Estudo do Metabolismo Intermediário e da Lipoperoxidação de *Hyalella curvispina* e *Hyalella pleoacuta* (Crustacea, Amphipoda, Dogielinotidae) e Padronização destas Espécies como Bioindicadores Ambientais

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FACULDADE DE BIOCIÊNCIAS

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOCIÊNCIAS - ZOOLOGIA

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DISSERTAÇÃO DE MESTRADO

PORTO ALEGRE, RS, BRASIL

2007

SUMÁRIO

AGRADECIMENTOS

Em primeiro lugar agradeço a Deus por minha vida e por tudo de bom ou ruim que tenha ocorrido em minha trajetória, pois com certeza cada um destes momentos me trouxe um aprendizado.

A minha mãe por ter acreditado em mim e nos meus sonhos, por todo o apoio, incentivo, apostas e certezas de que nada seria tão impossível e por me ensinar que todo o sacrifício um dia será recompensado. Espero ter sido motivo de orgulho para você como sempre fostes para mim. Sei que nossa caminhada até aqui teve várias subidas íngremes, mas conseguimos, terminamos mais uma etapa. Obrigada mãe!

Ao meu pai, João Alberto, que se não é o pai que me deu a vida de forma biológica, foi o pai que me possibilitou a conquista de meus sonhos, foi quem me ensinou que nada vida é conseguido sem esforço, que me puxou a orelha no momento certo. Obrigada Pai!

Ao meu marido pela paciência, pelo amor, pelas coletas e pelos intermináveis experimentos. Por ter me dado força mesmo nos meus momentos de nervosismo, sendo o meu anjo da guarda. Sem você esta jornada teria sido mais difícil, mas com certeza ela não acabou, pois ainda temos o Doutorado né?! Te amo muito!!!

À minha amiga e orientadora Guendalina Turcato Oliveira, o meu eterno agradecimento para a amiga que acreditou em mim como profissional. Obrigada por ter me mostrado a muitas vezes dura, mas gratificante vida de pesquisador. Obrigada por ter sido a minha mãe científica, por ter sido minha amiga nos momentos difíceis de minha vida e não ter me deixado desistir dos meus sonhos! Você sabe que muitas vezes rimos e choramos juntas e espero que esta amizade seja eterna. Obrigada!

A minha grande amiga Fernanda Severo Gering Fontoura que desde que nos conhecemos nos tornamos cúmplices nas alegrias e tristezas que esta jornada nos trouxe. Obrigada pela convivência, força, palavras de conforto e acima de tudo pela amizade.

Aos acadêmicos Renata Borges dos Santos, Luiz Ferrúa Farias de Oliveira e Arielle da Silva Dornelles que me auxiliaram nas coletas e no cultivo dos animais.

A professora Carla Denise Bonan pela doação do Carbofuran, sem o qual os experimentos de toxicologia não poderiam ter sido realizados. Obrigada!

Aos colegas Eduardo Rico e Denis Rosemberg do Laboratório de Neuroquímica e Psicofarmacologia pelo auxílio com a enzima, por sua paciência e amizade. Obrigada!

As Professoras Melissa Guerra Simões Pires e Berenice Alvares Rosito por suas palavras de incentivo e carinho, podem ter certeza que fizeram muita diferença nesta minha caminhada. Muito obrigada!

As Professoras Georgina Bond Buckup e Alessandra de Pádua Bueno pelas revisões dos textos taxonômicos e principalmente pelo incentivo em iniciar as pesquisas em ecotoxicologia utilizando este grupo taxonômico.

A Doutoranda Daniela da Silva Castiglioni pelo auxílio na identificação dos animais e das macrófitas.

A Faculdade de Biociências da PUCRS por ter me acolhido e permitido a realização de meu estágio docente.

Aos colegas de laboratório e professores do Laboratório da Fisiologia da Conservação pela convivência e amizade durante este período.

A secretária do Curso de Pós-graduação em Zoologia Maria Luiza Moreira que sempre se empenhou em resolver meus problemas com muita eficiência e paciência.Obrigada pela paciência com nossas angústias!!!

Aos animais que foram sacrificados em prol da ciência.

Por fim, agradeço a todos que, de uma maneira ou de outra, contribuíram para que este trabalho fosse executado com êxito.

Ao CNPq pela bolsa de mestrado.

"Se seus sonhos estiverem nas nuvens, não se preocupe, pois eles estão no lugar certo; agora construa os alicerces" (Autor desconhecido)

"O Futuro pertence àqueles que acreditam na beleza de seus sonhos"
(Elleanor Roosevelt)

Dedicatória

Dedico este trabalho a minha mãe Berenice por ter abdicado de muitas coisas em prol da minha vida. A Bisa Alzira, que de onde está sei que está olhando por mim. A meu pai João Alberto, por ter me acolhido como sua filha. A meu marido Felipe pelo amor, paciência e compreensão em todos os momentos.

RESUMO

Segundo a Environmental Protection Agency (EPA) para um organismo ser selecionado para testes de toxicológicos é necessário que se conheça: a distribuição da espécie, sua localização dentro da estrutura trófica, sua biologia, seus hábitos nutricionais, sua fisiologia e por fim que sejam desenvolvidas técnicas de manutenção de cultivo destes organismos em laboratório. Visando estas regras este trabalho foi dividido em quatro partes: No primeiro trabalho o amphipoda *Hyalella curvispina* foi coletado mensalmente de setembro 2003 a agosto 2005, na lagoa de Gentil, Tramandaí, Rio Grande do Sul, Brasil, e diferentes parâmetros bioquímicos e a lipoperoxidação foram medidos. Os resultados sugerem que estes animais armazenam e degradam de forma sazonal as reservas energéticas investigadas. Durante o verão, o glicogênio, os lipídios totais, e os triglicerídeos foram armazenados, e utilizados durante o outono e o inverno. As fêmeas armazenam proteínas na primavera e usam no verão; enquanto que os machos armazenam as proteínas na primavera e usam no inverno. Os níveis do lipoperoxidação durante o ano diferiram entre fêmeas e machos. Estas variações podem estar relacionadas aos fatores bióticos (ex. período reprodutivo) e aos fatores abióticos (ex. temperatura de água, salinidade). No segundo trabalho foi comparado o efeito de variações sazonais no metabolismo energético e nos níveis de lipoperoxidação de duas espécies simpátricas de amphipoda, *H. pleoacuta* e *H. castroi*. Os animais foram coletados mensalmente de abril 2004 a março 2006, no Vale das Trutas no município de São José dos Ausentes. As análises estatísticas revelaram diferenças sazonais significativas na composição bioquímica, bem como, diferenças entre sexos e espécies. Os fatores ambientais (ex., condições tróficas) e a reprodução pareceram ser os principais processos que influenciam os padrões sazonais da composição bioquímica. No terceiro trabalho nós comparamos variações no metabolismo energético, nos níveis de lipoperoxidação e de parâmetros reprodutivos de duas espécies de amphipoda, *H. pleoacuta* e *H. curvispina* mantido com duas dietas diferentes. Os animais foram coletados no inverno de 2004 e de 2005. No laboratório, os animais foram mantidos submersos em aquários sob circunstâncias controladas, alimentados *ad libitum* por 30 dias com dieta 1 (somente macrófitas) ou dieta 2 (macrófitas e ração). A análise estatística revelou diferenças significativas na composição bioquímica entre os sexos e as dietas. A dieta 1 mimetizou a restrição calórica, pois ocorreu uma depleção do glicogênio e das proteínas totais nas duas espécies e sexos, este fato é reforçado por uma diminuição nos níveis do lipoperoxidação. Nos amphipoda alimentados com a dieta 2, estes padrões metabólicos foram revertidos. A dieta 2 forneceu informações valiosas a respeito da manutenção adequada em laboratório para experimentos toxicológicos. As exigências calóricas das espécies foram supridas somente com a dieta 2, que forneceu mais carboidratos, proteínas e lipídios. O quarto estudo investigou os efeitos do Carbofuran no metabolismo energético, na lipoperoxidação e na atividade do Na^+/K^+ATPase, e em parâmetros reprodutivos nos amphipoda *H. pleoacuta* e *H.curvispina*. Os animais foram coletados no inverno de 2006. No laboratório, os animais foram mantidos submersos em aquários sob circunstâncias controladas e expostos ao Carbofuran numa dose de 5 ou 50μg/L por um período de 7 dias. A análise estatística revelou que o pesticida induz diminuições significativas no glicogênio, nas proteínas, nos lipídios, nos triglicerídeos, e na Na^+/K^+ATPase, bem como, um aumento significativo nos níveis de lipoperoxidação. O estudo dos parâmetros bioquímicos parece ser promissor, a fim de avaliar e predizer os efeitos dos pesticidas em organismos do não-alvo. Os resultados sugerem também que os parâmetros reprodutivos (formação dos pares, fêmeas ovígeras e número médio dos ovos) podem ser critérios sensíveis para avaliar efeitos ecotoxicológicos. Além disso, *H. pleoacuta* e *H. curvispina* são organismos apropriados para o uso em testes de toxicidade, e nós sugerimos que são espécies sensíveis que poderiam ser usadas para monitorar estudos.

Study of the Intermediate Metabolism and Lipoperoxidation of *Hyalella curvispina* and *Hyalella pleoacuta* (Crustacea, Amphipoda, Dogielinotidae) and Standardization of These Species as Environmental Indicators

ABSTRACT

According to the Environmental Protection Agency (EPA), for an organism to be selected for toxicology tests it is necessary to know: the distribution of the species, its nutritional place in the trophic structure, its biology, its habits, its physiology, and finally that laboratory culture techniques have been developed for it. Based on these guidelines, this project was divided into four parts. In the first study, *Hyalella curvispina* was collected monthly from September 2003 to August 2005, in Gentil Lagoon, Tramandaí, Rio Grande do Sul, Brazil, and the level of biochemical parameters and lipoperoxidation were measured. The results suggest that these animals have seasonal storage and degradation of the energy substrates investigated. During summer, glycogen, total lipids, and triglycerides were stored, and were utilized especially during autumn and winter. Females stored proteins especially in spring and used them in summer; whereas males stored proteins especially in spring and used them in winter. The levels of lipoperoxidation during the year differed between females and males. These variations may be related to biotic factors (e.g., reproduction period) and to abiotic factors (e.g., water temperature, salinity). In the second study, we compared the effect of seasonal variations in the energy metabolism and in the levels of lipoperoxidation of two sympatric species of amphipods, *H. pleoacuta* and *H. castroi*. The animals were collected monthly from April 2004 through March 2006, in the Vale das Trutas in the Municipality of São José dos Ausentes. Statistical analyses revealed significant seasonal differences in biochemical composition, as well as differences among sexes and species. Environmental conditions (e.g., trophic conditions) and reproduction appeared to be the main processes influencing the seasonal patterns of variation in biochemical composition. In the third study, we compared variations in the energy metabolism and in the levels of lipoperoxidation, and also reproductive parameters of two species of amphipods, *H. pleoacuta* and *H. curvispina* maintained on two different diets. The animals were collected in the winter of 2004 and 2005. In the laboratory, the animals were kept submerged in aquariums under controlled conditions; they were fed *ad libitum*, for 30 days with diet 1 or diet 2. Statistical analysis revealed significant differences in biochemical composition between the sexes and diets. Diet 1 (macrophytes only) mimicked caloric restriction, because this showed a depletion of the glycogen and total proteins in the two species and sexes, as reinforced by a decrease in the levels of lipoperoxidation. In amphipods fed on Diet 2 (macrophytes and ration), these metabolic patterns were reversed, and Diet 2 provided valuable information concerning adequate maintenance in the laboratory for toxicology experiments. The caloric requirements of both species were only supplied with Diet 2, which provided more carbohydrates, proteins, and lipids. The fourth study investigated the effects of carbofuran on the energy metabolism, lipoperoxidation and Na^+/K^+ATPase activity, and reproductive parameters in the amphipods *H. pleoacuta* and *H. curvispina.* The animals were collected in the winter of 2006. In the laboratory, the animals were kept submerged in aquariums under controlled conditions and exposed to carbofuran at a dose of 5 or 50µg/L for a period of 7 days. Statistical analysis revealed that carbofuran induces significant decreases in glycogen, proteins, lipids, triglycerides, and Na^+/K^+ATPase, as well as a significant increase in lipoperoxidation levels. Studies of all the biochemical parameters seem to be quite promising, in order to assess and predict the effects of toxicants on non-target organisms. The results also suggest that the reproductive parameters (formation of couples, ovigerous females and mean number of eggs) may provide sensitive

criteria for assessing ecotoxicological effects. Furthermore, *H. pleoacuta* and *H. curvispina* are suitable organisms for use in toxicity tests, and we suggest that they are sensitive species that could be used in monitoring studies.

Apresentação

1. Histórico do problema

1.1 Modelos experimentais em Ecotoxicologia

Atualmente, em várias regiões do mundo é possível observar que os ecossistemas vêm sofrendo alterações que podem estar associadas à atividade humana decorrente do processo de desenvolvimento industrial, urbano e agrícola. No que se refere aos ecossistemas aquáticos, às atividades antrópicas geram impactos, promovendo lentas e muitas vezes irreversíveis modificações nestes ambientes (Bohrer, 1995).

Tradicionalmente, as análises físicas e químicas são mais utilizadas na caracterização de resíduos líquidos, tanto de origem industrial como doméstica; porém, análises biológicas, tais como os testes de toxicidade com organismos aquáticos, têm sido cada vez mais utilizados no controle de despejos que podem causar impacto ao ambiente aquático (CESTEB, 1986 a,b).

Segundo Baudo ***et al.*** (1999) uma avaliação ambiental não deve ser realizada apenas com análises químicas isoladas, pois, normalmente, elas não estabelecem qual a porção química de determinado poluente é verdadeiramente biodisponível e capaz de interagir com organismos vivos. O autor sugere, então, que as análises químicas devem ser combinadas com testes biológicos.

De acordo com Soares (1991), a necessidade em monitorar os efeitos da ação antrópica, levou a criação da Ecotoxicologia, ciência preocupada em estudar os efeitos de agentes químicos tóxicos, em nível de indivíduo, e suas conseqüências na estrutura e funcionamento das populações, comunidades e ecossistemas.

Os ecossistemas aquáticos são formados por compartimentos abióticos básicos: a atmosfera, a coluna d'água e o sedimento. Estes ambientes recebem uma série de influências que devem ser monitoradas, através de avaliações ecotoxicológicas, a fim de avaliar a sua toxicidade.

Logo que surgiu a ciência Ecotoxicologia Aquática o enfoque principal era a qualidade da água, receptora de efluentes urbanos e industriais, já o sedimento era menos estudado por ser considerado um ambiente pouco contaminado (Baudo ***et al.***,1999). No entanto, a atual situação de prevenção da poluição hídrica, tem resultado em casos onde a coluna d'água pode estar impactada. Quanto aos sedimentos estes podem tornar-se poluídos, pois permanecem expostos a longos períodos de deposição de contaminantes.

Segundo Leppanen ***et al.***(1998), testes de toxicidade com sedimento determinam se o contaminante é danoso para os organismos bentônicos, sendo eficientes como recursos para avaliar a contaminação deste compartimento, pois fornecem informações complementares à caracterização química e às avaliações ecológicas.

Os efeitos ecológicos de contaminantes associados ao sedimento têm sido avaliados através do uso de testes de toxicidade em laboratório e através de ensaios de bioacumulação, utilizando-se organismos bentônicos ou epibentônicos (Benoit ***et al.*** 1993). A Environmental Protection Agency (EPA), em 1994, publicou uma norma para determinação da toxicidade aguda e bioacumulação de sedimentos associados à contaminantes com organismos de água doce. Estes métodos foram discutidos e desenvolvidos em resposta a "Estratégia de Manejo de Sedimentos Contaminados da EPA", propondo que estas avaliações sejam incorporadas aos programas de regulamentação ambiental (Burton ***et al.*** 1996).

De acordo com Ingersoll ***et al.*** (1998) uma variedade de métodos padronizados tem sido desenvolvida para avaliar a toxicidade de contaminantes, utilizando crustáceos (anfípodos e cladóceros), insetos (dípteros) e anelídeos (oligoquetas e poliquetas). Vários parâmetros são sugeridos nestes métodos, para medir os efeitos destes contaminantes, incluindo sobrevivência, crescimento, comportamento ou reprodução.

Os testes de toxicidade consistem em expor organismos a várias concentrações de uma ou mais substâncias, ou fatores ambientais, durante um determinado período de tempo (Ravera, 1984). Segundo Burton & Scott (1992) e Burton & MacPherson (1995) os organismos de água doce que têm sido mais comumente utilizados em avaliações de toxicidade aquática são os cladóceros *Daphnia magna* e *Ceriodaphnia dubia* (exposição de 7 dias), o peixe *Pimephales promelas* (exposição de 7 dias), o anfípodo *Hyalella azteca* (exposição de 7 e 21 dias), os insetos quironomídeos *Chironumus tentans* e *Chironomus ripanus* (exposição de 7 e 10 dias); e *Hexagenia bilineata* (exposição de 10 dias).

O número de espécies padronizadas em testes de toxicidade permanece limitado e na sua maioria são utilizados organismos alóctones, contrastando com a riqueza taxonômica da maioria dos ecossistemas naturais de nosso País. Este número reduzido deve-se, primariamente, ao fato de que a maioria dos testes de toxicidade implica em cultivos contínuos, com padronização das condições destes cultivos, em organismos-teste em estado saudável em número suficiente, o que limita a seleção das espécies utilizadas (Brendonck & Persoone, 1993).

A necessidade da criação de programas de monitoramento ambiental fazendo uso de espécies aquáticas autóctones reside, principalmente, em dois aspectos: primeiro, no impacto, direto ou indireto, que a introdução intencional ou não de uma espécie exótica teria sobre a dinâmica ambiental; segundo, na qualidade da resposta que uma espécie autóctone poderia fornecer, já que em geral, sua condição ideal de cultivo, bem como as condições de realização dos testes, estão próximas das características ambientais. O uso de espécies autóctones, adaptadas às características ambientais, certamente poderia prover resultados muito mais próximos da realidade do que os resultados obtidos com espécies exóticas (Arezon, 1996).

1.2. Amphipoda

Os crustáceos são um grupo dominante e de sucesso, representado por um elevado número de espécies, exibindo dessa forma uma grande variedade de estilos de vida e ocupando habitats diferentes, sendo esta diversidade resultado de seus padrões de vida e estratégias reprodutivas (Sastry, 1983).

A superordem Peracarida, juntamente com os Decapoda, representa a maioria dos Malacostraca e de fato a maioria dos crustáceos, correspondendo a 30% do total (Ruppert & Barnes, 1996). Os Peracarida estão representados por 7 ordens, das quais a Ordem Amphipoda é o grupo mais representativo dos ecossistemas aquáticos, sendo caracterizado por apresentarem o corpo lateralmente comprimido e os primeiros e segundos pares de pereópodos chamados de gnatópodos.

Os Amphipoda têm grande importância nas comunidades das quais fazem parte, pois possuem hábitos detritívoros e herbívoros, servindo de alimento para vários organismos (Schmitt, 1965; Muskó, 1993; Pilgrim & Burt, 1993). Constituem importante elo nas cadeias alimentares dos ecossistemas onde ocorrem (Moore, 1975) e possibilitam a transferência de energia produzida pelas algas e vegetais superiores para os consumidores de níveis tróficos mais elevados.

Os Amphipoda pertencentes à Infraordem Gammaridea são um grupo muito grande e diversificado de espécies distribuídas em 69 famílias. Existem representantes marinhos, na água doce e uma única família, Talitridae, que reúne as espécies terrestres. Nos ambientes marinhos e límnicos do Rio Grande do Sul são encontrados representantes de 7 famílias de Gammaridea, das quais se destacam Corophidae, Stenothoidae, Hyalidae, Ischyriceridae, Gammaridae, Talitridae e Dogielinotidae (Bento & Buckup, 1990).

A família Dogielinotidae segundo Bulycheva, (1957) abriga os gêneros *Hyalella, Parhyalella, Allorchestes, Insula, Chiltonia, Phreatochiltonia, Afrochiltonia* e *Austrochiltonia* (Zeilder, 1991; Bousfield, 1996).

Ao discutir a classificação de Bulycheva (1957) Barnard (1969) fez a mudança de *status* de Hyalellidae para Hyalellinae, onde alocou os gêneros *Hyalella*, *Parhyalella, Insula, Chiltonia, Afrochiltonia, Neobule* e *Austrochiltonia*. Entretanto, Bowman & Abele (1992) ao publicar uma classificação dos crustáceos recentes, consideraram como válida a Família Hyalellidae Bulycheva (1957).

Mais tarde, Boulsfield (1996) ao fazer uma revisão sistemática da Família Hyalellidae criou os subgêneros: *Austrohyalella* (para espécies dos Andes e regiões ao sul da América do Sul), *Mesohyalella* (para espécies da região nordeste da América do sul) e *Hyalella* (para espécies da América do Norte e Central).

Recentemente, Serejo (2004) publicou uma revisão dos anfípodos Talitroidea, propondo, com base em comparações morfológicas, uma nova classificação para este grupo. A autora fez uma análise taxonômica baseada em uma matriz de caracteres com 34 taxa terminais e 43 caracteres morfológicos. Sendo assim, a Superfamília Talitroidea foi elevada à Infraordem Talitrida *s.s*., e as Famílias Hyalellidae e Najnidae foram sinonimizadas com Dogielinotidae, e tratadas como Subfamílias. Serejo (*op. cit.)* ainda mantém a validade dos Subgêneros *Austrohyalella, Hyalella* e *Mesohyalella* propostos por Bousfield (1996).

O gênero *Hyalella* é bastante comum na América do Norte, ocorrendo também na América Central e América do Sul. É conhecido somente em regiões biogeográficas neárticas e neotropicais, sendo que 44 espécies já foram descritas (Gonzáles & Watling, 2002). As espécies pertencentes ao gênero *Hyalella* são geralmente encontradas numa variedade de habitats de água doce, como reservatórios permanentes, lagos, tanques e riachos, estando muitas vezes aderidas à vegetação, nadando na coluna d'água ou em buracos cavados no sedimento, sendo importantes membros da fauna bentônica (Kruschwitz, 1978; Wellborn, 1995; Grosso & Peralta, 1999).

As informações sobre as espécies do gênero *Hyalella* que ocorrem nas bacias hidrográficas do Brasil são esparsas e muitas com problemas na sua identificação. Registros anteriores mencionam a presença de 11 espécies para bacias hidrográficas do Brasil: *H. longistila* (Faxon, 1876), *H. gracilicornis* (Faxon, 1876), *H. meinerti* Stebbing, 1899, *H. warmingi*

Stebbing, 1899, *H. curvispina* Shoemaker, 1942, *H. pampeana* Cavalieri, 1968, *H. caeca* Pereira, 1989, H. *brasiliensis* Bousfield, 1996, *H. montenegrinae* Bond-Buckup and Araujo, 1998, *H. pseudoazteca* González and Watling, 2003, *H. dielaii* Pereira, 2004. No Rio Grande do Sul há registros de ocorrência das últimas três espécies citadas, acrescidas de *H.pleoacuta*, *H. castroi*, *H.* sp.n., *H. curvispina.* A *Hyalella pleoacuta* e a *Hyalella castroi* foram encontradas no município de São José dos Ausentes, na localidade do Vale das Trutas, em regiões de elevada altitude (1.100 metros). Já a *H. curvispina* é encontrada principalmente em regiões de planície do Rio Grande do Sul (González *et al*, 2005).

Bento & Buckup (1990) destacam que o gênero *Hyalella* tem ampla distribuição pelas Américas e pelo Brasil. No Rio Grande do Sul, a espécie *Hyalella curvispina* foi encontrada em diversos ambientes de água doce (Vacaria, Caxias do Sul, Cruz Alta, Montenegro, Triunfo, São Francisco de Paula, Taquara, Viamão, Gravataí, Caçapava do Sul, Quaraí, Estação Ecológica do Taim – Rio Grande); *Hyalella montenegrinae* foi registrada somente para a localidade-tipo, em arroio próximo ao morro Monte Negro, Silveira, município de São José dos Ausentes e *Hyalella peseudoazteca* foi registrada apenas na região do Taim.

O dimorfismo sexual das espécies de *Hyalella* é caracterizado pela presença do segundo par de gnatópodos alargados nos machos. O segundo par de gnatópodos dos machos são usados para o manuseio das fêmeas durante o comportamento de cópula e os primeiros pares pequenos são utilizados para carregá-las (Kruschwitz, 1978). As fêmeas maduras de espécies de Amphipoda são facilmente identificadas pelos seus ovários desenvolvidos, os quais são externamente visíveis, pela presença de um marsúpio e pela presença de ovos dentro do marsúpio (Kruschwitz, 1978).

Os crustáceos são freqüentemente utilizados como bioindicadores e biomonitores em vários sistemas aquáticos. Uma razão para isto, é que eles são um grupo de animais bem sucedidos, distribuídos em diferentes habitats, incluindo os ambientes marinho, terrestre e dulceaquícola. Alguns aspectos dos crustáceos, principalmente as estratégias reprodutivas, podem ser muito importantes para a interpretação de dados sobre estudos da bioindicação e para o desenvolvimento de estudos ecotoxicológicos. Não somente os aspectos reprodutivos, mas também outras respostas comportamentais dos crustáceos, como as mudanças na alimentação, na locomoção ou no comportamento de pré-cópula podem providenciar respostas preditivas com respeito à bioindicação de toxicidade em determinado ambiente (Rinderhagen *et. al.,* 2000). Os

Amphipoda dulceaquícolas comumente utilizados em testes de toxicidade são *Hyalella azteca* e *Gammarus lacustris* (Buyle, 1989; Nelson & Brunson, 1995; Duan ***et al.***, 1997); espécies exóticas em nosso meio ambiente.

Os Dogielinotidae, por outro lado, por serem organismos bentônicos, são muito utilizados em testes de toxicidade e bioensaios para avaliação da qualidade do sedimento dos ecossistemas aquáticos. Este sedimento serve ao mesmo tempo, como depósito e fonte de matéria orgânica e inorgânica, pelo fato de sua camada superficial ser mais permanente que a coluna de água, servindo, portanto, como melhor testemunho das atividades ocorridas recentemente na bacia hidrográfica (Wetzel, 1983; Burton, 1991).

Com relação aos estudos de ecotoxicologia, os Dogielinotidae até então utilizados têm uma série de características que os tornam adequados como organismos experimentais: são coletados com facilidade, ocorrem em altas densidades, facilmente mantidos em laboratório, apresentam ciclo de vida curto e apresentam grande sensibilidade a contaminantes (Sampaio, 1988). Diversos estudos sobre a utilização do Dogielinotidae, *Hyalella azteca,* em testes de toxicidade tem sido realizados (Nebeker & Miller, 1988; Collyard ***et al.***, 1994; Burton ***et al.***, 1996; Smith ***et al.***, 1997; Ingersold ***et al.***, 1998; Brent & Henicks, 1998; Defoe & Ankley, 1998; Leppanen & Maier, 1998; Kemble ***et al.***, 1999). Pesquisas usando *Hyalella curvispina* como organismo-teste ainda são desconhecidas para a ciência.

Muito pouco se conhece sobre a biologia e a ecologia das espécies do Rio Grande do Sul. Informações sobre a biologia dos Dogielinotidae são restritas a *Hyalella azteca,* espécie que ocorre na América do Norte e México.

1.3. Crustáceos e metabolismo

Tanto pelo número de espécies existentes, como pela diversidade de habitats nos quais vivem, os crustáceos estão entre os animais com maior êxito durante sua história evolutiva. Esta diversidade é resultado de seus padrões de vida e estratégias reprodutivas (Sastry, 1983).

O estudo do metabolismo intermediário em Crustáceos tem demonstrado grande variabilidade inter e intra espécies, o que torna difícil a determinação de um perfil metabólico padrão. Desconsiderando as diferenças entre os métodos bioquímicos empregados pelos diversos autores, esta variabilidade pode ser atribuída a fatores múltiplos, tais como seu habitat (terrestre, marinho, estuarino ou de água doce), estágio do ciclo de muda, maturidade sexual (especialmente

em fêmeas), estado alimentar, dieta oferecida e sazonalidade, visto que estes fatores determinam um padrão diferencial de resposta metabólica (Oliveira ***et al.***, 2003).

Dados da literatura sobre o metabolismo de carboidratos em Crustáceos confirmam a presença das vias de glicogênese, de glicogenólise, de glicólise e da gliconeogênese em diferentes tecidos (Meenaski & Scheer, 1968; Chang & O'Connor, 1983; Oliveira e Da Silva, 1997; Oliveira *et al*, 2001). As peculiaridades da biologia e da ecologia das diferentes espécies de crustáceos contribuem para a grande variabilidade e controvérsia dos resultados sobre os tecidos responsáveis pela gliconeogênese em caranguejos. As brânquias, os hemócitos, o hepatopâncreas e o músculo, têm sido propostos como sítios para ocorrência da via gliconeogênica (Thabrew ***et al.***, 1971; Johnston ***et al.***, 1973).

Os principais tecidos de reserva de glicogênio em crustáceos são o músculo, 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; Oliveira ***et al.***, 2003). A ausência de um depósito central de glicogênio parece ser, segundo Hochachka & Somero (1984), uma adaptação de várias classes de animais às mudanças nos fatores ambientais. Nery & Santos (1993) sugerem que esta independência em relação aos depósitos centrais de glicose seria muito importante em animais de circulação aberta, já que seu fluxo sangüíneo é lento e se dá sob baixa pressão, o que conduziria a uma distribuição menos efetiva da glicose para os tecidos. O glicogênio armazenado é utilizado nos processos de muda, hipóxia e/ou anoxia, osmorregulação, crescimento, diferentes estágios de reprodução e durante períodos de jejum (Chang & O'connor, 1983; Kucharski & Da Silva, 1991a,b; Oliveira ***et al.***, 2001 e 2004 a,b; Hervant *et al*, 1997, 1999 a,b).

Segundo Chang & O'connor (1983) a glicose é o principal monossacarídeo presente na hemolinfa de crustáceos, tendo seis destinos principais: a síntese de mucopolissacarídeos, a síntese de quitina, a síntese de ribose, a síntese de nicotinamida adenina dinucleotídeo fosfato reduzido (NADPH), a formação de piruvato e a síntese de glicogênio (Hochachka ***et al.,*** 1970; Herreid & Full, 1988).

Em crustáceos as concentrações de lipídios são bastante elevadas, apesar de não existir um tecido adiposo diferenciado. Os principais locais de armazenamento de lipídios são o músculo e o hepatopâncreas (O'connor & Gilbert, 1968; Chang & O'connor, 1983; Herreid & Full, 1988; Kucharski & Da Silva, 1991a, Oliveira ***et al.***, 2006). Herreid & Full (1988) verificaram que os

níveis de lipídios no hepatopâncreas excediam em dez vezes os níveis de glicogênio. Nesta Classe, a síntese de ácidos graxos, diacilglicerídeos e triacilglicerídeos é semelhante àquela encontrada em mamíferos. Diversos estudos têm demonstrado que durante períodos de grande demanda energética, como a muda e a gametogênese, ocorre uma marcante mobilização de lipídios, principalmente aqueles presentes no hepatopâncreas (Ferreira ***et al.***, 2005; Oliveira ***et al.***, 2006). No caranguejo *Chasmagnathus granulata*, foi evidenciada uma variação sazonal dos níveis de lipídios musculares, sendo estes mais elevados no verão, porém os níveis de lipídios totais no hepatopâncreas só diminuem no período reprodutivo (Kucharski & Da Silva, 1991a). Nessa mesma espécie de caranguejo, os lipídios musculares também são mobilizados durante processos de osmorregulação (Nery & Santos, 1993).

O músculo parece ser a principal fonte de proteínas nos crustáceos e, em decápodos os níveis de aminoácidos livres nos tecidos atingem valores dez vezes maiores que àqueles encontrados em vertebrados. Diversos trabalhos sugerem que estes aminoácidos estariam envolvidos nos processos de osmorregulação, estando principalmente ligados ao controle do volume celular (Huggins e Manday, 1968; Gilles, 1982; Chang e O'Connor, 1983). Dall (1981) verificou uma variação sazonal dos níveis de proteínas totais no músculo abdominal de *N. norvergicus* estando estas diminuídas durante períodos de jejum. Alguns estudos mostram uma variação no conteúdo de proteínas durante o desenvolvimento ovariano de crustáceos; estas variações poderiam resultar de um aumento na biossíntese de varias proteínas incluindo hormônios, enzimas e lipoproteínas envolvidas com a maturação gonadal (Rosa e Nunes, 2003a; Yehezkel ***et al.***, 2000). Hervant *et al* (1999c) verificaram que em períodos prolongados de jejum espécies de anfípodos de águas profundas (*Niphargus rhenorhodanensis* e *Niphargus virei*) e rasas (*Gammarus fossarum*) apresentaram mobilização de suas reservas proteicas.

Sabe-se que variáveis como a temperatura e a dureza da água podem influenciar no cultivo dos organismos (Lewis e Marki, 1981; Persoone ***et al.,*** 1989), entretanto, dentre todas as variáveis a dieta a qual os organismos estão submetidos tem se mostrado como fator determinante no seu desenvolvimento (Kersting e Van der Leeuw, 1976; Lewis e Marki, 1981; Vijverberg, 1989; Lei ***et al.*** 1990; Kawbata e Urabe, 1998; Beatrici, 2000). A importância na quantidade e na qualidade do alimento do alimento fornecido pode ser avaliada através do número de filhotes produzidos nos cultivos, uma vez que a dieta pode influenciar diretamente na capacidade reprodutiva dos indivíduos. Herbert (1978) ao estudar o gênero *Daphnia* (cladócero), constatou

que o número de neonatos produzidos por fêmeas ovígeras depende diretamente de sua ingestão de alimento. O número de filhotes, juntamente com a sensibilidade de um organismo a uma substância de referência e o teor de lipídios acumulados são critérios que podem ser adotados para a avaliação da qualidade dos cultivos de organismos utilizados em ensaios ecotoxicológicos (Zagatto, 1988).

Beatrice (2000), ao comparar a resposta de *Daphnia similis* a três diferentes dietas constatou que os indivíduos a uma dieta combinada da alga *Selenastrum caprcornutum* com um complemento alimentar a base de *Artemia salina* reproduziam significativamente mais do que quando cultivadas apenas com alga. Platte (1993) ao buscar uma forma de aumentar a produtividade dos cultivos de *Ceriodaphnia dubia* obteve resultados semelhantes ao testar o complemento alimentar a base de artêmia como uma forma de incrementar a dieta a base de algas dos organismos. Em um caranguejo estuarino muito estudado, *Chasmagnathus granulata*, diferentes dietas alteram significativamente as concentrações de glicose, glicogênio e lipídios nos tecidos e na hemolinfa deste caranguejo (Kucharski e Da Silva, 1991a). Hernandez-Vergara ***et al.,*** (2003) avaliaram o efeito de diferentes concentrações de lipídios em dietas artificiais oferecidas para o lagostim *Cherax quadricarinatus*, e concluíram que os machos investem suas reservas metabólicas para o crescimento, enquanto que as fêmeas, com um alto índice hepatossomático, investem no desenvolvimento gonadal e vitelogênese. Dutra ***et al.*** (2007) estudando a espécie *Parastacus brasiliensis* verificaram que independente da dieta oferecida (rica em carboidratos ou rica em proteínas) aos animais em condições controladas de laboratório (temperatura e fotoperíodo) por 15 dias, as marcas metabólicas trazidas de campo pelos animais não são retiradas, bem como, as alterações observadas em seus níveis metabólicos estão relacionadas ao período reprodutivo. Padrão semelhante foi encontrado por Ferreira ***et al.*** (2005) com as mesmas dietas porém estudando o anomura *Aegla platensis.*

A enzima Na^+K^+ATPase utiliza a energia derivada da hidrólise do ATP para bombear para fora da células $3Na^+$ transferindo $2K^+$ da parte externa para o citosol (Shepherd, 1994); funcionando assim como um *antiporter*, sendo um importante instrumento para restaurar o gradiente iônico nas células nervosas seguindo períodos de atividade elétrica como impulsos nervosos e potenciais sinápticos (Shepherd, 1994). Esta enzima dimérica existe em diversas isoformas no cérebro e consome grande parte do ATP disponível (Shepherd, 1994; Bertorello ***et al.***, 1991). Em crustáceos a Na^+K^+ATPase exerce um importante papel na manutenção de

gradientes iônicos entre o meio interno (animal) e o habitat; sendo por isto fundamental para a sobrevivência de animais osmorreguladores. Contudo, muito poucos estudos sobre esta enzima são encontrados em crustáceos dulce-aquícolas (Gilles, 1982; Castilho ***et al.***, 2001). Esta enzima de membrana requer fosfolipídios para sua atividade e é altamente vulnerável ao dano oxidativo visto que sob tais circunstâncias observa-se uma inativação que pode envolver o rompimento dos fosfolipídios do microambiente da enzima ou danos diretos à proteína causados por radicais do oxigênio ou por produtos gerados na lipoperoxidação (Fleuranceau-Fleuranceau-Morel ***et al.***, 1999; Lehtosky ***et al.***, 1999).

Conceitualmente, radicais livres são definidos como um átomo, grupo de átomos ou molécula com um elétron desemparelhado ocupando o orbital mais externo, o que os torna extremamente reativos. Esta definição inclui íons de metais de transição, o átomo de hidrogênio, o óxido nítrico e o dióxido de nitrogênio (Dröge, 2002). Os radicais livres são continuamente produzidos pela fosforilação oxidativa e por outros sistemas biológicos reagindo rapidamente com a maioria das moléculas orgânicas. Estes radicais podem reagir com lipídios de membrana, proteínas, DNA e também glicídios (Meerson ***et al.***, 1981). Quando reagem com os lipídios de membrana, causam lipoperoxidação destes através de uma série de reações, com a conseqüente formação de malondialdeído e outras substâncias que, quando aquecidas na presença de ácido tiobarbitúrico, formam um composto rosado, que pode ser medido espectrofotometricamente (Buege & Aust, 1978; Ohkawa ***et al.,*** 1979; Halliwell & Gutteridge, 1995). Sabe-se da literatura que o aumento do estresse oxidativo, principalmente, em períodos de alta demanda energética pode aumentar proporcionalmente a formação de espécies reativas ao oxigênio e com isto, aumentar a ocorrência do dano oxidativo (Llesuy *et al*, 1985). Portanto, a utilização e a padronização de uma medida de dano oxidativo (TBARS) em crustáceos podem refletir alterações biológicas, como as que ocorrem no período reprodutivo, assim como alterações no meio ambiente.

1.4. Pesticidas Carbamatos

O uso de produtos químicos para o controle de insetos prejudiciais às plantações contribuiu para o aumento da eficiência agrícola durante o século passado. Tradicionalmente, os inseticidas têm como alvo funções do sistema nervoso que são comuns a muitas espécies, inclusive aos seres humanos. O uso destes inseticidas neurotóxicos tem levado à morte indiscriminada de insetos benéficos e proporcionado riscos sérios a outros animais e aos humanos

através da exposição ao ambiente. Além disso, evidências crescentes têm sugerido uma possível ligação entre a exposição de humanos a ambientes contaminados com inseticidas e doenças neurológicas, tal como o mal de Parkinson. (Le Couteur *et al*, 1999). Desta forma, existe uma enorme necessidade de se desenvolver inseticidas que apresentem maior seletividade em relação ao alvo e, assim, um risco reduzido às espécies não alvo.

Acredita-se que os inseticidas organofosforados e N-metilcarbamatos atuam inibindo a atividade da Acetilcolinesterase (AChE) (Crosby, D.G., 1998; Timbrell, J., 2000). Eles conseguem isso agindo como pseudo-substratos e formando um aduto covalente com o sítio ativo da Serina. Isto resulta no acúmulo de acetilcolina na sinapse, sobre-estimulação dos receptores da AChE e, por último, morte por falência respiratória. O paration e o malation transformam-se em inibidores da AChE muito mais potentes após a oxidação, em uma reação catalisada pelas monooxigenases do citocromo P450.

Carbofurano (2,3-diidro-2,2-dimetil-7-benzofuranil-N-metilcarbamato) é um inseticida nematicida do grupo dos carbamatos, que apresenta curta persistência no ambiente e pequeno deslocamento para regiões adjacentes, sendo efetivo por contato, ingestão e por ação sistêmica (Kuhr, R. J. & Dorough, H. W., 1976; ILSI BRASIL, 1995). É um inseticida muito eficiente no controle de uma ampla gama de pragas agrícolas e que atua por contato ou após ingestão (FMC, 1977). O comportamento ambiental de um agrotóxico pode ser estimado pelas suas características físico-químicas e pelos seus metabólitos ou produtos de degradação formados (FMC, 1977).

O carbofurano é um composto relativamente solúvel em água e é hidrolisado com facilidade em meio básico formando dióxido de carbono, 7- hidroxicarbofurano e metilamina. O principal metabólito do carbofurano tanto em plantas quanto por ação microbiológica é um produto de oxidação, o 3-hidroxicarbofurano, que também pode sofrer outras transformações e ser eliminado por exsudação ou sofrer conjugações (Esquema 1) (FMC, 1977). O uso na agricultura de produtos comerciais contendo carbofurano nas dosagens recomendadas fornece níveis detectáveis desses metabólitos (FMC, 1977). Johnson & Lavy (1995) observaram que em tabuleiros de arroz do Arkansas, a meia vida de carbofurano em água foi de aproximadamente 3 dias e que 3-hidroxicarbofurano estava presente em 2% das amostras de água.

O inseticida é aplicado no solo em culturas de algodão, amendoim, arroz, milho, trigo, feijão, banana, batata, café, cana-de-açúcar, cenoura, repolho, tomate, e utilizado no tratamento

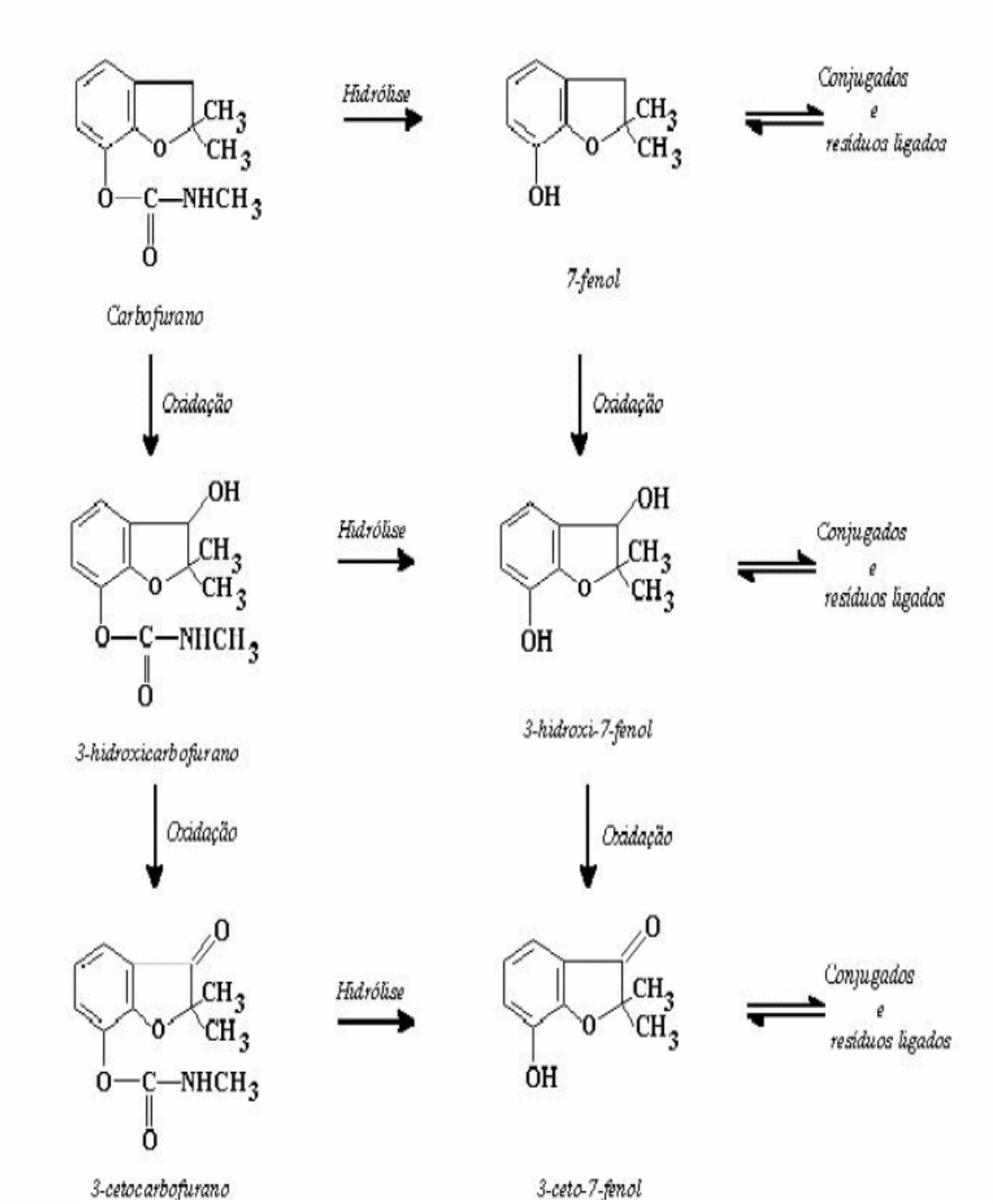
de sementes de algodão, arroz, trigo, milho, fumo e feijão, na época do plantio (ILSI BRASIL, 1995).

No ambiente, a permanência do carbofurano é controlada por processos de degradação que, dependendo do meio (solo, planta ou água), pode ser química ou biológica (FMC, 1977; Trotter ***et al.***, 1991). O carbofurano é altamente tóxico para peixes, pássaros e humanos, sendo a sua ingestão diária aceitável de até 0,002 mg/kg/peso corpóreo, o que estabelece o seu limite para um padrão de qualidade de água potável em 7mg L^{-1} (WORLD HEALTH ORGANIZATION, 1996). Embora possa ser facilmente degradado, pode induzir efeitos deletérios a espécies não alvo, antes que ocorra uma dissipação ambiental.

No ambiente, a biodegradação do carbofurano (Esquema 1) é dependente da temperatura, da umidade, do pH do solo, da biomassa disponível, assim como da atividade degradativa da mesma (Parkin, & Shelton, 1994). Aplicações periódicas de carbofurano, no mesmo solo, aumentam a atividade degradativa do composto (Felsot *et al*, 1981; Felsot, 1989), o que é devido ao maior crescimento de microrganismos capazes de utilizá-lo como fonte de carbono e nitrogênio (Charnay & Fournier, 1994).

O valor da DL50 do carbofurano, reportado para mosca doméstica (*Musca domestica*) é de 4,6 - 6,7 mg/kg pc (exposição tópica) e de 2,0 mg/kg pc para camundongos (exposição oral) (Metcalf *et al*, 1967; Metcalf *et al*, 1968; Yu *et al*, 1972). O Comitê de Peritos da FAO/OMS sobre Resíduos de Pesticidas (Joint Meeting on Pesticide Residues – JMPR) (JMPR, 1996) relatou valores de DL50 (exposição oral) de 14,4 mg/kg pc para camundongos e de 6,0 -18,0 mg/kg pc para ratos.

O carbofurano é absorvido rapidamente, independentemente da via de administração, e distribui-se nos órgãos, tecidos moles e esqueleto, sendo excretado como carbofurano nas fezes e seus metabólitos pelas vias urinária e pulmonar (Dorough, 1968; Metcalf *et al*, 1968; Shah *et al*, 1981; Ahdaya & Guthrie, 1982). Os processos de detoxificação ocorrem por hidrólise, N-, S- e O e alquilação e hidroxilação do anel fenólico. (Metcalf *et al*, 1967). Bioensaios com a mosca *Drosophila melanogaster* (Meig.) têm sido usados por alguns pesquisadores como indicador de resíduos de agrotóxicos em alimentos (Puga &Rubano, 1987; Bagdonas *et al*, 1988; Almeida & Reyes, 1999) e da presença no ambiente de substâncias químicas com potencial mutagênico (Zimmering, 1975).



Esquema 1: Degradação e Metabolismo do Pesticida Carbofurano (FMC, 1977)

2. Justificativa

Tendo em vista a crescente preocupação com as alterações provocadas no ambiente aquático resultante das diversas atividades humanas cresce também a utilização de bioindicadores e de testes de toxicidade ou bioensaios para avaliação destes impactos. Vários organismos são utilizados nestes testes, como algas, microcrustáceos, poliquetos, oligoquetos, larvas de insetos e peixes (Plate 1993). Entre os critérios para a seleção destes organismos, destacam-se um amplo conhecimento da distribuição da espécie, localização dentro da estrutura trófica, conhecimento da biologia, hábitos nutricionais e fisiologia, manutenção e cultivo em laboratório (EPA, 1989).

Muitos hyalellídeos são elos importantes na cadeia alimentar dos sistemas límnicos, além de serem facilmente cultivados em laboratório. Devido a esta característica são utilizados como bioindicadores em testes de toxicidade e bioensaios na avaliação dos impactos causados pelas diversas atividades humanas nos ambientes aquáticos (Cooper, 1956; Strong, 1972; Borowsky, 1991; Severo, 1997).

Com relação aos métodos de cultivo de Amphipoda, existem técnicas padronizadas apenas para *Hyalella azteca*, visto que a história de vida desta espécie é bastante conhecida. No entanto, para espécies típicas da América do Sul e, especialmente do Brasil, os estudos nesse sentido são inexistentes.

O Centro Tecnológico de Saneamento Básico (CETESB), órgão responsável pelo controle e prevenção da poluição em São Paulo, cultiva e utiliza *Hyalella azteca* em testes de toxicidade. Estudos moleculares desenvolvidos por Duan ***et al.*** (1997) e González & Watling (2002), constataram que esta espécie é resultado da miscigenação de espécies distintas. O seu uso em testes de toxicidade realizados no Brasil deve-se provavelmente a ausência de conhecimento sobre a bioecologia das espécies nativas.

Esta pesquisa visou, portanto, contribuir para o conhecimento do ciclo de vida e do metabolismo de *Hyalella curvispina*, *Hyalella pleoacuta* e *Hyalella castroi* em campo frente a variações sazonais; de *Hyalella curvispina* e *Hyalella pleoacuta* em cultivo experimental frente a diferentes dietas e frente a testes de toxicidade aguda com diferentes concentrações de carbofurano. Cabe salientar que o pesticida utilizado foi escolhido, porque é utilizado em lavouras nas regiões de planície e de planalto do Rio Grande do Sul, onde são encontradas as espécies supracitadas. Pretende-se, portanto, fornecer subsídios de cunho bioecológico e

fisiológico que permitam o uso de espécies nativas como modelo experimental em estudos de toxicologia e monitoramento ambiental.

3. Estrutura do Trabalho

O presente trabalho foi dividido em quatro artigos:

1. Seasonal Variations in the Biochemical Composition and Lipoperoxidation of *Hyalella curvispina* (Crustacea, Amphipoda, Dogielinotidae) este artigo está *in press* na Comparative Biochemistry and Physiology A – Molecular and Integrative Physiology, que possui conceito A na CAPES e índice de impacto 1.351 em 2005.
2. Seasonal Variations of the Energy Metabolism of Two Sympatric Species of *Hyalella* (Crustacea, Amphipoda, Dogielinotidae) in the Southern Brazilian Highlands. Este artigo está submetido à Comparative Biochemistry and Physiology A – Molecular and Integrative Physiology, que possui conceito A na CAPES e índice de impacto 1.351 em 2005.
3. Variations in the Biochemical Composition and Lipid Peroxidation of *Hyalella pleoacuta* and *Hyalella curvispina* (Crustacea, Amphipoda, Dogielinotidae) Maintained on Different Diets in the Laboratory este artigo está submetido à Aquaculture Nutrition, que possui conceito A na CAPES e índice de impacto 1.441 em 2005.
4. Carbofuran-Induced Alterations in Biochemical Composition, Lipoperoxidation and Na^+/K^+ATPase activity of *Hyalella pleoacuta* and *Hyalella curvispina* (Crustacea, Amphipoda, Dogielinotidae) in Bioassays este artigo está submetido à Aquatic Toxicology, que possui conceito A na CAPES e índice de impacto 2.719 em 2005.

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Seasonal Variations in the Biochemical Composition and Lipoperoxidation of *Hyalella curvispina* (Crustacea, Amphipoda, Dogielinotidae)

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ABSTRACT

We assessed the effect of seasonal variations on the biochemical composition and lipoperoxidation an amphipod crustacean, *Hyalella curvispina*, which inhabits the coastal plain of the state of Rio Grande do Sul, and correlated this with information on the biology of the species. The animals were collected monthly from September 2003 to August 2005, in Gentil Lagoon, Tramandaí, Rio Grande do Sul, Brazil. Glycogen levels, total proteins, total lipids, triglycerides, and lipoperoxidation were measured for each sex separately. The results suggest that these animals have seasonal storage and degradation of the energy substrates investigated. During summer, glycogen, total lipids, and triglycerides were stored, and were utilized especially during autumn and winter. Proteins were utilized differently in males and females. Females stored proteins especially in spring and used them in summer; whereas males stored proteins especially in spring and used them in winter. The levels of lipoperoxidation during the year differed between females and males. These variations may be related to biotic factors (e.g., reproduction period and others) and to abiotic factors (e.g., water temperature, salinity and others).

Keyword: Crustacea, Amphipod, Energy Metabolism, Seasonality

INTRODUCTION

Members of the genus *Hyalella* are common in the Nearctic and Neotropical regions, with 45 described species (Gonzáles & Watling, 2001). They are found in a variety of freshwater habitats, such as permanent reservoirs, lakes, impoundments, and streams, and often cling to the vegetation, swim in the water, or burrow in the sediment, where they are important members of the benthic fauna (Kruschwitz, 1978; Wellborn, 1995; Grosso & Peralta, 1999).

Amphipods are important members of their communities: they are detritivorous, carnivorous and herbivorous, and serve as food for many other organisms (Schmitt, 1965; Muskó, 1993; Pilgrim & Burt, 1993; Casset ***et al.***, 2001). Therefore amphipods constitute important links in the food web, serving to transfer energy from algae and macrophytes to higher-level consumers (Moore, 1975; Vassilenko, 1991; Pilgrim & Burt, 1993).

Studies of the intermediate metabolism in crustaceans have revealed great inter- and intra-species variability, which has made it difficult to establish a standard metabolic profile. Without considering differences among the biochemical methods used by various investigators, this variability can be attributed to multiple factors, such as habitat (terrestrial, marine, estuarine, or freshwater), period of life cycle, sexual maturity (especially in females), nutritional state, available food, and seasonality, because all these factors lead to different patterns of metabolic responses (Chang & O'Connor, 1983; Kucharski & Da Silva, 1991a and b; Oliveira ***et al.***, 2003; Rosa & Nunes, 2003a; Ferreira ***et al.***, 2005).

Biological responses to seasonal variations have a profound effect on the biochemical composition of the organisms, for instance in the tissues of the hepatopancreas and gonads (Ferreira ***et al.***, 2005; Oliveira ***et al.***, 2006). Biochemical changes in the dynamics and levels of total lipids during the reproductive cycle have been observed in some species of brachyurans

(Pillay & Nair, 1973) and in other decapods (Read & Caulton, 1980; Castille & Lawrence, 1989; Rosa & Nunes, 2003b).

Lipid concentrations are high in crustaceans, although they have no differentiated adipose tissue but store lipids mainly in muscle tissue and in the hepatopancreas (O'Connor & Gilbert, 1968; Chang & O'Connor, 1983; Herreid & Full, 1988; Kucharski & Da Silva, 1991a). During periods of high energy demand, such as molting and gametogenesis, large amounts of lipids are mobilized, especially from the hepatopancreas (Kucharski & Da Silva, 1991a; Rosa & Nunes, 2003a).

Copepods living in superficial water of temperate and abyssal oceans, and freshwater copepods and cladocerans have triacylglycerol as the main form of energy reserve. These compounds can reach up to 40% of body biomass. These reserves can be used by the adults to satisfy their metabolic needs during periods of food limitation (Goulden & Henry, 1988). The energy reserves of freshwater species, such as *Daphnia*, are also mainly triacylglycerols (Goulden & Horning, 1980). According to Goulden & Henry (1988), the energy reserves of cladocerans seem to be used in two different ways: a) the adults use them for their own metabolic needs; or b) the reserves are transferred to the offspring through eggs and are used by the young animals in their initial development.

In crustaceans, glycogen is stored mainly in the muscles, hepatopancreas, gills, and hemocytes; however, the storage locations vary among different species (Parvathy, 1971; Johnston & Davies, 1972; Herreid & Full, 1988). The stored glycogen is used in the processes of change, hypoxia and/or anoxia, osmoregulation, growth, different periods during reproduction, and during periods of starvation (Chang & O'Connor, 1983; Kucharski & Da Silva, 1991a; Rosa & Nunes, 2003b; Oliveira ***et al.***, 2001 and 2004).

The muscle is apparently the main protein-storage location in crustaceans. Proteins are a structural, functional and energy constituent of tissues and play an important role in spawning, fertilization and normal development of embryo in decapods (Garcia-Guerrero ***et al.***, 2003a; Rosa and Nunes 2003a and b; Rodriguez-González ***et al.***, 2006). Other studies have demonstrated variation in protein content during periods of starvation (Yehezkel ***et al.*** 2000). Some studies have reported variation in protein content during ovarian development in crustaceans. These variations may result from an increase in the biosynthesis of various proteins, including hormones, enzymes, and lipoproteins involved with gonadal maturation (Yehezkel ***et al.***, 2000; Rosa & Nunes, 2003b).

Malondialdehyde, a breakdown product of lipid endoperoxides, is an expression of lipid peroxidation and has been used with success in aquatic invertebrates as a general indicator of toxicant stress derived from various types of contamination (Zwart ***et al.***, 1999; Livingstone, 2001; Wilhelm Filho ***et al.***, 2001; Timofeyev ***et al.***, 2006). Neuparth ***et al.,*** 2005 described that in *Gammarus locusta* maintained with sediments have high levels of organic matter content present higher levels of lipoperoxidation. Effectively, some authors agree that endogenous variables like nutritional status, age, sex, growth and reproduction influence the peroxidation status of organisms (Viarengo ***et al.,*** 1991; Correia, 2002; Correia ***et al.,*** 2003).

The objective of the present work was to characterize the seasonal variations of the biochemical composition (total lipids, triglycerides, total proteins, and glycogen) and of the levels of lipoperoxidation (TBARS) in *Hyalella curvispina*. We also investigated the possible correlations between physiological, biological, and ecological parameters in these animals.

MATERIAL AND METHODS

The animals were cared for in accordance with guidelines such as the *Guide for the Care and Use of Laboratory Animal* (1996, published by National Academy Press, 2101 Constitution

Ave. NW, Washington, DC 20055, USA). The animal were used with the permission of the Ethic Committee of the Pontifícia Universidade Católica do Rio Grande do Sul (License 0004/03).

Collection of Hyallela curvispina

In order to establish the profile of seasonal variation, the collections were initiated in September 2003 and extended until August of 2005. The seasons were defined as Spring (September, October and November), Summer (December, January and February), Autumn (March, April and May) and Winter (June, July and August). The adult animals (males 0.80± 0.002 mm and females 0.64 ± 0.001 mm of cephalic capsule length) were collected monthly, always at the same time of day during two years. Individuals of *Hyalella curvispina* were collected in Gentil Lagoon (29º56'30"S, 50º07'50"W), in Tramandaí, Rio Grande do Sul, Brazil. The animals were collected by means of fish traps and bottom grabs.

The animals were transported on ice in insulated containers to the Laboratory of Conservation Physiology of PUCRS (Pontifícia Universidade Católica do Rio Grande do Sul). There, they were separated by sex, weighed on an electronic balance (± 0.001), and then stored frozen at -80°C until they were used to determine the biochemical parameters.

Biochemical Analyses

The metabolic determinations were made from homogenized monthly pools of five animals, the parameters were determined in quadruplicate, and the mean of the season was doing with results of each month. Three pools of males and females each were used monthly for determination of (1) glycogen and total proteins, (2) of total lipids and triglycerides, and (3) of lipoperoxidation levels.

a. Glycogen was extracted by the Van Handel (1965) method, and quantified as glucose after acid hydrolysis (HCl) and neutralization (Na_2CO_3), following the method of Geary ***et al.*** (1981).

Glucose was quantified using a Bioclin kit (God-clin glucose) (glucose oxidase). The results were expressed in mmol/g.

b. Proteins were quantified as described by Lowry ***et al.***, (1951), with bovine albumen (Sigma Chemical Co., St. Louis, MO) as the standard. The results were expressed in mg/ml of homogenate.

c. Total lipids and triglycerides were extracted by the method of chloroform: methanol (2:1) (Folch ***et al.***, 1957). Lipids were determined by the sulfophosphovanillin method (Frings & Dunn, 1970). Triglycerides were determined by means of a Labtest kit (Triglycerides GPO-ANA). The results were expressed in mg/g of animal.

d. Lipoperoxidation levels were quantified through the method of Buege & Aust (1978), by measuring thiobarbituric acid reactive substances (TBARS), using the extraction method of Llesuy ***et al.*** (1985). The results were expressed in nmols of TBA/mg of protein.

Statistical Analyses

The results were expressed as mean ± standard error. The seasonal results were evaluated by one-way ANOVA and a post-hoc Bonferroni test. The curves obtained for the different sexes and seasons were compared by two-way ANOVA. All the metabolic parameters passed the Levene test for homogeneity and the Kolmogorov-Smirnov test for normality. The SPSS program (Statistical Package for the Social Sciences) for Windows (11.5) was used; differences were considered significant at $p<0.05$.

RESULTS

Figure 1 shows the seasonal variation in glycogen content in females and males. In both sexes, glycogen levels were higher in summer, after decreasing 3.5 times and 5.4 times in autumn

in females and males respectively. There was no significant difference between glycogen levels in males and females ($p>0.05$).

During the winter, total protein levels in females increased (Figure 2), and then decreased significantly (2.6 times) in the rest of the seasons. In males, the protein content was highest in spring and decreased gradually in other seasons until reaching the lowest values in winter ($p<0.05$). There was a significant difference between total protein content in males and females ($p<0.05$).

The annual concentrations of total lipids in males and females are shown in Figure 3. Both sexes contained higher amounts of total lipids during the summer. Females and males showed decreases of 22 and 10 times, respectively, in lipid concentration during autumn, and then these values increased gradually during winter and spring. There was no difference between total lipid levels in males and females ($p>0.05$).

Figure 4 shows the seasonal fluctuations in triglyceride levels in both sexes. In males, triglycerides were generally 23 times lower in autumn an in summer. A similar yearly cycle occurred in females, although triglyceride levels were 30 times lower in autumn. There was no significant difference in triglyceride levels between males and females ($p>0.05$).

In females, the values of lipoperoxidation were highest in summer (Figure 5). In contrast, males showed two peaks of lipoperoxidation, during winter and summer. There was a significant difference in lipoperoxidation levels between males and females ($p<0.05$).

Seasonal variations in triglycerides proportions ranged from 8.76% to 60.04% of the total lipid content in females, and from 7.63% to 17.52% in males. Females showed a peak of the triglycerides proportion during summer and autumn and intense decrease in winter and spring, already in males this proportion is maintained constant (Figure 6).

Table 1 shows the seasonal variation in the oxygen content, pH, water temperature, air temperature, and salinity in the natural environment. The values of water and air temperature were highest in summer and lower in winter, and salinity levels were highest in autumn. The pH and oxygen content not showed variations ($p>0.05$).

DISCUSSION

In this study, the levels of total glycogen of females and males of *H. curvispina* ranged from 2.86 ± 0.63 to 17.37 ± 2.99 mmol/g. These values are similar to those obtained for the anomuran *Aegla platensis* and the crabs *Chasmagnathus granulata* maintained in laboratory conditions on a high-protein diet. The values are different from those observed in the same species maintained on a carbohydrate-rich diet (Kucharski & Da Silva, 1991a; Ferreira ***et al.***, 2005). We can therefore infer that the natural diet of *H. curvispina* may have a high protein and low carbohydrate content. Other amphipods, such as *Hyalella azteca*, are detritivorous and herbivorous (Wen, 1992). Casset ***et al.*** (2001) studying *Hyalella curvispina* in a river of the Argentina showed that these amphipod is a herbivorous, where the principal food is phitobenthos and eventual food is sediment. Although, work developed by Dutra ***et al.,*** (2006) showed that *H. curvispina* are omnivorous and detritivorous, feeding algae and bacteria associated with sediments and macrophytes, and detritus present in sediment.

In summer, the highest concentrations of glycogen, total lipids, and triglycerides were found in both females and males, and were significantly higher than those found in the autumn, winter, and spring. It is possible that in summer, food is more plentiful in the environment and this species increased feeding and motor activity. Similar results were found for other species of crustaceans, including amphipods (Hervant ***et al.***, 2001; Poretti ***et al.***, 2002). The accumulation of

energy reserves in species dependent upon unstable food resources has been reported by several authors (Lee ***et al.***, 1971; Griffiths, 1977; Oliveira ***et al.***, 2003; Rosa & Nunes, 2003b).

These results of activity and feeding may be related to increase of the levels of lipoperoxidation in males and females. Díaz-Muñoz ***et al.***, (1985) showed in rat cerebral cortex that the glutathione enzymatic activity decreased during the subjective night when cerebral cortex lipid peroxidation increased because of feeding and motor activity rhythmus. In the study of the Fanjul-Moles ***et al.***, (2003) lipid peroxidation of the hepatopâncreas was not determined in crayfish *Procambarus clarkii* , but this animal is a nocturnal species, and respiratory rhythmus showed produced a metabolic burst in the dark period, coincident with increasing levels of oxidized glutathione and the activation of other protective antioxidant scavengers.

During autumn, the levels of glycogen, total lipids, and triglycerides all decreased significantly in both males and females. These decreases may be related to the increased salinity and lower water volume and temperature during this period (Table 1). These changes in abiotic factors increase the need for energy. Furthermore, the exploratory activity of *Hyalella curvispina* is reduced, and this is reflected in the difficulty of collecting them at this time of year.

The reduction in lipid and triglyceride levels in autumn may also be related to the beginning of the reproductive period, when lipid reserves may be used in gametogenesis. In females, the sharp decrease in lipid reserves, approximately 23-fold, may be related to vitellogenesis; moreover, the proportion of triglycerides in total lipids was higher than in males in summer and autumn. This hypothesis is also supported by the fact that crustacean vitellin is a glycolipoprotein containing LDL and HDL fractions, and these fractions contain different proportions of proteins, phospholipids, triglycerides, and carotenoids (Vinagre ***et al.***, 2006). Also in favor of this hypothesis is the occurrence of ovigerous females in winter and spring, where the reproductive period to extend to winter until spring (Santos ***et al.***, 2005).

Clarke ***et al.***, (1985) study the correlation of the lipids content and reproductive conditions in Gammaridae and showed the straiten correlation between lipids accumulation and ovaric maturation.

The seasonal fluctuations in the levels of proteins in females and males were different during the year. In females, proteins decreased in summer and appeared to be the principal reserve for synthesis of ATP at this season. In males, the sharp decrease in protein levels in winter may be related to reproduction. In this amphipod, the males clasp the females for 3 to 5 days during copulation. This hypothesis is reinforced by the peak in lipoperoxidation levels observed in winter.

Robinson and Doyle (1985) showed that in *Gammarus lawrencianus*, the males fed less while they were in precopula, but the feeding rates of females were unaffected. Barclay ***et al.*** (1983), working with *Penaeus esculentus*, showed that during the period of starvation the abdominal muscle makes the largest contribution of protein to energy metabolism, where small changes in this tissue are sufficient to make a substantial contribution to the overall animal maintenance. The same response was observed by Dall (1981), working with *Nephrops norvegicus*, during seasonal variation of the feeding activity, where a decrease in feeding rates may be linked with a decrease in protein content.

The reproductive period in this specie remain of the winter until spring, with peak of the ovigerous females in winter. Females showed a peak of the triglycerides proportion during summer and autumn and intense decrease in winter and spring, already in males this proportion is maintained constant. This response of the triglycerides proportions may be related to reproductive events in females.

In females, we observed a peak of lipoperoxidation in summer. The results may be related with the behavior the carry the juveniles in the marsupium for 12 days after the eggs hatch,

during spring, and elevation of water temperature during summer. The males showed two peaks of lipoperoxidation, one during the period of precopulation and copulation (winter), and the other after the reproductive period during elevetion of the temperature (summer).

In *Perna perna*, the thiobarbithuric acid reactive substances content observed in May (autumn) were approximately double those found in the rest of the year, despite the increase seen in the enzymatic activities (superoxide dismutase and catalase). During May, *P. perna* usually display a maximal annual gamete emission. Their gonads have a higher lipid and carbohydrate mobilization and protein synthesis, and need a longer period to recover from this high metabolic activity (Wilhelm-Filho ***et al.***, 2001). The same pattern of the response was observed to *Bathymodiolus azoricus*, vent mussel, for Company ***et al.*** (2006).

The increased of the levels of lipoperoxidation in months of the summer may be related too to increase of light in environmental. Durán-Lizarraga ***et al.***, (2001) and Fanjul-Moles ***et al.***, (2003) observed in crayfish that light (12 and 20 hours of high irradiance) induced oxidative stress, as well as, changed in the antioxidant mechanisms that control and prevent the formation of reactive oxygen species.

In conclusion, these findings suggest that in *Hyalella curvispina*, the lipid reserves seem to be an important source of energy used during reproduction, in both males and females; whereas glycogen and proteins may be used during periods of intense activity or intense variation in environmental conditions. The levels of lipoperoxidation may be related to reproductive behavior, motor and feeding activity, and variation of the photoperiod.

ACKNOWLEDGEMENTS

This work was supported by grants from the Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS – process n° 03/0681.9 – ARD; process n° 05501490) and the Conselho Nacional de Desenvolvimento Tecnológico (CNPq – process n° 300075/2003-0; process n° 131721/2005-

4; process n° 103334/2005-0). The Pontifícia Universidade Católica do Rio Grande do Sul supported this work. The experiments were performed according to current Brazilian laws.

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FIGURE CAPTIONS:

Figure 1: Seasonal variations of glycogen in males and females of *Hyalella curvispina*. Columns represent the mean, and bars the standard error of the mean. The number of animals represented by each point varied between 15 and 30. Results are expressed in mmol/g. The same letter represents significant difference between the seasons.

Figure 2: Seasonal variations of total proteins in males and females of *Hyalella curvispina*. Columns represent the mean, and bars the standard error of the mean. The number of animals represented by each point varied between 15 and 30. Results are expressed in mg/ml of homogenate. The same letter represents significant difference between the seasons. & significant difference between sexes.

Figure 3: Seasonal variations of total lipids in males and females of *Hyalella curvispina*. Columns represent the mean, and bars the standard error of the mean. The number of animals represented by each point varied between 15 and 30. Results are expressed in mg/g of animal. The same letter represents significant difference between the seasons.

Figure 4: Seasonal variations of triglycerides in males and females of *Hyalella curvispina*. Columns represent the mean, and bars the standard error of the mean. The number of animals represented by each point varied between 15 and 30. Results are expressed in mg/g of animal. The same letter represents significant difference between the seasons.

Figure 5: Seasonal levels of lipoperoxidation in males and females of *Hyalella curvispina*. Columns represent the mean, and bars the standard error of the mean. The number of animals represented by each point varied between 15 and 30. Results are expressed in nmol/mg of protein. The same letter represents significant difference between the seasons. & significant difference between sexes.

Figure 6: Percentage of triglycerides in relation to the amount of total lipids in males and females. Results are expressed in %. The same letter represents significant difference between the seasons. & significant difference between sexes.

TABLE LEGEND

Table 1: Seasonal variations of the oxygen content (mg/L), pH, water temperature (°C), air temperature (°C) and salinity ($^{o}/_{oo}$) in the lagoon. Results represent the mean $\pm$ standard error of the mean. The same letter represents significant difference between the seasons.

Figure 1

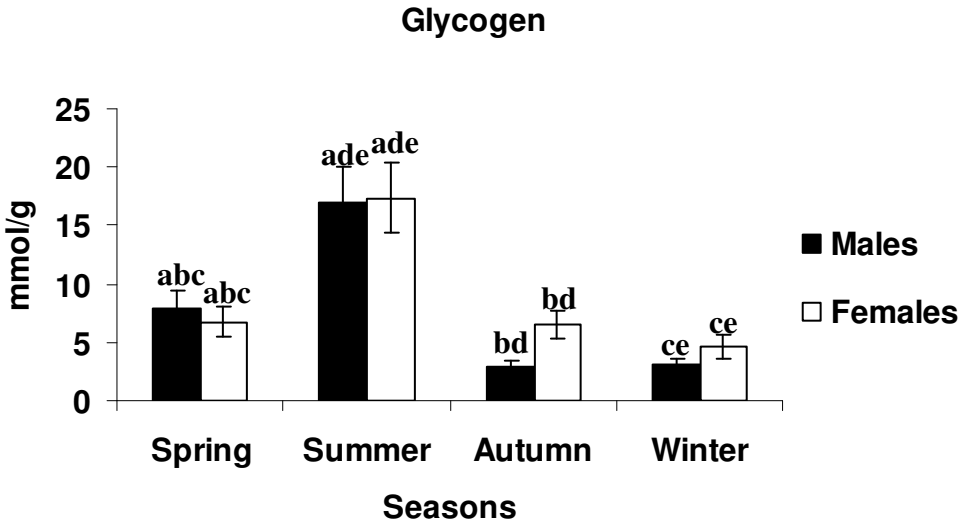


Figure 2

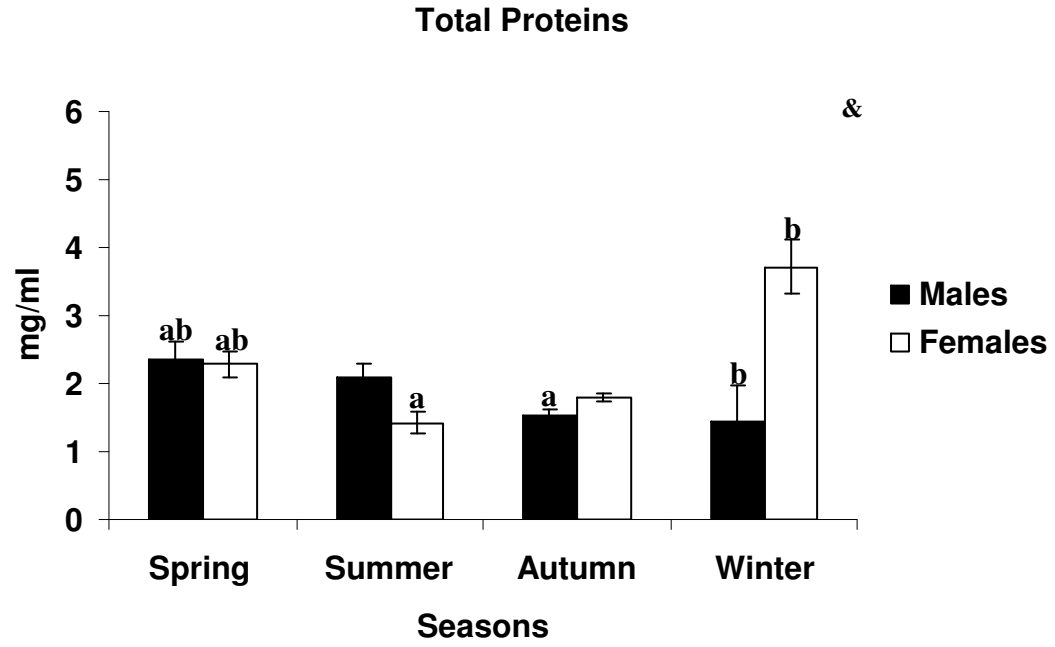


Figure 3

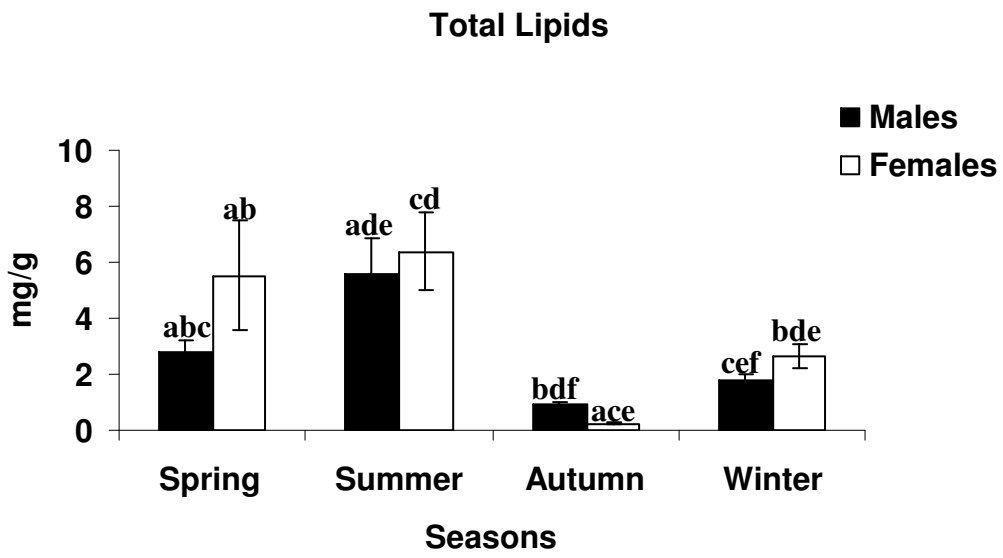


Figure 4

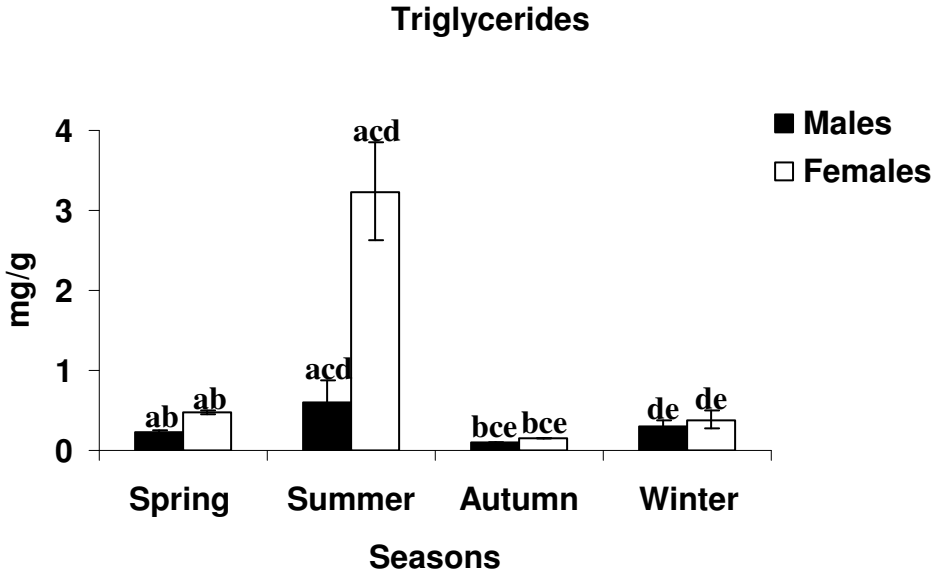
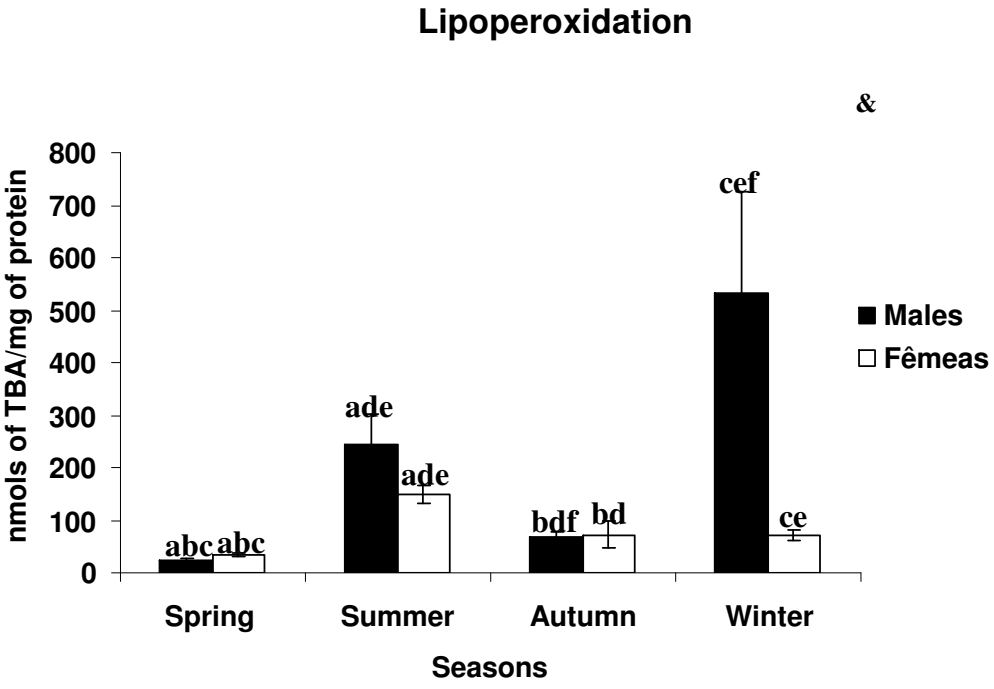


Figure 5


Lipoperoxidation
&
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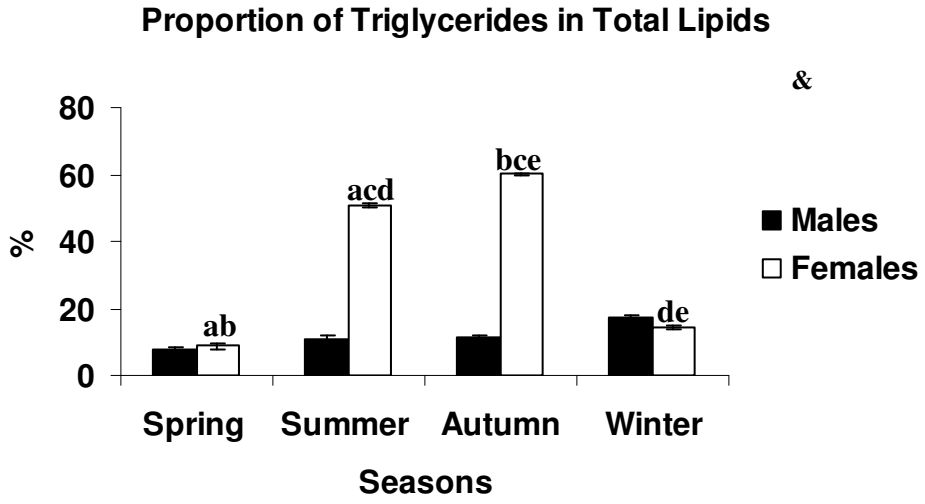
Autumn
Winter
Seasons


Proportion of Triglycerides in Total Lipids
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Spring
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Autumn
Winter
Seasons

Table 1

Abiotic Factors	**Spring**	**Summer**	**Autumn**	**Winter**
Oxygen content (mg/L)	7.18 ± 0.19	8.43 ± 0.05	9.75 ± 0.49	9.75 ± 0.28
pH	7.13 ± 0.04	8.53 ± 0.21	7.75 ± 0.33	7.03 ± 0.03
Water temperature (°C)	22.3 ± 0.25^{ab}	27.6 ± 0.24^{acd}	23.3 ± 0.25^{ce}	18.0 ± 0.35^{bde}
Air temperature (°C)	18.4 ± 1.68^{ab}	24.1 ± 0.52^{acd}	20.5 ± 2.27^{ce}	15.3 ± 0.75^{bde}
Salinity ($^{o}/_{oo}$)	0.00 ± 0.00^{a}	1.63 ± 0.13^{ac}	2.75 ± 0.85^{bd}	0.00 ± 0.00^{cd}

Seasonal Variations of the Energy Metabolism of Two Sympatric Species of *Hyalella* (Crustacea, Amphipoda, Dogielinotidae) in the Southern Brazilian Highlands

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ABSTRACT

Aquatic organisms exist in a constantly fluctuating habit, with changes in photoperiod, temperature, pH, dissolved organic content, dissolved oxygen and food supply. Organisms must alter past of their physiological and biochemical processes in order to cope with these changes. We compared the effect of seasonal variations in factors related to energy metabolism of two species of sympatric amphipods, *Hyalella pleoacuta* and *Hyalella castroi*. The animals were collected monthly from April 2004 through March 2006. Contents of glycogen, proteins, lipids, triglycerides and the levels of lipoperoxidation were determined in males and females throughout the year by used spectrophotometric methods. Observations revealed significant seasonal differences in biochemical composition, as well as differences among sexes and species. Environmental conditions (e.g., trophic conditions) and reproduction are supposed to be the main processes influencing the seasonal patterns of variation in biochemical composition. Both species of *Hyalella* show ecological and behavioral differences, especially by resources such as food, space and seasonal variations of the energy metabolism, which might facilitate coexistence at the environmental natural.

Keyword: Crustacea, Amphipod, *Hyalella pleoacuta*, *Hyalella castroi*, Energy Metabolism, Lipoperoxidation, Seasonality, Sympatric

INTRODUCTION

Members of the genus *Hyalella* are common in the Nearctic and Neotropical regions, with 45 described species (Gonzáles and Watling 2001). They are found in a variety of freshwater habitats, such as permanent reservoirs, lakes, ponds and streams, and often clinging to the vegetation, swimming in the water, or burrowing in the sediment (Kruschwitz 1978; Wellborn 1995; Grosso and Peralta 1999). Amphipods are important members of their communities; they are detritivorous and herbivorous, and serve as food for many other organisms (Schmitt 1965; Muskó 1993; Pilgrim and Burt 1993). Therefore amphipods constitute important links in the food web, serving to transfer energy from algae and macrophytes to higher-level consumers (Moore 1975; Vassilenko 1991; Pilgrim and Burt 1993).

Aquatic organisms exist in a constantly fluctuating habit, with changes in photoperiod, temperature, pH, dissolved organic content, dissolved oxygen and food supply (Reid and Wood 1976). Organisms must alter past of their physiological and biochemical processes in order to cope with these changes (Hochachka and Somero 1984). Nutritional deprivation is a natural part of the life cycle of many aquatic organisms, as the result of winter torpor, seasonal elimination of a food source or behavioral modifications during mating and spawning (Schirf ***et al.*** 1987).

Studies of the intermediate metabolism in crustaceans have revealed great inter- and intra-species variability, which has made it difficult to establish a standard metabolic profile. Without considering differences among the biochemical methods used by various investigators, this variability can be attributed to multiple factors, such as habitat (terrestrial, marine, estuarine, or freshwater), period of life cycle, sexual maturity (especially in females), nutritional state, available food, and seasonality, because all these factors lead to different patterns of metabolic responses (Chang and O'Connor 1983; Kucharski and Da Silva 1991a and b; Oliveira ***et al.*** 2003; Rosa and Nunes 2003b; Ferreira ***et al.*** 2005).

Crustaceans contain high concentrations of lipids, although they have no differentiated adipose tissue but store lipids mainly in muscle tissue and the hepatopancreas (O'Connor and Gilbert 1968; Chang and O'Connor 1983; Herreid and Full 1988; Kucharski and Da Silva 1991a). During periods of high energy demand, such as molting and gametogenesis, large amounts of lipids are mobilized, especially from the hepatopancreas (Kucharski and Da Silva 1991a; Quigley ***et al.*** 1992; Rosa and Nunes 2003b; Oliveira ***et al.*** 2006).

In crustaceans, glycogen is stored mainly in the muscles, hepatopancreas, gills, and hemocytes; however, the storage locations vary among different species (Parvathy 1971; Johnston and Davies 1972; Herreid and Full 1988). The stored glycogen is used in the processes of change, hypoxia and/or anoxia, osmoregulation, growth, different periods during reproduction, and during periods of starvation (Chang and O'Connor 1983; Kucharski and Da Silva 1991a and b; Rosa and Nunes 2003a; Oliveira ***et al.*** 2001 and 2004).

Some studies have reported variations in protein content during ovarian development in crustaceans. These variations may result from an increase in the biosynthesis of various proteins, including hormones, enzymes, and lipoproteins involved in gonadal maturation (Rosa and Nunes 2003a; Yehezkel ***et al.*** 2000). The muscle is apparently the main protein-storage location in crustaceans. Proteins are a structural, functional and energy constituent of tissues and play an important role in spawning, fertilization and normal development of embryo in decapods (Garcia-Guerrero ***et al.***, 2003; Rosa and Nunes 2003a and b; Rodriguez-González ***et al.***, 2006). Other studies have demonstrated variation in protein content during periods of starvation (Yehezkel ***et al.*** 2000).

Malondialdehyde, a breakdown product of lipid endoperoxides, is an expression of lipid peroxidation and has been used with success in aquatic invertebrates as a general indicator of toxicant stress derived from various types of contamination (Zwart ***et al.***, 1999; Livingstone, 2001; Wilhelm Filho *et al.*, 2001; Timofeyev *et al*., 2006). Neuparth ***et al.,*** 2005 described that in *Gammarus locusta* maintained with sediments have high levels of organic matter content present higher levels of lipoperoxidation. Effectively, some authors agree that endogenous variables like nutritional status, age, sex, growth and reproduction influence the peroxidation status of organisms (Viarengo ***et al.,*** 1991; Correia, 2002; Correia ***et al.,*** 2003).

The aim of the present work was to characterize the seasonal variations in the intermediate metabolism (total lipids, triglycerides, proteins, and glycogen) and in the levels of lipoperoxidation in two sympatric species of hyalellids in the natural environment. We also investigated the possible relationships between biochemical, biological and ecological parameters to comprehend the sympatric relation in these animals.

MATERIAL AND METHODS

The animals were cared for in accordance with guidelines such as the Guide for the Care and Use of Laboratory Animal (1996, published by National Academy Press, 2101 Constitution

Ave. NW, Washington, DC 20055, USA). The animal were used with the permission of the Ethic Committee of the Pontifícia Universidade Católica do Rio Grande do Sul (License 0004/03).

Collection of *H. pleoacuta* and *H. castroi*

In order to establish the profile of seasonal variation, the collections were initiated in April 2004 and continued through March 2006. Thirty individuals of each species (fifteen males and fifteen females), *H. pleoacuta* (Gonzáles, Bond-Buckup and Araújo 2006) and *H. castroi* (Gonzáles, Bond-Buckup and Araújo 2006) were collected monthly, always at the same time of day (1 pm until 3 pm) in São José dos Ausentes Municipality (28°47'00"S – 49°50'53"W), Rio Grande do Sul, Brazil. The amphipods were collected by means of fish traps and bottom grabs.

The amphipods were transported on ice in insulated containers to the Laboratory of Conservation Physiology of the Pontifícia Universidade Católica do Rio Grande do Sul. They were separated by sex, weighed on an electronic balance (±0.001g), and then stored frozen at -80°C until they were used to determine the biochemical parameters.

In order to characterize the habitat and its possible seasonal variations, the following abiotic parameters were measured: dissolved oxygen, pH, water temperature, and air temperature. Dissolved oxygen was measured with aid of a portable term-oxymeter (OXI 330/SET-WTW), pH with a portable pHmeter, and water temperature with a thermometer of internal scale.

Biochemical Analyses

Metabolic determination for *H. pleoacuta* and *H. castroi* was done in total homogenates of three pools of five males and five females monthly. One pool of each species, *H. pleoacuta* and *H. castroi*, was used for determination of glycogen and proteins, the second pool for quantification of lipids and triglycerides, and the third pool for quantification of lipoperoxidation levels. Metabolic parameters were determined in quadruplicate by used spectrophotometric methods.

a. Glycogen was extracted from tissues following the method described by Van Handel 1965, and glycogen levels in the animals were determined as glucose equivalent, 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 mmol/g of animal.

b. Proteins were quantified as described by Lowry ***et al.***, 1951, with bovine albumin (Sigma Co.) as the standard. Results are expressed in mg/ml of the total homogenate.

c. 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 (Meyer and Walter 1981). This method consists of oxidizing cellular lipids to small fragments after chemical digestion with hot concentrated sulfuric acid. After the addition of a solution of vanillin and phosphoric acid, a red complex is formed witch is measured with spectrophometer (530nm). Triglycerides were measured by the reactions of lipase, glycerokinase, 1-P-glycerol oxidase, and peroxidase enzymes (Biodiagnostic Kit / GPO Trinder). Results are expressed as mg/g of animals.

d. Lipoperoxidation levels were quantified by the method of Buege and Aust (1978) by measuring reactive substances to Thiobarbituric Acid (TBA-RS), using the extraction method of Llesuy ***et al.*** (1985). Results are expressed in nmol of TBA-RS/mg of protein.

Statistical Analysis

The results are expressed as mean ± standard error. For statistical analysis of the seasonal variations, a one-way ANOVA test was used, followed by a Bonferroni test. For comparisons between different species and sexes, a two-way ANOVA was used. All the metabolic parameters were homogeneous (Levene test), and were normally distributed (Kolmogorov-Smirnov test). 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:

Table 1 shows the seasonal variation in dissolved oxygen, pH, water temperature, and air temperature at the collection locality. Mean dissolved oxygen varied between 4.88 ± 0.65 mg/L (autumn) and 7.24 ± 0.07 mg/L (winter). Water temperature was warmest in summer (19.80 ± 0.59°C) and coolest in winter (11.03 ± 1.18°C). pH remained constant during the year.

Table 2 shows the seasonal variation in glycogen content in females and males in both species. In females of *H. pleoacuta*, glycogen levels were highest in autumn, and then decreased until reaching values approximately 10 times lower in spring; in males, glycogen levels were highest in autumn, decreasing in winter and then remaining steady until the end of summer. There was no significant difference between glycogen levels in females and males of *H. pleoacuta* during the year.

In females of *H. castroi*, glycogen levels were highest in winter, decreasing to approximately 7 times lower in autumn; in males, glycogen levels were highest in winter, decreasing in the following seasons until reaching minimum values in summer (20 times lower). There was a significant difference in the glycogen levels in females and males during the year.

Protein concentrations (Table 3) were high in all seasons in males and females of *H. pleoacuta*; except in autumn when protein levels decreased 50% in both sexes. There was a significant difference between total protein levels in females and males during the year.

During spring and autumn, the levels of total protein in females of *H. castroi* increased; and then decreased significantly in summer and winter. In males, the protein content was highest in autumn and decreased in spring (1.9 times). There was a significant difference between total protein content in females and males during the year.

The annual concentrations of total lipids in males and females in both species are shown in Table 4. Both sexes of *H. pleoacuta* contained highest amounts of total lipids during autumn, and minimum amounts in summer. There was a significant difference between total lipid levels in females and males during the year.

Both sexes of *H. castroi* contained higher amounts of total lipids during spring; females and males showed decreases of 13 and 27 times, respectively, in lipid concentrations during summer, and then these values increased during autumn and winter in females, whereas in males, lipid levels continued to decrease until winter. There was a significant difference between total lipid content in females and males during the year.

Table 5 shows the seasonal fluctuations in triglyceride levels in both sexes and species. The highest values of triglycerides in females of *H. pleoacuta* were found in spring, and then declined until autumn (2.1 times), rising somewhat in winter. In males, the highest values were found in winter, declined by approximately 3.3 times in spring, and then remained steady at this low level in the other seasons. There was a significant difference between triglyceride levels in females and males.

In females of *H. castroi*, the highest concentrations of triglycerides were found in spring, and then gradually declined until autumn (3 times). In males, the highest concentrations of triglycerides were found in summer, with a reduction of 3.2 times in autumn; these levels remained constant in the other seasons. There was a significant difference between triglyceride levels in females and males.

When we compared the metabolic pattern for glycogen, proteins and lipids level between the two species, in both sexes, there was a significant difference. But, in triglycerides content there was no significant difference between females of either species and in males, there was a significant difference between triglyceride contents of both species.

Table 6 shows the proportion of triglycerides in the total lipid reserve in males and females during the year in both species. This proportion increased in females of *H. pleoacuta* and *H. castroi* in summer, and in males of both species increased in winter.

In females of *H. pleoacuta*, as well as females of *H. castroi*, the values of lipoperoxidation (table 7) were highest in autumn. In females of *H. pleoacuta*, lipoperoxidation levels declined until summer; *H. castroi* showed a reduction in lipoperoxidation levels only in winter. In males of *H. pleoacuta*, the peak of lipoperoxidation occurred during autumn and winter (Table 7); these levels decreased about 2.6 times in spring and summer. In males of *H. castroi*, there was a peak of lipoperoxidation in autumn, with a reduction of 4.6 times in winter; the levels continued steady in other seasons (Table 7). There was a significant difference between lipoperoxidation levels in females and males of *H. pleoacuta*. There was a significant difference between lipoperoxidation levels in females and males of *H. castroi*.

There was a significant difference between lipoperoxidation levels in females of both species. A similar pattern was observed between levels of lipoperoxidation in males of both species.

DISCUSSION

The metabolic profile showed by the females and males of *H. pleoacuta* and *H. castroi* seems to be synchronized with the reproductive period (formation of gametes, reproductive behavior, and synthesis of vitellin) and abiotic factors. The levels of proteins, carbohydrates, and lipids are an expression of an animal's adaptive characteristics and its strategies for adaptation. Many biotic factors (e.g., maturation, reproduction, and food availability, and age) and abiotic factors (e.g., photoperiod, temperature, pH, and dissolved oxygen in the water) can strongly affect the biochemistry and physiology of crustaceans (Company and Sardà 1998; Hervant ***et al.*** 1999a and b; Rosa and Nunes 2003 a and b; Vinagre ***et al.*** 2006).

H. pleoacuta and *H. castroi* have been found only in the northeast region of the state, where they live in sympatry. In the Vale das Trutas, individuals of *H. pleoacuta* are found associated

mainly with macrophytes that have its floating roots. *H. castroi* are found among macrophytes rooted in the sediment. The latter species occurs in lower numbers than the former (Araújo ***et al.*** 2005).

The results for glycogen levels in females of *H. pleoacuta* indicated that they invest in the accumulation of this polysaccharide in the autumn months, during the reproductive peak; and this reserve is used in subsequent months for reproductive behavior (Araújo ***et al.*** 2005). According Castiglioni and Bond-Buckup (2007) this sympatric species showed the reproductive peak in different seasons, for *Hyalella peloacuta* this period occurred in autumn, while for *Hyalella castroi* this event occurred in winter. A similar response was seen in the females of *H. castroi* (Table 1) this polysaccharide was accumulated in winter (reproductive peak) for later use as energy for reproductive behaviors such as maternal care in subsequent season.

Maternal care in amphipod crustaceans involves passive carriage of embryos in an external brood pouch, from which fully developed young emerge. However, more active brood care activities have recently been identified in this group (Dick ***et al.*** 1998; Thiel 1999). Post-emergence care involves, for example, defense of juveniles from cannibals and predators (Aoki 1997; Thiel 1997, 1998, 1999); whereas pre-emergence care, identified in *Crangonyx pseudogracilis* involves brood ventilation, egg cycling, and ejection of nonviable eggs (Dick ***et al.*** 1998). Such pre-emergence brood care activities may be a feature of amphipods found in harsh environments (Dick ***et al.*** 1998). In *H. pleoacuta* and *H. castroi*, maternal care lasts for 12 days in the embryonic period and 3 days in the post-emergence period. The female carries the juveniles in her marsupium, and the juveniles explore the environment and return to their mother (Araújo ***et al.*** 2005).

In the anomuran *Aegla platensis* and the crab *Chasmagnathus granulata* maintained in laboratory conditions on a high-protein diet, total glycogen levels of females and males ranged from 0.54 ± 0.10 to 3.09 ± 0.54 mg/g (Kucharski and Da Silva 1991a; Ferreira ***et al.*** 2005). In the present study, similar values (0.032 ± 0.004 to 0.591 ± 0.07 mg/g) of glycogen levels were observed for females and males of *H. castroi*, suggesting that the natural diet may have a high-protein and low-carbohydrate content. In *H. pleoacuta*, the levels of glycogen ranged from 0.32 ± 0.04 to 1.58 ± 0.57, which suggests that these animals have a high-carbohydrate and low-protein diet. Notably we measured higher levels of this polysaccharide and triglycerides in *H. pleoacuta* than in *H. castroi*.

This difference between these species can be explained by the behavior of *H. castroi* in exploiting the sediment predominantly, where it finds more organic matter of animal origin; whereas *H. pleoacuta* exploits the water column more, where more organic matter of vegetal origin is available. The feeding habits of *H. pleoacuta* and *H. castroi* are unknown. For *H. azteca*, Hargrave (1970) reported that it is an omnivorous deposit feeder, primarily feeding on algae and bacteria associated with the sediments and aquatic macrophytes. It has been recorded feeding on dead animal and plant matter (Cooper 1965). Byrén ***et al.*** (2002) showed in two species of the amphipods *Monoporeia affinis* and *Pontoporeia femorata* that the settled phytoplankton and detrital organic matter are considered their main found source but bacteria, meiofauna and temporary meiofauna are also included in the diet.

The reserves of glycogen in *H. pleoacuta* seem to be related with levels of dissolved oxygen in water, because these species to be more sensitive to the reduction of dissolved oxygen that occurs in the autumn, when females and males depleted this reserve during winter, reaching values approximately three fold lower.

In females of *H. pleoacuta* and *H. castroi*, possibly the reductions in protein levels that occur in autumn and winter, respectively, may be related to maternal care and vitellogenesis: in these periods the number of ovigerous females in the natural environment is higher (Araújo ***et al.*** 2005). Studies in crustaceans have shown seasonal variations in protein content, related to ovarian development, resulting in an increase in biosynthesis of some proteins, including hormones, enzymes, and lipoproteins involved with gonadal maturation (Yehezkel ***et al.*** 2000; Rosa and Nunes 2003). The yolk protein, vitellin, is a glycolipoprotein found in many crustacean species (Riley and Tsukimura 1988; Tseng ***et al.*** 2001). Proteins, besides composing embryonic tissues, are also used in the final period of embryonic development, as observed in *Cherax quadricarinatus* (von Martens 1868) by García ***et al.*** (2003) who reported that proteins are the main components of eggs. Sastry (1983) showed that oogenesis involves an intense biochemical synthesis with the mobilization of lipids and proteins for the development of the eggs.

In males of both species of *Hyalella*, proteins can be used in gametogenesis and reproductive behaviors. The peaks of the protein reserves occurred in spring and autumn in *H. pleoacuta* and *H. castroi*, respectively. These results can be related with stocking of this metabolite for use in the autumn in *H. pleoacuta* and winter in *H. castroi* for gametogenesis,

courting, and copulation, because in this season the reproductive peak of the species occurs (Araújo ***et al.*** 2005), with a possible concurrent decrease in feeding rates.

Precopulatory mate guarding is thought to have evolved as a male mating strategy in species in which female receptivity is limited to a short period (Parker 1974; Ridley 1983). Mate guarding is common among crustaceans, and energetic costs associated with it are thought to promote size-associative pairing in such species; although direct evidence is lacking (Plaistow ***et al.*** 2003). Plaistow ***et al.*** (2003) observed that lipid and glycogen reserves of paired males of the *Gammarus pulex* are both significantly higher than those of unpaired males, and indicated that such a cost is more likely to result from pair formation than from the cost of carrying the female, as had previously been assumed. Males benefit from precopulatory mate guarding by maximizing their chances of fertilizing females eggs once they become receptive. However, the optimal time a male spends guarding each female will depend upon the costs associated with precopulatory mate guarding. Finally, precopulatory mate guarding may be energetically costly (Robinson and Doyle 1985; Elwood and Dick 1990; Sparkes ***et al.*** 1996; Jormalainen ***et al.*** 2001). For example, Robinson and Doyle (1985) showed that in *Gammarus lawrencianus* the feeding rates of males were reduced while they were in precopula, but female feeding rates were unaffected. However, Sparkes ***et al.*** (1996) showed that in the isopod *Lirceus fontinalis*, precopulatory mate guarding was associated with only a short-term (<36 h) reduction in glycogen reserves, and there was no reduction when pairs were given access to food. Precopulatory mate guarding had no impact on lipid reserves in *L. fontinalis*, suggesting that the energy cost of precopulatory mate guarding does not impact on growth in this species (Sparkes ***et al.*** 1996).

A similar response was observed for males of *H. pleoacuta*, in which glycogen was depleted and lipids were conserved during precopula and copula (autumn). In *H. castroi*, both metabolites were depleted during precopula and copula (winter). In both species of amphipods, lipids may be the principal energy reserve during summer, when levels were significantly decreased. This supposition is reinforced by the peak of lipoperoxidation observed in the autumn months.

Lipids are a source of energy in marine invertebrates, including shrimps, and are involved in the fundamental processes of growth, molting, and reproduction (ovarian maturation, egg laying, etc.). Lipids are also found as lipoproteins in the hemolymph (Yepiz-Plascentia ***et al.*** 2000). However, in freshwater crustaceans, little is known about the intermediate metabolism,

including lipids. Rosa and Nunes (2003b) and Oliveira ***et al.*** (2006) demonstrated that triglycerides and other forms of lipids are allocated to the synthesis of sexual hormones and to vitellogenesis.

H. pleoacuta and *H. castroi*, like other crustaceans, produce large eggs. Egg size is related to maternal investment, mainly the lipid metabolism (Rosa and Nunes 2003 a and b). Lipids are the main source of energy throughout embryonic development, and the amount of lipids is generally correlated with the size of eggs and the time interval between spawning and hatching (Petersen and Anger 1997; Rainuzzo ***et al.*** 1997). The very sharp decrease in lipids after spring observed in males and females of the *Hyalella castroi* may be related with the high temperatures and with the increase of the energy necessities.

The highest values of triglycerides in females of *H. pleoacuta* were observed in spring and the lowest values in autumn (reproductive period). In females of *H. castroi*, the highest concentrations of triglycerides were found in spring, showing a gradual reduction in subsequent seasons until the autumn (pre-reproductive period).

Zhukova ***et al.*** (1998) showed that the triglyceride content varied from 3.9 to 35.4% of total lipids in Artemia salina (Leach 1819) a marine crustacean. In *Daphnia laevis* (Birge, 1879) and *Moina micrura* (Kurz, 1874), two freshwater crustaceans, Macedo and Pinto-Corilho (2001) verified that most of the lipids reserve in both cladocerans consisted of triglycerides, corresponding to 49.8% to 68.4% of total lipids. However, these studies were performed in laboratory conditions with food supplied daily (algal diets). In the present study, there were variations in triglycerides from 3.74 to 17.79% in *H. pleoacuta*; and from 0.14 to 14.81% in *H. castroi* of the total lipid content (Table 6). These results suggest that triglycerides not are the main lipid reserves in these species of the hyalellids. Future studies should be performed to clear this point.

Percentage of triglycerides in relation to amount of total lipids showed an increase in energy demand, possibly allocated to gamete production in summer, incubation and egg laying in winter and spring, and parental care in winter and spring for *H. pleoacuta*. There was no obvious pattern in *H. castroi*.

The levels of lipoperoxidation may be related to reproductive events in both species. In females of *H. pleoacuta*, we observed a peak in autumn. In this season, females carry the juveniles in the marsupium for three days after the eggs hatch (Araújo ***et al.*** 2005). The males

showed two peaks of lipoperoxidation, one during the period of precopulation and copulation (autumn), and the other after the reproductive period (winter). In females of *H. castroi*, we observed a peak in autumn. In this season, prepared for the peak of reproduction that occurred in winter (Araújo ***et al.*** 2005), the males showed a peak of lipoperoxidation, during the period of precopulation and copulation (autumn) because they consume more energy for carrying females during this period.

Dutra ***et al.*** (2006), working with *H. curvispina* (Shoemaker 1942), reported the same pattern observed in the present work for levels of lipoperoxidation. In females, this author observed a peak in summer, because during spring the females carry the juveniles in the marsupium for five days after the eggs hatch. The males showed two peaks of lipoperoxidation, one during the period of precopulation and copulation (winter), and the other after the reproductive period (summer). In *Perna perna*, the thiobarbithuric acid reactive substances content observed in autumn were approximately double those found in the rest of the year, despite the increase seen in the enzymatic activities (superoxide dismutase and catalase). During May, *P. perna* usually display a maximal annual gamete emission. Their gonads have a higher lipid and carbohydrate mobilization and protein synthesis, and need a longer period to recover from this high metabolic activity (Wilhelm-Filho *et al.*, 2001). The same pattern of the response was observed to *Bathymodiolus azoricus*, vent mussel, for Company *et al.* (2006).

An important difference between *H. pleoacuta* and *H. castroi* is that they show different exploratory activity. *H. pleoacuta* occurs in the water column, exploiting its entire volume, and thus expending energy for swimming. This species seems to be sensitive to dissolved oxygen content, and its diet seems to be richer in carbohydrates. Already, *H. castroi* exploits the sediment, in which it can burrow. These different activities require different levels of energy consumption, as well as allowing consumption of organic substances of different origins, which may in part explain the different metabolic patterns of these two species, although they live in the same environment.

Byrên ***et al.*** (2002) studying two sympatric species of amphipods observed that there are differences in feeding depths between the two species, with *Monoporeia affinis* mainly a surface deposit-feeder and *Pontoporeia femorata* mainly a subsurface deposit-feeder, there must also be use of partly different food resources. *M. affinis* is more fecund, is more active swimmer and has a higher respiration rate. It is found closer to the sediment surface and feeds more rapidly than *P.*

femorata (Quigley ***et al.*** 1992). In addition, *P. femorata* in field has a more even lipid content over the year indicating less effect of starvation and high biomass probably related with the lower metabolic rate and locomotory activity (Quigley ***et al.*** 1992).

This work showed that the energy reserves of these hyalellids seem to be used in two different ways: (a) the adults use them for their own metabolic needs in response the simultaneously acting environmental factors such as temperature, food availability and her composition, feeding rhythmus and others; or (b) the reserves are transferred to behavioral reproductive and to the offspring through eggs and are used by the young animals in their development. Reproductive events are important in the life cycles of these animals, leading to high energy expenditures and a close correlation with their lipoperoxidation levels. Observations revealed significant seasonal differences in biochemical composition, as well as differences among sexes and species. Environmental conditions (e.g., trophic conditions) and reproduction are supposed to be the main processes influencing the seasonal patterns of variation in biochemical composition. Both species of *Hyalella* show ecological and behavioral differences, especially by resources such as food or space and seasonal variations of the energy metabolism, which might facilitate coexistence at the environmental natural.

Acknowledgements

This work was supported by grants from the Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS – process n° 05501490), the Conselho Nacional de Desenvolvimento Tecnológico (CNPq – process n° 131721/2005-4), and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). The Pontifícia Universidade Católica do Rio Grande do Sul and the Universidade Federal do Rio Grande do Sul supported this work. The experiments were performed according to current Brazilian laws.

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Table 1

	Spring	*Summer*	*Autumn*	*Winter*
Oxygen content (mg/L)	7.18 ± 0.07	6.26 ± 0.34	4.88 ± 0.65	7.24 ± 0.07
pH	7.04 ± 0.05	6.87 ± 0.04	7.09 ± 0.21	6.38 ± 0.26
Water temperature (°C)	18.27± 1.03	19.80± 0.59	15.48± 1.04	11.03± 1.18
Air temperature (°C)	18.93±1.98	22.73±1.71	17.73±1.49	10.37±0.55

Table 2

	Spring	*Summer*	*Autumn*	*Winter*
Hyalella pleoacuta				
Females#	0.139 ± 0.023^{a}	0.419 ± 0.117^{b}	1.447 ± 0.177^{abc}	0.599 ± 0.122^{c}
Males#	0.327 ± 0.041^{a}	0.329 ± 0.046^{b}	1.579 ± 0.571^{abc}	0.319 ± 0.058^{c}
Hyalella castroi*				
Females	0.248 ± 0.03^{b}	0.107 ± 0.001^{c}	0.078 ± 0.009^{a}	0.527 ± 0.093^{abc}
Males	0.119 ± 0.01^{c}	0.032 ± 0.004^{bd}	0.202 ± 0.033^{ab}	0.591 ± 0.073^{acd}

Table 3

	Spring	*Summer*	*Autumn*	*Winter*
Hyalella pleoacuta*				
Females#	3.421 ± 0.229^{a}	4.183 ± 0.435^{b}	1.847 ± 0.213^{abc}	3.645 ± 0.283^{c}
Males#	4.852 ± 0.397^{a}	4.434 ± 0.408^{b}	2.462 ± 0.359^{abc}	4.279 ± 0.414^{c}
Hyalella castroi*				
Females	2.840 ± 0.136^{cd}	1.154 ± 0.037^{bd}	2.521 ± 0.268^{ab}	1.289 ± 0.134^{ac}
Males	1.502 ± 0.138^{a}	1.807 ± 0.061^{b}	2.902 ± 0.318^{ab}	2.076 ± 0.092

Table 4

	Spring	*Summer*	*Autumn*	*Winter*
Hyalella pleoacuta*				
Females#	2.244 ± 0.261	1.199 ± 0.134^{a}	3.627 ± 0.916^{a}	2.167 ± 0.407
Males#	1.217 ± 0.168^{a}	0.657 ± 0.151^{bc}	2.081 ± 0.246^{ab}	1.563 ± 0.242^{c}
Hyalella castroi*				
Females	13.476 ± 1.450^{abc}	1.217 ± 0.079^{a}	3.089 ± 0.421^{b}	2.741 ± 0.223^{c}
Males	27.645 ± 1.334^{abc}	1.893 ± 0.676^{c}	1.517 ± 0.136^{a}	0.567 ± 0.056^{b}

Table 5

	Spring	*Summer*	*Autumn*	*Winter*
Hyalella pleoacuta*				
Females	0.301 ± 0.014^{a}	0.258 ± 0.016^{b}	0.139 ± 0.017^{abc}	0.274 ± 0.012^{c}
Males#	0.088 ± 0.008^{a}	0.081 ± 0.004^{b}	0.078 ± 0.015^{c}	0.278 ± 0.012^{abc}
Hyalella castroi*				
Females	0.219 ± 0.040^{a}	0.103 ± 0.006	0.074 ± 0.019^{a}	0.122 ± 0.024
Males	0.041 ± 0.003^{a}	0.191 ± 0.016^{abc}	0.057 ± 0.003^{b}	0.084 ± 0.019^{c}

Table 6

	Spring	*Summer*	*Autumn*	*Winter*
Hyalella pleoacuta				
Females	13.41	21.52	3.83	12.64
Males	7.23	12.33	3.75	17.79
Hyalella castroi				
Females	1.63	8.46	2.39	4.45
Males	0.15	10.09	3.43	14.81

Table 7

	Spring	*Summer*	*Autumn*	*Winter*
Hyalella pleoacuta*				
Females[#]	30.34 ± 4.206^{ad}	10.126 ± 0.669^{bd}	52.99 ± 5.285^{abc}	21.521 ± 2.549^{c}
Males	14.818 ± 1.913^{ac}	10.384 ± 1.683^{bd}	35.813 ± 4.326^{ab}	36.808 ± 1.936^{cd}
Hyalella castroi*				
Females	20.27 ± 2.344^{a}	18.76 ± 4.774^{b}	44.76 ± 6.712^{abc}	8.04 ± 0.706^{c}
Males	28.22 ± 6.622^{a}	24.39 ± 5.401^{b}	74.51 ± 7.959^{abc}	16.235 ± 0.693^{c}

CAPTIONS TO TABLES:

Table 1: Seasonal variations of the oxygen content (mg/L), pH, and water temperature (°C) in the natural environment. Results represent the mean ± standard error of the mean.

Table 2: Seasonal concentration of glycogen in females and males of *Hyalella pleoacuta* and *Hyalella castroi* collected in the natural environment. All results represent the mean ± standard error of the mean, and are expressed in mg/g. The number of animals represented by each point varied between 15 and 30. The same letter represents significant difference between the seasons. # significant difference between species. * significant difference between sexes.

Table 3: Seasonal concentration of total proteins in females and males of *Hyalella pleoacuta* and *Hyalella castroi* collected in the natural environment. All results represent the mean ± standard error of the mean, and are expressed in mg/ml. The number of animals represented by each point varied between 15 and 30. The same letter represents significant difference between the seasons. # significant difference between species. * significant difference between sexes.

Table 4: Seasonal concentration of total lipids in females and males of *Hyalella pleoacuta* and *Hyalella castroi* collected in the natural environment. All results represent the mean ± standard error of the mean, and are expressed in mg/g. The number of animals represented by each point varied between 15 and 30. The same letter represents significant difference between the seasons. # significant difference between species. * significant difference between sexes.

Table 5: Seasonal concentration of triglycerides in females and males of *Hyalella pleoacuta* and *Hyalella castroi* collected in the natural environment. All results represent the mean ± standard error of the mean, and are expressed in mg/g. The number of animals represented by each point varied between 15 and 30. The same letter represents significant difference between the seasons. # significant difference between species. * significant difference between sexes.

Table 6: Percentage of triglycerides in relation to the amount of total lipids. Results are expressed in %.

Table 7: Seasonal levels of lipoperoxidation in females and males of *Hyalella pleoacuta* and *Hyalella castroi* collected in the natural environment. All results represent the mean ± standard error of the mean, and are expressed in nmols of TBA-RS/mg of protein. The number of animals represented by each point varied between 15 and 30. The same letter represents significant

difference between the seasons. # significant difference between species. * significant difference between sexes.

Variations in the Biochemical Composition and Lipid Peroxidation of *Hyalella pleoacuta* and *Hyalella curvispina* (Crustacea, Amphipoda, Dogielinotidae) Maintained on Different Diets in the Laboratory

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ABSTRACT

We compared the effect of different diets in the energy metabolism and in the levels of lipoperoxidation of *Hyalella pleoacuta* and *Hyalella curvispina*. These crustaceans live in limnetic environments of the plateau and coastal plain, respectively, of the state of Rio Grande do Sul in southern Brazil. The animals were collected in the winter of 2004 and 2005 in São José dos Ausentes, and Tramandaí. In the laboratory, the animals were kept submerged in aquariums under controlled conditions and were fed *ad libitum*, for 30 days with different diets. At the end of this period, the animals were immediately frozen for determination of biochemical parameters. Statistical analysis revealed significant differences in biochemical composition between the sexes and diets. Diet 1 (macrophytes only) mimicked caloric restriction, because this showed a depletion of the glycogen and proteins in two species and sexes, this fact reinforced by a decrease in the lipoperoxidation. In amphipods fed on Diet 2 (macrophytes and ration), these metabolic patterns were reversed, and Diet 2 provided valuable information concerning adequate maintenance in the laboratory for toxicology experiments. The caloric requirements of both species were only supplied with Diet 2, which provided more carbohydrates, proteins and lipids.

Keyword: Crustacea, Amphipod, *Hyalella pleoacuta*, *Hyalella curvispina*, Intermediate Metabolism, Lipoperoxidation, Diet

INTRODUCTION

Members of the genus *Hyalella* are common in the Nearctic and Neotropical regions, with 45 described species (Gonzáles & Watling, 2001). They are found in a variety of freshwater habitats, such as permanent reservoirs, lakes, impoundments, and streams, and often cling to the vegetation, swim in the water, or burrow in the sediment, where they are important members of the benthic fauna (Kruschwitz, 1978; Wellborn, 1995; Grosso & Peralta, 1999).

Aquatic organisms exist in a constantly fluctuating habit, with changes in photoperiod, temperature, pH, dissolved organic matter, dissolved oxygen and quality of the food and food supply (Reid and Wood 1976). Organisms must alter all or part of their physiological and biochemical processes in order to cope with these changes (Hochachka and Somero, 1984).

The number of species available for standardized toxicology tests is limited, and almost all of the species used are not native to Brazil. The reason for this low number is that the majority of toxicology tests require continuous cultures in order to supply the test organisms in sufficient number; this requirement has sharply limited the choice of species to be utilized (Brendonck & Persoone, 1993). According to Hebel ***et al.*** (1997), it is necessary to include multiple measurements of variables in the experimental design, to better assess the effects of toxicants on crustaceans.

Crustaceans contain high concentrations of lipids, although they have no differentiated adipose tissue but store lipids mainly in muscle tissue and the hepatopancreas (O'Connor & Gilbert, 1968; Chang & O'Connor, 1983; Herreid & Full, 1988; Kucharski & Da Silva, 1991a). During periods of high energy demand, such as molting and gametogenesis, large amounts of lipids are mobilized, especially from the hepatopancreas (Kucharski & Da Silva, 1991a; Rosa & Nunes, 2003b; Oliveira ***et al.***, 2006). Rosa and Nunes (2003b) and Oliveira ***et al.*** (2006) demonstrated that triglycerides and other forms of lipids are allocated to the synthesis of sexual

hormones and to vitellogenesis. Zhukova ***et al.*** (1998) showed that the triglyceride content varied from 3.9 to 35.4% of total lipids in *Artemia salina* (Leach 1819), a marine crustacean. In *Daphnia laevis* (Birge, 1879) and *Moina micrura* (Kurz, 1874), two freshwater crustaceans, Macedo and Pinto-Corilho (2001) verified that most of the lipid reserve in both cladocerans consisted of triglycerides, corresponding to 49.8% to 68.4% of total lipids.

In crustaceans, glycogen is stored mainly in the muscles, hepatopancreas, gills, and hemocytes; however, the storage locations vary among different species (Parvathy, 1971; Johnston & Davies, 1972; Herreid & Full, 1988). The stored glycogen is used in the processes of change, hypoxia and/or anoxia, osmoregulation, growth, different periods during reproduction, and during periods of starvation (Chang & O'Connor, 1983; Kucharski & Da Silva, 1991a; Kucharski & Da Silva, 1991b; Rosa & Nunes, 2003a; Oliveira ***et al.***, 2001 and 2004).

The muscle is apparently the main protein-storage location in crustaceans. Proteins are a structural, functional and energy constituent of tissues and play an important role in spawning, fertilization and normal development of embryo in decapods (Garcia-Guerrero ***et al.***, 2003a; Rosa and Nunes 2003a and b; Rodriguez-González ***et al.***, 2006). Other studies have demonstrated variation in protein content during periods of starvation (Yehezkel ***et al.,*** 2000).

Malondialdehyde, a breakdown product of lipid endoperoxides, is an expression of lipid peroxidation and has been used with success in aquatic invertebrates as a general indicator of toxicant stress derived from various types of contamination (Zwart ***et al.***, 1999; Livingstone, 2001; Wilhelm Filho ***et al.***, 2001; Timofeyev ***et al.***, 2006). Neuparth ***et al.*** (2005) reported that in *Gammarus locusta* maintained with sediments having high levels of organic matter, showed higher levels of lipoperoxidation. Effectively, some investigators agree that endogenous variables such as nutritional status, age, sex, growth and reproduction influence the peroxidation status of organisms (Viarengo ***et al.,*** 1991; Correia, 2002; Correia ***et al.,*** 2003).

Some variables, such as temperature and hardness of water, can influence the culture of organisms (Lewis and Marki, 1981; Persoone ***et al.,*** 1989). However, among all variables, the diet on which organisms are maintained is a determining factor in their development (Kersting and Win van der Leeuw, 1976; Lewis and Marki, 1981; Vijverberg, 1989; Lei ***et al.,*** 1990; Kawabata and Urabe, 1998; Beatrice, 2000).

Dutra ***et al.*** (2006), studying the crayfish *Parastacus brasiliensis,* observed that independent of the diets offered to the animals (high-carbohydrate or high-protein diet) and the controlled conditions (temperature and photoperiod) for 15 days, the indications of seasonality were unchanged. Also, the observed changes seemed to be related to the reproductive period, and the lipid reserves were high in crayfish on both diets. Similar patterns were found by Kucharski and Da Silva (1991b) and Ferreira ***et al.*** (2005) with an estuarine crab and a freshwater anomuran, *Chasmagnathus granulata* and *Aegla platensis* respectively, maintained on the same diets. However, the reserves of carbohydrates were very high in animals that received a high-carbohydrate diet.

Beatrice (2000) compared the response of *Daphnia similis* submitted to three different diets, and observed that individuals maintained on a combined diet, algae *Selenastrum caprcornutum* with a food supplement based on *Artemia*, reproduced significantly more than the animals cultured on algae alone. Platte (1993), seeking to increase the production of *Ceriodaphnia dubia* cultures, found similar results when testing a food supplement based on *Artemia*. Hernandez-Vergara ***et al.*** (2003) evaluated the effect of different concentrations of lipids in artificial diets offered to the crayfish *Cherax quadricarinatus*, and concluded that males invest their lipid reserves in growth, whereas females, with a higher hepatosomatic index, invest in gonadal development and vitellogenesis.

The importance of the quantity and quality of the food provided can be evaluated through

the offspring produced in the cultures, because the diet can directly influence the reproductive capacity of individuals. Herbert (1978), studying the genus *Daphnia*, observed that the number of neonates produced by ovigerous females depends directly on the food ingestion. The number of juveniles, together with the sensitivity of the organism to some reference substance and the course of accumulation of lipids course, can be adopted as criteria to evaluate the quality of the cultures of organisms used in ecotoxicological bioassays (Zagatto, 1988).

The present study had the objective of evaluating the effect of different diets on the energy metabolism of the freshwater amphipods *Hyalella curvispina* and *Hyalella pleoacuta* maintained for 30 days in the laboratory. The purpose was to obtain basic physiological data to support the development of a new model for toxicology tests in the laboratory.

MATERIAL AND METHODS

The animals were cared for in accordance with guidelines such as the *Guide for the Care and Use of Laboratory Animals* (1996, published by the National Academy Press, 2101 Constitution Ave. NW, Washington, DC 20055, USA) and Brazilian laws. The animals were used with the permission of the Ethics Committee of the Pontifícia Universidade Católica do Rio Grande do Sul (License 0004/03).

Collection of *Hyallela curvispina* and *Hyalella pleoacuta*

In order to establish the profile of variation in the biochemical composition of amphipods, *Hyalella curvispina* and *Hyalella pleoacuta*, submitted to two different diets, the collections were made in the winter (July, June and August). The animals were collected together with aquatic macrophytes from each locale (*Hyallela curvispina* with *Salvinia bilob* and *Hyalella pleoacuta* with *Callitriche rimosa*). *Hyallela curvispina* (120 males and females) was collected in the Gentil Lagoon (29º56'30"S, 50º07'50"W – 0 meters), in Tramandaí; and *Hyallela pleoacuta* (120 males and females) was collected in São José dos Ausentes Municipality (28°47'00"S,

49°50'53"W - 1100 meters a.s.l), Rio Grande do Sul, Brazil. The animals were collected by means of fish traps and bottom grabs. They were transported on ice in insulated containers to the Laboratory of Conservation Physiology of PUCRS.

In order to characterize the collection locale, the pH, temperature and hardness of the water were measured during the months of collection. The pH was determined with a portable pH meter, and water temperature with an internal-scale thermometer. The hardness of the water was determined using a classic method of volumetric complexation (Adad, 1982).

Experimental procedure:

After 24 hours of adaptation, adult animals were separated and kept submerged in aerated aquariums (70L) in small cages (15 animals per cage). The males and females of each species were kept separately. The mean temperature was 23±1ºC and the photoperiod 14:10 hours light/dark.

The amphipods were divided into two groups fed *ad libitum* in the late afternoon, when the majority of amphipods were active, for a period of 30 days. The first group received only macrophytes. The second group received macrophytes plus commercial fish food (ALCOM). (Table 1 and Table 2)

When the culture was begun, and then after 15 and 30 days of culture, 15 males and 15 females were cryoanesthetized, weighed on an electronic balance (± 0.001), and stored frozen at -80°C until they were used to determine the biochemical parameters. The cultures were repeated for three months (winter) in two years.

Biochemical Analyses

Metabolic determination for *H. curvispina* and *H. pleoacuta* was done in total homogenates of three pools of five males and five females in each point. One pool of each

species, *H. curvispina* and *H. pleoacuta*, was used for determination of glycogen and proteins, the second pool for quantification of lipids and triglycerides, and the third pool for quantification of lipoperoxidation levels. Metabolic parameters were determined in quadruplicate by spectrophotometric methods.

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

b. Proteins were quantified as described by Lowry (1957), with bovine albumin (Sigma Co.) as the standard. Results are expressed in mg/ml of the total homogenate.

c. 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, 1970; Meyer and Walter, 1980). This method consists of oxidizing cellular lipids to small fragments after chemical digestion with hot concentrated sulfuric acid. After the addition of a solution of vanillin and phosphoric acid, a red complex is formed which is measured with a spectrophometer (530 nm). Triglycerides were measured by the reactions of lipase, glycerokinase, 1-P-glycerol oxidase, and peroxidase enzymes (Biodiagnostic Kit / GPO Trinder). Results are expressed as mg/g of animals.

d. Lipoperoxidation levels were quantified by the method of Buege and Aust (1978), by measuring reactive substances to Thiobarbituric Acid (TBA-RS), using the extraction method of Llesuy ***et al.*** (1985). Results are expressed in nmol of TBARS/mg of protein.

Analyses of behavior

Reproductive behavior

After a period of 24 hours, 10 couples were placed in each 20-liter aquarium, for a total of eight aquariums (four aquariums for each group). The animals were observed each day for 30 days, and the number of couples and ovigerous females was counted (Plaistow, 2003).

Survival and Mortality

The survival and mortality of the animals during the experimental cultures were recorded.

Statistical Analysis

The results are expressed as mean ± standard error. For statistical analysis of the diet, a one-way ANOVA test was used, followed by a Bonferroni test. For comparisons between different species and sexes, a two-way ANOVA was used. All the metabolic parameters were homogeneous (Levene test), and were normally distributed (Kolmogorov-Smirnov test). 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 environmental and experimental culture abiotic factors are shown in Table 4. The temperatures in the collection locales were significantly lower than the temperature of the experimental cultures. The pH and hardness of the water were constant in both situations. The environmental pH was 6.38 ± 0.21, and in the experimental culture it was 7.00 ± 1.00. The hardness of the water in the environment was 0.97 ± 0.23 ppm $CaCO_3$ and in the experimental culture the water hardness was 1.08 ± 0.35 ppm $CaCO_3$.

The two diets were analyzed by the Instituto de Ciências e Tecnologia dos Alimentos (ICTA) of the Universidade Federal do Rio Grande do Sul (UFRGS). The diets were quite

different in their total caloric values. Diet 1 provided 37 (*Hyalella pleoacuta*) and 26 (*Hyalella curvispina*) times lower caloric levels than did Diet 2 (Tables 1 and 2).

The number of the couples and the ovigerous females fed on Diet 1 and Diet 2 are shown in Table 3. *Hyalella pleoacuta* fed on diet 2 (macrophytes and fish food) paired 2.8 times more often than did the animals maintained on Diet 1 (macrophytes alone). The females fed on Diet 2 showed a higher proportion of ovigerous females than did the females maintained on Diet 1, and bore approximately twice as many eggs. *Hyalella curvispina* fed on Diet 2 paired 2.7 times more often than did the animals maintained on Diet 1. The females fed on Diet 2 showed a higher proportion of ovigerous females than did the females maintained on Diet 1, and bore about three times as many eggs.

The survival rates of the males and females of both species fed on Diets 1 and 2 are presented in Table 2. During the period of the experimental culture, males and females fed on Diets 1 and 2 showed no significant differences in their survival rates.

Figure 1 shows the variation in glycogen content in females (A) and males (B) of *Hyalella pleoacuta* fed on the different diets. In females of *H. pleoacuta* on Diet 1, glycogen levels were highest in the beginning of the experiment, then decreased 14 times after 15 days of diet, and then increases 2 times in relation to day 15 days by the end of the culture period (30 days). The females on Diet 2 showed no significant difference in their levels after 15 days of experiment, and decreased after 30 days of experiment (4 times approximately).

In males of *H. pleoacuta* fed on Diet 1, glycogen levels were highest in the beginning of the experiment, decreased approximately 15 times after 15 days on the diet, and then increased by approximately 2 times after 30 days on the diet. Males fed on Diet 2 showed no significant difference in their levels after 15 days of cultivation; the levels then increased after 30 days of the experiment (by approximately 1.13 times).

Figure 1 shows the variation in glycogen content in females (C) and males (D) of *Hyalella curvispina* fed on the different diets. In females of *H. curvispina* on Diet 1, glycogen levels decreased by approximately 50% after 15 days of experiment, and remained lower after 30 days of experiment. The females fed on Diet 2 showed an increase of 50% after 15 days of experimental culture, and these values then remained constant until the end of the experiment.

In males of *H. curvispina* fed on diet 1, glycogen levels decreased approximately 30% after 15 days of experiment, and decreased again after 30 days (6.6 times approximately). In the males fed on diet 2, glycogen levels were higher at the beginning of the experiment, decreased after 15 days, and then increase 1.7 times after 30 days of culture.

When the two diets were compared in both species, in females and in males, there was a significant difference in the levels of glycogen ($p<0.05$). The same pattern was shown when the sexes were compared ($p<0.05$).

Figure 2 shows the variation in total protein content in females (A) and males (B) of *Hyalella pleoacuta* fed on the two diets. In females of *H. pleoacuta* fed on Diet 1, total protein levels were highest in the beginning of the experiment, then decreased 130 times after 15 days, and then remained lower after 30 days of culture. The females fed on Diet 2 showed no significant difference in their levels during the same experimental period.

In males of *H. pleoacuta* fed on Diet 1, total protein levels were highest in the beginning of the experiment, decreased approximately 122 times after 15 days of diet, and then remained stable during the next 15 days of culture. The males fed on Diet 2 showed no significant difference in their levels of this metabolite during the experimental period. There was a significant difference in the behavior of total protein levels only in comparing the animals on the different diets (Diet 1 versus Diet 2) ($p<0.05$).

Figure 2 shows the variation in total protein content in females (C) and males (D) of *Hyalella curvispina* fed on the different diets. In females of *H. curvispina* fed on Diet 1, total protein levels decreased 1.2 times after 15 days of experiment, and decreased further (11.4 times approximately) by the end of the experiment. The females fed on diet 2 showed a decrease of 50% after 15 days of experimental culture, and then these values remained constant until the end of the experiment.

In males of *H. curvispina* fed on Diet 1, total protein levels decreased approximately 1.4 times after 15 days of experiment, and decreased again after 30 days of experiment. In males fed on Diet 2, total protein levels showed no significant difference after 15 days of culture, but after 30 days showed a decrease of 50% from the level at 15 days of culture, and then these values remained constant until the end of the experiment.

When Diet 1 versus Diet 2 were compared, the levels of proteins were different ($p<0.05$). There was difference ($p>0.05$) in the behavior of total proteins between the sexes only in Diet 2.

Figure 3 shows the variation in total lipid content in females (A) and males (B) of *Hyalella pleoacuta* fed on the two diets. In females of *H. pleoacuta* fed on Diet 1, total lipid levels were highest in the beginning of the experiment, and then decreased 1.7 times after 15 days on the diet, remaining constant until the end of the experiment. In the females fed on Diet 2, total lipid levels were highest in the beginning of the experiment, and then increased 0.8 times after 15 days on the diet; these levels then remained constant until the end of the experiment. In males of *H. pleoacuta* fed on both Diets 1 and 2, total lipid levels increased after 15 days of the experiment, and then decreased in animals on Diet 1 and remained steady in the amphipods on Diet 2.

Figure 3 shows the variation in total lipid content in females (C) and males (D) of *Hyalella curvispina* fed on the different diets. In females of *H. curvispina* fed on Diet 1, the levels of total lipids increased 50% after 15 days of experiment and then returned to the same

levels as at the beginning of the diet. In the females fed on Diet 2, protein levels increased 50% after 15 days on the diet and then remained constant until the end of the experiment.

The males of *H. curvispina* fed on Diet 1 showed a decrease until the minimum values were reached at the end of the experiment (30 days). The animals fed on Diet 2 showed no significant difference in their levels.

There was a significant difference in the behavior of total lipid levels between males and females fed on Diets 1 and 2 ($p<0.05$). The same behavior was observed when the different diets were compared ($p<0.05$).

Figure 4 shows the variation in triglyceride content in females (A) and males (B) of *Hyalella pleoacuta* fed on the different diets. In females of *H. pleoacuta* fed on Diet 1, triglyceride levels were highest in the beginning of the experiment, and then decreased 7.7 times after 15 days of diet, and then increased approximately 2.7 times after 30 days, compared with the levels at day 15. The females fed on Diet 2 showed no significant difference in their levels during the period of the experiment.

In males of *H. pleoacuta* fed on Diet 1, triglyceride levels were highest in the beginning of the experiment, decreased approximately 6.3 times after 15 days of diet, and decrease again (4.4 times approximately) after 30 days of diet. The males fed on Diet 2 showed a small decrease after 15 days of experiment in relation the beginning of the diet, and these levels then remained constant until the end of the experiment. There was a significant difference in the behavior of triglyceride levels in females and males fed on diet 1 and diet 2 ($p<0.05$). There was no significant difference in the behavior of triglycerides when the sexes were compared.

Figure 4 shows the variation in triglyceride content in females (C) and males (D) of *Hyalella curvispina* fed on different diets. In females of *H. curvispina* fed on Diet 1, the levels of triglycerides decreased 50% after 15 days of experiment, and after 30 days increased to similar

levels as at the beginning of the experiment. In the females fed on Diet 2, triglyceride levels decreased 50% after 15 days of diet, and then remained constant for the next 15 days.

In males of *H. curvispina* fed on Diet 1, triglyceride levels increased after 15 days of the experiment and then remained constant until the end of the experiment. In males fed on Diet 2, triglyceride levels increased 50% after 15 days, and then remained constant.

There was a significant difference in the behavior of triglyceride levels when the animals fed the different diets were compared, in both sexes ($p<0.05$). There was also a significant difference between the sexes ($p<0.05$).

Figure 5 shows the variation in levels of lipoperoxidation in females (A) and males (B) of *Hyalella pleoacuta* fed on the different diets. In females of *H. pleoacuta* fed on Diet 1, levels of lipoperoxidation were highest in the beginning of the experiment, and then decreased 4.9 times after 15 days, then remaining constant until the end of the experiment. In females fed on Diet 2, levels of lipoperoxidation were highest in the beginning of the experiment, increased 1.3 times after 15 days, and decreased after 30 days of the experiment (2.9 times approximately).

In males of *H. pleoacuta* fed on Diet 1, levels of lipoperoxidation were highest in the beginning of the experiment, decreased by approximately 8.2 times after 15 days of the diet, and then remained constant until the end of the experiment. Males fed on Diet 2 showed high levels of lipoperoxidation in the beginning of the experiment, then after 15 days these levels decreased 4.4 times, and decreased further by day 30 (1.7 times approximately). There was a significant difference in the behavior of the levels of lipoperoxidation between females and between males fed on Diet 1 or Diet 2 ($p<0.05$). There was a significant difference in the behavior only between females and males fed on diet 2 ($p<0.05$).

Figure 5 shows the variation in levels of lipoperoxidation in females (C) and males (D) of *Hyalella curvispina* fed on the different diets. In females of *H. curvispina* fed on Diet 1, levels of

lipoperoxidation were approximately 2.3 times lower after 15 days, and increased after 30 days to the same levels as in the beginning. In females fed on Diet 2, levels of lipoperoxidation increased gradually until they reached a maximum at 30 days of experiment (5.7 times higher approximately).

In males of *H. curvispina* fed on Diet 1, levels of lipoperoxidation increased 50% after 15 days and decreased again after 30 days to the same values at in the beginning of the experiment. In males fed on Diet 2, lipoperoxidation levels were higher in the beginning of the experiment, decreased at 15 days, and increased at the finish of the experiment. There was a significant difference in the behavior of levels of lipoperoxidation between females and between males fed with Diet 1 or Diet 2 ($p<0.05$). There was a significant difference in the levels of lipoperoxidation between the sexes given the same diets ($p>0.05$).

DISCUSSION

The physiological condition of animals is determined, among other factors, by their nutritional state, which is, therefore, a main factor controlling overall metabolic performance and capacity. This is shown by the decrease in respiration rates of different crustacean species under food-limited conditions (Aldrich, 1975; Regnault, 1981; Du Preez, 1983; Hiller-Adams and Childress, 1983).

Diet 1 (macrophytes alone) mimicked a caloric restriction in both species and sexes, because they showed depletion of glycogen and total proteins, which was reinforced by the decrease in the levels of lipoperoxidation. These responses were probably a result of the low caloric input (9.52 or 13.92 Kcal/100g). In the animals that received Diet 2 (macrophytes and fish food), these responses were the reverse, the animals showed higher levels of lipoperoxidation than those on Diet 1, and Diet 2 maintained the energy reserves. Animals fed on Diet 2 showed more activity in the aquariums during the experimental culture than did the animals that received

Diet 1. These results suggest that these species are omnivorous, feeding on algae and bacteria associated with the sediments and aquatic macrophytes, and on detritus present in the sediment, like other amphipods.

A common denominator in crustaceans is their constant feeding activity (Cuzon ***et al.***, 2000). Furthermore, they alternate episodes of feeding and fasting during development, which occurs through molting (ecdysis) and results in growing by sequential steps. For *Hyalella azteca* (Saussure 1858), Hargrave (1970) reported that it is an omnivorous deposit feeder, primarily feeding on algae and bacteria associated with the sediments and aquatic macrophytes. It has also been recorded feeding on dead animal and plant matter (Cooper 1965). Byrén ***et al.*** (2002) showed in two species of amphipods, *Monoporeia affinis* (Lindström 1855) and *Pontoporeia femorata* (Kröyer, 1842), that the settled phytoplankton and detrital organic matter are their main food source, but that bacteria, meiofauna and temporary meiofauna are also included in the diet. Casset ***et al.*** (2001), studying *Hyalella curvispina* in a river in Argentina, showed that this amphipod is herbivorous, feeding mainly on the phytobenthos and occasionally on sediment.

The animals that received Diet 1 showed lower levels of glycogen than the animals that received Diet 2. This difference may be explainable because of the low levels of carbohydrate in this diet (2.04 - *Callitriche rimosa*; 0.67 – *Salvinia bilob*) and protein (1.24 - *Callitriche rimosa*; 1.19 – *Salvinia biloba*). The two macrophytes are composed mainly of water, whereas the balanced diet had amounts of carbohydrate (45.23 – diet given to *Hyalella pleoacuta*; 43.86 – diet given to *Hyalella curvispina*) and protein (32.12 – diet given to *Hyalella pleoacuta*; 32.07 – diet given to *Hyalella curvispina*) sufficient to maintain the levels of this metabolite and probably also the hemolymphatic glucose levels.

Carbohydrates are often included in crustacean artificial diets for their protein-sparing effect. By supplying energy to support routine metabolism, a greater quantity of protein is

directed towards somatic growth (Shiau and Peng, 1992; Rosas ***et al.***, 2000). However, although glucose is the main sugar circulating in the hemolymph of crustaceans, inclusion of this monosaccharide in the diet of penaeid shrimps was associated with low growth rates, increased mortality and poor protein conversion efficiencies (Abdel-Rahman ***et al.***, 1979; Rosas ***et al.***, 2001; Cuzon ***et al.***, 2001). Hemolymphatic glucose is the result of the influx of intestinal glucose (Verri ***et al.***, 2001), from the balance between anabolic (gluconeogenic, glycogenesis) and catabolic (glycogenolysis, glycolysis) processes (Chang and O'Connor 1983; Oliveira and Da Silva, 1997; Hall & Van Ham, 1998).

When the curves of total protein are compared, it can be observed that this metabolite decreased sharply in the animals fed on Diet 1. In the animals maintained on Diet 2, this reserve was not used. This may explain why we observed a lower fecundity rate and a decrease in the number of pairs in both species (Table 3).

Several studies have evaluated maturation, eye ablation (Sagi ***et al.***, 1997; Wongprasert ***et al.***, 2006), use of hormones (Abdu ***et al.,*** 2001), fecundity (King, 1993), and reproductive cycle (Villarreal ***et al.***, 1999; Serrano-Pinto ***et al.***, 2004) of the redclaw crayfish under laboratory conditions. Different diets have been used in these studies. Diet plays an important role in crayfish broodstock condition (Holdich, 2002). Broodstock nutrition is important for reproductive success, because egg and larval production are strongly dependent on the diets offered (Bromage, 1995; Harrison, 1997; García-Ulloa, 2000). According to Harrison (1997), the amount of protein required in broodstock diets for maturation and production of eggs is higher than the level required for growout, because gonad maturation is a process of intense protein synthesis, mainly during vitellogenesis (Abdu ***et al.***, 2000; Cortés-Jacinto ***et al.***, 2003; Thompson ***et al.,*** 2005).

Studies in crustaceans have shown seasonal variations in protein content, related to ovarian development, resulting in an increase in biosynthesis of some proteins, including hormones, enzymes, and lipoproteins involved with gonadal maturation (Yehezkel ***et al.,*** 2000; Rosa and Nunes, 2003). The yolk protein, vitellin, is a glycolipoprotein found in many crustacean species (Riley and Tsukimura, 1988; Tseng ***et al.,*** 2001). Proteins, as well as being structural components of embryonic tissues, can also be used as energy in the final stages of development. This was observed in the embryonic development of *Cherax quadricarinatus* by García-Guerrero ***et al.*** (2003b), who reported that proteins are the principal components of the eggs. According to Sastry (1983), oogenesis involves an intense biochemical synthesis, with the mobilization of lipids and proteins for egg development.

When insufficient energy is available in a diet from non-protein sources, protein may be catabolized to meet the energy requirements at the cost of nutrient supply for somatic growth (Capuzzo and Lancaster, 1979; Sedgwick, 1979). The most efficient diets contain sufficient non-protein energy sources (lipid and carbohydrate) that are metabolized preferentially to protein to meet general energy requirements, leaving an organism to direct the maximum level of available dietary protein into growth (Sedgwick, 1979; Bautista, 1986).

Integral to the development of a feed for any species is the identification of their protein, lipid and energy requirements. Protein is required to provide the fundamental units, amino acids, for growth, while dietary lipid provides both essential fatty acids for the energy needed for the metabolic processes of growth (D'Abramo ***et al.***, 1997). Further energy can also be derived from the metabolism of protein and some dietary carbohydrates. Overall, the identification of the protein and lipid requirements for rapid growth and development of the animal allows the formulation of suitable diets from a range of ingredients (Tacon, 1990).

Most studies on nutrition of freshwater crustaceans are focused on proteins (Corte´s-Jacinto ***et al.,*** 2003, 2004; Thompson ***et al.,*** 2004), and little information is available on carbohydrates and lipids (Hernandez-Vergara ***et al.,*** 2003). These two nutrients have important roles, not only as energy sources, but also in the development and reproduction of crustaceans

Robinson and Doyle (1985) showed that in *Gammarus lawrencianus*, the males fed less while they were in precopula, but the feeding rates of females were unaffected. Barclay ***et al.*** (1983), working with *Penaeus esculentus*, showed that during the period of starvation the abdominal muscle makes the largest contribution of protein to energy metabolism, where small changes in this tissue are sufficient to make a substantial contribution to the overall animal maintenance.

Comparing the curves for total lipids, we observed no clear response of this metabolite in animals fed on Diet 1. In the animals maintained on Diet 2, there was an increase in this reserve, and this reserve was maintained. The triglycerides were less used than the lipids, principally in the animals fed on Diet 2. This may also explain why the couples fed on Diet 1, showed lower fecundity and fewer paired animals.

Hervant ***et al.*** (1999) observed that the metabolic response to prolonged food deprivation (28 days) was monophasic in surface-dwelling amphipods, showing an immediate, linear and large decline in all of the energy reserves. *Gammarus fossarum* displayed a different energetic strategy: during a 28-day starvation period, proteins comprised 56% of the energy losses, total lipids 39%, and glycogen reserves only 5%.

Lipids are also found as lipoproteins in the hemolymph (Yepiz-Plascentia ***et al.,*** 2000). However, in freshwater crustaceans, little is known about the intermediate metabolism, including

lipids. Rosa and Nunes (2003b) and Oliveira ***et al.*** (2006) demonstrated that triglycerides and other forms of lipids are allocated to the synthesis of sexual hormones and to vitellogenesis.

Although the males and females were maintained in separate cages in the same aquariums during the experiment, the ovaries of the females matured. Several authors have reported a decrease in total or specific lipids in the hepatopancreas during maturation, and have assumed that they are transferred to the ovary (Galois, 1984; Teshima ***et al.***, 1988; Castille and Lawrence, 1989; Millamena and Pascual, 1990). Depletion of triglycerides could also be explained by a decrease in dietary lipid (Clarke, 1982) or by an increase in energy consumption (Teshima ***et al.***, 1988; Harrison, 1990).

Plaistow ***et al.*** (2003) observed that lipid and glycogen reserves of paired males of *Gammarus pulex* (Linnaeus 1758) are both significantly higher than those of unpaired males, and indicated that such a cost is more likely to result from pair formation than from the cost of carrying the female, as had previously been assumed. Males benefit from precopulatory mate guarding by maximizing their chances of fertilizing females' eggs once they become receptive. However, the optimal time that a male spends guarding each female will depend upon the costs associated with precopulatory mate guarding. Finally, precopulatory mate guarding may be energetically costly (Robinson and Doyle, 1985; Elwood and Dick, 1990; Sparkes ***et al.,*** 1996; Jormalainen ***et al.***, 2001). For example, Robinson and Doyle (1985) showed that in *Gammarus lawrencianus*, the feeding rates of males were reduced while they were in precopula, but female feeding rates were unaffected. However, Sparkes ***et al.*** (1996) showed that in the isopod *Lirceus fontinalis* (Rafinesque-Schmaltz 1820), precopulatory mate guarding was associated with only a short-term (<36 h) reduction in glycogen reserves, and there was no reduction when pairs were given access to food. Precopulatory mate guarding had no impact on lipid reserves in *L.*

fontinalis, suggesting that the energy cost of precopulatory mate guarding does not impact on growth in this species (Sparkes ***et al.***, 1996).

Levels of lipoperoxidation in the animals that received Diet 1 are lower in relation of the animals that received Diet 2. This reinforces the hypothesis that Diet 1 sends the amphipods into a process of caloric restriction.

The ability of an organism to survive and recover from long periods of starvation is vital. Changes in food intake during development may have important consequences for life history (Brzek and Konarzewski, 2001). Although any instantaneous ecological consequences of poor or intermittent nutrition are sometimes difficult to distinguish, the reproductive potential of any organism experiencing such conditions may be reduced. Animals that experience natural cycles of food intake may have interesting adaptations for use and storage of energy sources. Starvation can lead to a severe deficiency of nutrients. Therefore, starvation studies may be useful predictors to determine energetic and metabolic requirements (Guderley, 2003). Since crustaceans experience starvation periods during their growing process, artificially induced fasting and starvation may shed some light on the metabolic routes used, and in what order, during the molt, and may describe novel biochemical and physiological adaptation mechanisms (Barclay ***et al.***, 1983). Furthermore, the knowledge derived from the understanding of their biochemical processes may form a basis to optimize crustacean pond rearing efforts (Ribeiro ***et al.***, 2001).

On the other hand, it may happen that macrophytes produce anti-herbivore chemicals (Hay, 1985; Brawley, 1992) to defend against herbivory (Lubchenco and Gaines, 1981). In systems of intense herbivory (Hay, 1997), palatable macroalgae are more susceptible to elimination (Hay, 1996; Cronin, 2001). Therefore, it is not surprising that many tropical algal

species produce such chamicals (Hay and Fenical, 1988; Hay, 1996). This phenomenon may be related to the possible caloric restriction observed in animals fed on Diet 1.

Our results showed that these diets changed the biochemical patterns of the animals taken from the natural environment. Animals fed on Diet 1 showed two distinct phases of biomass degradation. Initially, energy-rich carbohydrate and protein reserves are preferentially mobilized, reflected in the slight alteration in total lipid levels. After this intense utilization of glycogen and proteins, there was a decrease in the lipoperoxidation and activity levels, suggesting a decrease in metabolic rate. This suggests that carbohydrate, protein and lipids are all important energy stores, although the lipid reserve may be more important for reproduction, as in other crustaceans. Diet 2 reversed these responses. The response to this diet provided valuable information concerning the maintenance of these animals in the laboratory, as for instance for toxicology experiments. Both species showed caloric requirements that were only supplied by Diet 2, which was higher in carbohydrates, proteins and lipids.

ACKNOWLEDGEMENTS

This study was supported by grants from the Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS, process no. 03/0681.9 – ARD, and process no. 05501490) and the Conselho Nacional de Desenvolvimento Tecnológico (CNPq, process nos. 300075/2003-0, 131721/2005-4, and 103334/2005-0). The Pontifícia Universidade Católica do Rio Grande do Sul supported this study.

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Figure Legends

Figure 1: Concentration of glycogen of *Hyalella pleoacuta* and *Hyalella curvispina* in environment and maintained in experimental culture. Columns represent the mean, and bars represent the standard error of the mean. Results are expressed in mg/g.

Figure 2: Concentration of total proteins of *Hyalella pleoacuta* and *Hyalella curvispina* in environment and maintained in experimental culture. Columns represent the mean, and bars represent the standard error of the mean. Results are expressed in mg/ml.

Figure 3: Concentration of total lipids of *Hyalella pleoacuta* and *Hyalella curvispina* in environment and maintained in experimental culture. Columns represent the mean, and bars represent the standard error of the mean. Results are expressed in mg/g.

Figure 4: Concentration of triglycerides of *Hyalella pleoacuta* and *Hyalella curvispina* in environment and maintained in experimental culture. Columns represent the mean, and bars represent the standard error of the mean. Results are expressed in mg/g.

Figure 5: Levels of lipoperoxidation of *Hyalella pleoacuta* and *Hyalella curvispina* in environment and maintained in experimental culture. Columns represent the mean, and bars represent the standard error of the mean. Results are expressed in nmol of TBARS/mg of protein.

Tables Legends

Table 1 and 2: Centesimal composition of the diets 1 and diet 2.

Table 3: Indices of the survival of the males and females of the *Hyalella pleoacuta* and *Hyalella cusrvispina* maintained in experimental culture with different diets.

Table 4: Number of the couples and ovigerous females of the *Hyalella pleoacuta* and *Hyalella curvispina* maintained in experimental culture with different diets.

Table 5: Water temperature (°C), pH, and Hardness of water (ppm de $CaCO_3$) in natural environmental and experimental conditions. The results are expressed as mean ± standard error of the mean.

Table 6: Percentage of triglycerides in relation to the amount of total lipids in males and females. Results are expressed in %.

Table 1

	Diet 1	Diet 2
Compound	*Callitriche rimosa*	*Callitriche rimosa* and Ration
Water content (g/100g)	93,84	98,97
Ashes (g/100g)	1,26	12,28
Protein (g/100g)	1,24	32,12
Fat (g/100g)	0,28	6,47
Fiber (g/100g)	1,34	5,29
Carbohydrates (g/100g)	2,04	45,23
Total Caloric Value (Kcal/100g)	**9,52**	**361,51**

Table 2

	Diet 1	Diet 2
Compound	*Salvinia bilob*	*Salvinia bilob* and Ration
Water content (g/100g)	84,00	**89,13**
Ashes (g/100g)	11,07	**22,09**
Protein (g/100g)	1,19	**32,07**
Fat (g/100g)	0,72	**6,91**
Fiber (g/100g)	0,71	**4,66**
Carbohydrates (g/100g)	0,67	**43,86**
Total Caloric Value (Kcal/100g)	**13,92**	**365,91**

Table 3

Hyalella pleoacuta	**1º Culture**	**2º Culture**	**3º Culture**	**Mean**
Males –Diet 1	96.20%	95.55%	96.63%	96.13%
Females –Diet 1	97.72%	96.36%	97.20%	97.09%
Males –Diet 2	97.46%	98.01%	98.45%	97.97%
Females –Diet 2	97.72%	95.54%	95.89%	96.38%
Hyalella curvispina				
Males –Diet 1	97.67%	96.99%	97.89%	97.51%
Females –Diet 1	98.27%	97.95%	97.89%	98.04%
Males –Diet 2	99.64%	98.71%	99.54%	99.29%
Females –Diet 2	98.94%	98.42%	99.86%	98.56%

Table 4

Hyalella pleoacuta	Number of couples	Ovigerous Females	Eggs
Diet 1	9	5	15±5
Diet 2	25	20	32±7
Hyalella curvispina			
Diet 1	8	7	12±3
Diet 2	22	18	35±6

Table 5

Abiotic Factors	Environmental	Experimental Culture
Temperature	11.03± 0.07	20.00 ± 1.00
pH	6.38 ± 0.26	7.00 ± 1.00
Hardness of Water	0.97 ± 0.23	1.08 ± 0.35

Table 6

Proportion of Triglycerides in Total Lipids (%)	Days of culture	*Hyalella*	*pleoacuta*	*Hyalella*	*curvispina*
		Females	**Males**	**Females**	**Males**
Diet 1	0	24.38	20.38	30.46	54.56
	15	9.03	9.59	5.36	23.74
	30	27.59	12.61	28.08	27.55
Diet 2	0	24.38	20.38	30.46	58.63
	15	22.68	18.97	4.50	8.33
	30	22.74	17.16	4.45	7.88

Figure 1

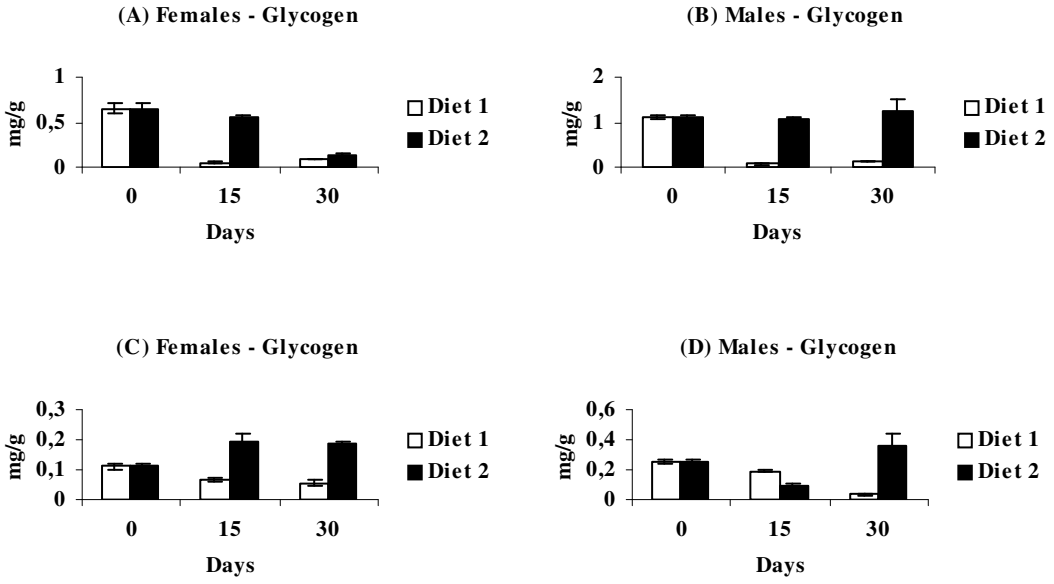

(A) Females - Glycogen
mg/g
1
0,5
0
0
15
30
Days
Diet 1
Diet 2
(B) Males - Glycogen
mg/g
2
1
0
0
15
30
Days
Diet 1
Diet 2
(C) Females - Glycogen
mg/g
0,3
0,2
0,1
0
0
15
30
Days
Diet 1
Diet 2
(D) Males - Glycogen
mg/g
0,6
0,4
0,2
0
0
15
30
Days
Diet 1
Diet 2

Figure 2

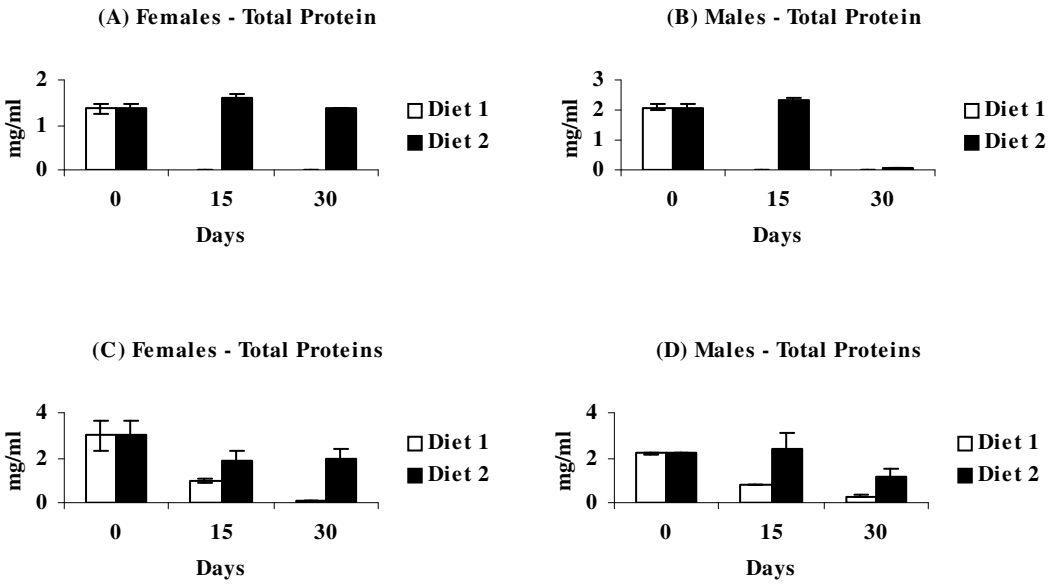

(A) Females - Total Protein
mg/ml
2
1
0
0
15
30
Days
Diet 1
Diet 2
(B) Males - Total Protein
mg/ml
3
2
1
0
0
15
30
Days
Diet 1
Diet 2
(C) Females - Total Proteins
mg/ml
4
2
0
0
15
30
Days
Diet 1
Diet 2
(D) Males - Total Proteins
mg/ml
4
2
0
0
15
30
Days
Diet 1
Diet 2

Figure 3

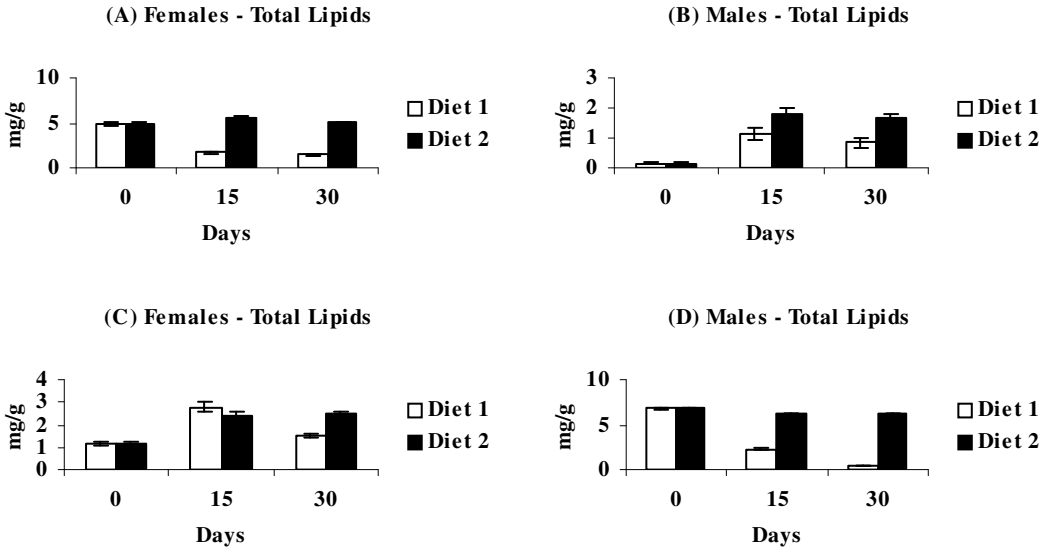

(A) Females - Total Lipids
mg/g
10
5
0
0
15
30
Days
Diet 1
Diet 2
(B) Males - Total Lipids
mg/g
3
2
1
0
0
15
30
Days
Diet 1
Diet 2
(C) Females - Total Lipids
mg/g
4
3
2
1
0
0
15
30
Days
Diet 1
Diet 2
(D) Males - Total Lipids
mg/g
10
5
0
0
15
30
Days
Diet 1
Diet 2

Figure 4

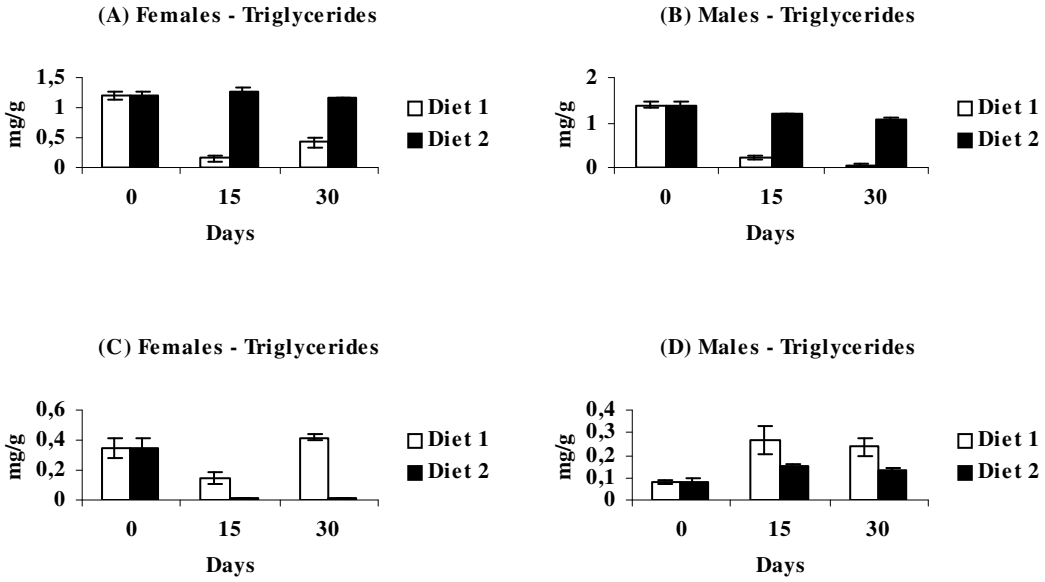
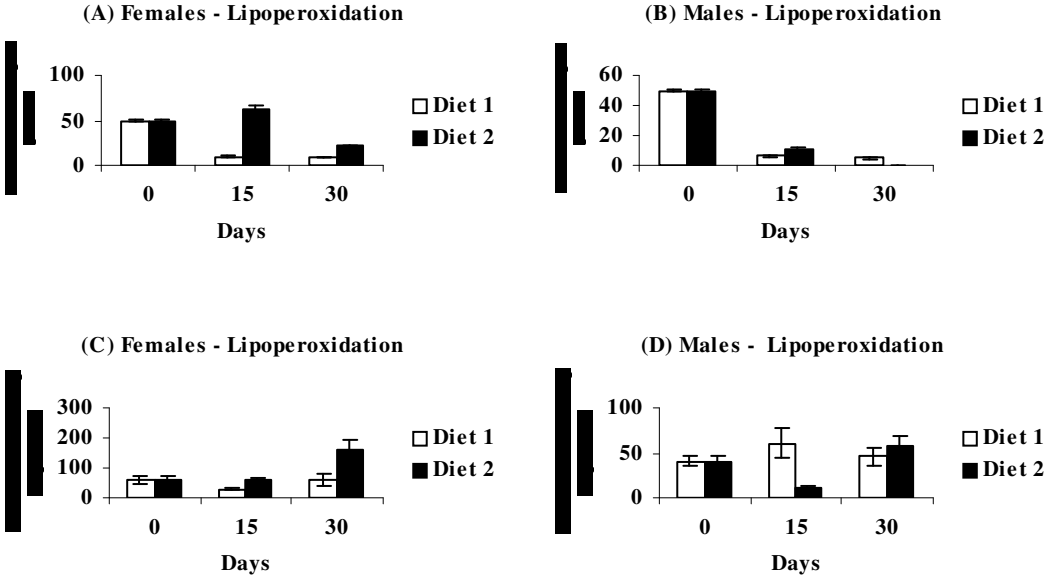

(A) Females - Triglycerides
1,5
1
0,5
0
mg/g
0
15
30
Days
Diet 1
Diet 2
(B) Males - Triglycerides
2
1
0
mg/g
0
15
30
Days
Diet 1
Diet 2
(C) Females - Triglycerides
0,6
0,4
0,2
0
mg/g
0
15
30
Days
Diet 1
Diet 2
(D) Males - Triglycerides
0,4
0,3
0,2
0,1
0
mg/g
0
15
30
Days
Diet 1
Diet 2

Figure 5


(A) Females - Lipoperoxidation
100
50
0
0
15
30
Days
Diet 1
Diet 2
(B) Males - Lipoperoxidation
60
40
20
0
0
15
30
Days
Diet 1
Diet 2
(C) Females - Lipoperoxidation
300
200
100
0
0
15
30
Days
Diet 1
Diet 2
(D) Males - Lipoperoxidation
100
50
0
0
15
30
Days
Diet 1
Diet 2

Carbofuran-Induced Alterations in Biochemical Composition, Lipoperoxidation and Na^+/K^+ATPase activity of *Hyalella pleoacuta* and *Hyalella curvispina* (Crustacea, Amphipoda, Dogielinotidae) in bioassays

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ABSTRACT

The present study investigated the effects of carbofuran on the energy metabolism (levels of glycogen, total proteins, total lipids, triglycerides, and lipoperoxidation), Na^+/K^+ATPase activity, and reproductive parameters (formation of couples, ovigerous females, and mean number of eggs) in the freshwater amphipods *Hyalella pleoacuta* and *Hyalella curvispina.* These crustaceans live in limnetic environments of the plateau (*H. pleoacuta*) and coastal plain (*H. curvispina*) of the state of Rio Grande do Sul in southern Brazil. The animals were collected in the winter of 2006 in the Vale das Trutas (28°47'00"S – 49°50'53"W) in the Municipality of São José dos Ausentes, and in Gentil Lagoon (29º56'30"S, 50º07'50"W) in the Municipality of Tramandaí. In the laboratory, the animals were kept submerged in aquariums under controlled conditions of photoperiod (14h light: 10h dark), temperature (23°C ± 1), and constant oxygenation. Animals were exposed to carbofuran at a dose of 5 or 50 mg/L for a period of 7 days. At the end of this period, the animals were immediately frozen for determination of the biochemical parameters, lipoperoxidation levels (TBARS), and enzyme Na^+/K^+ATPase activity. During each day of culture, several reproductive parameters were observed. Statistical analysis (ANOVA) revealed that carbofuran induces significant decreases in glycogen, proteins, lipids, triglycerides, and Na^+/K^+ATPase, as well as a significant increase in lipoperoxidation levels. Studies of all the biochemical parameters seem to be quite promising, in order to assess and predict the effects of toxicants on non-target organisms. The results also suggest that the reproductive parameters (formation of couples, ovigerous females and mean number of eggs) may provide sensitive criteria for assessing ecotoxicological effects. Furthermore, *H. pleoacuta* and *H. curvispina* are suitable organisms for use in toxicity tests, and we suggest that they are sensitive species that could be used in monitoring studies.

Keyword: *Hyalella pleoacuta*, *Hyalella curvispina*, Intermediate Metabolism, Lipoperoxidation, Na^+/K^+ATPase, Toxicology bioassay

INTRODUCTION

Members of the genus *Hyalella* are common in the Nearctic and Neotropical regions, with 45 described species (Gonzáles & Watling, 2001). They are found in a variety of freshwater habitats, such as permanent reservoirs, lakes, impoundments, and streams, and often cling to the vegetation, swim in the water, or burrow in the sediment, where they are important members of the benthic fauna (Kruschwitz, 1978; Wellborn, 1995; Grosso & Peralta, 1999).

The number of species used in standard toxicology tests is quite limited, and almost none of the species is native to Brazil. The reason for this small number of available species is that the majority of toxicology tests require continuous cultures to provide sufficient numbers of organisms. This rule has sharply limited the choice of species that can be utilized (Brendonck & Persoone, 1993).

Continuous or pulse exposure to pesticides may cause serious problems for non-target organisms, leading to a number of pathological and disturbed biochemical processes, including changes in energy budgets. The organisms may have direct energy costs to resist the toxicant by avoidance, exclusion, or removal; moreover, they may need energy for repair mechanisms and eventually pathological effects. All these energy expenses reduce the amount of energy left to invest in normal life, and therefore increase the probability of dying from additional stress (Calow, 1989).

Recently, a great deal of attention has been devoted to the use of physiological and energetic processes of non-target organisms (e.g., feeding parameters, growth, respiration, reproduction, and energy allocation mechanisms) as sensitive indicators in situations of toxic stress from exposure to metals (Donker, 1992; Donker ***et al.***, 1993; Drobne and Hopkin, 1994; Khalil ***et al.***, 1995) and chemicals (Van Straalen and Verweij, 1991; Mohamed ***et al.***, 1992; Van Brummelen ***et al.***, 1996a). The ecological relevance of these parameters is clear, because short-

term exposures can cause long-term effects on the life cycles of non-target organisms, even though some compounds do not persist for long in the soil (Ribeiro ***et al.***, 2001).

Disturbance of the homeostasis of an organism leads to compensatory, adaptive and finally pathological processes which are mostly energy-demanding. Therefore, the metabolic rate of an organism must increase under toxic stress (Calow, 1989). Because the energy resources of organisms are limited, the additional metabolic costs result in a reallocation of energy resources, and can only be met at the expense of other energy-demanding processes (Beyers ***et al.***, 1999) or by increased energy intake.

The toxicity of chemicals to aquatic organisms can also be modified by changes in environmental conditions. For example, increasing salinity has been reported to have varying effects on the toxicity of chemical compounds to aquatic biota (Hall and Anderson, 1995), while oxygen stress and carbaryl (1-napthyl methylcarbamate) have been reported to act in a more than additive fashion in *Daphnia pulex* (Hanazato and Dodson, 1995). Hardness, pH, dissolved organic carbon (DOC), and other water-quality characteristics have been shown to modulate the toxicity of metals (Erickson ***et al.***, 1996). Although the effects of feeding in acute studies with *Daphnia magna* using organic toxicants are not predictable (Adema, 1973), food availability has been shown to decrease some of the toxic effects of lead (Enserink ***et al.***, 1995) and cadmium (Kluttgen and Ratte, 1994) on chronically exposed *D. magna.*

Carbofuran is a carbamate nematicide and insecticide. Its mode of action, like other carbamate and organophosphate pesticides, is via acetylcholinesterase (AChE) inhibition (Kuhr and Dorough, 1976; US EPA, 1988). In invertebrates, AChE inhibitors exert their toxicity on the central nervous system (Eldefrawi, 1985). AChE inhibition in insects causes hyperactivity, loss of coordination, convulsions, paralysis, and death (Kuhr and Dorough, 1976). Consequently, intoxicated organisms experiencing a loss of coordination or orientation may be additionally

compromised while coping with environmental stresses, such as suspended solids, which require changes in physical activity. Increased metabolic activity (e.g., Samson ***et al.***, 1984) coupled with increased physical stress may also deplete energy stores in an organism (e.g., Rambabu and Rao, 1994). If energy stores and/or physical activity are affected by exposure to both carbofuran and suspended solids, and energy availability affects the toxic response of *D. magna*, both stressors could affect similar physiological processes. Therefore, it is reasonable to hypothesize that the toxic effects of carbofuran and suspended solids could combine in an additive manner.

The toxicity of carbofuran to aquatic life has been reviewed by Elser (1985) and by the Canadian Water Quality Guidelines (Anonymous, 1989). LC_{50} (24-48hr) values for fish range between 280 and 8500 µg/liter (Bakhavathsalam and Reddy, 1982; Carter and Graves, 1972; Davey ***et al.***, 1976 Hejduk and Svobodova, 1980; Stephenson ***et al.***, 1984; Verma ***et al.,*** 1981, 1982), while amphibians, mollusks, oligochaetes, higher plants, and algae are generally less sensitive (Dad ***et al.***, 1982; Hartman and Martin, 1985; Kar and Singh, 1979; Khangarot ***et al.***, 1985; Pawar and Katdare, 1983). On the other hand, crustaceans and insect larvae are among the most susceptible groups of organisms, with acute toxicities in the range of 1.6-500 µg/liter (Chitra and Pillai, 1984; Hartman and Martim, 1985; Johnson, 1986; Karnak and Collins, 1974; Parsons and Surgeoner, 1991; Pawar and Katdare, 1983).

ATPases play important roles in intracellular functions and in all types of physiological activities. Satyavathi and Prabhakara Rao (2000) reported four different ATPase activities in the plasma membrane/mitochondrial fractions of *Penaeus indicus*: Mg^{2+}ATPase, $Na^{+/}K^{+}$ATPase, Na^{+}ATPase and K^{+}ATPase, although the stimulation caused by Na^{+} and/or K^{+} could be due to the effect of each cation acting on a single ATPase. The sodium pump or Na^{+}/K^{+}ATPase is a membrane-bound enzyme found in animal cells, with its most important feature being the coupling of the free energy stored within the ATP molecule to the translocation of Na^{+} ions.

Often, ATPase activity is used as a sensitive indicator of heavy metal toxicity (Haya and Waiwood, 1983), although there is evidence that organic pollutants can inhibit ATPase activity in concentration-based experiments (Reddy ***et al.***, 1992). The measurement of feeding rate has been proposed as a general, although sensitive, physiological indicator of toxic stress in both freshwater and marine species (Naylor ***et al.***, 1989; Roddie ***et al.,*** 1996). Furthermore, a toxicant-induced reduction in feeding rate is relevant from the ecological point of view, because it could be related to reductions in an organism's energy assimilation, which, in turn, could lead to a reduction in resource allocation to growth, reproduction, and survival, and finally translate into effects at the population level (Maltby and Naylor, 1990; Maltby, 1994; Maltby ***et al.***, 2001; Irving ***et al.***, 2003). Calow and Sibly (1990) suggested that this commonly used index of environmental stress could lead to differences in the intrinsic rate of population growth, depending on whether the reduction in reproduction was due to a reduction in food intake or to an increase in metabolic cost.

Some studies have reported that the peroxidation of membrane phospholipids induced by reactive oxygen species and/or free radicals leads to alterations in the membrane structure and functions (Halliwell and Gutteridge, 1986; Vercesi ***et al.,*** 1997; Milatovic ***et al.,*** 2005). These degenerative changes can affect dynamic properties of the membranes such as fluidity and permeability, and consequently the activity of various membrane-associated enzymes (Meccoci ***et al.,*** 1997). Several investigators have reported that lipid peroxidation products disrupt neuronal ion homeostasis by impairing the function of membrane-bound ion-motive ATPases such as Na^+/K^+ATPase (Keller ***et al.,*** 1997; Mark ***et al.,*** 1997).

The aims of the present study were to quantify biochemical responses of ecological importance (energy reserves, lipid peroxidation and Na^+/K^+ATPase activity, as well as reproductive parameters) in the organisms exposed to carbofuran; and to relate data obtained for

the biochemical parameters, to the physiological status of the animals. The purpose was to obtain basic physiological data to support the development of a new model for toxicology tests in the laboratory.

MATERIAL AND METHODS

The animals were cared for in accordance with guidelines such as the *Guide for the Care and Use of Laboratory Animals* (1996, published by National Academy Press, 2101 Constitution Ave. NW, Washington, DC 20055, USA) and Brazilian laws. The animals were used with the permission of the Ethics Committee of the Pontifícia Universidade Católica do Rio Grande do Sul (License 0004/03).

Collection of *Hyallela pleoacuta* and *Hyalella curvispina*

In order to establish the profile of variation in the biochemical composition, lipid peroxidation levels and levels of activity of Na^+/K^+ATPase in the amphipods *Hyalella curvispina* and *Hyalella pleoacuta*, submitted to two concentrations of carbofuran (5 or 50 μg/L). The collections were made in the winter (June, July and August). The animals were collected together with macrophytes from each locale (*Hyallela curvispina* with *Salvinia bilob,* and *Hyalella pleoacuta* with *Callitriche rimosa*). *Hyallela curvispina* (120 males and females) were collected in Gentil Lagoon (29º56'30"S, 50º07'50"W), in Tramandaí; and *Hyallela pleoacuta* (120 males and females) in São José dos Ausentes Municipality (28°47'00"S – 49°50'53"W), Rio Grande do Sul, Brazil. The animals were collected by means of fish traps and bottom grabs, and were transported on ice in insulated containers to the Laboratory of Conservation Physiology of PUCRS. Twenty animals of each sex and each species were cryoanesthetized, in order to compare whether there was any difference between the animals collected in the environment and the animals that received artificial diets and were exposed to the pesticide.

In order to characterize the collection locales, the pH, temperature, and hardness of the water were measured during the months of collection. The pH was determined with a portable pH meter, and water temperature with an internal-scale thermometer. The hardness was determined using a classic method of volumetric complexation (Adad, 1982).

Experimental procedure:

Adult animals of each species were kept submerged in aerated aquariums (20L), divided with netting in order to maintain contact with the chemical but to prevent any physical interaction (the water passed by the two sides of the aquarium) between males and females; some early studies in our laboratory demonstrated that this arrangement is important to keep the animals alive. The mean temperature was 23 ± 1ºC and the photoperiod was 14:10 hours light/dark. The animals were acclimated in the aquariums for seven days, during which they received only food (macrophytes and ration) *ad libitum*, daily during periods when most of the animals were active (Tables 1 and 2 show the composition of the diets for each species). After this acclimation period, 20 animals of each sex and each species were cryoanesthetized, in order to compare whether there was any difference between the animals that only received the diet and the animals that received the diet and were exposed to the pesticide.

After seven days, the remaining amphipods were divided into four groups, and fed *ad libitum* for a further seven days. Group 1 was the control (natural environment). Group 2 was treated with dimethyl sulfoxide (DMSO), a solvent used to dissolve the pesticide; in the aquarium the DMSO concentration was 0.0000175%. Group 3 was exposed to 5 μg/L carbofuran. Group 4 was exposed to 50 μg/L carbofuran. All of the animals were exposed for a period of 7 days.

When the bioassay period ended, all the amphipods were cryoanesthetized, weighed on an electronic balance (± 0.001), and stored frozen at -80°C until they were used to determine the biochemical parameters.

Analyses of Behavior

Reproductive behavior

After a period of 24 hours, 10 couples were placed in each 20-liter aquarium, for a total of eight aquariums and 80 animals (two aquariums for each group). The animals were observed each day, for 7 days when they were only fed the diet, and for an additional 7 days with the control, DMSO or the 5 or 50 μg/L carbofuran treatments. The number of couples and ovigerous females was counted each day (Plaistow, 2003).

Survival and Mortality

The survival and mortality of the animals were recorded during the course of the experimental cultures.

Biochemical Analyses

The metabolic determinations for *H. curvispina* and *H. pleoacuta* were done in total homogenates of four pools of five males and five females for each point. One pool of each species, *H . curvispina* or *H. pleoacuta*, was used for determination of glycogen and proteins; the second pool for quantification of lipids and triglycerides; the third pool for quantification of lipoperoxidation levels; and the fourth pool for quantification of the levels of activity of Na^+/K^+ATPase. Metabolic parameters and the enzyme activity were determined in quadruplicate by spectrophotometric methods.

a. Glycogen was extracted from tissues following the method described by Van Handel (1965), and glycogen levels in the animals were determined as glucose equivalent, 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 animal.

b. Proteins were quantified as described by Lowry (1957), with bovine albumin (Sigma Co.) as the standard. Results are expressed in mg/ml of the total homogenate.

c. 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, 1970; Meyer and Walter, 1981). This method consists of oxidizing cellular lipids to small fragments after chemical digestion with hot concentrated sulfuric acid. After the addition of a solution of vanillin and phosphoric acid, a red complex is formed which is measured with a spectrophometer (530 nm). Triglycerides were measured by the reactions of lipase, glycerokinase, 1-P-glycerol oxidase, and peroxidase enzymes (Biodiagnostic Kit / GPO Trinder). Results are expressed as mg/g of animals.

d. Lipoperoxidation levels were quantified by the method of Buege and Aust (1978) by measuring reactive substances to Thiobarbituric Acid (TBA-RS), using the extraction method of Llesuy ***et al.*** (1985). The results are expressed in nmol of TBARS/mg of protein.

e. The membrane was extracted from five animals, according to Barnes (1993). The pool were homogenized (10% W/V) in cold buffer Tris (40mM) and phenylmethylsulfonyl fluoride (1 mM; from Sigma, St. Louis, MO) with pH adjusted to 7.40. The homogenate was centrifuged at 10000X*g* at 4°C, and the supernatant was collected and centrifuged at 40.000X*g* (4°C). The pellet was resuspended in the same buffer and centrifuged again at 40.000X*g* (4°C). This last supernatant was then used as the source of Na^+/K^+ATPase. Na^+/K^+ATPase activity was measured according to the method described by Esmann (1988). Incubation medium A contained ATP (5 mM; from Sigma), NaCl (60 mM), KCl (10 mM) and MgCl (40mM), with the pH adjusted to 7.40. In the incubation medium B, KCl was replaced by ouabain (1 mM; from Sigma). Aliquots of homogenate were incubated at 30°C in both mediums A and B, for 30 min with the equivalent of 10 mg of the proteins. The enzymatic reaction was stopped by addition of 10% trichloroacetic acid. The inorganic phosphorus released was determined using the method of Chan (1986), in a

spectrophotometer at 630 nm. Any difference in phosphorus concentration between medium A and B was attributed to Na^+/K^+ATPase activity. All determinations were done at least in quadruplicate. Results are expressed in μmoles of the P*i*.mg protein^{-1}.min^{-1}. These experimental procedures were used after testing different incubation temperatures (20, 25, 30 and 37ºC), times (15, 30, 45 and 60 minutes), and protein concentrations (5, 10, 15 and 20 mg).

Statistical Analysis

The results are expressed as mean ± standard error. All the metabolic parameters were homogeneous (Levene test), and were normally distributed (Kolmogorov-Smirnov test). For statistical analysis of the different treatments, a one-way ANOVA test was used, followed by a Bonferroni test. For comparisons between different species and sexes, 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 environmental and experimental culture water parameters are listed in Table 2. The mean temperatures of the collection locales were significantly lower than the temperature of the experimental cultures. The pH and hardness of the water were constant in both situations; the environmental pH was 6.50 ± 0.50, and 7.00 ± 1.00 in the experimental culture. The water hardness in the environment was 1.07 ± 0.43 ppm $CaCO_3$ and in the experimental culture it was 0.98 ± 0.43 ppm $CaCO_3$. The two diets were analyzed by the Instituto de Ciências e Tecnologia dos Alimentos (ICTA) of the Universidade Federal do Rio Grande do Sul (UFRGS). These analyses verified that the diets were isocaloric (Table 1).

The numbers of couples and ovigerous females of the two species submitted to the different treatments are listed in Table 3. The females of the control groups for both species were collected in the natural environment, and they bore similar numbers of eggs. *Hyalella pleoacuta*

maintained on the diet for 7 or 14 days showed similar numbers of couples and ovigerous females. These parameters were also similar in the animals from the natural environment, and between the periods when they were maintained on the diet (7 and 14 days). Females submitted to DMSO formed fewer couples and showed a lower proportion of ovigerous females than did the females maintained on the diet alone or collected in the environment. These females also showed a decrease in the number of eggs (approximately 50% lower). No couples, ovigerous females, or eggs were observed in the groups treated with the pesticide: the animals never paired in the laboratory during treatment with pesticide.

The survival rates of the males and females of both species submitted to the different treatments are shown in Table 4. We observed that in both species and both sexes, the animals exposed to carbofuran showed lower survival rates in relation to the other groups. Although the survival rate of the DMSO groups was also lower than the control groups, this difference was not significant.

Figure 1 shows the variation in glycogen content in females and males of *Hyalella pleoacuta* (A) and *Hyalella curvispina* (B). In *H. pleoacuta* females, the group collected in the environment, maintained on the diet for 7 or 14 days, showed similar levels of glycogen. When these animals were treated with DMSO, glycogen decreased 38%. When the females were submitted to carbofuran (5 and 50 μg/L), glycogen levels decreased approximately 8 times. In males of *H. pleoacuta* collected in the environment, maintained on the diet for 7 or 14 days or treated with DMSO, glycogen levels were higher than in the other groups. When the amphipods were treated with the two concentrations of pesticide, the levels decreased approximately 16 times. There was a significant difference in the behavior curve of glycogen levels in females and males of *Hyalella pleoacuta* submitted to the different treatments ($p<0.05$).

In females of *H. curvispina,* the group collected in environment and maintained on the diet for 7 or 14 days showed similar glycogen levels. When these animals were submitted to the treatment with DMSO, their glycogen levels decreased by 4 times. In the females submitted to both concentrations (5 and 50 μg/L) of carbofuran, the glycogen levels decreased approximately 17.4 times in relation to the control groups and periods on the diet. In males of *H. curvispina* collected in environment or maintained on the diet for 7 or 14 days, glycogen levels were higher then in the animals treated with DMSO or 5 and 50 μg/L carbofuran. In the group treated with the solvent, the values of glycogen in males decreased 2.0 times, and the values decreased 4.0 (5 μg/L) and 8.5 times (50 μg/L), in relation to the control groups when the animals were treated with carbofuran. There was no significant difference in the behavior of glycogen levels in females and males of *Hyalella curvispina* submitted to the different treatments ($p>0.05$).

There was a significant difference in the behavior of the glycogen levels in males of *Hyalella pleoacuta and Hyalella curvispina* submitted to the different treatments ($p<0.05$). The same pattern was found for the females of the two species submitted to the different treatments ($p<0.05$).

Figure 2 shows the variation in total protein content in females and males of *Hyalella pleoacuta* (A) and *Hyalella curvispina* (B). In females of *H. pleoacuta,* the group collected in the environment and maintained on the diet for 7 or 14 days showed similar levels of total proteins. When these animals were submitted to treatment with DMSO, there was a decrease of 50% in their values. In females submitted to either concentration of carbofuran, the levels decreased approximately 10 times. In males of *H. pleoacuta* collected in the environment or maintained on the diet for 7 or 14 days, total protein levels were higher ($p<0.05$) than in the other groups (DMSO, 5 and 50 μg/L carbofuran). There was a significant difference in the behavior of total-

protein levels in females and males of *Hyalella pleoacuta* submitted to the different treatments ($p<0.05$).

In females of *H. curvispina,* the group collected in the environment and maintained on the diet for 7 or 14 days showed similar levels of total proteins. When these animals were submitted to treatment with DMSO, the levels of this reserve decreased 3.8 times. These levels decreased even more in the animals exposed to carbofuran (5 and 50 μg/L), by approximately 23.3 times. In males of *H. curvispina* collected in the environment and maintained on the diet for 7 or 14 days, these values (total proteins) were similar. When these animals were submitted to treatment with DMSO, total proteins decreased fourfold. The same pattern of response was observed with the different concentrations of carbofuran. There was a significant difference in the behavior of total protein levels in females and males of *Hyalella curvispina* submitted to the different treatments ($p<0.05$).

There was a significant difference in the levels of total proteins in males of *Hyalella pleoacuta* and *Hyalella curvispina* submitted to the different treatments ($p<0.05$). The same pattern was found in females of the two species submitted to the different treatments ($p<0.05$).

Figure 3 shows the variation in total lipid content in females and males of *Hyalella pleoacuta* (A) and *Hyalella curvispina* (B). The females of *H. pleoacuta,* in the groups collected in the environment and maintained on the diet for 7 or 14 days or submitted to the solvent, showed similar levels of total lipids. When these animals were treated with the different concentrations of carbofuran, their lipid levels decreased approximately 64%. In males of *H. pleoacuta* collected in the environment and maintained on the diet for 7 or 14 days, or treated with DMSO, total lipid levels were higher than in the males treated with carbofuran in both concentrations, the values decreased approximately 1.62 times. There was a significant difference

in the behavior of total lipid levels in females and males of *Hyalella pleoacuta* submitted to the different treatments ($p<0.05$).

In females of *H. curvispina,* the group collected in the environment and maintained on the diet for 7 or 14 days showed similar total lipid levels, but when these animals were submitted to treatment with DMSO there was a decrease of 50%. In the females submitted to the two concentrations of carbofuran (5 μg/L and 50 μg/L), the lipid levels decreased 2 times and 5 times, respectively, in relation to the control. In the males of *H. curvispina* collected in the environment and maintained on the diet for 7 or 14 days, or treated with DMSO, the lipid concentrations were higher than in the animals exposed to the different concentrations of carbofuran (5.2 times and 2.3 times, respectively). There was a significant difference in the behavior of total-lipid levels in females and males of *Hyalella curvispina* submitted to the different treatments ($p<0.05$).

There was a significant difference in the levels of total lipids in both males and females of *Hyalella pleoacuta* and *Hyalella curvispina* submitted to the different treatments ($p<0.05$.

Figure 4 shows the variation in triglyceride content in females and males of *Hyalella pleoacuta* (A) and *Hyalella curvispina* (B). In females of *H. pleoacuta*, the group collected in the environment and maintained on the diet for 7 or 14 days, or submitted to the DMSO, showed similar levels of triglycerides. When these animals were submitted to treatment with carbofuran, the levels decreased by approximately 5 times. In males of *H. pleoacuta* collected in the environment and maintained on the diet for 7 or 14 days, or treated with DMSO, triglyceride levels were higher than in the animals treated with carbofuran (5 and 50 μg/L). There was a significant difference in the behavior of triglyceride levels in females and males of *Hyalella pleoacuta* submitted to the different treatments ($p<0.05$).

In *H. curvispina* females, the group collected in the environment and maintained on the diet for 7 or 14 days, or treated with DMSO, showed similar levels of triglycerides. When these

animals were submitted to the two concentrations of carbofuran, these levels decreased by approximately 8.67 times. In males of *H. curvispina* collected in the environment or maintained on the diet for 7 or 14 days, triglyceride levels were higher than in the other groups (DMSO, 5 and 50 µg/L carbofuran). There was a significant difference in the behavior of triglyceride levels in females and males of *Hyalella curvispina* submitted to the different treatments ($p<0.05$).

There was a significant difference in the levels of triglycerides in males of *Hyalella pleoacuta* and *Hyalella curvispina* submitted to the different treatments ($p<0.05$). The same pattern shown by males was also found in the females of both species submitted to the different treatments ($p<0.05$).

Figure 5 shows the variation in levels of lipoperoxidation in females and males of *Hyalella pleoacuta* (A) and *Hyalella curvispina* (B). In females of *H. pleoacuta*, the group collected in the environment, maintained on the diet for 7 or 14 days showed similar levels of lipoperoxidation. In contrast, when these animals were submitted to treatment with DMSO or carbofuran, lipoperoxidation increased by 6 times and 22 times, respectively. In males of *H. pleoacuta* collected in the environment or maintained on the diet for 7 or 14 days, lipid peroxidation levels were higher than the levels in the animals treated with DMSO or 5 and 50 µg/L carbofuran. In animals exposed to the solvent, the values of lipoperoxidation in males increased 7.5 times, and the values in females increased 34 and 31 times, respectively, in relation to the control groups. There was a significant difference in the behavior of lipoperoxidation levels in females and males of *Hyalella pleoacuta* submitted to the different treatments ($p<0.05$).

In females of *H. curvispina,* the group collected in the environment, maintained on the diet for 7 or 14 days showed similar levels of lipoperoxidation. When the animals were submitted to treatment with DMSO or 5 and 50 µg/L of carbofuran, lipoperoxidation increased 5.54, 31.5 and 29.30 times, respectively. In males of *H. curvispina* collected in the environment or

maintained on the diet for 7 or 14 days, lipid peroxidation levels were lower than the levels in animals treated with DMSO (3.4 times) or 5 μg/L carbofuran (9.4 times) and 50 μg/L carbofuran (9.1 times). There was a significant difference in the behavior of lipoperoxidation levels in females and males of *Hyalella curvispina* submitted to the different treatments ($p<0.05$).

There was a significant difference in the levels of lipoperoxidation in males of *Hyalella pleoacuta* and *Hyalella curvispina* submitted to the different treatments ($p<0.05$). The same response was found for females of both species submitted to the different treatments ($p<0.05$).

Figure 6 shows the variation in Na^+/K^+ATPase activity in females and males of *Hyalella leoacuta* (A) and *Hyalella curvispina* (B). The females of *H. pleoacuta* showed, in the groups collected in the environment, maintained on the diet for 7 or 14 days, or treated with solvent, similar levels of enzyme activity (Na^+/K^+ATPase). When these animals were treated with both concentrations of carbofuran, the levels of activity decreased by 32 times and 15.7 times respectively. In males of *H. pleoacuta* collected in the environment or maintained on the diet for 7 or 14 days, the levels of Na^+/K^+ATPase activity were higher than in the males treated with DMSO or carbofuran. There was a significant difference in the behavior of the levels of Na^+/K^+ATPase activity in females and males of *Hyalella pleoacuta* submitted to the different treatments ($p<0.05$).

In females of *H. curvispina,* the group collected in the environment, maintained on the diet for 7 or 14 days, showed similar levels of Na^+/K^+ATPase activity. When these animals were exposed to DMSO, the levels of activity decreased by 1.68 times, and decreased more when the amphipods were exposed to both concentrations of carbofuran (approximately 15.5 times). In males of *H. curvispina* collected in the environment, maintained on the diet for 7 or 14 days showed similar values of Na^+/K^+ATPase activity. When these animals were treated with DMSO and the two concentrations of carbofuran, the activity levels decreased 1.14 and 7.67 times,

respectively. There was a significant difference in the behavior of levels of Na^+/K^+ATPase activity in females and males of *Hyalella curvispina* submitted to the different treatments ($p<0.05$).

There was a significant difference in the levels of Na^+/K^+ATPase activity in males of *Hyalella pleoacuta* and *Hyalella curvispina* submitted to the different treatments ($p<0.05$); the same response occurred in females of both species submitted to the different treatments ($p<0.05$).

DISCUSSION

The results of the present study showed that the diets were capable of sustaining the energetic homeostasis of animals, such as the formation of couples, the number of ovigerous females, and the mean number of eggs. However, exposure to different concentrations (5 and 50 μg/L) of carbofuran altered all the metabolic parameters (glycogen, total proteins, total lipids, triglycerides, lipoperoxidation and Na^+/K^+ATPase activity), as well as the reproductive parameters analyzed.

Disturbance of the homeostasis of an organism leads to compensatory, adaptive, and finally pathological processes, which are mostly energy-demanding. Therefore, the metabolic rate of an organism should increase under toxic stress (Calow, 1989). Because the energy resources of organisms are limited, the additional metabolic costs result in reallocation of energy resources, and can only be met at the expense of other energy-demanding processes (Beyers ***et al.***, 1999) or by increased energy intake. Probably, this reallocation of energy accounts for the reproductive response observed in these animals (*H. pleoacuta* and *H. curvispina*), in both sexes.

The solvent (DMSO) used to dilute the pesticide showed biological effects on the reproductive parameters and the levels of glycogen, total proteins, total lipids, lipoperoxidation and Na^+/K^+ATPase.

The animals treated with carbofuran showed lower levels of glycogen and total lipids than the animals collected in the environment or maintained only on the diet, independent of the length of time that they remained on the diet (7 or 14 days). Depletions in glycogen and lipid contents were also observed after exposure to organophosphate and organochloride insecticides, in mice (Naqvi and Vaishnavi, 1993), eels (Sancho ***et al.***, 1998), terrestrial isopods (Ribeiro ***et al.***, 2001), and snails (Rambabu and Rao, 1994), and after exposure to metals, in isopods (Donker, 1992; Sørensen ***et al.***, 1997). According to Ribeiro ***et al.*** (2001), the depletion of glycogen may result from direct utilization of this compound to generate energy, as a result of parathion- and endosulfan-induced hypoxia. This polysaccharide is rapidly catabolized, resulting in a rapid decrease in this energy reserve. The lipid content also decreased during exposure to both pesticides, because of its use as an energy reserve, parallel to glycogen (Sancho ***et al.***, 1998; Rambabu and Rao, 1994).

The decrease in protein content of parathion-intoxicated isopods also indicates a physiological adaptability to compensate for pesticide stress. To overcome the stress situation, animals require high amounts of energy, and this energy demand may have stimulated protein catabolism (Ribeiro ***et al.***, 2001). Similar decreases in protein content were observed by Van Brummelen and Stuijfzand (1993) in *Porcellio scaber* and *Oniscus asellus* exposed to benzo(*a*)pyrene, and by Van Brummelen ***et al.*** (1996) in *O. asellus* exposed to fluorine and benz(*a*)anthracene; this decrease was followed by a reduction in growth. Furthermore, this decrease in protein content might also be due to formation of lipoprotein, which will be used to repair damaged cell and tissue organelles (Sancho ***et al.***, 1998; Rambabu and Rao, 1994). The sharp decrease in proteins, as well as the increase in lipoperoxidation levels observed in this study, which were independent of species and sex, reinforced the suggestions of Sancho ***et al.*** (1998) and Rambabu and Rao (1994).

It is well established that carbofuran exerts its effects through cholinergic hyperactivity because of the accumulation of acetylcholine (Gupta ***et al.,*** 2001). According to Yang and Dettbarn (1996), there is a correlation between the accumulation of acetylcholine and the extent of lipid peroxidation. Milatovic ***et al.*** (2005) have shown that carbofuran produces oxidative stress, as measured by increased generation of reactive oxygen and nitrogen species in skeletal muscles. Peroxidation of membrane phospholipids induced by reactive oxygen species and/or free radicals leads to alterations in the membrane structure and functions (Halliwell and Gutteridge, 1986; Vercesi ***et al.,*** 1997). These degenerative changes can affect dynamic properties of the membranes such as fluidity and permeability, and consequently the activity of various membrane-associated enzymes (Mecocci ***et al.***, 1997). Several investigators have reported that lipid peroxidation products disrupt neuronal ion homeostasis by impairing the function of membrane-bound ion-motive ATPases such as Na^+-K^+ATPase and Ca^{2+}ATPase (Keller ***et al.***, 1997; Marc ***et al.***, 1997; Kamboj ***et al.***, 2006). The lower activity of Na^+/K^+ATPase and higher rates of lipoperoxidation shown by the two species and sexes can be explained by the association of this mechanism.

Kamboj ***et al.*** (2006) administered carbofuran to rats at a dose of 1 mg/kg body weight for 28 days, and found a significant increase (65.0%) in lipoperoxidation, decreases in total lipids (12.0%) and in phospholipid levels (15.6%), and an increase of cholesterol in the cerebral cortex. Gupta ***et al.***(2001) also demonstrated a decrease in phospholipid fractions in the brains of carbofuran-treated mice, whereas the levels of total lipids and cholesterol remained unaltered. These studies, together with the results shown in amphipods, demonstrate the action of the pesticide on lipid metabolism. This action is correlated with energy production and lipoperoxidation levels.

The animals submitted to the carbofuran (5 and 50 μg/L) showed a lower survival rate compared to the control animals, and the concentration of 500μg/L caused 100% mortality in 24 hours. Wayland and Boag (1990), working with amphipod crustaceans in cages, observed mortalities in this group over 3-4 days at initial carbofuran exposure concentrations in the range of 9-32 μg/liter, which is one of the most sensitive responses known. Mattessen ***et al.*** (1995) showed that crustaceans and insect larvae are among the most susceptible groups of organisms, with acute toxicities in the range of 1.6 - 500 mg/L.

Our results revealed that carbofuran induces a significant depression of glycogen, proteins, lipids, triglycerides, and Na^+/K^+ATPase, as well as a significant increase in lipoperoxidation rates. Studies of all these biochemical parameters seem quite promising to assess and predict the effects of toxicants on non-target organisms. The results also suggest that the reproductive parameters (formation of couples, ovigerous females and mean number of eggs) may provide sensitive criteria for assessing ecotoxicological effects. Furthermore, the amphipods *Hyalella pleoacuta* and *Hyalella curvispina* are suitable organisms for use in toxicity tests, and we suggest that they are sensitive species which could be useful in monitoring studies.

ACKNOWLEDGEMENTS

This study was supported by grants from the Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS, Process no. 05501490) and the Conselho Nacional de Desenvolvimento Tecnológico (CNPq, Process nos. 300075/2003-0, 131721/2005-4, and 103334/2005-0). The Pontifícia Universidade Católica do Rio Grande do Sul supported this work. The experiments were performed according to current Brazilian laws.

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Figures Legends

Figure 1: Concentrations of glycogen in *Hyalella pleoacuta* and *Hyalella curvispina* submitted to the different treatments. Columns represent the mean, and bars represent the standard error of the mean. Results are expressed in mg/g.

Figure 2: Concentrations of total proteins of *Hyalella pleoacuta* and *Hyalella curvispina* submitted to the different treatments. Columns represent the mean, and bars represent the standard error of the mean. Results are expressed in mg/ml.

Figure 3: Concentrations of total lipids in *Hyalella pleoacuta* and *Hyalella curvispina* submitted to different treatments. Columns represent the mean, and bars represent the standard error of the mean. Results are expressed in mg/g.

Figure 4: Concentrations of triglycerides in *Hyalella pleoacuta* and *Hyalella curvispina* submitted to the different treatments. Columns represent the mean, and bars represent the standard error of the mean. Results are expressed in mg/g.

Figure 5: Lipoperoxidation rates in *Hyalella pleoacuta* and *Hyalella curvispina* submitted to the different treatments. Columns represent the mean, and bars represent the standard error of the mean. Results are expressed in nmol TBARS/mg protein.

Figure 6: Activity levels of Na^+/K^+ATPase in *Hyalella pleoacuta* and *Hyalella curvispina* submitted to the different treatments. Columns represent the mean, and bars represent the standard error of the mean. Results are expressed in nmol TBARS/mg protein.

Table Legends

Table 1: Centesimal composition of the diets.

Table 2: Water temperature (°C), pH, and hardness (ppm $CaCO_3$) in the natural environment and in the experimental conditions. The results are expressed as mean ± standard error of the mean.

Table 3: Number of couples and ovigerous females of *Hyalella pleoacuta* and *Hyalella curvispina* maintained with the different treatments.

Table 4: Survival rates of males and females of *Hyalella pleoacuta* and *Hyalella curvispina* maintained with the different treatments.

Table 1

Compound	*Hyalella pleoacuta* *Callitriche rimosa* and ration	*Hyalella curvispina* *Salvinia biloba* and ration
Water content (g/100 g)	98.97	89.13
Ash (g/100 g)	12.28	22.09
Protein (g/100 g)	32.12	32.07
Fat (g/100 g)	6.47	6.91
Fiber (g/100 g)	5.29	4.66
Carbohydrates (g/100 g)	45.23	43.86
Total Caloric Value (Kcal/100 g)	361.51	365.91

Table 2

Abiotic Water Factors	Environment	Experimental Culture
Temperature	12.04 ± 0.30	20.00 ± 1.00
pH	6.50 ± 0.50	7.00 ± 1.00
Hardness	1.07 ± 0.43	0.98 ± 0.53

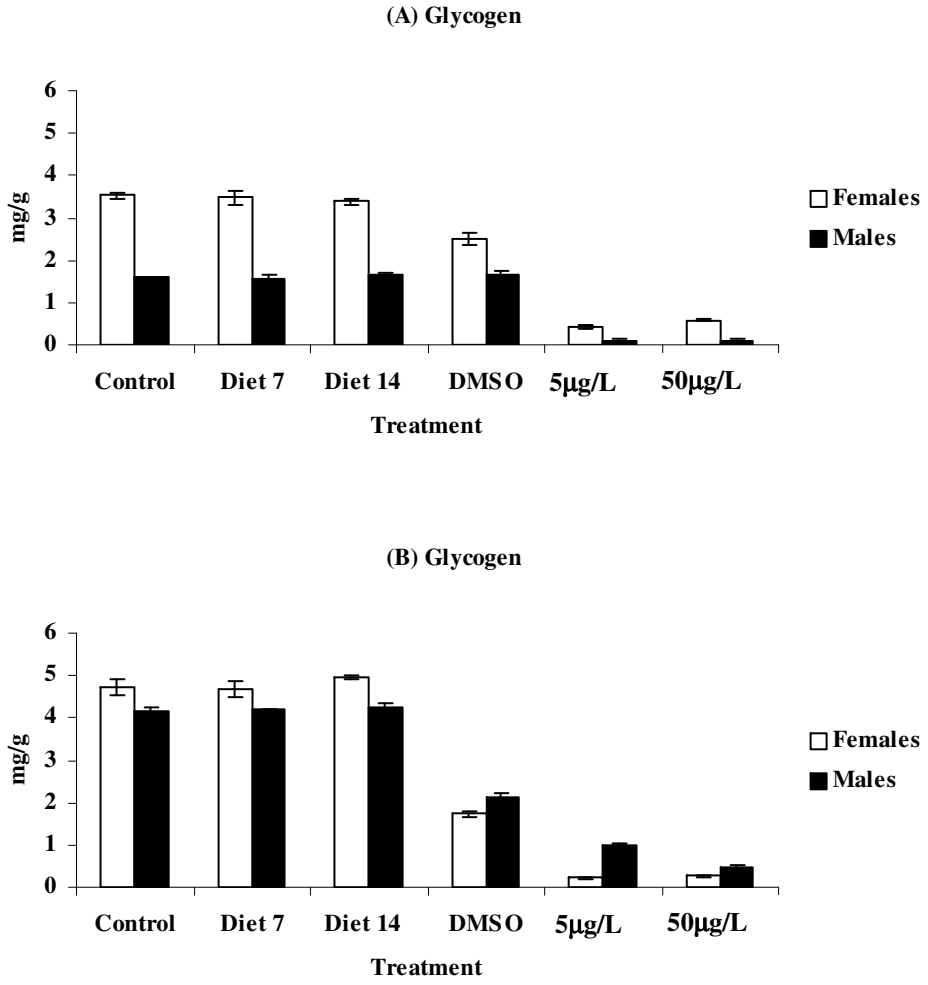
Table 3

Hyalella pleoacuta	Number of Couples	Ovigerous Females	Mean Number of Eggs
Control (Natural Environment)	-	20	30 ± 5
Diet, 7 days	15	8	30 ± 8
Diet, 14 days	13	10	29 ± 3
DMSO	6	4	20 ± 4
5 µg/L	-	-	-
50 µg/L	-	-	-
Hyalella curvispina			
Control (Natural Environment)	-	20	33 ± 7
Diet, 7 days	13	9	30 ± 5
Diet, 14 days	11	7	28 ± 3
DMSO	8	4	15 ± 5
5 µg/L	-	-	-
50 µg/L	-	-	-

Table 4

Hyalella pleoacuta	Culture 1	Culture 2	Culture 3	Mean
Diet, 7 days	97.92%	96.60%	97.80%	97.44%
Diet, 14 days	98.67%	98.41%	98.74%	98.61%
DMSO	92.72%	93.73%	94.25%	93.57%
5 µg/L	79.65%	82.63%	80.98%	81.09%
50 µg/L	69.96%	71.85%	70.52%	70.78%
Hyalella curvispina				
Diet, 7 days	98.52%	97.39%	97.71%	97.87%
Diet, 14 days	99.74%	98.99%	99.49%	99.41%
DMSO	94.98%	94.42%	95.68%	95.03%
5 µg/L	80.54%	78.69%	82.47%	80.57%
50 µg/L	75.98%	70.96%	72.65%	73.20%

Figure 1


(A) Glycogen
mg/g
6
5
4
3
2
1
0
Control
Diet 7
Diet 14
DMSO
5µg/L
50µg/L
Treatment
Females
Males
(B) Glycogen
mg/g
6
5
4
3
2
1
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Control
Diet 7
Diet 14
DMSO
5µg/L
50µg/L
Treatment
Females
Males

Figure 2

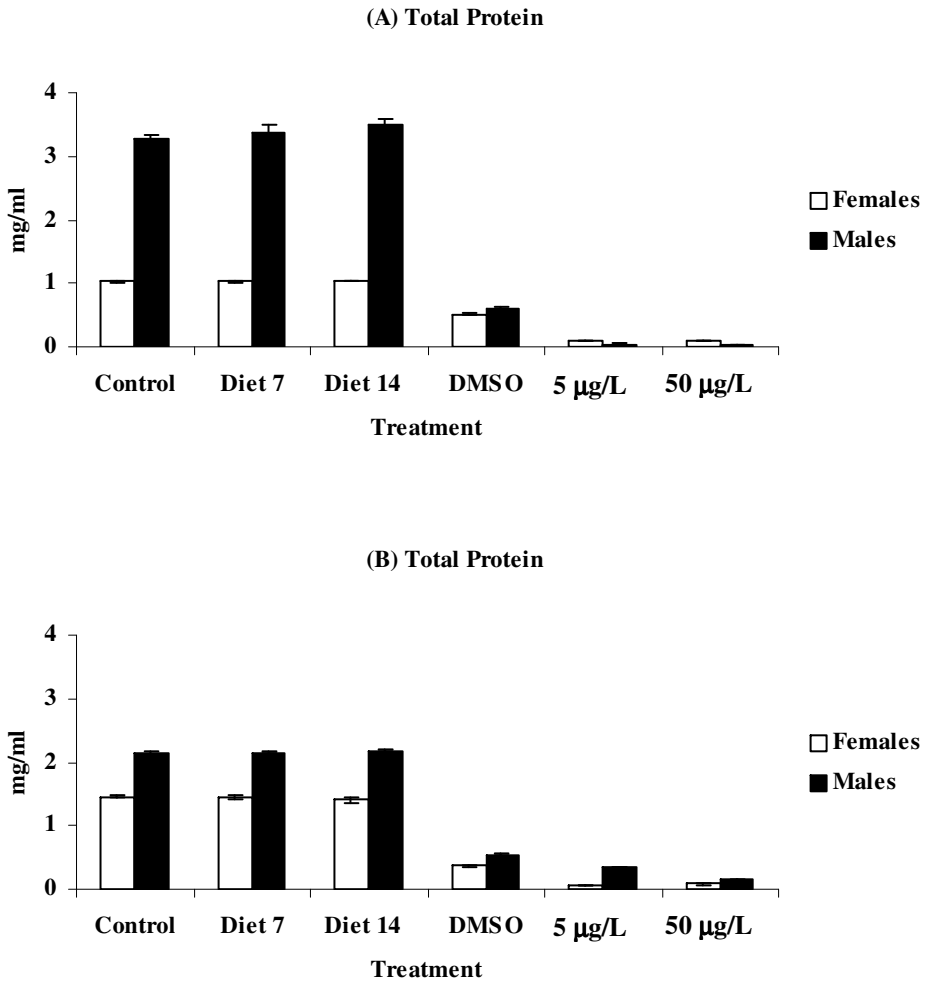

(A) Total Protein
mg/ml
4
3
2
1
0
Control
Diet 7
Diet 14
DMSO
5 µg/L
50 µg/L
Treatment
Females
Males
(B) Total Protein
mg/ml
4
3
2
1
0
Control
Diet 7
Diet 14
DMSO
5 µg/L
50 µg/L
Treatment
Females
Males

Figure 3

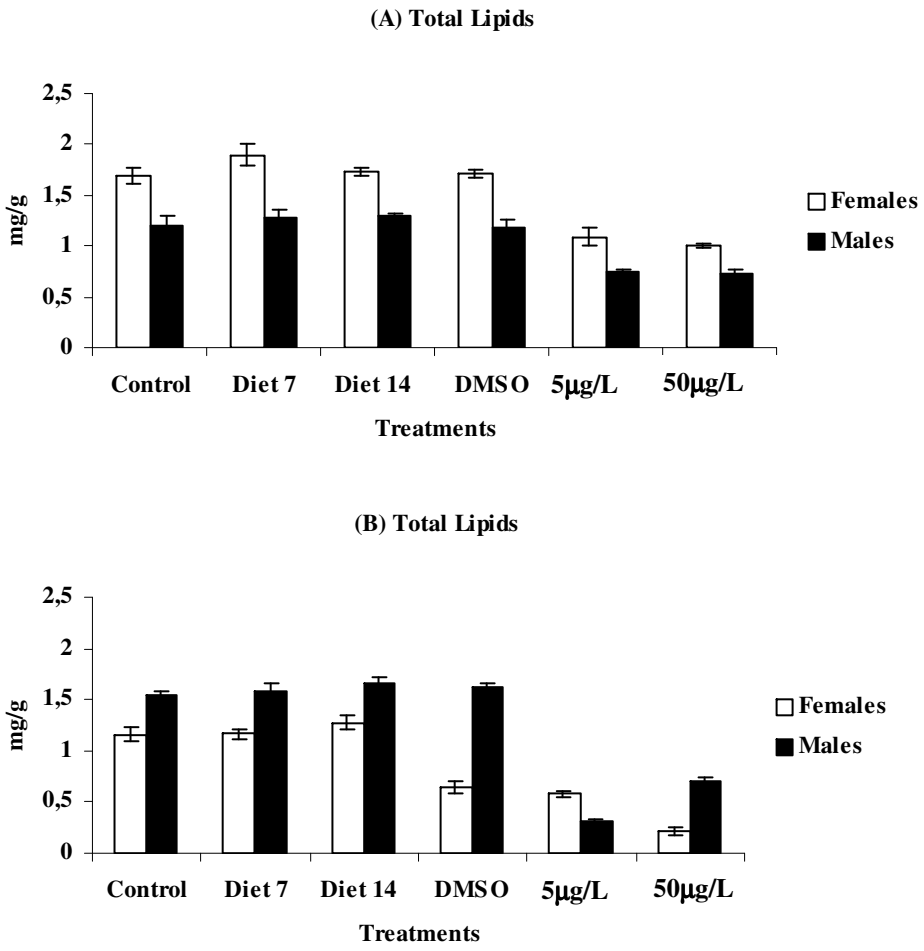

(A) Total Lipids
2,5
2
1,5
1
0,5
0
mg/g
Females
Males
Control
Diet 7
Diet 14
DMSO
5µg/L
50µg/L
Treatments
(B) Total Lipids
2,5
2
1,5
1
0,5
0
mg/g
Females
Males
Control
Diet 7
Diet 14
DMSO
5µg/L
50µg/L
Treatments

Figure 4

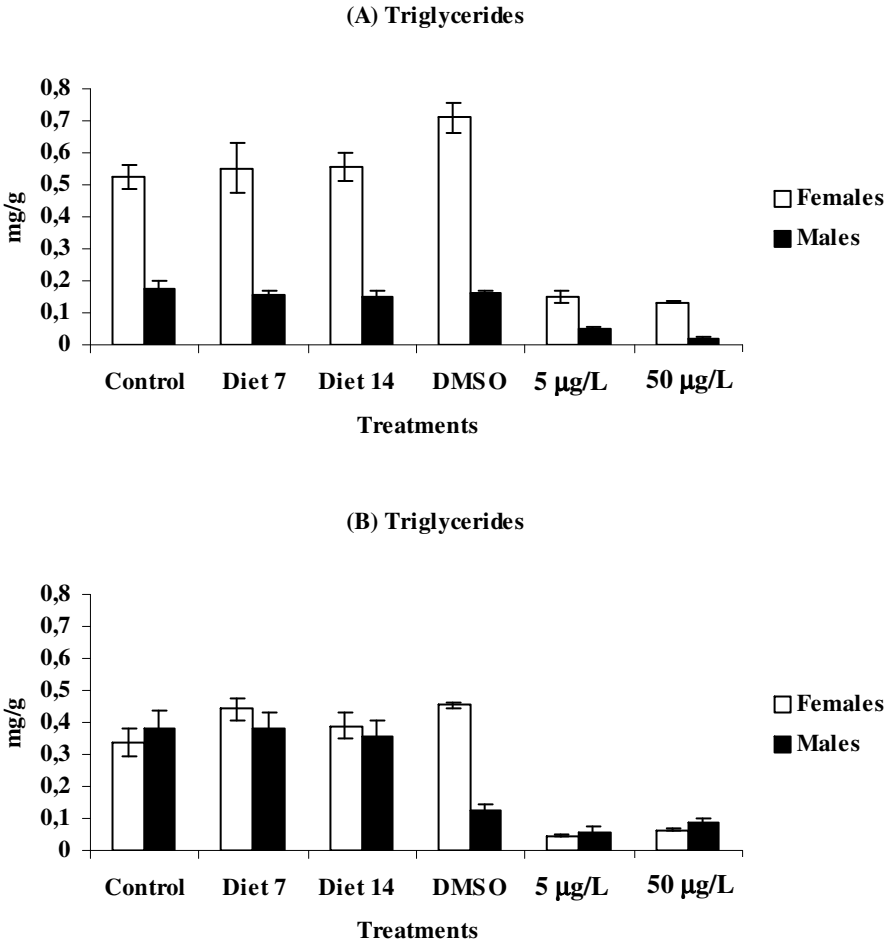

(A) Triglycerides
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
mg/g
Females
Males
Control
Diet 7
Diet 14
DMSO
5 µg/L
50 µg/L
Treatments
(B) Triglycerides
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
mg/g
Females
Males
Control
Diet 7
Diet 14
DMSO
5 µg/L
50 µg/L
Treatments

Figure 5

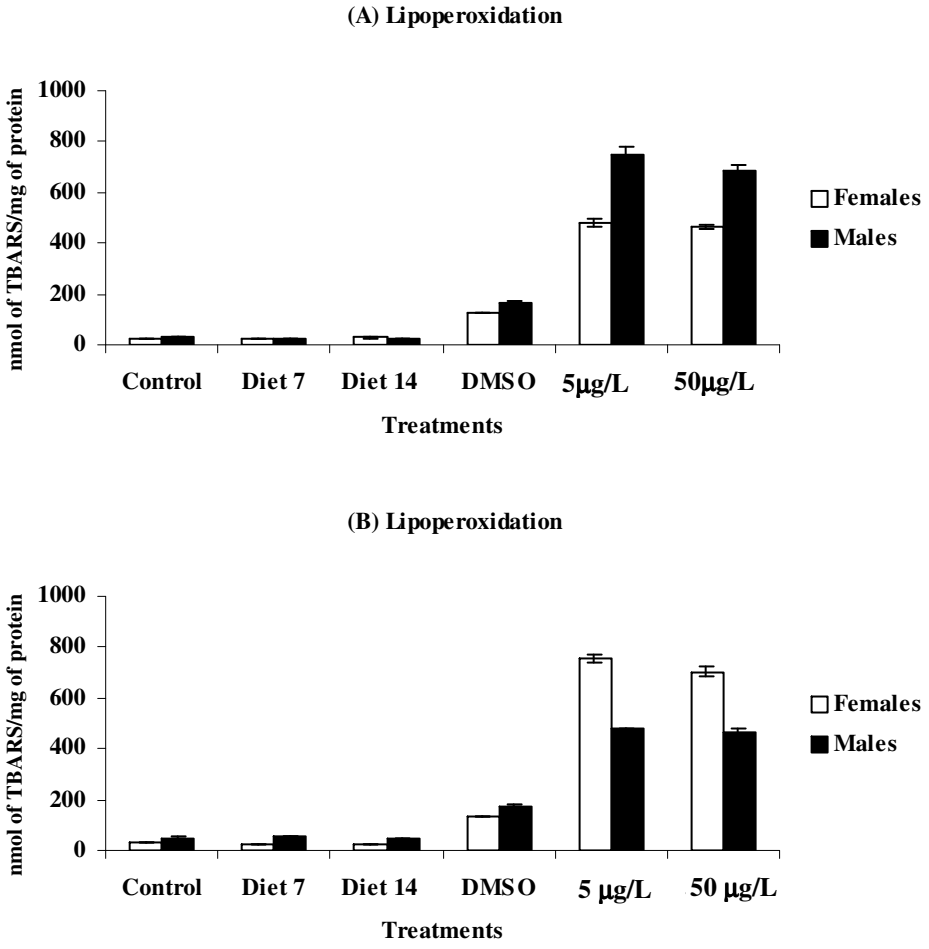
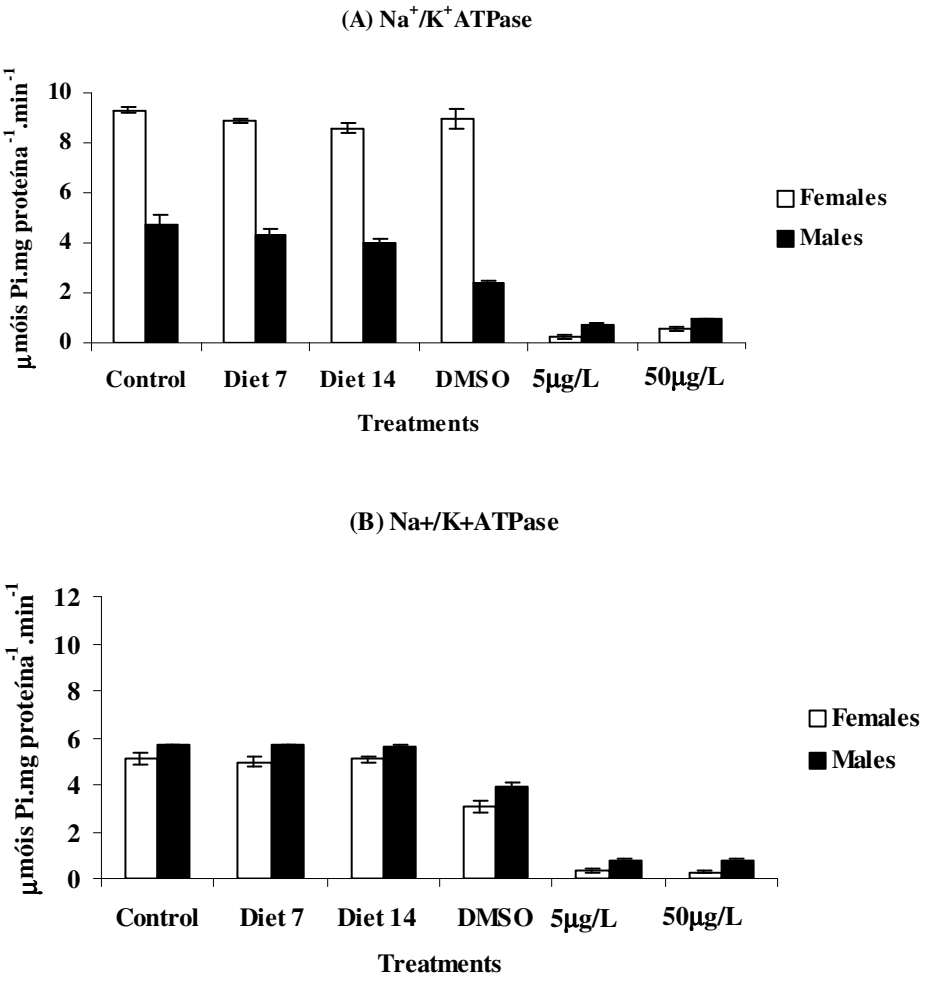

(A) Lipoperoxidation
nmol of TBARS/mg of protein
1000
800
600
400
200
0
Control
Diet 7
Diet 14
DMSO
5µg/L
50µg/L
Treatments
Females
Males
(B) Lipoperoxidation
nmol of TBARS/mg of protein
1000
800
600
400
200
0
Control
Diet 7
Diet 14
DMSO
5 µg/L
50 µg/L
Treatments
Females
Males

Figure 6


(A) Na+/K+ATPase
µmóis Pi.mg proteína -1 .min -1
10
8
6
4
2
0
Control
Diet 7
Diet 14
DMSO
5µg/L
50µg/L
Treatments
Females
Males
(B) Na+/K+ATPase
µmóis Pi.mg proteína -1 .min -1
12
10
8
6
4
2
0
Control
Diet 7
Diet 14
DMSO
5µg/L
50µg/L
Treatments
Females
Males

CONSIDERAÇÕES FINAIS

O presente estudo pesquisou duas espécies de Hyalella em três diferentes situações: no seu ambiente natural, em cultivo experimental em laboratório e por fim frente a testes de toxicidade. De acordo com os objetivos propostos e resultados obtidos neste trabalho, podemos concluir que:

O amphipoda *Hyalella curvispina* tem seu metabolismo intermediário, bem como, seus níveis de lipoperoxidação afetados por distintos fatores sendo eles tanto abióticos quanto bióticos. Dentre todos os fatores destacamos a reprodução, pois os dados parecem mostrar que o animal gasta grande parte de suas reservas para estas as atividades.

Os amphipoda *Hyalella pleoacuta* e *Hyalella castroi* apresentaram uma resposta semelhante a *Hyalella curvispina*, pois estes animais também padrões de resposta metabólica que parecem estar intimamente correlacionados com suas atividade reprodutivas, contudo a forma como estas espécies simpátricas exploram o ambiente em busca da alimentação é distinto o que parece fazer com que *Hyalella pleoacuta* tenha uma dieta mais rica em carboidratos, enquanto que a *Hyalella castroi* possui uma dieta mais rica em proteínas.

Das duas dietas administradas aos amphipoda, *Hyalella pleoacuta* e *Hyalella curvispina*, apenas a dieta mista (macrófitas mais ração) mostrou-se capaz de suprir as necessidades calóricas dos animais, mostrando assim que experimentos realizados com estes animais, onde lhes é fornecido apenas macrófitas podem os remeter a restrição calórica.

Os resultados apresentados pelas espécies submetidas ao tratamento com Carbofuran, nos permitem verificar que as espécies são sensíveis aos efeitos dos pesticidas mesmo não sendo os organismos alvo. Os resultados sugerem também que os parâmetros reprodutivos (formação dos pares, fêmeas ovígeras e número médio dos ovos) podem ser critérios sensíveis para avaliar efeitos ecotoxicológicos.

Estes resultados sugerem que *Hyalella pleoacuta* e *Hyalella curvispina* são organismos apropriados para o uso em testes de toxicidade, bem como, são espécies sensíveis que poderiam ser usadas em estudos de monitoramento.

APÊNDICE I

INSTITUTO DE CIÊNCIA E TECNOLOGIA DE ALIMENTOS
DEPARTAMENTO DE CIÊNCIAS DOS ALIMENTOS
RELATÓRIO DE ENSAIO

NÚMERO**: 172B/2004** **DATA ENTRADA: 11/10/2005**
DATA SAÍDA: 27/10/2005

SOLICITANTE: **Bibiana Kaiser Dutra**
Fone: 9191.1954

AMOSTRA: Macrófita *Salvinia bilob*

ENSAIOS:

Umidade.....85,64g/100g
Cinzas.....11,07g/100g
Proteina 1,19g/100g
Lípidios 0,72g/100g
Fibra Bruta.....0,71g/100g
Carboidratos 0,67g/100g
VCT 13,92Kcal/100g

Prof. **Adriano Brandelli**
Coordenador Prestação de Serviços

Heloisa H. Chaves Carvalho
Nutricionista

CRN 1484

Referências: Ministério da Agricultura. Laboratório Nacional de Referência Animal. Métodos analíticos para análise e seus ingredientes. Brasília, 1981. V.2: Métodos físicos e químicos.

Official methods of the Association of Official Analytical Chemists. AOAC, 1995. Cap. 4, seção 4.5.0.1.

Portaria n. 108 de 04 e 11 de setembro de 1991, Método n. 04. Diário Oficial da União, Brasília, p. 11813-19819, 17 setembro de 1991. Seção 1.

Normas Analíticas do Instituto Adolfo Lutz. 3 Ed. São Paulo, 1985. V. 1: Métodos Químicos e Físicos para análise de alimentos.

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL -INSTITUTO DE CIÊNCIA E TECNOLOGIA DE ALIMENTOS
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Prestação de serviços - telefone: (051) 3316-6248 - Fax: (051) 316-7048
E MAIL BROMO.ICTA @UFRGS.BR

INSTITUTO DE CIÊNCIA E TECNOLOGIA DE ALIMENTOS
DEPARTAMENTO DE CIÊNCIAS DOS ALIMENTOS
RELATÓRIO DE ENSAIO

NÚMERO**: 171B/2004**

DATA ENTRADA: 10/10/2005
DATA SAÍDA: 27/10/2005

SOLICITANTE: **Bibiana Kaiser Dutra**
Fone: 9191.1954

AMOSTRA: Macrófita *Callitriche rimosa*

ENSAIOS:

Umidade.....93,84g/100g
Cinzas.....1,26g/100g
Proteina 1,24g/100g
Lípidios 0,28g/100g
Fibra Bruta.....1,34g/100g
Carboidratos 2,04g/100g
VCT 9,52Kcal/100g

Prof. **Adriano Brandelli**
Coordenador Prestação de Serviços

Heloisa H. Chaves Carvalho
Nutricionista
CRN 1484

Referências: Ministério da Agricultura. Laboratório Nacional de Referência Animal. Métodos analíticos para análise e seus ingredientes. Brasília, 1981. V.2: Métodos físicos e químicos.

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RELATÓRIO DE ENSAIO

NÚMERO**: 170B/2004**

DATA ENTRADA: 10/10/2005
DATA SAÍDA: 27/10/2005

SOLICITANTE: **Bibiana Kaiser Dutra**
Fone: 9191.1954

AMOSTRA: Ração em Flocos para Peixes

ENSAIOS:

Umidade.....5,13g/100g
Cinzas.....11,02g/100g
Proteina 30,88g/100g
Lípidios 6,19g/100g
Fibra Bruta.....3,59g/100g
Carboidratos 43,19g/100g
VCT 351,99Kcal/100g

Prof. **Adriano Brandelli**
Coordenador Prestação de Serviços

Heloisa H. Chaves Carvalho
Nutricionista
CRN 1484

Referências: Ministério da Agricultura. Laboratório Nacional de Referência Animal. Métodos analíticos para análise e seus ingredientes. Brasília, 1981. V.2: Métodos físicos e químicos.

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APÊNDICE II

-----Mensagem original-----
De: T.P. Mommsen [mailto:tpmom@zeus.econ.umd.edu]
Enviada em: sábado, 14 de abril de 2007 04:56
Para: Guendalina Turcato Oliveira
Cc: CBPjrnl@interchange.UBC.ca; tpmom@uvic.ca
Assunto: CBP: decision on revised ms. 14400

Dear Dr. Oliveira:

Thank you for submitting your revised manuscript 14400 to CBP.

The editors and one of the previous reviewers have examined your revisions in light of the comments made during the review process, and I am happy to report that your manuscript is now accepted for publication in CBP.

At this point, I am sure you are familiar with the remainder of the process: Within the next week, the Elsevier Log-in site will confirm via e-mail that your accepted manuscript has been logged-in and a few days later, Elsevier will post your accepted manuscript on the CBP Science Direct website. At this point, your manuscript will be citable via D.O.I. The proofs of your manuscript will be sent to you via e-mail within the next three weeks.

If you have any queries or comments, please contact the CBP office at CBPjrnl@interchange.UBC.ca

Thank you again for submitting your manuscript to CBP.

Best regards,

Tom Mommsen & Patrick Walsh
Editors-in-Chief : Comparative Biochemistry and Physiology
<CBPjrnl@interchange.UBC.ca>

New CBP submission sites (Aug. 2006):
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part B: http://gemini.econ.umd.edu/cbp_b
part C: http://gemini.econ.umd.edu/cbp_c
part D: http://gemini.econ.umd.edu/cbp_d

-----Mensagem original-----
De: T.P. Mommsen [mailto:tpmom@zeus.econ.umd.edu]
Enviada em: sábado, 14 de abril de 2007 04:56
Para: Guendalina Turcato Oliveira
Cc: CBPjrnl@interchange.UBC.ca; tpmom@uvic.ca
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part B: http://gemini.econ.umd.edu/cbp_b
part C: http://gemini.econ.umd.edu/cbp_c
part D: http://gemini.econ.umd.edu/cbp_d

-----Mensagem original-----
De: an@nifes.no [mailto:an@nifes.no]
Enviada em: segunda-feira, 22 de janeiro de 2007 14:11
Para: Guendalina Turcato Oliveira; bibianakaiser@yahoo.com.br
Assunto: Aquaculture Nutrition - Manuscript ANU-07-009

22-Jan-2007

Dear Dr. Oliveira,

Your manuscript entitled "Variations in the Biochemical Composition and Lipid Peroxidation of Hyalella pleoacuta and Hyalella curvispina (Crustacea, Amphipoda, Dogielinotidae) Maintained on Different Diets in the Laboratory" has been successfully submitted online and is presently being given full consideration for publication in Aquaculture Nutrition.

Your manuscript ID is ANU-07-009.

Please mention the above manuscript ID in all future correspondence or when calling the editorial office with questions. If there are any changes to your mailing address or e-mail address, please log into Manuscript Central at http://mc.manuscriptcentral.com/anu and edit your user information as appropriate.

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Thank you for submitting your manuscript to Aquaculture Nutrition.

Yours sincerely,

Aquaculture Nutrition Editorial Office
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N-5817 Bergen

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E-mail Address: an@nifes.no

APÊNDICE III

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COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY - PART A: MOLECULAR & INTEGRATIVE PHYSIOLOGY

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The journal publishes original articles emphasizing comparative and environmental aspects of the physiology, biochemistry, molecular biology, pharmacology, toxicology and endocrinology of animals. Adaptation and evolution as organizing principles are encouraged. Studies on other organisms will be considered if approached in a comparative context.

Part A. Molecular and Integrative Physiology deals with molecular, cellular, integrative, and ecological physiology. Topics include bioenergetics, circulation, development, excretion, ion regulation, endocrinology, neurobiology, nutrition, respiration, and thermal biology. Studies on regulatory mechanisms at any level or organization such as signal transduction and cellular interactions and control of behaviour are encouraged.

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mechanisms and evolutionary principles.

Part C. Toxicology and Pharmacology is concerned with chemical and drug action at different levels of organization, biotransformation of xenobiotics, mechanisms of toxicity, including reactive oxygen species and carcinogenesis, endocrine disruptors, natural products chemistry, and signal transduction. A molecular approach to these fields is encouraged.

Part D. Genomics and Proteomics covers the broader comprehensive approaches to comparative biochemistry and physiology that can be generally termed as "-omics", e.g., genomics, functional genomics (transcriptomics), proteomics, metabolomics, and underlying bioinformatics. Papers dealing with fundamental aspects and hypotheses in comparative physiology and biohcemistry are encouraged rather than studies whose main focus is purely technical of methodological.

Naturally, a certain degree of overlap exists between the different sections, and the final decision as to where a particular manuscript will be published after passing the rigorous review process lies with the editorial office.

Submission and review of manuscripts

Online submission of papers
Manuscripts are to be submitted to the CBP Editorial Office electronically at http://gemini.econ.umd.edu/cbp_a.
After registration, authors are asked to upload their article and associated artwork, preferably in a single file. If necessary, the CBP Editorial Office will generate a PDF file to be used for the reviewing proces.

Full instructions on how to use the online submission tool will be available at the above web address or can be requested by e-mail from the CBP Editorial Office (CBPjrnl@interchange.UBC.ca).

During the submission proces, authors are asked to select an appropriate section of CBP and to provide names and address (including e-mail) of at least five researchers of recognized competence who may be considered as reviewers.

Review articles
Before writing their manuscripts, potential authors of review

articles should contact one of the Editors who, after consultations with the other editor and/or members of the Editorial Board, will provide feedback on suitability of the topic. Reviews should be topical, and serve as critical appraisals of areas of research. They should provide an up-to-date analysis of concepts and point out future directions. For manuscript preparation, follow the instructions below.

Revision of manuscripts: Revised manuscripts must be submitted within two months of the authors' receipt of the referees' reports. Otherwise they will be considered as new submissions.

Proofs: The corresponding author will receive proofs by e-mail or post. Proofs must be checked immediately and returned to Elsevier. Corrections to the proofs should be restricted to printer's errors only. Substantial alterations may be charged to the author. Elsevier will do everything possible to get your article corrected and published as quickly and accurately as possible. *Therefore, it is important to ensure that all of your corrections are sent back to us in one communication.* Subsequent corrections will not be possible, so please ensure your first sending is complete.

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Page charges: **CBP has no page charges.**

Preparation of manuscripts

Sections: Manuscripts should be subdivided into the following sections: Title page, abstract, introduction, materials and methods, results, discussion, acknowledgements, references, captions to figures, tables.

Format: All sections of the manuscript must be 1.5-spaced with 2.5 cm (1 inch) margins. Pages should be numbered consecutively. Avoid footnotes. Underline only words or letters that will be printed in italics. Mark the position of each figure and table in the margin. The full Latin name of all species used in the study must be supplied.

Title page: The title should be short, concise and informative. Consult a recent issue of CBP for author format (http://www/elsevier.com/locate/cbp). The author's name

should be followed by his/her department, institution, city, and country. Indicate the author to whom correspondence and proofs should be addressed, and supply full postal address as well as phone and fax numbers, and an e-mail address. If submitting a review article, write "REVIEW" at the top of the title page.

Abstract: The second page of the manuscript must contain only the abstract and the key words. The abstract should be a single paragraph not exceeding 200 words. Non-standard abbreviations and reference citations should be avoided.

Key words: Up to eight key words, which may or may not appear in the title, should be listed in alphabetical order after the abstract. Only these key words, together with the title, will be used to compile the subject index.

References:
1. All publications cited in the text should be presented in alphabetical order in a list following the text of the manuscript.
2. In the text refer to the author's name and year of publication.
3. If reference is made in the text to a publication written by more than two authors the name of the first author should be used followed by "et al.". In this list names of first authors and all co-authors should be mentioned.
4. References cited together in the text should be arranged chronologically.
5. The List of references should be arranged alphabetically on authors' names, and chronologically per author. Names of all authors must be included. *Do not use et al*. Publications by the same author(s) in the same year should be listed as 2000a, 2000b, etc.
Follow the relevant examples below.

Axelsson, M., Farrell, A.P., 1993. Coronary blood flow in vivo in the coho salmon *(Oncorhynchus kisutch).* Am. J. Physiol. 264, R963 - 971.
Hiramatsu, N., Cheek, A.O., Sullivan, C.V., Matsubara, T., Hara, A., 2005. Vitellogenesis and endocrine disruption. In: Mommsen, T.P., Moon, T.W. (Eds.), Biochemistry and Molecular Biology of Fishes, vol. 6. Environmental Toxicology, Elsevier, Amsterdam, pp. 431-471.
Lindsley, J.E., Rutter, J., 2004. Nutrient sensing and metabolic decisions. Comp. Biochem. Physiol. B 139, 543-559.

Moyle, P.B., Cech, J.J., 2004. Fishes. An introduction to ichthyology. 5th ed. Prentice Hall, Upper Saddle River, NJ.

Tables: Tables should be prepared as follows:
(a) Refer to current tables in the journal, for required spatial layout. If possible, a Times Roman font should be used.
(b) Each table, including heading and legend should be typed on a separate sheet. If possible, a Times Roman font should be used.
(c) Insert heavy rules at the head and foot of each table, and fine rules below column headings.

Italics: Genus and species names, and other words normally italicized, should be typed in italics or underlined.

Illustrations: Photographs, charts and diagrams are to be referred to as "figs" and should be ordered consecutively.

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As only one figure caption is used for both colour and black and white versions of figures, please ensure that the figure captions are meaningful for both versions, if applicable.

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Authors must check accession numbers very carefully. **An error in a letter or number can result in a dead link.**

In the final version of the ***printed*** **article**, the accession number text will not appear bold or underlined (see Example 2 below).

Example 2: "B-cell tumor from a chronic lymphatic leukemia (GenBank accession no. BE675048), and a T-cell lymphoma (GenBank accession no. AA361117)".

In the final ***electronic*** **copy**, the accession number text will be linked to the appropriate source in the NCBI databases enabling readers to go directly to that source from the article (see Example 3 below).

Example 3: "B-cell tumor from a chronic lymphatic leukemia (GenBank accession no. BE675048), and a T-cell lymphoma (GenBank accession no. AA361117)".

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1. Designate the corresponding author and provide telephone and fax numbers, and an e-mail address.
2. Provide an abstract of less than 200 words; append up to eight key words to the abstract page.
3. Check the style in which references are cited; unpublished work will not be listed in this section unless it is "in press".
4. If referencing manuscripts "in press", these must be uploaded as supplementary material during the manuscript

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5. Provide names and addresses (including phone and fax numbers & e-mail addresses) of at least five researchers of recognized competence who may be considered as referees.

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