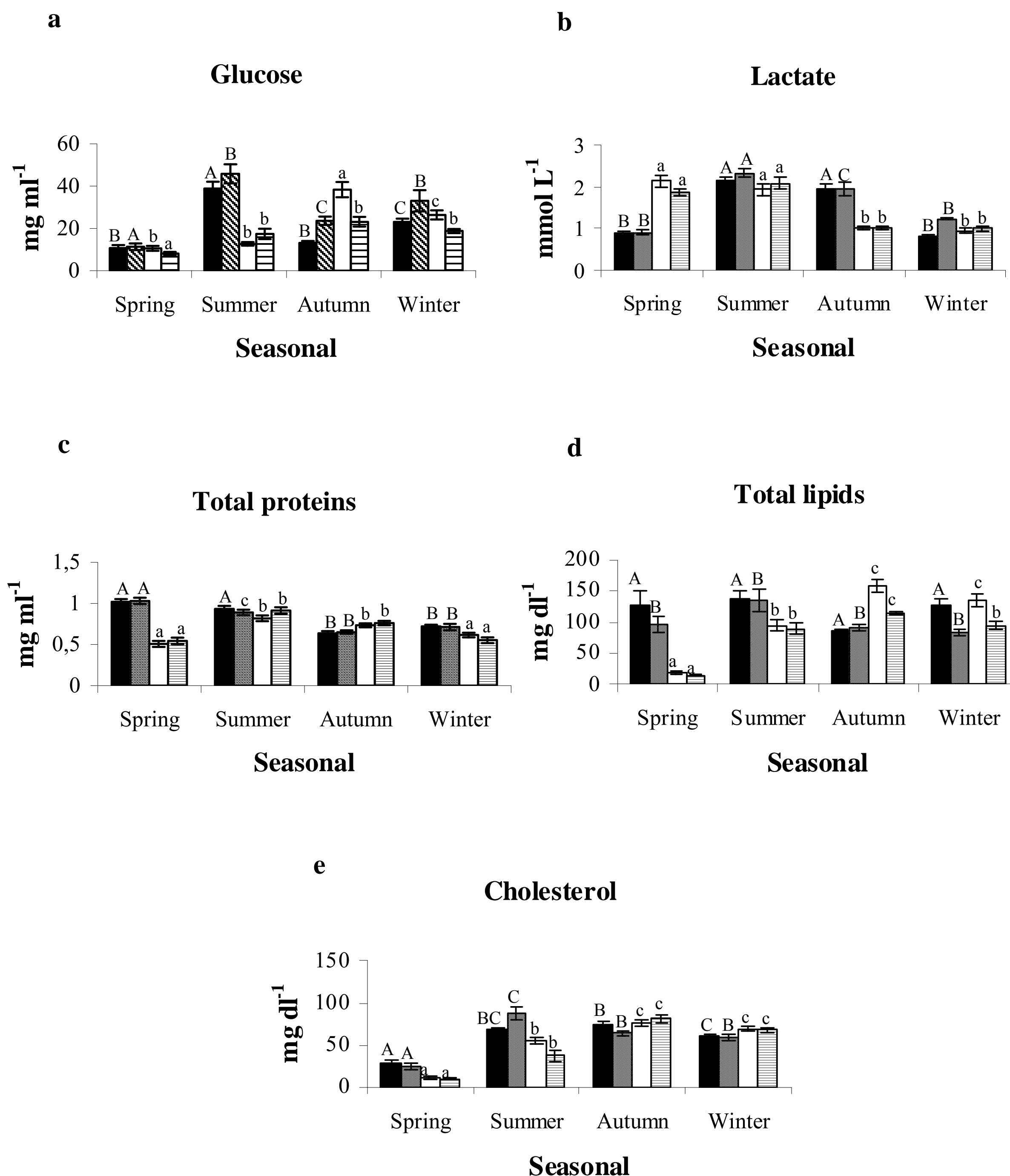



**Figure 1**. Seasonal concentrations of metabolites in the hemolymph of *Parastacus defossus* and *Parastacus brasiliensis*. *P. defossus* (males): black bar; *P. defossus* (females): striped bar; *P. brasiliensis* (males): white bar; *P. brasiliensis* (females): gray bar. The columns show the mean; vertical bars show the standard error of the mean. The different letters indicate significant differences (capital letters for *P. defossus* and small letters for *P. brasiliensis*) ($p<0.05$).

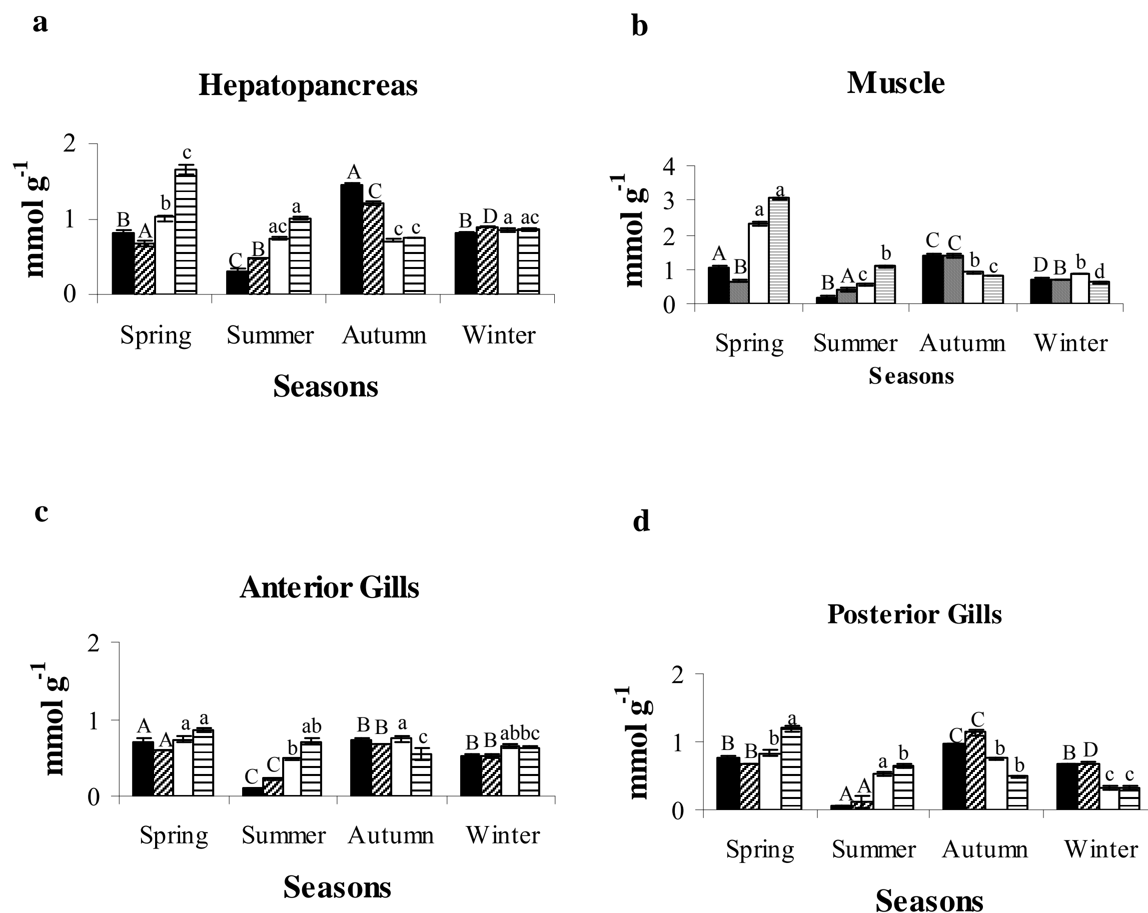



**Figure 2**. Seasonal concentrations of arginine in all tissues of *Parastacus defossus* and *Parastacus brasiliensis*. *P. defossus* (males): black bar; *P. defossus* (females): striped bar; *P. brasiliensis* (males): white bar; *P. brasiliensis* (femables): gray bar. The columns show the mean; vertical bars show the standard error of the mean. The different letters indicate significant differences (capital letters for *P. defossus* and small letters for *P. brasiliensis*) ($p<0.05$).

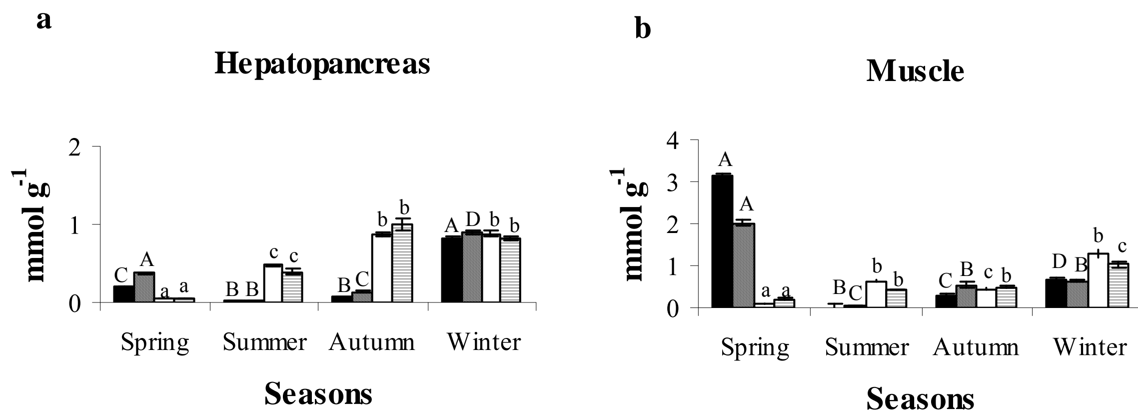



**Figure 3**. Seasonal concentrations of arginine phosphate in the hepatopancreas and muscle of *Parastacus defossus* and *Parastacus brasiliensis*. *P. defossus* (males): black bar; *P. defossus* (females): striped bar; *P. brasiliensis* (males): white bar; *P. brasiliensis* (females): gray bar. The columns show the mean; vertical bars show the standard error of the mean. The different letters indicate significant differences (capital letters for *P. defossus* and small letters for *P. brasiliensis*) ($p<0.05$).

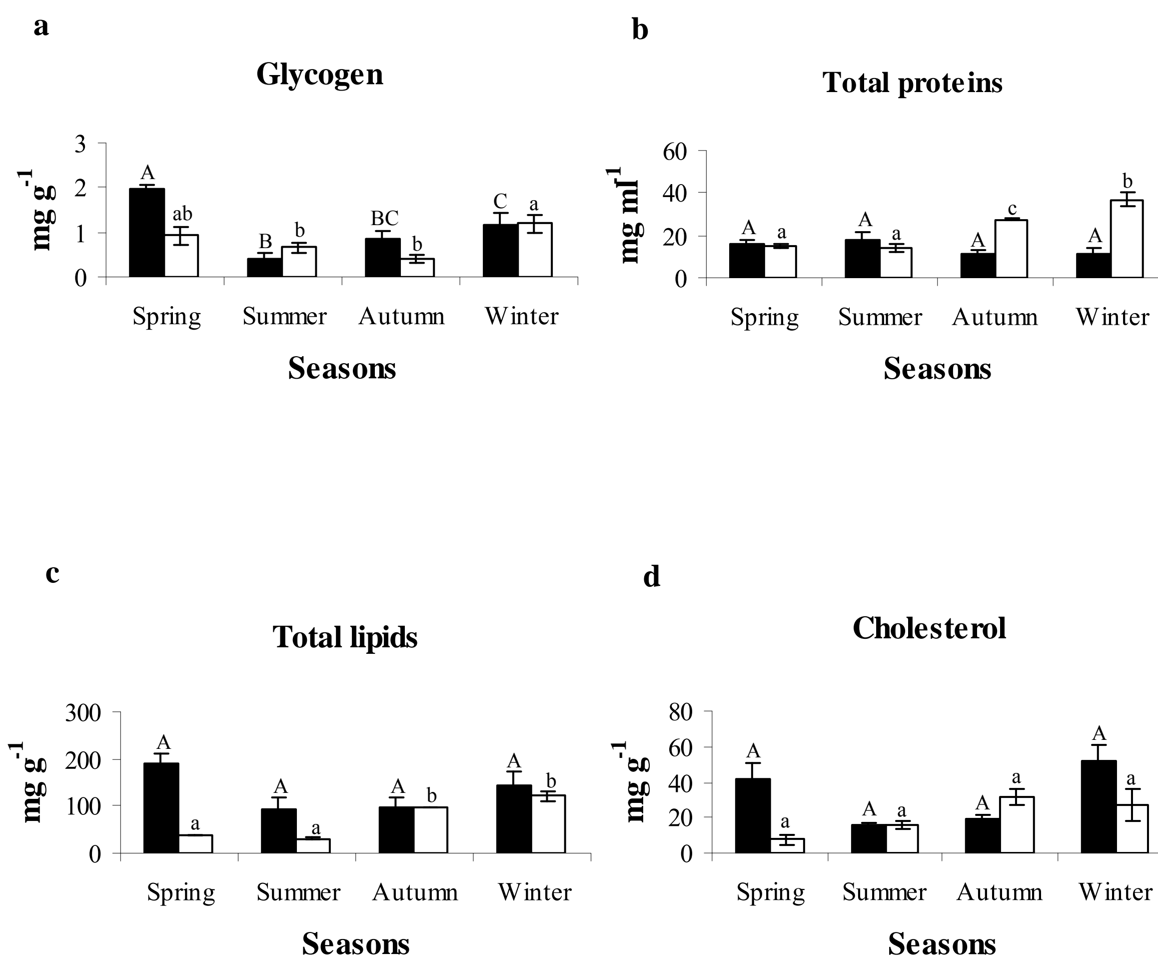
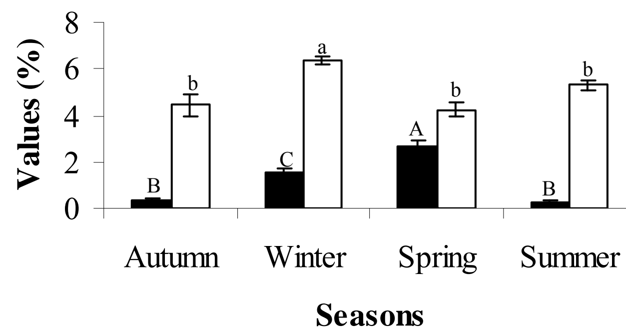



**Figure 4**. Seasonal concentrations of metabolites examined in the gonads of *Parastacus defossus* and *Parastacus brasiliensis*. *P. defossus*: black bar; *P. brasiliensis*: white bar. The columns show the mean; vertical bars show the standard error of the mean. The different letters indicate significant differences (capital letters for *P. defossus* and small letters for *P. brasiliensis*) ($p<0.05$).

**a**




**b**

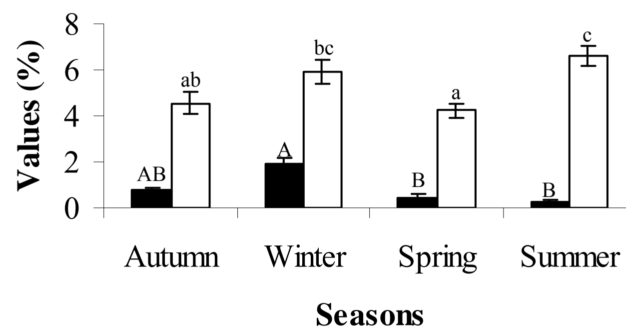



**Figure 5.** Gonad index (GI) and hepatopancreatic index (HI) of *Parastacus defossus* (a) and *Parastacus brasiliensis* (b) (represented in percentage). GI: black bar; HI: white bar. The columns show the mean; vertical bars show the standard error of the mean. The different letters indicate significant differences (capital letters for GI and small letters for HI) ($p<0.05$).

# Capítulo II

**Metabolic responses of *Parastacus defossus* and *Parastacus brasiliensis* (Crustacea, Decapoda, Parastacidae) to hypoxia**

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**Abstract.** The metabolic responses of two crayfish species living in different habitats, *P. defossus* and *P. brasiliensis,* were analyzed after different periods of hypoxia (2.0 mg $O_2$/L of oxygen). In the laboratory, groups of animals were subjected to hypoxia for 1, 2, 4, and 8 h. The hemolymph, hepatopancreas, muscle, and anterior and posterior gills were removed for determination of glucose, free glucose, glycogen, lactate, total proteins, total lipids, total cholesterol, arginine, and arginine phosphate. In both species, glucose and lactate increased significantly after 4 h of hypoxia, but decreased after 8 h ($p > 0.05$) from the beginning of the experiment. Reductions of glycogen, lipids, and cholesterol were recorded in hepatopancreas and muscle tissue, especially of *P. defossus*, after 4 h of hypoxia. Free glucose levels decreased in all tissues of *P. brasiliensis*, mainly in the hepatopancreas and muscle ($p<0.05$), while *P. defossus* showed smaller reductions of these reserves. All reserves in the anterior and posterior gills, with exception of the glycogen reserves, behaved similarly in both species. Both crayfishes stored and used arginine phosphate, mainly *P. defossus,* which also showed higher concentrations of all metabolites than did *P. brasiliensis*. Both species showed metabolic adaptations to hypoxia, but, as expected, *P. defossus* appeared to be better adapted.

**Keywords:** Crayfish, Crustacea, Hypoxia, Metabolism.

## 1. Introduction

All organisms are adapted to their natural habitat, and additionally have the ability to acclimate to environmental changes. The process of acclimation involves physiological, biochemical, behavioral, and other responses that allow the animal to adjust to such changes (McMahon, 2002). Aquatic invertebrates encounter periodic environmental hypoxia or anoxia, and evolve effective anaerobic mechanisms to cope with low ambient oxygen concentrations (Hochachka, 1980; Lutz and Storey, 1997; Childress and Seidel, 1998; Hochachka and Lutz, 2001).

Among invertebrates, mollusks are the most tolerant to hypoxia, and crustaceans are the most sensitive (Vaquer-Sunyer and Duarte, 2008). These invertebrates show the largest number of species tolerant to hypoxia and/or anoxia, according to the study of Zwaan and Eertman (1996). Adaptations to hypoxia are very variable among crustaceans. This variability may be due to the number of species and also to the diversity of habitats in which crustaceans live, particularly freshwater crayfish.

Burrowing species of decapod crustaceans usually show higher tolerance to hypoxia than do free-swimming species, because they maintain large stores of energy sources such as glycogen and phosphoarginine, and these species can reduce their metabolic rate when submitted to prolonged hypoxia (Hervant et al., 1999). Species of the crayfish genus *Parastacus* display the greatest range of adaptations to extreme environmental conditions. Some species live in flowing water, and others prefer water with little or no current such as small streams, lakes, reservoirs, and swamps. Many species live in subterranean burrows, and some pass their entire lives within these burrows (Buckup and Rossi, 1980; Hogger, 1988; Buckup, 1999). According to Noro (2007) the dissolved oxygen of the burrow water of *P. defossus* remained at very low levels (1.6 mg/L) throughout the period of observation, indicating nearly anaerobic conditions in the burrows. However, other crayfish species such as *Parastacus brasiliensis* live in better-oxygenated lotic environments (Fontoura and Buckup 1989, Silva-Castiglioni et al., unpublished observations).

In order to survive in hypoxic or anoxic conditions, animals may evolve adaptive strategies such as the use of anaerobic pathways for the production of ATP, maintenance of high glycogen and phosphate concentrations in all the tissues, and metabolic depression (Storey and Storey, 1990; Hervant et al., 1995; Lutz and Storey, 1997; Childress and Seidel, 1998; Hochachka and Lutz, 2001). The intermediary metabolism of freshwater crustaceans subjected to hypoxia or anoxia has not been studied in Brazil. In Brazil, the only investigations were carried out by Oliveira et al. (2001, 2004) and Marqueze et al. (2006) with the estuarine crab *Neohelice granulatus*, maintained on different diets, and the study of Oliveira et al. (2005) on the oxidative balance of the gills of this same species. Therefore, the objective of the present study was to analyze the metabolism of two species of freshwater crayfish with different habitats, *Parastacus defossus* and *Parastacus brasiliensis*, subjected to different periods of hypoxia. Our hypothesis was that *P. defossus*, which lives in subterranean burrows, will show more extensive adaptation to hypoxia than will *P. brasiliensis,* which lives in better-oxygenated surface waters.

## 2. Materials and Methods

The animals were used with the permission of the Ethics Committee of the Pontifícia Universidade Católica do Rio Grande do Sul (Permit No. 0002/03) and according to Brazilian laws.

### 2.1. Experimental procedure

*Parastacus defossus* and *Parastacus brasiliensis* were collected in the winter of 2008. *P. defossus* was collected in the Lami region, Porto Alegre, Rio Grande do Sul, Brazil (30°11’41”S, 51°06’00”W), with a suction pump due to its fossorial habit. *P. brasiliensis* was collected in Mariana Pimentel in Rio Grande do Sul (30°20’32.27”S, 51°34’02.87”W), with traps baited with liver, set in its typical lotic environments. After they were collected, the animals were taken to the laboratory and acclimatized at a constant temperature of 19 °C and a 12 h light: 12 h dark photoperiod (Hama 47656 CE electric timer). The approximately 40 specimens of both species were

fed two or three times per week for 10 days with commercial fish feed.

After this 10-day acclimatization period, the crayfish were separated and placed in different aquariums during the hypoxia experiment. The aquariums were aerated with nitrogen gas in order to reduce the concentration of oxygen to 2 mg/L. During each experiment, the gas pumping was stopped and the time was recorded. The level of oxygen was monitored with an oximeter (OXI 330/SET-WTW). The oxygen concentration of 2 mg/L was used because this level was measured in the burrows of *P. defossus* by Noro (2007) and Silva-Castiglioni et al. (unpublished observations). According to Diaz and Rosenberg (1995), hypoxia is defined as an oxygen level below 2.8 mg $O_2$/L.

Groups of animals were maintained in the aquariums for 1, 2, 4, and 8 h. Part of the animals were maintained in normoxic conditions (control groups). Samples of hemolymph were collected with a syringe containing 10% potassium oxalate (anti-clotting substance). The hepatopancreas, abdominal muscle, and anterior and posterior gills were removed and stored in a freezer at -80°C for determination of the levels of glucose, lactate, free glucose, glycogen, total proteins, total lipids, total cholesterol, arginine, and arginine phosphate. The metabolic parameters were determined in triplicate using the same spectrophotometric methods that were previously used for other crustaceans in the Laboratório de Fisiologia da Conservação of the Pontifícia Universidade Católica do Rio Grande do Sul (Oliveira et al., 2003; Silva-Castiglioni et al., 2007; Dutra et al., 2008).

### 2.2. Hemolymph determinations

**2.2.1.** Glucose levels were measured by the glucose-oxidase method, using a Bioclin Kit (Ref. 84) (glucose GOD-CLIN). Results are expressed in mmol/L.

**2.2.2.** For the lactate determination, the samples were deproteinized with perchloric acid. The concentration of lactate was measured using a Bioclin kit (Ref. K084-2), by the formation of pyruvate (L-lactate + $NAD^+ \overset{LDH}{\leftrightarrow}$ Pyruvate + NADH + $H^+$). Results are expressed in mmol/L.

**2.2.3.** Total proteins were measured according to Lowry et al. (1951), using bovine albumin as the reference substance. Results are expressed in mg/ml.

**2.2.4.** Total lipids were measured by the sulfophosphovanillin method (Frings and Dunn 1970), with the results expressed in mg/dl.

**2.2.5.** Total cholesterol was measured using a Labtest kit (Total Cholesterol, Liquiform Catalog number 76), with the results expressed in mg/dl.

**2.3. Tissue determinations**

**2.3.1.** Free glucose was determined according to Carr and Neff (1984). Tissues were weighed and homogenized with Ultra-Turrax. To separate the lipid fraction, the samples were mixed in a chloroform-methanol solution and centrifuged. The concentration of free glucose was determined by the colorimetric glucose-oxidase method (Biodiagnóstico Kit) in an intermediate fraction obtained after centrifugation. The results are expressed in mg/g of tissue.

**2.3.2.** The glycogen was extracted from tissues following the method described by Van Handel (1965), and glycogen levels in the animals were determined as glucose equivalent (glucose-oxidase method), after acidic hydrolysis (HCl) and neutralization ($Na_2CO_3$), following the method of Geary et al. (1981). Glucose was quantified using a Biodiagnostic kit (glucose-oxidase). Results are presented as mg/g of tissue.

**2.3.3.** Levels of total proteins were measured as described by Lowry et al. (1951), with bovine serum albumin (Sigma) as the reference standard. The results are expressed in mg/ml of homogenate.

**2.3.4.** Total lipids were extracted using a 2:1 (v/v) chloroform-methanol solution, according to Folch et al**.** (1957), and were determined by the sulfophosphovanillin method (Frings and Dunn 1970). Results are expressed in mg/g of tissue.

**2.3.5.** Total cholesterol was measured by the reactions of cholesterol esterase, cholesterol oxidase,

and peroxidase enzymes (Labtest Kit/Liquiform, Catalog number 76 ). The results are presented as mg/g of tissue.

**2.3.6.** Arginine and arginine phosphate were determined using the method of Bergmeyer (1985). Arginine was determined by the change in absorbance at 339 nm in the reaction catalyzed by octopine dehydrogenase: arginine + pyruvate + NADH + $H^+$ ↔ octopine + $NAD^+$ + $H_2O$. To hydrolyze arginine and arginine phosphate to phosphate, 100 µl of HCl (1 mol/L) was added to 100 µl of tissue (homogenate) and incubated in tightly capped tubes for 90 s in boiling water. The hydrolysates were then cooled and neutralized with 100 µl NaOH (1 mol/L). The arginine assay was repeated and the previous concentration of arginine subtracted to obtain the arginine phosphate level. Results are expressed in mmol/g.

## 2.4. Statistical Analyses

All the metabolic parameters were homogeneous (Levene test), and were normally distributed (Kolmogorov-Smirnov test). For statistical analysis of the different periods of hypoxia, a one-way ANOVA test was used, followed by a Bonferroni test. For comparison between species, a two-way ANOVA was used. The significance level adopted was 5%. All the tests were done with the program Statistical Package for the Social Sciences (SPSS- 11.5) for Windows.

## 3. Results

Males and females were pooled for metabolic dosages because they showed the same behavior. This pooling increased the number of animals in each group. During the experiments, no animals died.

## 3.1. Hemolymph

**3.1.1. Glucose:** The two species showed different responses in the glucose concentrations ($p<0.05$).

In hypoxia, glucose levels increased in *P. defossus* with the highest concentration after 4 h of hypoxia. This level was approximately 132% higher than the levels recorded in the normoxia group ($p<0.05$). The levels of glucose were reduced ($p>0.05$) after 8 h of hypoxia. *P. brasiliensis* showed similar responses, except that the increase in glucose after 4 h of hypoxia (approximately 285%) was higher than in *P. defossus* (Figure 1).

**3.1.2. Lactate:** The two species showed similar responses ($p>0.05$). In hypoxia, their lactate concentrations progressively increased after 1, 2, and 4 h. Increases of 170 and 250% were observed after 4 h in *P. defossus* and *P. brasiliensis*, respectively, compared with the control group ($p<0.05$). Both species reduced lactate levels after 8 h of hypoxia, compared with the group exposed for 4 h (Figure 1).

**3.1.3. Total proteins:** The species showed different responses in their protein reserves ($p<0.05$). Significant variations were not recorded in different groups of *P. defossus* subjected to hypoxia, whereas in *P. brasiliensis* we observed a reduction of 24% after 1 h of hypoxia, compared with the control group (Figure 1).

**3.1.4. Total lipids:** The lipid reserves behaved similarly between the species ($p>0.05$). In *P. defossus* we recorded reductions in total lipids after 4 and 8 h, approximately 21 and 32%, compared with the control group ($p>0.05$). The hemolymph lipids of *P. brasiliensis* increased after 2 and 4 h, with an increase of 66% after 4 h ($p<0.05$). However, the concentrations observed after 8 h were similar to those recorded in the control group (Figure 1).

**3.1.5. Total cholesterol:** The species showed different responses in the cholesterol levels ($p<0.05$). The cholesterol in the hemolymph of *P. defossus* showed the same behavior as the lipid reserves. *P. brasiliensis* showed a decrease in total cholesterol after 1 h of hypoxia ($p>0.05$). Compared with the group exposed for 1 h, the reserves increased in other periods of hypoxia, but to levels lower than those recorded in the control group ($p>0.05$) (Figure 1).

### 3.2. Hepatopancreas

**3.2.1. Glycogen:** The species responded differently ($p<0.05$). Glycogen in the hepatopancreas of *P. defossus* decreased 38, 57, and 50% after 1 ($p>0.05$), 2, and 4 h of hypoxia ($p<0.05$), respectively, and after 8 h increased again ($p<0.05$). Significant decreases were observed in the levels of glycogen in hepatopancreas of *P. brasiliensis* after all the periods of hypoxia; the lowest concentrations were recorded after 2 h, with a reduction of 68% ($p<0.05$) (Figure 2).

**3.2.2. Free glucose:** The free glucose levels in the hepatopancreas of the species behaved similarly ($p>0.05$); both species showed reductions in all the periods. These reductions were not significantly different (Figure 3).

**3.2.3. Total proteins:** The species responded differently ($p<0.05$). In *P. defossus*, protein levels increased 42 and 26% ($p<0.05$) after 1 and 2 h of hypoxia, respectively. Total proteins in the hepatopancreas of *P. brasiliensis* increased in all periods of hypoxia, mainly after 1 and 2 h when they increased significantly (50%) compared with the normoxia group ($p<0.05$) (Table I).

**3.2.4. Total lipids:** The species differed in their responses ($p<0.05$). After 2 and 4 h of hypoxia, the lipids in the hepatopancreas of *P. defossus* decreased aproximately 37 and 30%, respectively, compared with the normoxia group ($p>0.05$). In *P. brasiliensis* the lipid levels did not change significantly, but did decrease after 2 h and 8 h of hypoxia ($p>0.05$). (Table I).

**3.2.5. Total cholesterol:** The species responded differently in relation to the cholesterol reserves ($p<0.05$). In *P. defossus*, similar levels of cholesterol to the control group were recorded after 1 h of hypoxia. The levels then decreased to 2 h, by approximately 32% compared with the controls ($p>0.05$). In the other periods, small reductions were recorded ($p>0.05$). In *P. brasiliensis* the cholesterol concentration decreased in all periods, but decreased significantly only after 2 h of hypoxia (47%) ($p<0.05$) (Table I).

**3.2.6. Arginine:** The species showed different responses in the arginine levels ($p<0.05$). *P. defossus* showed reductions in arginine levels in all periods of hypoxia, mainly after 2 h with a decrease of

74% ($p<0.05$). In *P. brasiliensis* we recorded an increase of 41% in arginine levels after 2 h of hypoxia ($p<0.05$). However, when the crayfish were subjected to 4 and 8 h of hypoxia, we observed reductions of 32 and 52% respectively, but only the difference after 8 h was significant ($p<0.05$) (Figure 4).

**3.2.7. Arginine phosphate:** The species responded differently ($p<0.05$). In the hepatopancreas of *P. defossus,* arginine phosphate increased 25% after 2 h of hypoxia ($p>0.05$), decreased significantly (52%) after 4 h, and then increased after 8 h. The arginine phosphate reserves of *P. brasiliensis* did not show significant variations, but tended to decrease in all the periods ($p>0.05$) (Figure 4).

**3.3. Muscle**

**3.3.1. Glycogen:** The species responded differently ($p<0.05$). Glycogen levels decreased in the muscle tissue of *P. defossus* in all periods of hypoxia, with significant differences only after 4 and 8 h. In *P. brasiliensis*, we observed similar levels to animals in normoxia in all the periods (Figure 2).

**3.3.2. Free glucose**: The species responded differently ($p<0.05$). The free glucose in muscle of *P. defossus* did not vary significantly in the different periods of hypoxia. In *P. brasiliensis,* the free glucose concentration also did not vary significantly (Figure 3).

**3.3.3. Total proteins:** The protein reserves changed differently in the two species ($p<0.05$). The proteins in the muscle of *P. defossus* did not vary significantly in the different periods, except after 8 h when the levels were higher than after all the other periods ($p<0.05$). In the muscle tissue of *P. brasiliensis,* no significant variations were observed (Table II).

**3.3.4. Total lipids:** The lipid levels responded differently in the two species ($p<0.05$). After the different periods of hypoxia, *P. defossus* showed higher lipid levels, although these concentrations were not significantly different. Reductions of 53 and 62% in total lipids were recorded in *P. brasiliensis* after 1 and 2 h of hypoxia, respectively ($p<0.05$). After 4 and 8 h of hypoxia, the lipid

concentrations were higher than those recorded after 1 and 2 h, and did not differ significantly from the control group (Table II).

**3.3.5. Total cholesterol:** The responses of the two species differed significantly. Reductions of cholesterol in the muscle tissue of *P. defossus* were recorded in all the periods of hypoxia, but a significant variation was observed only after 8 h (decrease of 59%). Cholesterol in the muscle tissue of *P. brasiliensis* also decreased, approximately 43 and 68% after 2 and 8 h, respectively ($p<0.05$) (Table II).

**3.3.6. Arginine:** The species behaved differently ($p<0.05$). *P. defossus* showed reductions of arginine levels of 64% after 1 h of hypoxia ($p<0.05$), which then remained stable until 8 h after the beginning of the experiment, approximately 75% lower than in the control group ($p<0.05$). In the muscle of *P. brasiliensis,* the arginine concentrations did not vary significantly (Figure 4).

**3.3.7. Arginine phosphate:** The species showed different responses in the arginine phosphate reserves ($p<0.05$). In the muscle tissue of *P. defossus*, decreases of 20 and 16% were recorded after 2 and 4 h, respectively, but a significant difference was recorded only after 2 h. The concentrations increased after 8 h of hypoxia, to the highest levels observed, significantly higher than the levels after 2 and 4 h ($p<0.05$). *P. brasiliensis* showed reductions in arginine phosphate reserves in all the periods of hypoxia, compared to the control ($p<0.05$). The concentrations decreased approximately 85% after 8 h, compared with the control group (Figure 4).

**3.4. Anterior gills (AG)**

**3.4.1. Glycogen:** The species behaved similarly ($p>0.05$). The glycogen reserves of *P. defossus* were similar ($p>0.05$) to those in the animals in normoxia after 1 h of hypoxia until the end of the experiment (8 h). The anterior gills of *P. brasiliensis* showed no significant variations in the different periods of hypoxia (Figure 2).

**3.4.2. Free glucose:** The species showed different responses in relation to the free glucose reserves

($p<0.05$), but not during the different periods of hypoxia. In *P. defossus,* reductions were observed in all periods; the lowest level was reached after 2 h, approximately 30% lower than that in the control ($p>0.05$). Reductions were also recorded in *P. brasiliensis* ($p>0.05$), with the lowest concentration observed after 1 h of hypoxia. Free glucose decreased less in *P. brasiliensis* than in *P. defossus* (Figure 3).

**3.4.3. Total proteins:** The protein levels responded differently in the two species ($p<0.05$). The anterior gills of *P. defossus* showed no significant variations in all periods of hypoxia. In the anterior gills of *P. brasiliensis*, total proteins were higher in all the periods of hypoxia, increasing 52, 57, and 53% after 1, 2, and 8 h, respectively, compared with the control group ($p<0.5$) (Table III).

**3.4.4. Total lipids:** The species differed in relation to lipid reserves ($p<0.05$). *P. defossus* showed no significant variations. In the anterior gills of *P. brasiliensis*, reductions occurred in all the periods, but the concentrations did not differ significantly (Table III).

**3.4.5. Total cholesterol:** The cholesterol levels responded differently in the species ($p<0.05$). Total cholesterol did not vary significantly, but the levels observed in *P. defossus* were higher than in *P. brasiliensis* (Table III).

### 3.5. Posterior gills (PG)

**3.5.1. Glycogen:** The species responded differently in relation to glycogen levels ($p<0.05$). Glycogen decreased significantly in the posterior gills of *P. defossus* in all periods, except after 1 h of hypoxia. The concentrations observed after 2 h of hypoxia were approximately 37% lower than those in the control ($p<0.05$). In *P. brasiliensis,* we observed no significant differences from the control group after the different periods of hypoxia (Figure 2).

**3.5.2. Free glucose:** The species responded differently ($p<0.05$). In *P. defossus* free glucose decreased in all the periods of hypoxia, although not significantly. The same response was observed

in the PG of *P. brasiliensis* (Figure 3).

**3.5.3. Total proteins:** Protein levels responded differently in the two species ($p<0.05$). Total protein reserves in *P. defossus* increased 29 and 24% after 1 and 2 h of hypoxia, respectively ($p<0.05$), but after 4 and 8 h were similar to the controls. In *P. brasiliensis*, proteins increased significantly in all the periods of hypoxia, except after 4 h (Table IV).

**3.5.4. Total lipids**: The species responded differently ($p<0.05$). The lipids of the PG of *P. defossus* showed no significant variations. In the PG of *P. brasiliensis* we recorded reductions in all periods of hypoxia, although these reductions were not significantly different (Table IV).

**3.5.5. Total cholesterol:** The species showed different responses in relation to cholesterol reserves ($p<0.05$). Reductions were observed in all the periods of hypoxia in both species, although these reductions were not significantly different (Table IV).

## 4. Discussion

Crustacean species live in a variety of habitats, and they are often adapted to very different and extreme environments, including some with regularly hypoxic conditions. Species living in extreme conditions can develop metabolic adaptations, as observed in this study mainly in *P. defossus*. Among these metabolic adaptations is the utilization of anaerobic pathways when environmental oxygen is low (hypoxia) or absent (anoxia) (Hochachka and Somero, 2002). Anaerobic metabolism is seen in bivalves (Brooks et al., 1991) and crustaceans (Anderson et al., 1994; Zou et al., 1996; Hervant et al., 1995, 1996, 1999), and also in other groups. Both species of *Parastacus* investigated in this study used the anaerobic pathway, as observed in the increase of lactate levels in the hemolymph, and also in the increase in the rate of glycogen utilization in different tissues.

The two species of *Parastacus* showed the highest levels of lactate in the hemolymph after 4 h of hypoxia. In normoxia, *P. defossus* showed higher concentrations of lactate than *P. brasiliensis,*

as well as in all the periods of hypoxia. This response pattern was expected, because *P. defossus* lives in burrows with low oxygen levels compared to the environment of *P. brasiliensis.* Lactate production in *P. defossus* also increased more rapidly than in *P. brasiliensis*, although not significantly. This may also suggest an adaptation of *P. brasiliensis* to the hypoxic environment.

As expected, under hypoxic conditions the lactate concentrations increased from 0.95 to 2.57 mmol/L in *P. defossus* and from 0.65 to 2.28 mmol/L in *P. brasiliensis* after exposure to hypoxia. This indicated activation of the anaerobic metabolism, as also observed in the freshwater crayfishes *Procambarus clarkii* and *Orconectes limosus* by Mauro and Thompson (1984) and Gäde (1984), respectively. However, Hagerman and Vismann (1995) did not observe an increase of lactate levels in the crab *Crangon crangon* after 60 or 80 min of hypoxia, suggesting that this crab has a low tolerance to hypoxia compared with other crustaceans. This low tolerance may be due to a low anaerobic capacity, since this species seems to be rarely found in hypoxic or anoxic environments. Spicer et al. (2002) found a similarly low anaerobic capacity in the freshwater amphipod *Gammarus pulex*.

Glucose levels in the hemolymph of the two *Parastacus* species increased during the hypoxia experiment, more intensely in *P. brasiliensis***,** mainly after 4 h of hypoxia. An increase of the hemolymph glucose is a metabolic adaptation to a hypoxic or anoxic environment, and appears to be a kind of physiological preparation for the substrate-fermentation demand when anaerobic pathways are used (Zou et al., 1996). An increase of glucose during hypoxia was observed in other crustacean species such as *Eriocheir sinensis, Stenasellus virei,* and *Neohelice granulata* studied by Zou et al. (1996), Hervant et al. (1997), and Oliveira et al. (2001), respectively. Therefore, hyperglycemia in crayfish may be related to the need for an increased supply of substrate for glycolysis, and it appears to be a response to hypoxia.

According to Lorezon (2005), hyperglycemia is a typical response of many aquatic animals to adverse physical and chemical changes in the environment. Hyperglycemia may not be a specific

response to hypoxia, but rather a general response to stress. This was suggested by Taylor and Spicer (1987) for *Palaemon elegans* and *Palaemon serratus,* although these species have a low tolerance to anoxia.

*P. brasiliensis* produced more lactate and glucose. In this species the lactate levels increased by 71% and glucose levels by 74% in the hemolymph, whereas in *P. defossus* the lactate and glucose increased 63% and 56%, respectively. This difference between the species is likely related to their different natural environments. These concentrations also increased in the lobster *Nephrops norvegicus* subjected to hypoxia by Schmitt and Uglow (1998), but the increase was higher, approximately 750% for lactate and 210% for glucose. The freshwater crab *Eriocheir sinensis* and the subterranean amphipod *Stenasellus virei* also showed high levels of lactate and glucose in the hemolymph (Zou et al., 1996; Hervant et al., 1997, respectively).

The two crayfish species studied here showed higher levels of glucose in all periods of hypoxia, except after 8 h when glucose decreased significantly. This decrease in *P. defossus* may be associated with the significant increase of the glycogen in the hepatopancreas, which suggests that this carbohydrate is captured to be used in glycogen synthesis. The capture of glucose and the synthesis of glycogen were observed in the crab *N. granulata* during anoxia by Oliveira et al. (2001).

*P. defossus* showed higher glycogen concentrations in all tissues than did *P. brasiliensis*. During hypoxia the rate of glycogen use increased markedly, mainly in *P. defossus* which showed reductions of glycogen in all tissues, whereas in *P. brasiliensis* glycogen decreased only in the hepatopancreas. This increase is called the Pasteur Effect, which is due to an activation of anaerobic glycolysis in hypoxia or anoxia in order to compensate for the lower production or synthesis of ATP. As glycogen is assumed to be the primary source of energy production in crustaceans in hypoxia or anoxia (Zebe, 1991) and is an important substrate for anaerobic glycolysis, the higher concentrations in *P. defossus* suggest that this species is better adapted to the hypoxic environment.

In the crayfish *Parastacus varicosus*, Silva-Castiglioni et al. (2007) observed a significant decrease of glycogen in the muscle tissue during the periods when the lower oxygen levels occurred (spring and summer) in the natural environment. The natural environment of *P. varicosus* is very similar to that of *P. defossus*.

The higher utilization of glycogen of the hepatopancreas in the first two hours of hypoxia reflected a transitory increase in glucose concentrations, mainly in *P. defossus.* This also occurs in other hypoxia-adapted species, such as the amphipod *Niphargus virei* and the isopods *Asellus aquaticus* and *Stenasellus virei* studied by Hervant et al. (1995, 1996), and also the crab *N. granulata* studied by Oliveira et al. (2001).

The high rate of glycogen consumption in the gills of *P. defossus* may be related to hypoxic stress allied to ion transport, because these are the main energy-consuming processes in the gills (Lyndon and Houlihan, 1998). All freshwater crayfish must closely regulate both their osmotic and ion levels because they are constantly exposed to a hypo-osmotic and hypo-ionic environment, and are subject to constant ion loss and water gain by diffusion across their body surface. Crayfish must therefore constantly regulate their internal ion composition in order to survive in fresh water (McMahon, 2002).

The study of the glycogen reserves is important to understand the metabolic adaptations used during hypoxic or anoxic periods, since glycogen can generate energy through a fermentation process. However, these crayfish species showed low glycogen concentrations compared to other crustaceans, indicating that this is a characteristic of *Parastacus*. These low reserves may be compensated by the high reserves of free glucose and arginine phosphate in all tissues of both species.

*P. defossus* showed higher levels of free glucose in all tissues than did *P. brasiliensis,* except in the hepatopancreas. Free glucose serves as a buffer so that the animals can respond more quickly to environmental variations (Oliveira et al., 2001, 2004). The higher concentrations may help to

maintain the metabolism in hypoxic conditions, because both species used these reserves when subjected to hypoxia. In the crab *N. granulata* fed a carbohydrate-rich diet, after 8 h of anoxia the free glucose levels decreased about 72% in all tissues (hepatopancreas, muscle, and gills), suggesting that the glucose reserve is a larger source of ATP synthesis during environmental anaerobiosis (Oliveira et al., 2001).

Santos and Colares (1986) found that in *N. granulata* under hypoxia, the changes in hemolymph glucose levels were controlled by the crustacean hyperglycemic hormone (CHH). This hormone is directly involved in the control of homeostasis of glucose in crustaceans, mobilizing glycogen reserves from both hepatopancreas and muscle (Santos and Keller 1993). Therefore, the CHH must also be involved in carbohydrate metabolism of these *Parastacus* species, although this remains to be confirmed by future studies.

High reserves of arginine phosphate were recorded in *P. defossus* in hepatopancreatic (1.12 mmol/g) and muscle tissue (3.83 mmol/g). This characteristic, together with the higher levels of glycogen in *P. defossus,* may partly explain the high resistance of this species to low oxygen levels in its burrows. Hervant et al. (1995, 1996, 1997) showed that the subterranean isopod *Stenasellus virei,* as in two species of *Niphargus,* contained higher amounts of stored arginine phosphate and glycogen than the epigean crustaceans *Gammarus fossarum* and *Asellus aquaticus*. According to these authors, the storage of large amounts of arginine phosphate and glycogen is the most adaptive characteristic of subterranean aquatic species.

Arginine phosphate is widely used by decapod crustaceans to provide energy during hypoxia (Hill et al., 1991; Speed et al., 2001). Different species of crustaceans maintain high concentrations of phosphate (arginine phosphate) under aerobic conditions as adaptive strategies to hypoxia or anoxia, approximately 25 μmol/g according to Hervant et al. (1995). Both *Parastacus* species, mainly *P. defossus*, stored and used arginine phosphate under hypoxic conditions*;* but *P. brasiliensis* when subjected to 8 h of hypoxia depleted its reserves in the muscle tissue. This

depletion may possibly provide more energy; since this species, because it lives in a more oxygenated environment, may be less adapted to hypoxic conditions.

We suggest that *Parastacus* species use arginine phosphate to maintain ATP synthesis under hypoxia. Holman and Hand (2009) showed that the pool of arginine phosphate in *Lepidophthalmus louisianensis* declined slowly as anoxia continued. Therefore, as well as using arginine phosphate, *P. defossus* is able to reestablish its reserves in the hepatopancreas and muscle after 8 h of hypoxia. The reestablishment in *P. brasiliensis* occurred only in the hepatopancreatic tissue.

Amino acids have many functions in biological processes; as well as being precursors in the biosynthesis of proteins, carbohydrates, and lipids, amino acids are also used as an energy source and as system buffer components (Kilberg and Haussinger, 1992). In the present study, the only amino acid analyzed was arginine. *P. defossus* showed reductions in the hepatopancreatic and muscle tissues, while *P. brasiliensis* decreased the arginine reserves only in the hepatopancreas. This amino acid seemed to be used as a source of energy, mainly in *P. defossus.* Simultaneous fermentation generally provides more ATP per mole of substrate metabolized, compared with classical anaerobic glycolysis (Fields, 1983). Simultaneous fermentation seemed to occur in *P. defossus,* which used its glycogen and arginine reserves. The utilization of glycogen and amino acid was recorded by Hervant et al. (1995, 1996), who also suggested that simultaneous fermentation was occurring in the species that they studied.

Carbohydrates, lipids, and proteins can be used as oxidative fuels for aerobic pathways, whereas in anaerobic conditions the organisms are restricted to carbohydrates and a few amino acids as fermentable fuels (Storey and Storey, 2004). Fats are metabolized only by oxidative phosphorylation, necessitating the "presence of oxygen", differently from the other metabolites. Therefore, total lipids and cholesterol are not used for anaerobic metabolism. The only investigation of the use of lipids under hypoxic conditions was carried out with the prawn *Litopenaeus vannamei* by Pérez-Rostro et al. (2004). These authors analyzed, among other metabolites, the lipid levels in

the hepatopancreas, and observed that the lipid reserves did not differ significantly between groups in normoxia of 6 mg $O_2$/L and in hypoxia of 0.4 mg $O_2$/L of oxygen.

Both these *Parastacus* species showed reductions in the levels of lipids and cholesterol in the hepatopancreas and muscle, at least during some periods of hypoxia. These metabolites were reduced in the early hours of hypoxia, except in the muscle tissue of *P. defossus* where they decreased more in the last hours. However, the gills did not show significant variations. This demonstrated that the store of total lipids in this tissue does not contribute significantly to the metabolism in animals in hypoxia.

In the periods of hypoxia, *P. brasiliensis* showed a greater decrease in total lipids in muscle than did *P. defossus.* This reinforces the hypothesis of the less-efficient adaptation of *P. brasiliensis* to a hypoxic environment, as observed by Silva-Castiglioni et al. (unpublished observations). This species probably uses fats in addition to carbohydrates, and the fat reserves are metabolized only in oxidative pathways. The utilization of fats by these species may be a factor of the low concentration of oxygen used in the experiments (2 mg/L). Therefore, the animals subjected to hypoxia of 2 mg/L of oxygen seemed to be able to metabolize the total lipids by aerobic metabolism.

Few studies have examined the utilization of lipids in environments with low levels of oxygen. Lipids were also analyzed in the mysid *Neomys integer* studied by Verslycke and Janssen (2002), who related the changes to abiotic factors. Among other results, they found that high temperatures associated with high salinity and low oxygen levels resulted in increased use of lipids.

The allocation and utilization of total lipids and cholesterol in *P. brasiliensis* and *P. defossus* may also be related to the reproductive period. The experiments on hypoxia were carried out in winter. According to Silva-Castiglioni et al. (unpublished observations), *P. brasiliensis* has its reproductive peak in winter, and *P. defossus* begins reproductive activity in this same period. The relationship of cholesterol to the reproductive period seems to be more evident in *P. defossus.* This species, according to Silva-Castiglioni et al. (unpublished observations) shows greater dependence

on the energy reserves in the tissues; whereas *P. brasiliensis* seems to depend more on dietary nutrients for direct allocation of the energy for reproduction.

Animals in hypoxic and anoxic environments use anaerobic glycolysis, as suggested in this investigation, but this pathway produces a low yield of ATP compared to aerobic glycolysis. Organisms can adopt strategies to maintain an energy charge during hypoxia/anoxia due to the low glycolytic efficiency, such increasing the rate of glycolysis (glycolytic strategy) and reducing activities that consume energy (strategy of metabolic depression) (Lutz and Nilsson, 1997). These strategies should be studied in crustacean species, mainly fossorial ones such as *Parastacus defossus,* for better understanding of the intermediary metabolism and its regulation in evolutionary and adaptive processes of the organisms to their environment.

Although these species of *Parastacus* did not show significant differences in lactate levels, they did show differences in hemolymph glucose and free glucose, glycogen, and arginine phosphate in the hepatopancreatic and muscular tissue, except for free glucose in the hepatopancreas. These metabolites are also important reserves in the hypoxic periods, and their behavior revealed that although *P. brasiliensis* showed adaptations to hypoxia, *P. defossus* was better adapted to these conditions. These observations, associated with the high efficiency of the anaerobic metabolism, may partly explain how the fossorial *P. defossus* tolerates the low oxygen levels in its burrows.

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**Table I.** Levels of metabolites in the hepatopancreas of *Parastacus defossus* and *Parastacus brasiliensis* to different times of hypoxia. The different letters indicate significant differences (capital letters for *P. defossus* and small letters for *P. brasiliensis*) ($p<0.05$).

| | Species | Time (h) | | | | |
|---|---|---|---|---|---|---|
| | | **0** | **1** | **2** | **4** | **8** |
| **Total proteins** (mg ml$^{-1}$) | ***P. defossus*** | 10.85 ± 0.49A | 15.43 ± 0.84B | 13.71 ± 0.75B | 10.80 ± 0.67A | 9.97 ± 0.57A |
| | ***P. brasiliensis*** | 12.75 ± 1.91a | 23.50 ± 1.61b | 24.25 ± 1.67b | 14.84 ± 1.60a | 13.99 ± 2.16a |
| **Total lipids** (mg g$^{-1}$) | ***P. defossus*** | 33.33 ± 5.73A | 35.06 ± 7.14A | 21.13 ± 3.38A | 23.46 ± 4.89A | 30.06 ± 5.53A |
| | ***P. brasiliensis*** | 13.88 ± 3.66a | 13.16 ± 1.52a | 8.14 ± 1.47a | 12.55 ± 1.25a | 9.30 ± 4.77a |
| **Total cholesterol** (mg g$^{-1}$) | ***P. defossus*** | 26.53 ± 4.78A | 26.71 ± 6.19A | 17.91 ± 3.71A | 23.90 ± 8.49A | 23.91 ± 4.10A |
| | ***P. brasiliensis*** | 2.70 ± 0.10b | 2.09 ± 0.27ab | 1.42 ± 0.26a | 2.20 ± 0.18ab | 2.51 ± 0.28b |

**Table II.** Levels of metabolites in the muscle tissue of *Parastacus defossus* and *Parastacus brasiliensis* to different times of hypoxia. The different letters indicate significant differences (capital letters for *P. defossus* and small letters for *P. brasiliensis*) ($p<0.05$).

| | Species | Time (h) | | | | |
|---|---|---|---|---|---|---|
| | | **0** | **1** | **2** | **4** | **8** |
| **Total proteins** (mg ml$^{-1}$) | ***P. defossus*** | 15.21 ± 1.03A | 14.70 ± 0.78A | 14.07 ± 0.31A | 13.91 ± 0.44A | 18.97 ± 0.93B |
| | ***P. brasiliensis*** | 17.19 ± 0.36a | 19.62 ± 1.31a | 19.98 ± 0.73a | 17.70 ± 0.95a | 18.90 ± 1.03a |
| **Total lipids** (mg g$^{-1}$) | ***P. defossus*** | 22.12 ± 2.00A | 30.44 ± 3.33A | 29.75 ± 2.05A | 27.01 ± 1.97A | 23.70 ± 2.96A |
| | ***P. brasiliensis*** | 1.37 ± 0.16a | 0.64 ± 0.04b | 0.52 ± 0.03b | 0.99 ± 0.15a | 0.95 ± 0.16ab |
| **Total cholesterol** (mg g$^{-1}$) | ***P. defossus*** | 19.07 ± 3.12A | 16.20 ± 0.88AB | 13.48 ± 1.66AB | 11.31 ± 0.96AB | 7.82 ± 1.14B |
| | ***P. brasiliensis*** | 0.82 ± 0.14ab | 0.85 ± 0.12a | 0.46 ± 0.04ab | 0.77 ± 0.17ab | 0.26 ± 0.04b |

**Table III.** Levels of metabolites in the anterior gills of *Parastacus defossus* and *Parastacus brasiliensis* to different times of hypoxia. The different letters indicate significant differences (capital letters for *P. defossus* and small letters for *P. brasiliensis*) (p<0.05).

| | Species | Time (h) 0 | 1 | 2 | 4 | 8 |
|---|---|---|---|---|---|---|
| **Total proteins** | ***P. defossus*** | 4.82 ± 0.26A | 5.62 ± 0.18A | 5.31 ± 0.12A | 5.35 ± 0.12A | 5.00 ± 0.20A |
| (mg $ml^{-1}$) | ***P. brasiliensis*** | 6.66 ± 0.12b | 13.90 ± 0.89a | 15.65 ± 0.49a | 9.76 ± 0.77b | 14.42 ± 0.44a |
| **Total lipids** | ***P. defossus*** | 70.11 ± 3.59A | 95.25 ± 3.77A | 90.79 ± 5.61A | 65.90 ± 5.41A | 66.71 ± 7.12A |
| (mg $g^{-1}$) | ***P. brasiliensis*** | 24.90 ± 1.65a | 20.14 ± 2.39a | 15.75 ± 1.88a | 22.46 ± 6.13a | 19.47 ± 2.82a |
| **Total cholesterol** | ***P. defossus*** | 60.51 ± 7.74A | 60.29 ± 6.4A | 56.87 ± 5.42A | 51.65 ± 6.30A | 53.65 ± 4.54A |
| (mg $g^{-1}$) | ***P. brasiliensis*** | 22.13 ± 2.00a | 14.97 ± 3.99a | 11.49 ± 2.90a | 19.02 ± 3.85a | 22.45 ± 3.89a |

**Table IV.** Levels of metabolites in the posterior gills of *Parastacus defossus* and *Parastacus brasiliensis* to different times of hypoxia. The different letters indicate significant differences (capital letters for *P. defossus* and small letters for *P. brasiliensis*) (p<0.05).

| | Species | Time (h) 0 | 1 | 2 | 4 | 8 |
|---|---|---|---|---|---|---|
| **Total proteins** | ***P. defossus*** | 4.47 ± 0.25A | 6.29 ± 0.18B | 5.87 ± 0.16B | 4.56 ± 0.11A | 4.22 ± 0.11A |
| (mg $ml^{-1}$) | ***P. brasiliensis*** | 6.58 ± 0.08b | 13.63 ± 0.82a | 15.53 ± 059a | 9.28 ± 0.86bc | 12.87 ± 1.22ac |
| **Total lipids** | ***P. defossus*** | 57.41 ± 5.55A | 59.56 ± 2.90A | 54.37 ± 2.75A | 52.94 ± 4.24A | 48.02 ± 7.82A |
| (mg $g^{-1}$) | ***P. brasiliensis*** | 19.91 ± 1.27a | 16.53 ± 3.16a | 9.09 ± 1.50a | 18.11 ± 0.66a | 13.84 ± 3.07a |
| **Total cholesterol** | ***P. defossus*** | 43.48 ± 3.14A | 41.69 ± 3.94A | 36.07 ± 3.54A | 33.18 ± 3.64A | 31.11 ± 4.17A |
| (mg $g^{-1}$) | ***P. brasiliensis*** | 19.78 ± 6.64a | 8.89 ± 2.60a | 6.81 ± 1.37a | 15.36 ± 2.29a | 12.43 ± 5.18a |

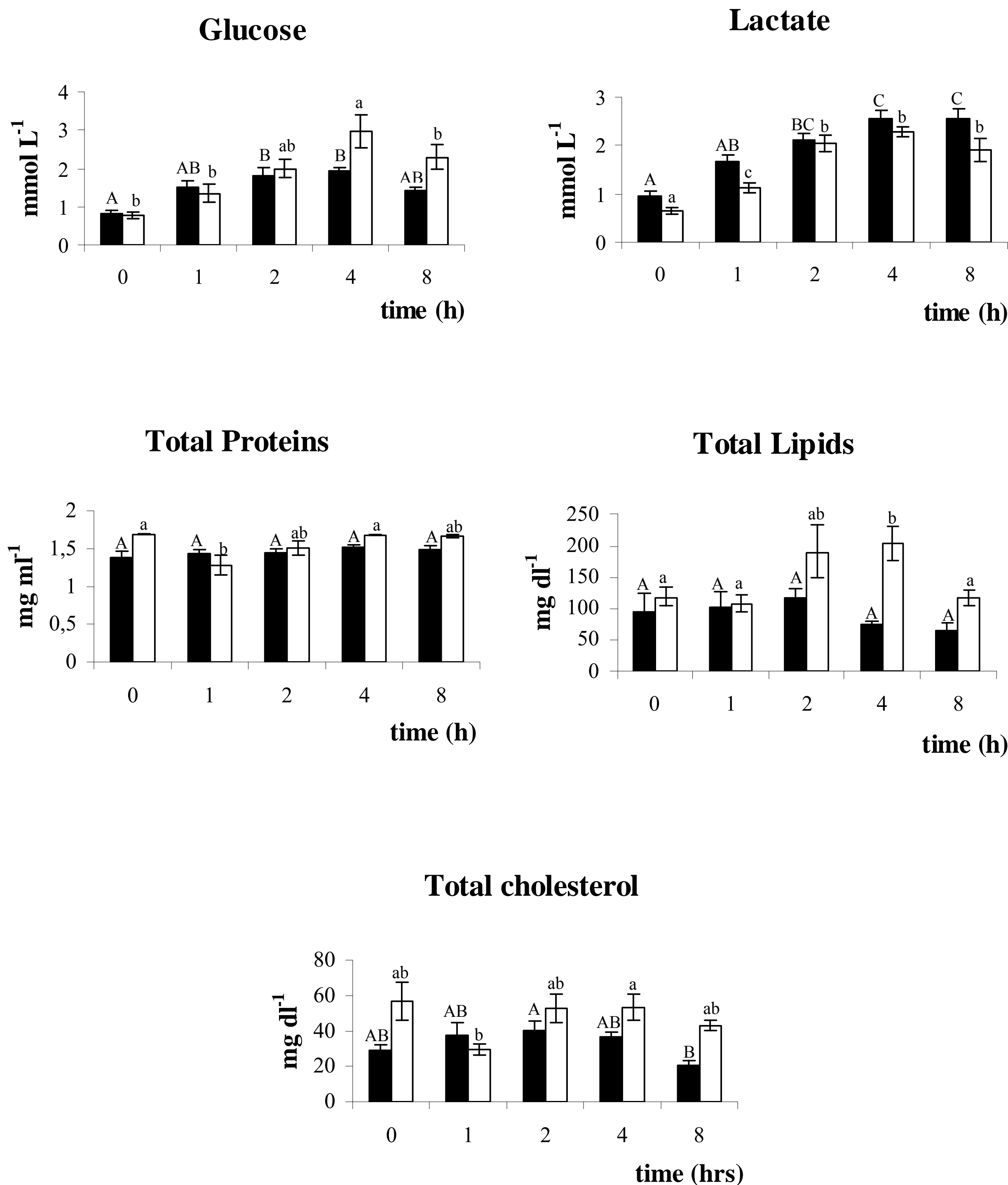


**Figure 1.** Levels of metabolites in the hemolymph of *Parastacus defossus* and *Parastacus brasiliensis* in hypoxia. *P. defossus*: black bar; *P. brasiliensis*: white bar. The columns show the mean; vertical bars show the standard error of the mean. The different letters indicate significant differences (capital letters for *P. defossus* and small letters for *P. brasiliensis*) ($p<0.05$).

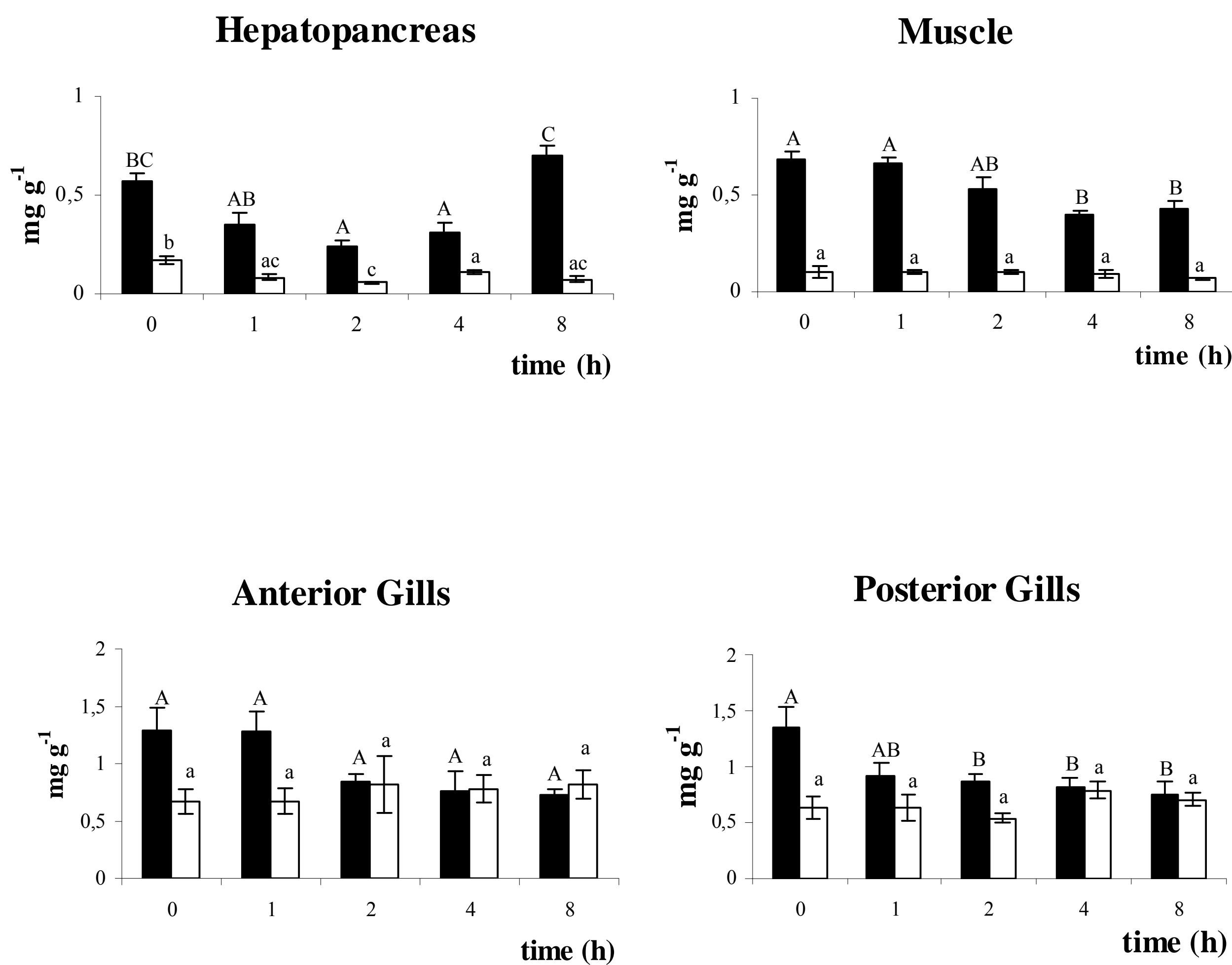


**Figure 2.** Glycogen levels of all tissues of *Parastacus defossus* and *Parastacus brasiliensis* in hypoxia. *P. defossus*: black bar; *P. brasiliensis*: white bar. The columns show the mean; vertical bars show the standard error of the mean. The different letters indicate significant differences (capital letters for *P. defossus* and small letters for *P. brasiliensis*) ($p<0.05$).

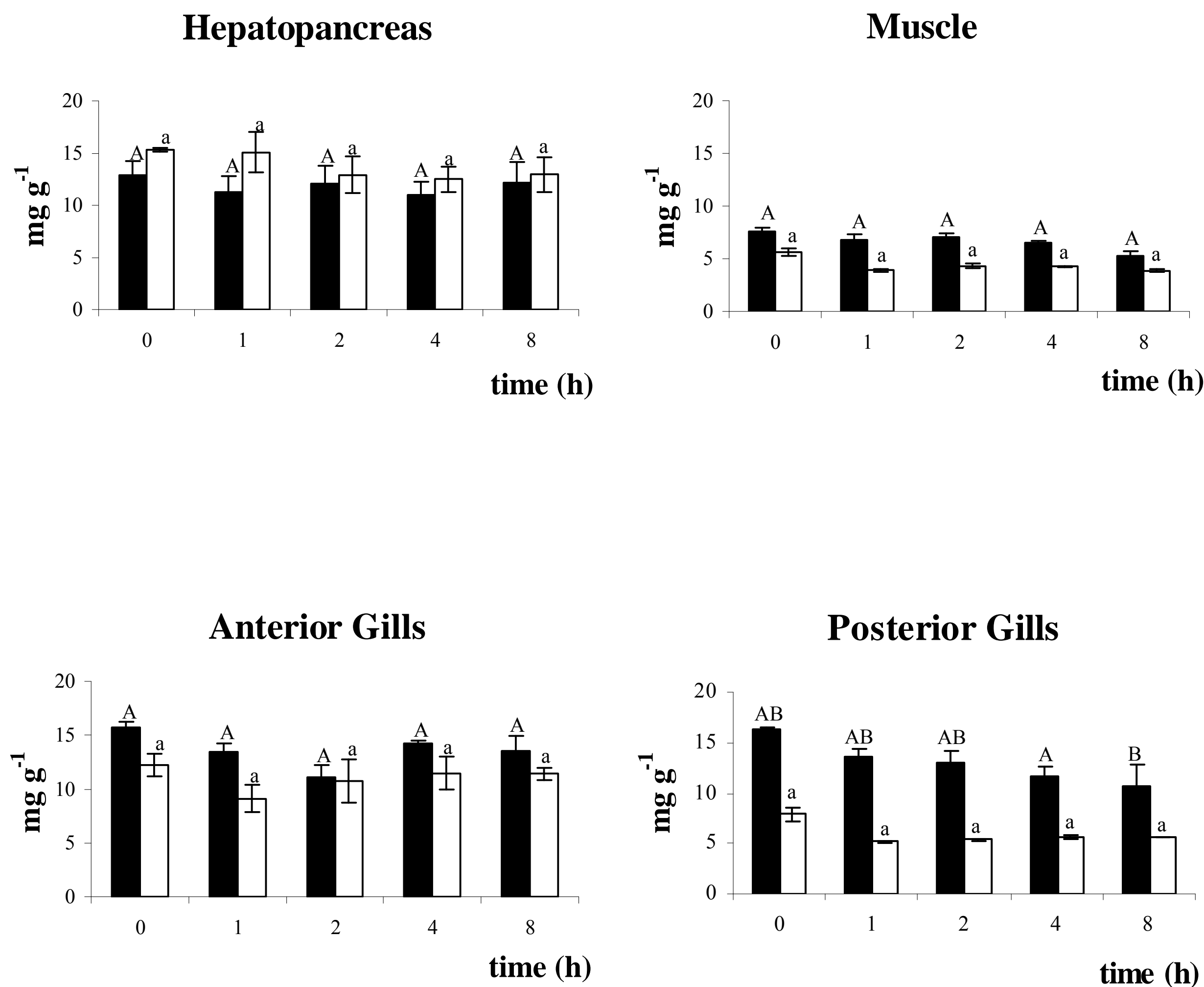


**Figure 3.** Free glucose levels of all tissues of *Parastacus defossus* and *Parastacus brasiliensis* in hypoxia. *P. defossus*: black bar; *P. brasiliensis*: white bar. The columns show the mean; vertical bars show the standard error of the mean. The different letters indicate significant differences (capital letters for *P. defossus* and small letters for *P. brasiliensis*) ($p<0.05$).

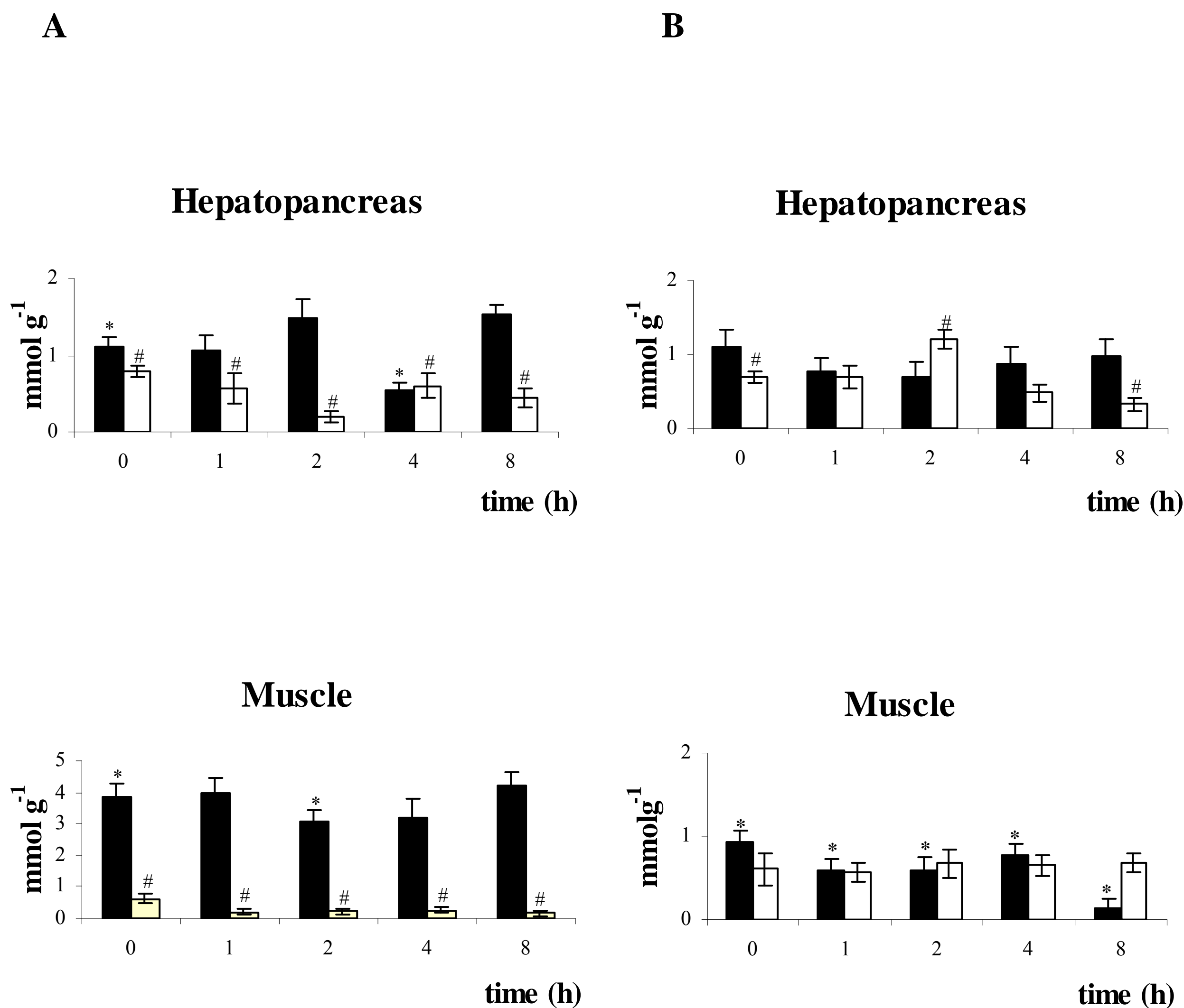


**Figure 4.** Levels of arginine phosphate and arginine during hypoxia, in *Parastacus defossus* (A) and *Parastacus brasiliensis* (B). Arginine phosphate: black bar; Arginine: white bar. The columns show the mean; vertical bars show the standard error of the mean. The symbols indicate significant differences with the control (* arginine phosphate; # arginine) ($p<0.05$).

# *Capítulo III*

**Metabolic responses of *Parastacus defossus* and *Parastacus brasiliensis* (Crustacea, Decapoda, Parastacidae) to post-hypoxia recovery**

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**Abstract.** The period of post-hypoxia recovery is essential for the rapid replenishment of energy reserves and for the removal of metabolic end products formed during hypoxia. Periods of post-hypoxia recovery were analyzed in two crayfish species of different habitats, *Parastacus defossus* and *Parastacus brasiliensis*. *P. defossus* is a fossorial species, and *P. brasiliensis* lives in lotic environments with higher oxygen levels. After 4 h of hypoxia (2 mg $O_2$/L), groups of animals were placed in tanks with oxygenated water and were then removed at intervals of 1, 3, 6, and 9 h. Hemolymph and tissues (hepatopancreas, muscle, and anterior and posterior gills) were extracted for the determination of glucose, lactate, free glucose, glycogen, total proteins, total lipids, total cholesterol, arginine phosphate, and arginine. As expected, lactate levels were restored more rapidly in *P. defossus* than in *P. brasiliensis*. *P. defossus* restored its glycogen reserves of the hepatopancreas and muscle tissue. Free glucose was quickly restored in all tissues of both species. In relation to arginine phosphate reserves, *P. defossus* showed a greater ability to restore this metabolite in the hepatopancreas. Both species recovered their arginine phosphate reserves, but they also used this metabolite in longer periods of recovery. In both species, the reserves of total lipids and cholesterol seem be an important source of energy during the recovery period, mainly in *P. brasiliensis*. The animals developed various metabolic adaptations to post-hypoxia recovery, mainly *P. defossus* which restored its reserves more completely and more rapidly than did *P. brasiliensis.*

**Keywords:** Crustacea, Hypoxia, Metabolism, Parastacidae, Recovery.

**Introduction**

Many species of freshwater and marine invertebrates encounter hypoxic or even anoxic conditions. These species are able to survive by developing adaptive mechanisms (Zebe, '82). In addition to changes to hypoxia, the animals must undergo periods of reoxygenation after hypoxic stress, known as the post-hypoxia recovery. This period occurs when oxygen is again available in the environment. Post-hypoxia recovery is functionally important for the organism because during this period, the reserves used during hypoxia are restored and the products of anaerobic metabolism are oxidized, excreted, or used for the gluconeogenic pathway (Ellington, '83; Hervant et al., '95; Oliveira et al., 2004).

Rapid recovery of the reserves can be an adaptive response of burrowing species for survival in hypoxic or anoxic conditions (Hervant and Renault, 2002). Several studies have evaluated the adaptations of animals during post-hypoxia, but the metabolism of species subjected to post-hypoxia recovery has been little investigated in Brazil. The only studies were with the estuarine crab *Neohelice granulata* Dana, 1851 by Oliveira et al. (2001, 2004a,b), Marqueze et al. (2006), and Maciel et al. (2008). In view of this scarcity of information, this study aimed to analyze and compare the metabolic reserves of two species of freshwater crayfishes, *Parastacus defossus* Faxon, 1898 and *Parastacus brasiliensis* (von Martens 1869), which are found in different habitats, subjected to different periods of post-hypoxia recovery.

*P. defossus* is a fossorial species that constructs burrows approximately 1.5 meters deep (Noro, Personal Communication). *P. brasiliensis* lives in lotic environments such as streams, rivers, and springs (Buckup, '99) with higher oxygen levels than in the habitat of *P. defossus* (Silva-Castiglioni, Personal Communication). Because of this difference in habitats, this study also examined the hypothesis that *P. defossus*, because it lives in poorly oxygenated subterranean burrows, would be able to recover its reserves more rapidly than would *P.*

*brasiliensis*.

**Materials and Methods**

Specimens of *Parastacus defossus* were collected with a partial-vacuum pump in the Lami region, Porto Alegre municipality, Rio Grande do Sul State, Brazil. Specimens of *Parastacus brasiliensis* were collected with traps in Mariana Pimentel municipality, Rio Grande do Sul. The animals were collected during the winter of 2008; approximately, 40 specimens of each species. The animals showed a variation of size of 18.8 - 30.3 mm in *P. defossus* and, 21.4 – 44.0 mm in *P. brasiliensis* (cephalothorax length).

In the laboratory, the crayfish were acclimated to a constant temperature of 19°C and fed with fish for 10 days. The animals were placed in individual flasks, and the air in each flask was displaced by pure nitrogen gas, to reduce the oxygen concentration to 2 mg/L. Oxygen levels were monitored with an oxymeter, and when the oxygen reached 2 mg/L the animals were maintained under hypoxia for 4 h. This length of time was chosen for the analysis of the lactate curve because we observed that during this period in hypoxia, the animals significantly increased their lactate reserves. These levels then decreased after 8 h of hypoxia, compared with the previous period (4 h).

After the period of hypoxia, groups of animals were placed in aquariums with oxygenated water (approximately 10 mg/L of oxygen), and were then removed at intervals of 1, 3, 6, and 9 h; all the groups were analised in same day. A control group was also monitored. Samples of hemolymph were collected with a syringe containing 10% potassium oxalate, an anti-clotting substance, and immediately frozen. The hepatopancreas, abdominal muscle, and anterior and posterior gills were removed and stored in a freezer at −80 °C until the determination of the metabolic parameters.

In the hemolymph we quantified the levels of glucose, lactate, total proteins, total

lipids, and total cholesterol. In the tissues, the glycogen, free glucose, total proteins, total lipids, total cholesterol, arginine and arginine phosphate levels were determined. All metabolites were quantified using spectrophotometric methods previously standardized for other species of crustaceans studied in the Laboratório de Fisiologia da Conservação da Pontifícia Universidade Católica do Rio Grande do Sul. For each metabolite, a specific spectrophotometric method was used:

**a) Glucose:** The levels were measured by the glucose-oxidase method, using a Bioclin Kit (Ref. 84) (glucose GOD-CLIN);

**b) Lactate:** Samples were deproteinized with perchloric acid, and the determination was carried out using the Boehringer-Mannheim kit (Ref. K084-2), for the formation of pyruvate (L-lactate + $NAD^+$ NADH + $H^+$);

**c) Free glucose:** The concentrations were determined according to Carr and Neff ('84). Tissues were weighed and homogenized with Ultra-Tura. The samples were mixed, to separate the lipid fraction, in a solution of chloroform-methanol and then centrifuged. Free glucose levels were determined by the colorimetric glucose-oxidase method (Kit Biodiagnóstico) in an intermediate fraction obtained after centrifugation;

**d) Glycogen:** The glycogen was extracted by the method described by Van Handel ('65). Glycogen reserves were determined as glucose-equivalent (glucose-oxidase method), after acidic hydrolysis (HCl) and neutralization ($Na_2CO_3$), following the method of Geary *et al.* ('81). Glucose levels were quantified using a Biodiagnostic kit (glucose-oxidase);

**e) Total proteins:** Total proteins were measured following the method described by Lowry *et al.* ('51), using bovine albumin (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) as the reference substance;

**f) Total lipids:** Total lipids were measured in the hemolymph by the sulfophosphovanillin method (Frings and Dunn, '70). In the tissues, the lipids were extracted from tissue

homogenized with an Omni Mixer Homogenizer in a 2:1 (v/v) chloroform-methanol solution, according to Folch *et al.* (1957). Total lipids in this homogenate were determined by the sulfophosphovanillin method (Frings and Dunn, '70);

**g) Total cholesterol**: Total cholesterol was measured using a Labtest kit (Total Cholesterol Liquiform);

**h) Arginine and arginine phosphate:** These metabolites were determined using the method of Bergmeyer ('85). Arginine was determined by the change in absorbance at 339 nm in the reaction catalyzed by octopine dehydrogenase: arginine + pyruvate + NADH + $H^+$ $\leftrightarrow$ octopine + $NAD^+$ + $H_2O$. To hydrolyze arginine and arginine phosphate to phosphate, 100 µl of 1 mol $l^{-1}$ HCl was added to 100 µl of tissue (homogenate) and incubated in tightly capped tubes for 90 s in boiling water. The hydrolysates were then cooled and neutralized with 100 µl of 1 mol l -1 NaOH. The arginine (assay) was repeated and the previous concentration of arginine subtracted to obtain the quantity of arginine phosphate.

**Statistical Analyses**

The metabolic parameters were homogeneous (Levene test), and were normally distributed (Kolmogorov-Smirnov test). For statistical analysis of the different periods of post-hypoxia recovery, a one-way ANOVA test was used, followed by a Bonferroni test. For comparison between species, a two-way ANOVA was used. The significance level adopted was 5%. All the tests were done with the program Statistical Package for the Social Sciences (SPSS- 11.5) for Windows.

**Results**

The hemolymph and tissues were removed from the animals subjected to 4 h of hypoxia and during the subsequent periods (1, 3, 6, and 9 h) of post-hypoxia recovery for the metabolic determinations. The control group was also examined at these same intervals.

Males and females were pooled for metabolic dosages because they showed the same behavior, thus increasing the number of animals in each group.

***Hemolymph measurements***

**Glucose:** Different responses were observed between the species ($p<0.05$). After 4 h of hypoxia, the levels in *P. defossus* increased 37% compared to the control group ($p>0.05$). In the recovery periods the concentrations decreased, but were not significantly different. Glucose levels of *P. brasiliensis* increased 60% in hypoxia compared to the control group ($p<0.05$). The concentrations decreased in the recovery periods, but the highest reductions, approximately 58%, were recorded after 6 and 9 h ($p<0.05$).

**Lactate:** The species showed different behavior ($p<0.05$). When subjected to hypoxia, *P. defossus* increased lactate levels 67% compared with normoxia ($p<0.05$). The reserves decreased in the recovery periods, but to 6 h we observed greater restoration of these reserves, approximately 52%, compared with hypoxia ($p<0.05$); however, the reserves did not return to the levels observed in normoxia. After 4 h of hypoxia, *P. brasiliensis* significantly increased its lactate reserves by 65% compared with the control group. The levels decreased during the recovery periods with the lowest concentration observed after 6 h, approximately 29% lower than the level recorded in hypoxia ($p<0.05$).

**Total proteins:** The species showed different responses in protein levels ($p<0.05$). When subjected to hypoxia and different periods of post-hypoxia, *P. defossus* did not show a significant difference. In *P. brasiliensis* also no significant differences were recorded, except after 9 h which showed an increase compared to the control ($p<0.05$).

**Total lipids:** Different behavior of the lipid reserves was recorded between the species ($p<0.05$). In *P. defossus* we did not observe significant variations when subjected to hypoxia and different periods of recovery. *P. brasiliensis* also did not show significant variations.

However, we observed an increase of 32% in lipid concentrations in hypoxia, compared to the control ($p>0.05$).

**Total cholesterol:** The species responded differently ($p<0.05$). The cholesterol reserves of *P. defossus* increased 10% during hypoxia ($p>0.05$). In the different periods of recovery we observed reductions of approximately 27% after 1 and 3 h ($p>0.05$) and 60% after 9 h ($p<0.05$), compared with hypoxia. *P. brasiliensis* in hypoxia showed similar levels to control group; the reserves increased 27 and 30% after 6, and 9 h of recovery, respectively, compared with hypoxia and normoxia ($p<0.05$).

***Hepatopancreas measurements***

**Glycogen:** The glycogen levels showed different responses between the species ($p<0.05$). In hypoxia, the glycogen levels of the hepatopancreas of *P. defossus* decreased 30% compared with the control, but the difference was not significant. After 1 and 3 h of recovery, the reserves also decreased, approximately 50% compared with the control group ($p<0.05$). However, after 6 and 9 h of post-hypoxia recovery the levels increased ($p>0.05$). In the hepatopancreas of *P. brasiliensis,* significant variations were not recorded.

**Free glucose:** The free glucose levels in the hepatopancreas of the species behaved similarly ($p>0.05$). *P. defossus* did not show significant variations in the free glucose reserves under hypoxia and during the different periods of post-hypoxia, but the concentrations decreased approximately 15% in hypoxia ($p>0.05$). In the hepatopancreas of *P. brasiliensis* also, significant variations were not recorded, but a 26% reduction occurred under hypoxia ($p>0.05$). During the recovery period, free glucose reserves increased, to levels higher than those observed in normoxia ($p>0.05$).

**Total proteins:** The species showed different behavior in the protein reserves ($p<0.05$). In *P. defossus* the reserves decreased 26% during hypoxia ($p<0.05$). The reserves increased during

the first hours of recovery, and during all periods of recovery these reserves were higher than in hypoxia. *P. brasiliensis* showed higher concentrations of total proteins in hypoxia compared with normoxia ($p>0.05$), and no significant variations occurred in the different recovery periods.

**Total lipids:** Different responses between the species were recorded in the levels of lipids ($p<0.05$). No significant differences of lipids were recorded in the hepatopancreas of *P. defossus* when subjected to hypoxia and after the different recovery periods. However, a 20% reduction was recorded in hypoxia and the reserves reaching higher levels when subjected to 6 and 9 h, compared with the control ($p>0.05$). In the hepatopancreas of *P. brasiliensis,* also no significant variations of lipids occurred during hypoxia, but a reduction was observed, approximately 16%, compared with normoxia. During post-hypoxia recovery, significant decreases occurred after 6 and 9 h, compared with all periods.

**Total cholesterol:** The species showed different behavior in relation to the concentrations of cholesterol ($p<0.05$). In the hepatopancreas of *P. defossus,* no significant differences were observed during hypoxia and recovery, although reductions were recorded in all periods ($p>0.05$). *P. brasiliensis* also showed no significant differences during hypoxia and recovery, but reductions were observed during recovery ($p>0.05$).

**Arginine:** No differential responses between the species were observed ($p>0.05$). Significant differences in arginine reserves were not recorded in *P. defossus*, but in hypoxia the reserves decreased 13%, and after 3 h of recovery the reserves decreased by approximately 25%. However, the reserves increased 12% after 6 h of recovery compared with the control ($p>0.05$). After 9 h, the concentrations were similar to the levels recorded in hypoxia. In *P. brasiliensis*, the levels of arginine decreased 37% after 4 h of hypoxia ($p<0.05$). The reserves were restored after 1 h, but after 3 h decreased 32% compared with the control ($p<0.05$). The levels of arginine were restored after 6 and 9 h, mainly after 6 h ($p<0.05$).

**Arginine phosphate:** The species showed different behavior ($p<0.05$). Arginine phosphate levels in *P. defossus* decreased 48% during hypoxia ($p<0.05$), and were restored after 1 and 3 h of recovery. However, they decreased significantly after 6 h, to levels lower than observed in hypoxia; and after 9 h the concentrations were higher than after 6 h ($p<0.05$). In *P. brasiliensis* no significant reduction occurred during hypoxia. In all periods of post-hypoxia recovery, the concentrations increased, but these reserves were restored after 3 and 6 h, to higher levels than observed in hypoxia ($p<0.05$). The largest increase occurred after 9 h, 48 and 62% higher than those observed in normoxia and hypoxia, respectively ($p<0.05$).

***Muscle measurements***

**Glycogen:** Glycogen levels showed different behavior between the species ($p<0.05$). In the muscle tissue of *P. defossus* we observed a reduction of 32% in hypoxia, compared with normoxia, but this reduction was not significant. After 1 and 3 h of post-hypoxia, the concentrations also decreased, with a significant difference from the control group. After 6 and 9 h the levels increased 35% and 44%, respectively, compared with hypoxia ($p>0.05$). In hypoxia, *P. brasiliensis* showed significant variation only after 9 h of recovery, with a decrease of the reserves compared with the control. In hypoxia, glycogen was reduced approximately 21% ($p>0.05$).

**Free glucose:** Different responses of free glucose were recorded between the species ($p<0.05$). Compared with control group, the reserves of *P. defossus* showed no significant variation when subjected to hypoxia and recovery periods. The levels increased in all recovery periods, compared with to hypoxia ($p<0.05$). In muscle tissue of *P. brasiliensis,* significant variations were not observed during hypoxia and post-hypoxia recovery.

**Total proteins:** Different behavior was recorded between the species ($p<0.05$). The muscle proteins of *P. defossus* in hypoxia showed levels similar to normoxia. During recovery, the

reserves remained constant. In hypoxia, *P. brasiliensis* showed similar levels to normoxia. In the different recovery periods, we recorded higher levels than in hypoxia, except after 6 h, but significant variations were not recorded in these periods.

**Total lipids:** The species showed different responses in the levels of lipids ($p<0.05$). After 4 h of hypoxia, the reserves increased approximately 24% in *P. defossus,* compared with normoxia ($p>0.05$). During the recovery periods, we recorded decreases compared with hypoxia and normoxia, except to 6 h when they were increased compared with normoxia ($>0.05$). The lipids of muscle tissue of *P. brasiliensis* decreased approximately 29% in hypoxia although this difference was not statistically significant. During the recovery periods, we observed no difference significant.

**Total cholesterol:** The species showed different responses in the reserves of cholesterol ($p<0.05$). Cholesterol in muscle tissue of *P. defossus* decreased 36% during hypoxia, but this reduction was not significant. During the different periods of recovery, *P. defossus* showed decreases in cholesterol levels after 1 and 3 h ($p>0.05$). However, after 6 and 9 h of recovery, the levels were higher ($p>0.05$). In *P. brasiliensis* the cholesterol levels decreased 37% in hypoxia ($p>0.05$). In the early hours the differences were not statistically significant, but after 9 h a significant reduction of 46% of the reserves occurred, compared with the control.

**Arginine**: Different behavior was recorded between the species ($p<0.05$). In *P. defossus*, arginine decreased 33% during hypoxia ($p<0.05$). Restoration of the reserves was not observed, although a significant increase of 27% occurred after 1 h compared with hypoxia ($p<0.05$). Arginine levels in the muscle of *P. brasiliensis* during hypoxia were similar to the control. During the periods of recovery, the concentrations decreased approximately 39% after 1 h compared with hypoxia ($p<0.05$), and increased in other periods, mainly after 3 h of recovery when arginine increased 45% compared to the reserves of the control and levels during hypoxia ($p<0.05$).

**Arginine phosphate:** The species showed different responses in arginine phosphate ($p<0.05$). The levels in the muscle tissue of *P. defossus* decreased 19% during hypoxia ($p>0.05$). The reserves were partly restored after 1 h, but after 6 and 9 h the concentrations decreased 10% compared with hypoxia ($p>0.05$). In hypoxia, in *P. brasiliensis*, arginine phosphate reserves decreased 22% ($p<0.05$). The reserves were restored after 6 h with values higher than those observed in the control ($p<0.05$). After 9 h of recovery, the levels were lower than those observed after 6 h, but were similar to those recorded after 3 h.

***Anterior gills measurements***

**Glycogen:** Different behavior of the glycogen in the anterior gills was observed between the species ($p<0.05$). After 4 h of hypoxia, the glycogen reserves of *P. defossus* decreased 33% ($p>0.05$). During post-hypoxia recovery, the levels remained constant. In *P. brasiliensis,* the levels did not vary significantly during hypoxia. In hypoxia the levels increased 25 %, but reductions were recorded in the recovery periods compared with the control group ($p>0.05$).

**Free glucose:** Different responses were observed between the species ($p<0.05$). The levels decreased 15% after 4 h of hypoxia in *P. defossus* ($p>0.05$). During recovery, the reserves were restored, after 6 h reaching similar values to those recorded in normoxia. During hypoxia and in the different periods of post-hypoxia recovery, *P. brasiliensis* showed similar levels of free glucose.

**Total proteins:** The species showed different responses ($p<0.05$). In *P. defossus* we observed an increase when submitted to hypoxia ($p>0.05$). In the early hours of recovery, the reserves increased, but after 6 and 9 h the levels decreased to similar values as in normoxia. The proteins of anterior gills of *P. brasiliensis* did not show significant variation.

**Total lipids:** Different responses were recorded for lipids ($p<0.05$). In hypoxia, the concentrations in *P. defossus* decreased approximately 14 % compared with normoxia

($p>0.05$). During the different periods of post-hypoxia recovery, the reserves decreased compared with hypoxia and normoxia ($p<0.05$). *P. brasiliensis* decreased only 7% of their reserves in hypoxia ($p>0.05$). During recovery, we observed reductions of lipids in all periods, compared with hypoxia, but recorded a significant reduction only after 9 h, compared with control group and hypoxia.

**Total cholesterol**: Cholesterol reserves showed different responses between the species ($p<0.05$). The cholesterol of *P. defossus* decreased 15% in hypoxia ($p>0.05$). In the recovery periods were observed similar levels compared with hypoxia. In hypoxia, significant variations were not recorded in *P. brasiliensis*, compared with the control, although a reduction of 10% was recorded ($p>0.05$). In the periods of recovery, except after 1 h, reductions were observed, with a significant difference only after 9 h, compared with the control.

***Posterior gills measurements***

**Glycogen:** Different responses between the species were not recorded ($p>0.05$). In the posterior gills of *P. defossus* we observed a reduction of 45% of the glycogen reserves after 4 h of hypoxia ($p<0.05$). In the early hours of recovery, decreases also were observed ($p<0.05$). However, the levels increased after 6 and 9 h, compared with hypoxia, but were not restored completely. The posterior gills of *P. brasiliensis* showed higher concentrations in hypoxia, but the levels did not differ significantly. During the recovery periods, also were not recorded significant variations.

**Free glucose:** The reserves of free glucose differed between the species ($p<0.05$). In hypoxia, *P. defossus* did not show significant variation, although free glucose decreased 29%. The reserves increased during recovery ($p>0.05$), after 6 h reaching similar values to normoxia. In hypoxia, *P. brasiliensis* lost approximately 23% of their reserves, although this

difference was not statistically significant. During the periods of recovery also, no significant variation was recorded.

**Total proteins:** Different behavior of proteins was observed between the species ($p<0.05$). *P. defossus* did not show significant variations during 4 h of hypoxia and also in all periods of post-recovery, compared with the control group. In all periods of recovery, we observed higher reserves than the control and hypoxia, except after 6 h ($p>0.05$). In hypoxia, the posterior gills of *P. brasiliensis* showed similar levels to normoxia. During recovery, no significant variations were recorded in the different periods.

**Total lipids:** Lipids showed different responses between the species ($p<0.05$). In hypoxia, *P. defossus* showed similar levels when subjected to hypoxia. Reductions were recorded in all the periods of recovery ($p<0.05$). The reserves of *P. brasiliensis* also did not show significant variation when subjected to hypoxia. In the early hours, the differences were not statistically significant; however, after 6 and 9 h significant reductions of 50 and 45% occurred, compared with the control and hypoxia, respectively.

**Total cholesterol:** Different responses were recorded between the species ($p<0.05$). The reserves of *P. defossus* decreased approximately 21% compared with normoxia ($p>0.05$). In the periods of recovery, except after 1 h, the concentrations decreased compared with those recorded in hypoxia, although difference significant was observed only to 3 h ($p<0.05$). The posterior gills of *P. brasiliensis* showed reductions of 19% in hypoxia, although this decrease was not significant. In the early hours, the changes were not significantly different compared with the control and hypoxia. However, after 6 and 9 h the reserves decreased compared with others periods ($p<0.05$).

**Discussion**

Variations of the environmental oxygen concentrations can affect an organism directly or indirectly, but according to Lutz and Storey ('97), organisms have evolved to use oxygen for the production of energy, developing efficient respiratory and circulatory systems. When the environmental oxygen levels are reduced or absent, or when these systems cannot deliver oxygen at an adequate rate to satisfy metabolic demand, adaptive strategies can be used. These strategies include the metabolic adaptations observed in *Parastacus defossus* and *Parastacus brasiliensis* by Silva-Castiglioni et al. (*in press*). Adaptations to post-hypoxia recovery are also used, as recorded in the present study, mainly by *P. defossus.*

Glycogen is the most important substrate of anaerobic metabolism (Zebe, '82). This metabolite is widely used in periods of hypoxia or anoxia, as recorded in *P. defossus* and *P. brasiliensis* by Silva-Castiglioni et al. (*in press*). Therefore, in periods of post-hypoxia recovery, glycogen levels must these concentrations need to be restored. During the recovery, *P. defossus* replenished its glycogen reserves in the hepatopancreas and muscle after 6 and 9 h, respectively. However, this restoration was only partial, as previously observed in the amphipods *Niphargus rhenorhodanensis* (Schellenberg, 1937) and *Gammarus fossarum* (Koch 1835) by Hervant et al. ('95). The same behavior was observed in the amphipod *Niphargus virei* Chevreux, 1896 and the isopod *Asellus aquaticus* (Linnaeus, 1758) by Hervant et al. ('96), and the subterranean isopod *Stenasellus virei* Dollfus, 1897 by Hervant et al. ('97).

The glycogen concentrations of *P. defossus* were restored in the hepatopancreas and muscle after 6 and 9 h, respectively. In *P. brasiliensis,* glycogen levels were restored only in the hepatopancreatic tissue, and this restoration was slower than in *P. defossus*. Hypogean species restore glycogen and arginine phosphate, as they do other metabolites, faster than epigean species (Hervant et al., '95, '96, '97). *P. defossus* lives in a subterranean environment;

however, its more rapid recovery of reserves may be related to its habitat. The high capacity of glycogen resynthesis is ecologically very advantageous for subterranean organisms, especially to resist a new hypoxic stress related to recurring low-water periods of "low-water". Restoration of glycogen also was faster in the subterranean isopods *Niphargus virei* and *Niphargus rhenorhodanensis* than in the epigean species studied by Hervant et al. ('95, '96, '97).

In the gill tissue of *P. defossus,* we observed no restoration of the glycogen reserves. This may be related to the high consumption of these reserves during hypoxia, as recorded by Silva-Castiglioni et al. (*in press*) and also observed in this study. High consumption of glycogen in the gills may be due to ion transport, because this process consumes more energy in the gills (Lyndon and Houlihan, '98), and probably this tissue is a main energy consumer in *P. defossus*. In the gills of *P. brasiliensis,* glycogen was not used in hypoxia, perhaps because of the lower energy requirement, since this species lives in oxygenated environments and its glycogen reserves are more accessible. However, *P. brasiliensis* used glycogen during post-hypoxia recovery, and may depend mainly on glycogenolysis in other tissues.

In both *Parastacus* species, we observed increased glucose levels in the hemolymph and subsequent reductions until they reached similar values to normoxia after 6 h, suggesting an increase in glucose uptake and subsequent glycogenesis, principally in the hepatopancreas and muscle. Restoration of glucose reserves to their initial levels was also observed in the first hours of recovery of the thalassinidean *Calocaris macandreae* Bell, 1853 and the crab *Eriocheir sinensis* H. Milne Edwards, 1853 studied by Anderson et al. ('94) and Zou et al. ('96), respectively. However, glucose concentrations did not differ significantly during post-hypoxia recovery in the amphipods and isopod studied by Hervant et al. ('96, '97), which suggests a normal glycolytic recovery rate.

Free glucose is the storage of glucose in the cells in different tissues of crustaceans,

and it serves as a buffer to allow the animals to respond more rapidly to environmental variations (Oliveira et al. 2004a). This response seems to occur in the crayfish species, because the free glucose reserves decreased after hypoxia and subsequently this metabolite were rapidly replenished in all the tissues of both *Parastacus* species during post-hypoxia recovery.

The species showed no significant differences in protein levels during post-hypoxia recovery, except in the hemolymph and gills of *P. defossus.* Hypoxia is one of the major factors that affect the relative proportions and total quantities of the hemolymph proteins of crustaceans (Depledge and Bjerregaard, '89). However, in other tissues, we found no significant variation in protein levels, as also reported by Hervant et al. ('99), who found no evidence of large-scale protein utilization for energy metabolism of the subterranean crustacean *Niphargus virei* during recovery.

Arginine phosphate is used by invertebrates, principally Mollusca and Crustacea, as a store of energy for ATP synthesis and inorganic phosphate during periods of hypoxia (Hill et al., '91; Speed et al., 2001). In periods of post-hypoxia recovery, it is expected that these reserves will be restored, as observed in this study. *P. defossus* showed a greater ability to restore arginine phosphate in the hepatopancreas, but they used these reserves by 6 h of recovery. However, the concentrations increased after 9 h, probably to maintain the energy source under these conditions. The muscle tissue also replenished the arginine phosphate reserves after 1 h of recovery, but in the other periods the concentrations decreased. Therefore, *P. defossus* restored its arginine phosphate reserves, but also used these concentrations in longer periods of post-hypoxia recovery. This utilization may be related to the stress that they undergo during this period. According to Beis and Newsholme ('75), arginine phosphate was quickly depleted in conditions of stress; and Speed et al. (2001) observed a reduction of these reserves in captive *Jasus edwardsii* (Hutton, 1875).

In relation to the arginine phosphate reserves of *P. brasiliensis*, we recorded a better ability to restore this metabolite than in *P. defossus*. *P. brasiliensis* restored its reserves in the hepatopancreas and muscle after 3 and 6 h, respectively with values higher than those recorded in normoxia. The use of this metabolite as recorded in *P. defossus* was not observed in the tissue of *P. brasiliensis*. Restoration of arginine phosphate was also reported in the crayfish *Orconectes limosus* (Rafinesque 1817*),* the shrimp *Macrobrachium japonicus* (De Haan), the isopod *Stenasellus virei,* and the thalassinid *Lepidophthalmus louisianensis* (Schimtt, 1935) (Gäde, '84; Abe et al., 2007; Hervant et al., '97; Holman and Hand, 2009, respectively).

Lactate is the main end product of anaerobic metabolism in decapod crustaceans. Therefore, many species have high lactate concentrations in hypoxic conditions, as observed in *P. defossus* and *P. brasiliensis* by Silva-Castiglioni et al. (*in press*) and also in this study. When the oxygen supply is restored, lactate can be oxidized to $CO_2$ and $H_2O$, excreted, or converted to glycogen (Ellington, '83), leading to reduction of the lactate levels. Here, we recorded a reduction in both species of crayfish during post-hypoxia recovery, with lower concentrations after 6 h, but these reserves were higher than recorded in normoxia, especially in *P. brasiliensis*, suggesting partial restoration of this metabolite.

During the periods of post-hypoxia recovery, similar levels of lactate to normoxia were reaching more rapidly in *P. defossus* than in *P. brasiliensis*. This may be related to the subterranean habitat of *P. defossus*, which showed a similar response as other hypogean crustaceans. According to Hervant et al. ('95), hypogean species recover their reserves more rapidly than epigean species: they observed faster recovery of lactate levels in the hypogean amphipod *Gammarus fossarum* compared with the epigean *Niphargus rhenorhodanensis*. The slower recovery of the lactate levels in *P. brasiliensis* suggests that this species has a slower system for metabolizing lactate than does *P. defossus*.

In addition to the restoration of metabolic reserves during post-hypoxia recovery, it is also important to analyze the removal of anaerobic end products. These products can be oxidized (Marquez et al., 2006), excreted (Ellington, '83), or used in the gluconeogenic pathway (Oliveira et al., '97; 2004a,b; Hill et al., 2001; Maciel et al., 2008). Gluconeogenesis is a pathway responsible for the synthesis of glucose from precursors such as lactate, glycerol, amino acids, pyruvate, and propionate (Moon, '88; Marks et al.,'96; Corssmit et al., 2001). In this study, the removal of the end products of metabolism of the *Parastacus* species was not directly investigated, but the results, increase in glycogen with depletion of lactate, suggest a gluconeogenic capacity of the muscle and hepatopancreas in *P. defossus.* Hypogean species preferentially use gluconeogenesis as the major mechanism for the removal of lactate, whereas epigean species use oxidation and excretion (Hervant et al., '95, '96, '97). However, the destination of the anaerobic end products should be investigated in *Parastacus* species to confirm the occurrence of gluconeogenesis, as well as studies of oxidation and excretion of lactate and also of other products that can be produced anaerobically by these organisms.

In relation to lipids, during recovery *P. brasiliensis* used total lipids and cholesterol from the hepatopancreas and muscle, respectively ($p<0.05$). *P. defossus* used cholesterol from 1 to 9 h in the hepatopancreas and total lipids and cholesterol in the muscle, although these differences were not significant. In the gills, significant reductions were recorded during the recovery period in the total lipids of *P. defossus* and the total lipids and cholesterol of *P. brasiliensis.* The difference recorded between these species, in all tissues, is very important because the total lipids and cholesterol seem to be an important source of energy during post-hypoxia recovery, mainly in *P. brasiliensis*.

Environmental oxygen levels play a significant role in the evolution of aquatic animals. These animals have developed various metabolic adaptations for survival in hypoxic environments, and they have also developed adaptations to post-hypoxia recovery, as

observed in the present study. During the recovery period, two basic processes allow the return of the organism or tissue to the previous metabolic condition (in normoxia): restoration of phosphagen and glycogen, and the use of products of anaerobic metabolism, probably by the gluconeogenic pathway. As was expected, *P. defossus* restored these reserves more rapidly and efficiently than did *P. brasiliensis*.

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**Table I.** Levels of metabolites in the hepatopancreas of *Parastacus defossus* and *Parastacus brasiliensis* during post-hypoxia recovery. The different letters indicate significant differences (capital letters for *P. defossus* and small letters for *P. brasiliensis*) ($p<0.05$). (0: Control group; H: Hypoxia; 1, 3, 6, and 9: periods (h) of recovery).

| | Species | 0 | H | 1 | 3 | 6 | 9 |
|---|---|---|---|---|---|---|---|
| **Total proteins** | ***P. defossus*** | 12.58 ± 0.6A | 9.24 ± 0.7A | 11.40 ± 0.7A | 10.27 ± 0.6A | 10.00 ± 0.4A | 10.07± 0.5A |
| (mg $ml^{-1}$) | ***P. brasiliensis*** | 10.34 ± 0.6a | 2.97 ± 0.4a | 12.17 ± 0.7a | 11.39 ± 0.6a | 11.23 ± 1.2a | 12.89 ± 1.1a |
| **Total lipids** | ***P. defossus*** | 35.44 ± 4.2A | 28.28 ± 4.3A | 34.02 ± 6.8A | 35.03 ± 6.3A | 39.38 ± 5.13A | 36.49 ±8.2A |
| (mg $g^{-1}$) | ***P. brasiliensis*** | 13.12 ±1.9a | 11.03 ± 1.5a | 12.50 ± 1.26a | 11.81 ± 1.5a | 5.50 ± 1.46b | 5.47 ± 0.6b |
| **Total cholesterol** | ***P. defossus*** | 22.4 ± 6.5A | 19.27 ± 3.5A | 12.55 ± 3.0A | 11.46 ± 2.8A | 13.78 ± 4.0A | 11.26± 0.8A |
| (mg $g^{-1}$) | ***P. brasiliensis*** | 3.83 ± 0.3a | 3.61 ± 0.5a | 3.07 ± 0.3a | 3.50 ± 0.3a | 2.46 ± 0.2a | 2.24 ± 2.4a |

**Table II.** Levels of metabolites in the muscle tissue of *Parastacus defossus* and *Parastacus brasiliensis* during post-hypoxia recovery. The different letters indicate significant differences (capital letters for *P. defossus* and small letters for *P. brasiliensis*) ($p<0.05$). (0: Control group; H: Hypoxia; 1, 3, 6, and 9: periods (h) of recovery).

| | Species | 0 | H | 1 | 3 | 6 | 9 |
|---|---|---|---|---|---|---|---|
| **Total proteins** | ***P. defossus*** | 13.92 ± 0.5A | 13.55 ± 0.6A | 13.37 ± 0.8A | 13.46 ± 0.7A | 13.82 ± 0.6A | 13.73 ±0.4A |
| (mg $ml^{-1}$) | ***P. brasiliensis*** | 15.08 ± 0.3a | 15.55 ± 0.3a | 18.91 ± 0.9a | 18.14 ± 1.7a | 15.36 ± 1.8a | 16.48 ± 0.8a |
| **Total lipids** | ***P. defossus*** | 21.87 ± 0.9A | 28.77 ± 4.7A | 18.28 ± 5.9A | 15.9 ± 3.5A | 22.50 ± 5.8A | 20.64± 4.9A |
| (mg $g^{-1}$) | ***P. brasiliensis*** | 1.49 ± 0.1a | 1.06 ± 0.03a | 1.30 ± 0.25a | 1.21 ± 0.3a | 1.33 ± 0.2a | 0.82 ± 0.09a |
| **Total cholesterol** | ***P. defossus*** | 21.15 ± 6.4A | 13.5 ± 1.3A | 11.66 ± 2.3A | 11.56 ± 2.3A | 17.73 ± 2.7A | 20.55± 8.0A |
| (mg $g^{-1}$) | ***P. brasiliensis*** | 0.65 ± 0.1a | 0.41 ± 0.02ab | 0.49 ± 0.06ab | 0.39 ± 0.12ab | 0.49 ± 0.05ab | 0.35 ± 0.01b |

**Table III.** Levels of metabolites in the anterior gills of *Parastacus defossus* and *Parastacus brasiliensis* during post-hypoxia recovery. The different letters indicate significant differences (capital letters for *P. defossus* and small letters for *P. brasiliensis*) (p<0.05). (0: Control group; H: Hypoxia; 1, 3, 6, and 9: periods (h) of recovery).

| | Species | 0 | H | 1 | 3 | 6 | 9 |
|---|---|---|---|---|---|---|---|
| **Total proteins** | ***P. defossus*** | 3.56 ± 0.1B | 4.01 ± 0.2AB | 4.63 ± 0.4A | 4.29 ± 0.2AB | 3.56 ± 0.15B | 3.47 ± 0.1B |
| (mg $ml^{-1}$) | ***P. brasiliensis*** | 6.91 ± 0.4a | 7.12 ± 0.6a | 7.70 ± 0.4a | 7.39 ± 0.6a | 6.98 ± 0.47a | 7.6 ± 0.4a |
| **Total lipids** | ***P. defossus*** | 60.59 ± 4.8A | 52.18 ± 16.1A | 44.79 ± 5.5B | 49.11 ± 7.8B | 42.66 ± 2.1B | 40.67 ±4.0B |
| (mg $g^{-1}$) | ***P. brasiliensis*** | 29.31 ± 4.7a | 27.15 ± 1.1a | 27.01 ± 3.1a | 21.84 ± 3.5a | 22.54 ± 5.2a | 19.87 ± 3.4b |
| **Total cholesterol** | ***P. defossus*** | 48.64 ± 2.7A | 39.52 ± 5.4A | 44.09 ± 4.0A | 38.39 ± 5.4A | 40.39 ± 7.0A | 38.6 ± 4.9A |
| (mg $g^{-1}$) | ***P. brasiliensis*** | 26.03 ± 3.4a | 23.40 ± 2.3ab | 26.14 ± 4.3ab | 21.07 ± 3.2ab | 18.62 ± 3.1ab | 17.43 ± 1.1b |

**Table IV.** Levels of metabolites in the posterior gills of *Parastacus defossus* and *Parastacus brasiliensis* during post-hypoxia recovery. The different letters indicate significant differences (capital letters for *P. defossus* and small letters for *P. brasiliensis*) (p<0.05). (0: Control group; H: Hypoxia; 1, 3, 6, and 9: periods (h) of recovery).

| | Species | 0 | H | 1 | 3 | 6 | 9 |
|---|---|---|---|---|---|---|---|
| **Total proteins** | ***P. defossus*** | 3.58 ± 0.1AB | 3.87 ± 0.1AB | 4.49 ± 0.43B | 4.33 ± 0.3B | 3.38 ± 0.08A | 4.04±0.05AB |
| (mg $ml^{-1}$) | ***P. brasiliensis*** | 6.52 ± 0.4a | 6.88 ± 0.7a | 6.81 ± 0.27a | 7.36 ± 0.5a | 6.67 ± 0.34a | 7.49 ± 0.27a |
| **Total lipids** | ***P. defossus*** | 53.68 ± 5.3A | 49.8 ± 4.6A | 33.41 ± 2.6B | 29.62 ± 2.4B | 38.54±4.1AB | 30.00 ± 2.6B |
| (mg $g^{-1}$) | ***P. brasiliensis*** | 20.49 ± 4.5a | 18.7 ± 2.1a | 18.00 ± 4.8a | 11.08 ± 3.2a | 10.47 ± 1.2b | 9.74 ± 0.6b |
| **Total cholesterol** | ***P. defossus*** | 35.43 ± 5.5AB | 25.78 ± 4.4A | 27.83 ±4.8AB | 18.12 ± 3.3B | 22.33±5.0AB | 23.13±4.3AB |
| (mg $g^{-1}$) | ***P. brasiliensis*** | 16.97 ± 2.9a | 11.70 ± 2.3a | 16.50 ± 3.3a | 6.17 ± 1.2b | 4.59 ± 1.1b | 3.11 ± 1.2b |

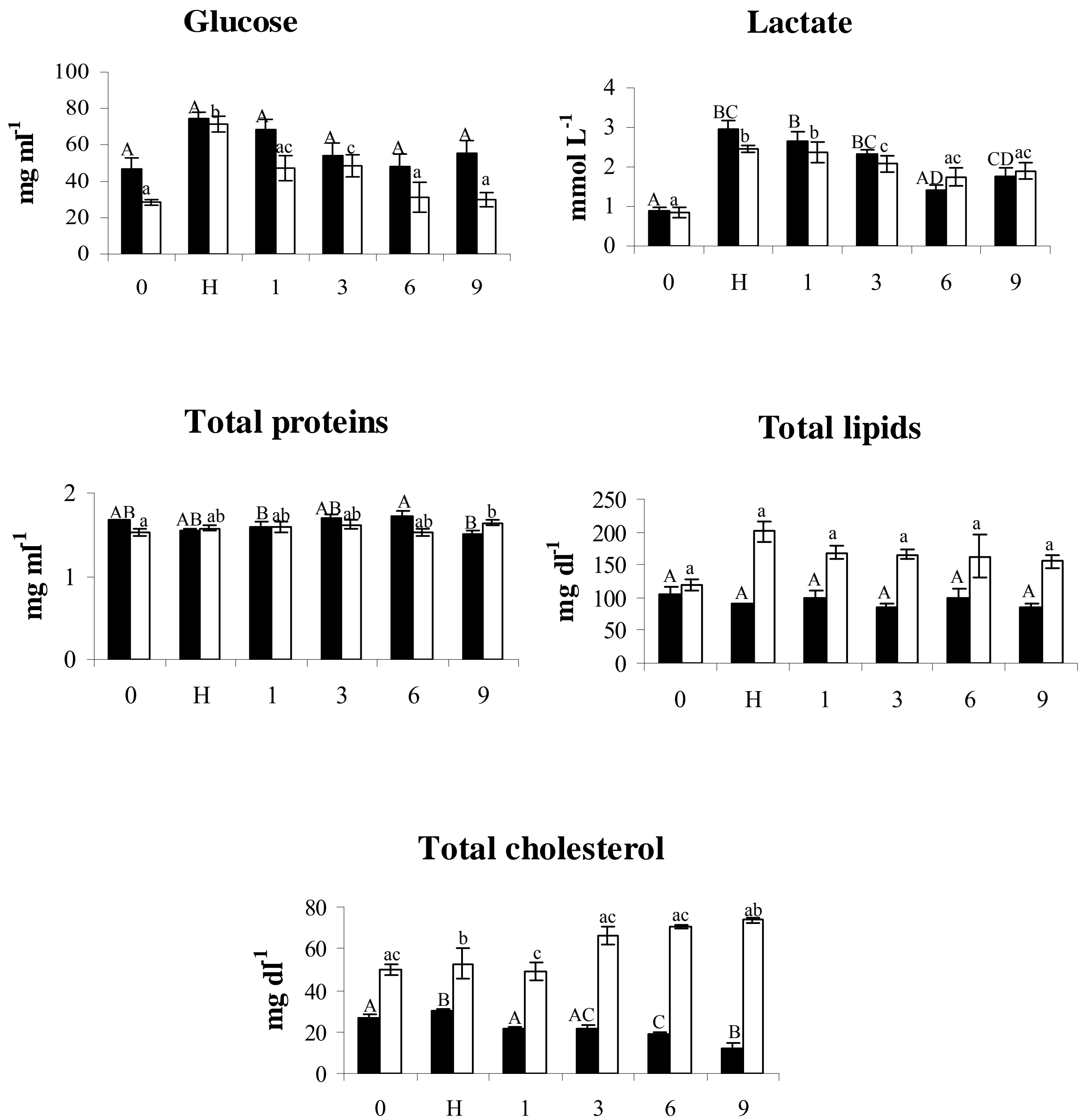



**Figure 1.** Levels of metabolites in the hemolymph of *Parastacus defossus* and *Parastacus brasiliensis* during post-hypoxia recovery. *P. defossus*: black bar; *P. brasiliensis*: white bar. The columns show the mean; vertical bars show the standard error of the mean. The different letters indicate significant differences (capital letters for *P. defossus* and small letters for *P. brasiliensis*) ($p<0.05$).

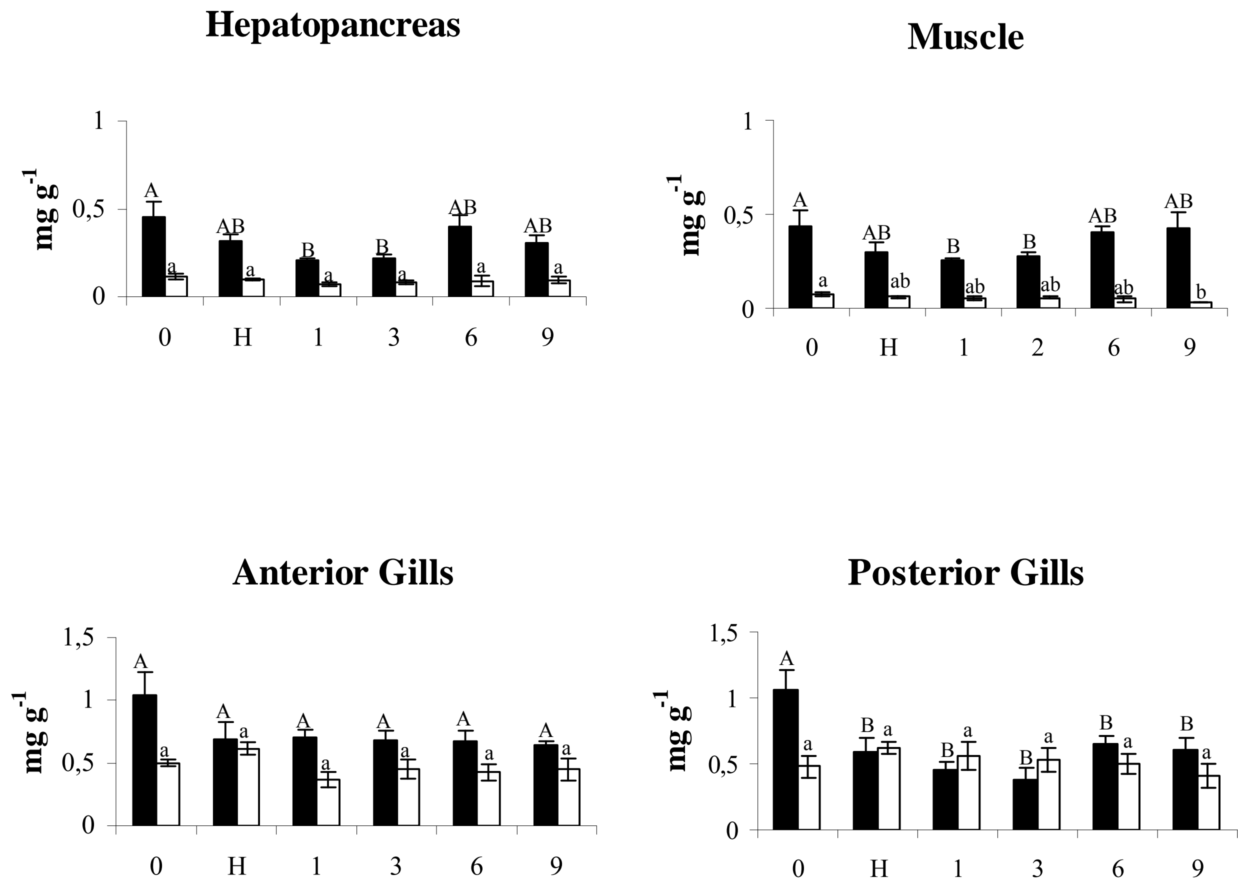



**Figure 2.** Glycogen levels of all tissues of *Parastacus defossus* and *Parastacus brasiliensis* during post-hypoxia recovery. *P. defossus*: black bar; *P. brasiliensis*: white bar. The columns show the mean; vertical bars show the standard error of the mean. The different letters indicate significant differences (capital letters for *P. defossus* and small letters for *P. brasiliensis*) ($p<0.05$).

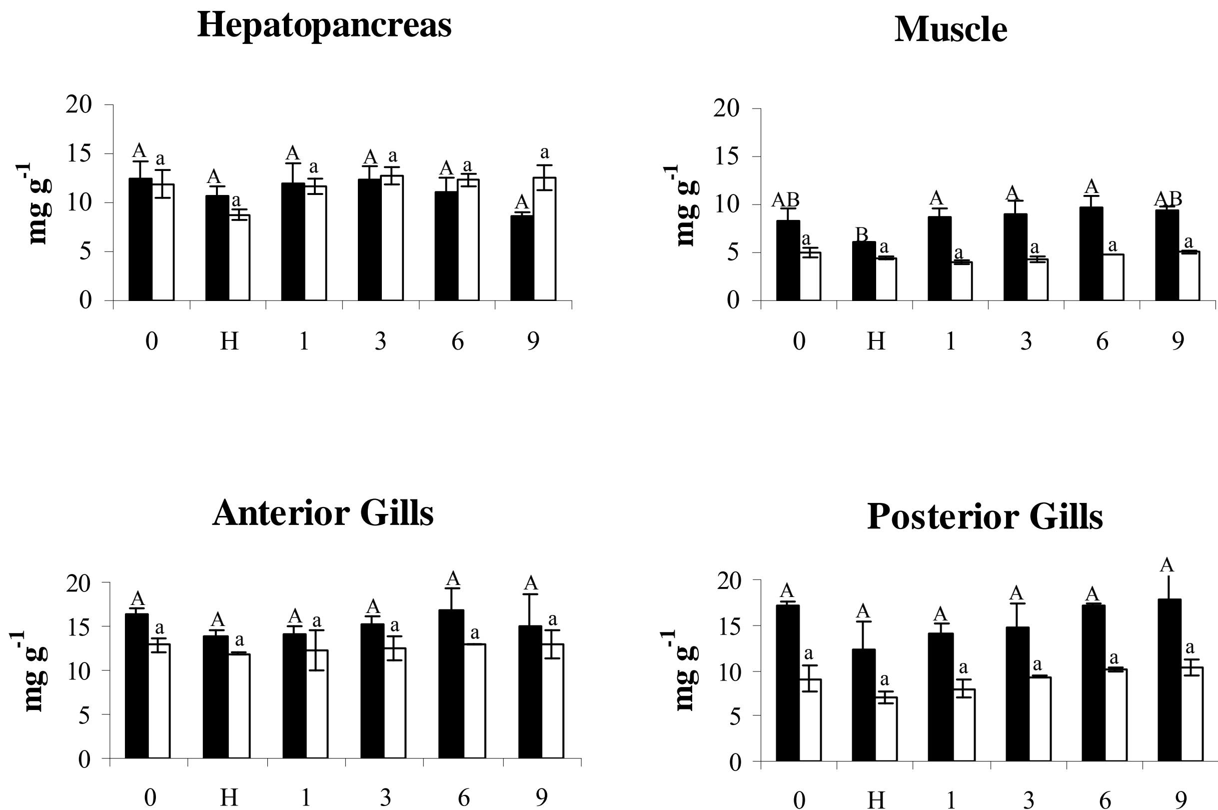
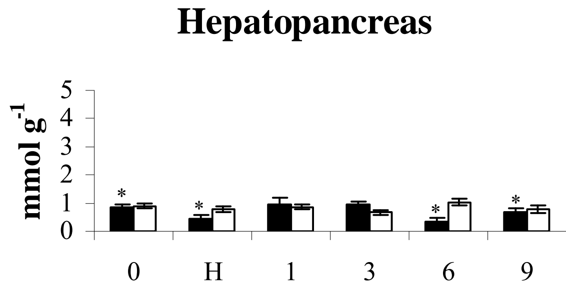
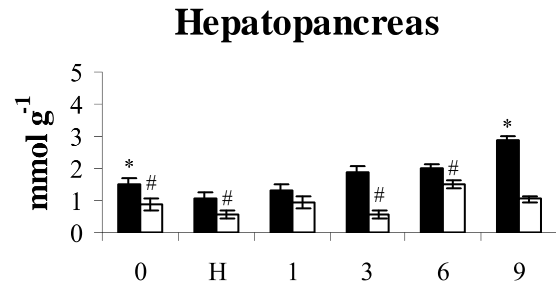
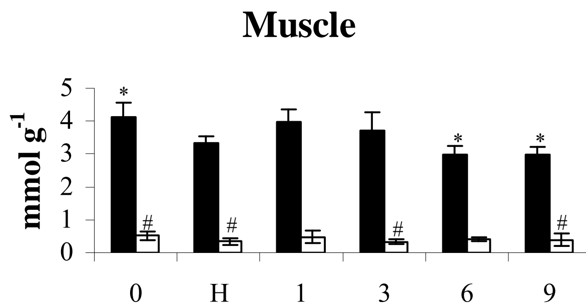



**Figure 3.** Free glucose levels of all tissues of *Parastacus defossus* and *Parastacus brasiliensis* during post-hypoxia recovery. *P. defossus*: black bar; *P. brasiliensis*: white bar. The columns show the mean; vertical bars show the standard error of the mean. The different letters indicate significant differences (capital letters for *P. defossus* and small letters for *P. brasiliensis*) ($p<0.05$).

**A)** **B)**



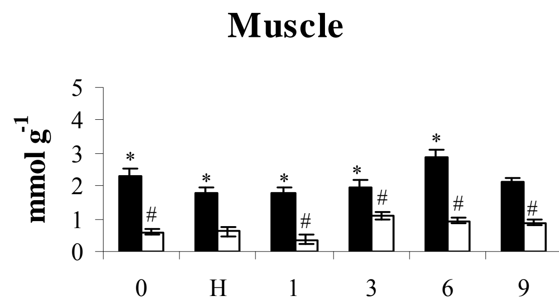




**Figure 4.** Levels of arginine phosphate and arginine during post-hypoxia recovery, in *Parastacus defossus* (A) and *Parastacus brasiliensis* (B). Arginine phosphate: black bar; Arginine: white bar. The columns show the mean; vertical bars show the standard error of the mean. The symbols indicate significant differences with the control (* arginine phosphate; # arginine) ($p<0.05$).

# *Considerações finais*

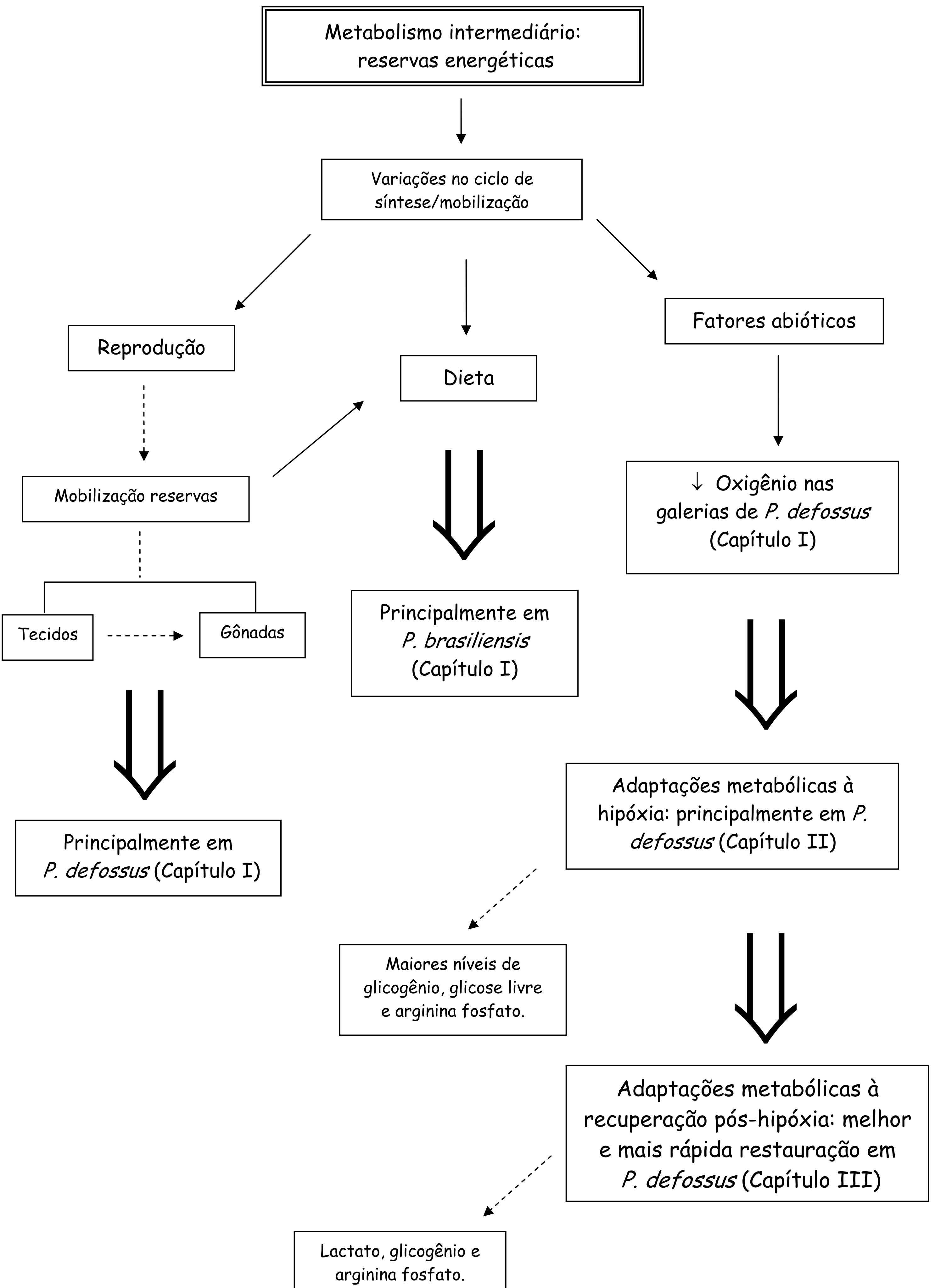
As variações sazonais do metabolismo intermediário de *Parastacus defossus* e *Parastacus brasiliensis* foram analisadas e discutidas no capítulo I. Em *P. defossus,* foi observado transferência de reservas principalmente dos tecidos para as gônadas durante o período reprodutivo e também variações sazonais relacionadas aos períodos de redução nos níveis de oxigênio em suas galerias subterrâneas. Em *P. brasiliensis,* as variações sazonais mostraram uma alocação significante de nutrientes da dieta para o período reprodutivo, com uma menor transferência das reservas de diferentes tecidos. Outros fatores como atividade exploratória e temperatura também parecem influenciar nas variações sazonais do metabolismo das espécies, principalmente em *P. brasiliensis*.

No capítulo I também foi observado maiores níveis de glicose e lactato nos períodos em que foram registradas as menores concentrações de oxigênio nas galerias de *P. defossus;* metabólitos importantes em períodos de hipóxia, pois são produzidos como estratégias adaptativas a essas condições. Esse fato foi corroborado no Capítulo II, pois se analisou o metabolismo das duas espécies de lagostins quando submetidos à hipóxia e, como esperado, devido ao hábito de viver em galerias subterrâneas foram registradas melhores adaptações metabólicas em *P. defossus* do que em *P. brasiliensis,* que também mostrou algumas adaptações. Além das estratégias adaptativas as condições hipóxicas, as espécies também mostraram adaptações metabólicas quando submetidas à recuperação pós-hipóxia no Capítulo III, mas *P. defossus* mostrou uma melhor e mais rápida restauração de lactato, glicogênio e arginina fosfato do que *P. brasiliensis.* Os principais resultados dessa pesquisa estão mostrados no esquema abaixo das considerações finais.

As principais diferenças entre os resultados das determinações metabólicas sazonais e do metabolismo de *P. defossus* e *P. brasiliensis* submetidos à hipóxia e a recuperação pós-hipóxia mostraram estar relacionadas com o habitat desses lagostins. No entanto, pesquisas sobre o estresse oxidativo, balanço ácido-básico e ecologia trófica poderão contribuir para o melhor entendimento

do metabolismo dessas espécies.

Além das adaptações metabólicas, os lagostins podem apresentar adaptações morfológicas branquiais em nível de Microscopia Eletrônica de Transmissão (MET) que poderão complementar os nossos resultados obtidos, em paralelo a essa pesquisa, com a microscopia de varredura das brânquias de *P. defossus* e *P. brasiliensis*. Portanto, possivelmente a MET será o próximo objetivo a ser investigado nesses lagostins com diferentes habitats.


Metabolismo intermediário:
reservas energéticas
Variações no ciclo de
síntese/mobilização
Reprodução
Dieta
Fatores abióticos
Mobilização reservas
↓ Oxigênio nas
galerias de P. defossus
(Capítulo I)
Tecidos
Gônadas
Principalmente em
P. brasiliensis
(Capítulo I)
Adaptações metabólicas à
hipóxia: principalmente em P.
defossus (Capítulo II)
Principalmente em
P. defossus (Capítulo I)
Maiores níveis de
glicogênio, glicose livre
e arginina fosfato.
Adaptações metabólicas à
recuperação pós-hipóxia: melhor
e mais rápida restauração em
P. defossus (Capítulo III)
Lactato, glicogênio e
arginina fosfato.

## *Anexos*

As normas dos periódicos escolhidos para a publicação dos artigos desta tese encontram-se nas páginas seguintes. As normas da Revista Brasileira de Zoologia no qual foi formatada a parte geral da tese também se encontram nos anexos.

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Tucker RP. 1991a. The sequential expression of tenascin mRNA in epithelium and mesenchyme during feather morphogenesis. Roux's Arch Dev Biol, 200:108-112.

Tucker RP. 1991b. The distribution of tenascin and its transcript in the developing avian central nervous system. J Exp Zool 259:78-91.

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# Comparative Biochemistry and Physiology A

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Axelsson, M., Farrell, A.P., 1993. Coronary blood flow in vivo in the coho salmon (*Oncorhynchus kisutch*). Am. J. Physiol. 264, R963 - 971.

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