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Biochemical Composition, Lipoperoxidation, Na^+/K^+ ATPase activity and Reproduction of *Hyalella castroi* (Amphipoda, Dogielinotidae) Fed With Different Diets

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ABSTRACT This study compared the effect of different diets on the biochemical composition (lipids, cholesterol, proteins and glycogen) and the levels of lipoperoxidation and activity of Na^+/K^+ ATPase of *Hyalella castroi*. We also investigated some patterns of the life cycle including survival, precopulatory mating pairs and number of ovigerous females after 21 days of culture with different diets. These crustaceans live in limnetic environments of the plateau (1,200 m a.s.l.) of the state of Rio Grande do Sul, in southern Brazil. Adult animals were collected in the autumn of 2006 in São José dos Ausentes. In the laboratory, the animals were kept submerged in aquariums, separated by sex, under controlled conditions. They were fed ad libitum for 21 days with different diets, and then some were used for biochemical determinations and others for observations of reproductive aspects. Statistical analysis revealed significant differences in the responses to glycogen, total proteins, lipoperoxidation levels and Na^+/K^+ATPase activity in both sexes of these amphipods and total lipids and total cholesterol levels only in females. These diets changed the biochemical patterns of the animals taken from the natural environment, and allowed a high survival rate but did not improve reproduction. *J. Exp. Zool. 311A:408–421, 2009.*

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Members of the amphipod genus *Hyalella* are found in a variety of freshwater habitats, such as permanent reservoirs, lakes, impoundments and streams. They often cling to the vegetation, swim in the water or burrow in the sediment, where they are important members of the benthic fauna (Kruschwitz, '78; Wellborn, '95; Grosso and Peralta, '99). In the state of Rio Grande do Sul, Brazil, six species of this genus occur. One is *Hyalella castroi*, found in the municipality of São José dos Ausentes (1,200 m a.s.l. in the region of Aparados da Serra) in the Vale das Trutas (28°47′00″S–49°50′53″W) (Gonzalez et al., 2006).

Dutra et al. (2007), studying the sympatric amphipods *H. pleoacuta* and *H. castroi* in the natural environment, observed similar values (0.18 ± 0.02–3.28 ± 0.39 mmol g^{-1}) of glycogen levels 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 1.81 ± 0.22 to 8.76 ± 3.16 mmol g^{-1}, suggesting a high-carbohydrate and a low-protein natural diet. This difference between these species can be explained by the behavior of *H. castroi* in exploiting the sediment

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predominantly, where it finds more organic matter of animal origin, whereas *H. pleoacuta* lives more in the water column, where more organic matter of plant origin is available. The precise feeding habits of *H. pleoacuta* and *H. castroi* are unknown. Although the nutritional ecology of amphipods and other mesograzers is poorly understood, some feed on a range of plant, animal and detrital foods, with a few species showing a strong preference for particular prey species or groups (Nelson, '79; Cruz-Rivera and Hay, 2000; Poltermann, 2001). Hargrave ('70) reported that *H. azteca* is an omnivorous deposit feeder, primarily feeding on algae and bacteria associated with the sediments and aquatic macrophytes. It has been recorded eating dead animal and plant matter (Cooper, '65). Byrén et al. (2002) showed that for the amphipods *Monoporeia affinis* and *Pontoporeia femorata*, sedimented phytoplankton and organic detritus are their main food sources, but bacteria, meiofauna and temporary meiofauna are also included in the diet.

Dutra et al. (2008a), working with *H. curvispina*, suggested that 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 wide variations in environmental conditions. This correlation was also found by Chang and O'Connor ('83), Kucharski and Da Silva ('91b), Rosa and Nunes (2003b) and Oliveira et al. (2007) in their studies with other crustaceans. Dutra et al. (2008a) showed in *H. curvispina* that levels of lipoperoxidation may be related to reproductive behavior, motor and feeding activity and variation of the photoperiod.

Diet plays an important role in crustacean broodstock condition (Holdich, 2002; Díaz and Fenucci, 2004). Broodstock nutrition is important for reproductive success because egg and larval production is strongly dependent on the diets offered (Bromage, '95; Harrison, '97; García-Ulloa, 2000). Protein is the most critical ingredient in practical diets because it is expensive and growth responses are affected (Cortés-Jacinto et al., 2003; Thompson et al., 2005). According to Harrison ('97), the amount of protein required in broodstock diets for maturation and production of eggs is higher than the level required for growth because gonad maturation is a process of intense protein synthesis, mainly during vitellogenesis (Abdu 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., '99; Livingstone, 2001; Wilhelm et al., 2001; Timofeyev et al., 2006). Neuparth et al. (2005) reported that *Gammarus locusta* maintained on highly organic sediments showed higher levels of lipoperoxidation. There is general agreement that endogenous variables such as nutritional status, age, sex, growth and reproduction influence the peroxidation status of organisms (Viarengo et al., '91; Correia et al., 2002, 2003).

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 function (Halliwell and Gutteridge, '86; Vercesi et al., '97; Milatovic et al., 2005). These degenerative changes can affect the dynamic properties of the membranes such as fluidity and permeability, and consequently the activity of various membrane-associated enzymes (Mecocci et al., '97). 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., '97; Mark et al., '97).

Dutra et al. (2008c) compared the effect of different diets on the biochemical composition, levels of lipid peroxidation, survival rate and reproductive aspects of *H. pleoacuta* and *H. curvispina*. When the animals were fed with macrophytes only (Diet 1), the metabolic response mimicked a caloric restriction in both species and sexes, because they showed depletion of glycogen and proteins, which was reinforced by the decrease in the levels of lipoperoxidation. These responses were probably a result of the low caloric input. In animals fed with macrophytes and commercial ration (Diet 2), these responses were reversed because the energy reserves were maintained and the levels of lipoperoxidation were higher than in those on Diet 1. The authors observed that animals fed on Diet 2 showed more activity, precopulatory mating pairs, ovigerous females and eggs per female; however, the rate of survival was similar for both diets. The authors attributed this difference to the likelihood that the caloric requirements of both species were probably supplied with Diet 2, which provided more carbohydrates, proteins and lipids. This diet was also important for the adequate maintenance in the toxicology experiments.

Amphipods are benthic organisms; they have short generation times, carry their eggs in an

external broodpouch and can be cultured relatively easily on different diets (Cruz-Rivera and Hay, 2000). Amphipods thus offer an excellent opportunity for evaluation of dietary effects or fitness, for toxicity tests, and for bioassays for evaluation of the quality of the water or sediment of an aquatic ecosystem. Recent studies demonstrated that *Corophium volutator*, a typical sediment-dwelling amphipod, is suitable to assess the sediment toxicity in limnetic environments (Gerhardt et al., 2005). The number of species used for standard toxicity tests is limited, and the majority is not native to Brazil, in particular *H. azteca*, in contrast with the taxonomic richness of the natural ecosystems in Brazil (Brendonck and Persoone, '93). Nutritional requirements for amphipod culture are poorly defined, although it is generally recognized that broodstock nutrition is an important factor in reproduction success or failure.

The aim of this study was to characterize the response of the intermediate metabolism (total lipids, cholesterol, proteins and glycogen), of the levels of lipoperoxidation (TBA-RS) and of Na^+/K^+ ATPase activity in the native Brazilian species *H. castroi* maintained in experimental culture with two different diets. We also investigated some aspects of the life cycle such as survival, number of precopulatory mating pairs and number of ovigerous females and juveniles hatched in order to standardize this species for future use in toxicity tests.

MATERIAL AND METHODS

Adult animals were used with the permission of the Ethics Committee of the Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS) (License 06/03423) and in accordance with Brazilian laws.

The animals were collected from April through June of 2006 (autumn) in a stream in São José dos Ausentes, Rio Grande do Sul (28°47′00″S–49°50′53″W), at a relatively pristine location. Three collection periods (April, May and June) allowed us to use the animals of the same population, but not the same generation, in order to be certain that the results are significant for the entire population. Animals and macrophytes (*Callitriche rimosa*) were collected by means of fish traps and bottom grabs at the same time of day.

Some of the animals were immediately frozen in the natural environment, and others were transported in cold water (5°C) in insulated containers to the Laboratory of Conservation Physiology of the PUCRS, where they were separated by sex and placed in aerated aquariums for 24 hr without food (Period 1).

In order to characterize the collection locality, the pH, water temperature and hardness of the water were measured each month during the study period. pH was determined with a portable pH meter (Quimis/400H, Diadema, São Paulo, Brazil), and water temperature with an internal-scale thermometer. The hardness of the water was determined using a classic method of volumetric complexation (Adad, '82).

Experimental procedure

After Period 1, some of the animals were kept submerged in aerated aquariums, at a density of one animal per liter of water, with a photoperiod of 14:10-hr light/dark. The amphipods were divided into two groups, which were fed ad libitum in late afternoon, when most of the animals were active, for a period of 21 days. Males and females were kept in the same aquariums; they were separated by a nylon fabric, but were in chemical contact, because the water passed through both parts of the aquarium. They were fed one of two diets. The first group (Diet 1) received macrophytes and commercial ration (ALCOM: fresh shrimp, fish flour, hydrolized soy protein, corn cream, wheat flour, marine algae flour, dehydrated carrots, yeasts, soy oil, vitamin C, mineral and vitamin supplement, inorganic minerals, additives for pigment and BHT antioxidant). The second group (Diet 2) received macrophytes and another commercial ration (ALCOM: fish flour, hydrolized soy protein, corn cream, wheat flour, marine algae flour, yeasts, soy oil, vitamin C, mineral and vitamin supplement, inorganic minerals, dehydrated spinach, antioxidant BHT spiruline and prebiotic additive).

The percentage composition of these rations was determined by the Instituto de Ciência e Tecnologia dos Alimentos of the Universidade Federal do Rio Grande do Sul and is shown in Table 1. The diets were isocaloric. After 7, 14 and 21 days of experimental culture, a group of each diet and each sex was cryoanesthetized, weighed on an electronic balance (± 0.001) and then stored frozen at −80°C until they were used to determine the biochemical parameters.

Reproductive aspects

After Period 1, other animals (10 males and 10 females) were placed in each of six 20-L aquariums (120 animals), three aquariums for Diet 1 and

TABLE 1. Percent composition of Diets 1 and 2

Compound	Diet 1 Macrophyte and Ration 1	Diet 2 Macrophyte and Ration 2
Water content (g/100 g)	5.30	7.26
Ashes (g/100 g)	11.02	14.15
Protein (g/100 g)	30.88	39.78
Fat (g/100 g)	6.19	4.99
Fiber (g/100 g)	3.59	4.83
Carbohydrates (g/100 g)	43.19	28.99
Total caloric value (kcal/100 g)	351.59	319.99

three for Diet 2. In this experiment, we allowed physical contact between males and females. The animals were observed each day in early morning and evening, for 21 days, and the number of precopulatory mating pairs and ovigerous females was counted (Plaistow et al., 2003).

Survival and mortality

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

Biochemical analyses

Metabolites

Metabolic determination for *H. castroi* was done in total homogenates of 3 pools of 12 males and 12 females each. One pool was used for the determination of glycogen and proteins, the second pool for the quantification of lipids and cholesterol and the third pool for the quantification of lipoperoxidation levels. Metabolic parameters were determined in quintuplicate in each homogenate by spectrophotometric methods:

(a) Glycogen was extracted from tissues following the method described by Van Handel ('65). Glycogen levels were determined as glucose-equivalent, after acidic hydrolysis (HCl) and neutralization (Na_2CO_3), following the method of Geary et al. ('81). Glucose was quantified using a Biodiagnostic Kit (glucose oxidase). Results are presented as $mmol\,g^{-1}$ of animal.
(b) Proteins were quantified as described by Lowry et al. ('51), with bovine albumin (Sigma Co., St. Louis, MO) as the standard. Results are expressed as $mg\,mL^{-1}$ of 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. ('57). After two washes with physiological solution and centrifugation (2,000 rpm), total lipids were determined by the sulfophosphovanillin method (Meyer and Walther, '80). 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 spectrophotometer (530 nm). The levels of total cholesterol were measured by the reactions of cholesterol esterase, cholesterol oxidase and peroxidase enzymes (Labtest Kit/Liquiform). Results are expressed as $mg\,g^{-1}$ of animals.
(d) Lipoperoxidation levels were quantified by the method of Buege and Aust ('78) by measuring reactive substances to thiobarbituric acid (TBA-RS), using the extraction method of Llesuy et al. ('85). Results are expressed in nmol TBA-RS mg^{-1} protein.

Activity of Na^+/K^+ATPase

The pool of animals was homogenized (10% w/v) in cold Tris buffer (40 mM) and phenylmethylsulfonyl fluoride (1 mM; Sigma) with the pH adjusted to 7.40. The homogenate was centrifuged at $10{,}000 \times g$ at 4°C, and the supernatant was collected and centrifuged at $40{,}000 \times g$ (4°C). The pellet was resuspended in the same buffer and centrifuged again at $40{,}000 \times g$ (4°C) (Barnes and Blackstock, '93). 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 ('88), as adjusted by Dutra et al. (2008b). Incubation medium A contained ATP (5 mM; Sigma), NaCl (60 mM), KCl (10 mM) and MgCl (40 mM), with the pH adjusted to 7.40. In incubation medium B, KCl was replaced by ouabain (1 mM; Sigma). Aliquots of the homogenate were incubated at 30°C in media A and B, for 30 min with the equivalent of 10 mg of protein. The enzymatic reaction was stopped by the addition of 10% trichloroacetic acid. The inorganic phosphate released was determined using the method of Chan and Swaminathan ('86) in a spectrophotometer at 630 nm. Any difference in phosphate concentration between media A and B was attributed to Na^+/K^+ATPase activity. All determinations were done in quadruplicate. Results are expressed in μmol of the Pi mg of $protein^{-1}\,min^{-1}$.

Statistical analysis

The results are expressed as mean ± standard error. For statistical analysis of the different periods of experimental culture, a one-way analysis of variance (ANOVA) test was used, followed by a Tukey test. For comparisons between different diets and sexes, a two-way ANOVA was used. The experimental cultures and data for the natural environment and the differences in the number of ovigerous females between different diets were compared with Student's *t*-test. 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

Abiotic condition analyses

The environmental temperature at the collection locality (16.4 ± 0.35°C) was much lower than the temperature in the experimental culture (23.0 ± 1.00°C). The pH and hardness of water were constant in both situations: the environmental pH was 7.09 ± 0.21 and 7.00 ± 0.40 in experimental culture; the hardness of the water in the environment was 1.12 ± 0.52 ppm $CaCO_3$ and in the culture was 0.98 ± 0.46 ppm $CaCO_3$. These differences were not significant ($P > 0.05$).

Reproductive parameters

The number of precopulatory mating pairs (pairs formed) and ovigerous females fed with Diet 1 and Diet 2 is given in Table 2. The amphipods fed with Diet 1 paired 22% more than the animals on Diet 2. The females fed with Diet 1 showed a higher proportion of ovigerous females than those on Diet 2. However, in both cultures, the maximum period that the females bore eggs was 4 days, and after this period no juveniles were observed hatching in the aquariums. The number of ovigerous females was low in relation to the number of couples formed, in animals on both diets.

Survival rate

The survival of the males and females fed with Diets 1 and 2 is given in Table 3. During the culture period, males and females fed with Diet 1 showed survival rates of 11.20 and 13.40%, respectively, higher than the animals fed with Diet 2. There was no significant difference between the sexes fed with the same diet, for either diet. The survival rate on Diet 1 ranged from 94.5 to 98.20%, and on Diet 2 from 83.64 to 89.10%, in both sexes in the three experiments.

TABLE 2. Number of precopulatory mating pairs and ovigerous females of Hyalella castroi maintained in experimental culture with different diets

Days	Precopulatory mating pairs Diet 1	Precopulatory mating pairs Diet 2	Ovigerous females Diet 1	Ovigerous females Diet 2
1	8	10	0	0
2	10	9	0	0
3	10	9	0	0
4	2	2	0	1
5	2	2	0	1
6	3	2	0	1
7	3	2	0	1
8	2	0	2	0
9	2	1	2	0
10	2	1	2	0
11	2	1	1	0
12	3	1	0	0
13	2	1	0	0
14	2	3	0	0
15	4	3	0	0
16	5	3	0	0
17	3	4	0	0
18	3	1	0	0
19	3	2	0	0
20	3	3	0	0
21	3	3	0	0
Total	77	63	2	1

TABLE 3. Survival indices of males and females of Hyalella castroi maintained in experimental culture with different diets

	1° Culture (%)	2° Culture (%)	3° Culture (%)	Mean (%)
Males—Diet 1	98.20	94.55	96.36	96.37
Females—Diet 1	98.20	96.36	98.20	97.59
Males—Diet 2	83.64	89.10	85.45	86.06
Females—Diet 2	87.27	85.45	85.45	86.06

Metabolic parameters

Glycogen

Figure 1A shows the glycogen concentrations in males and females in the natural environment and in culture with Diet 1. Males of *H. castroi* cultured for 7 days showed glycogen levels 1.3 times higher than in the animals collected in the natural environment. These levels continued to increase

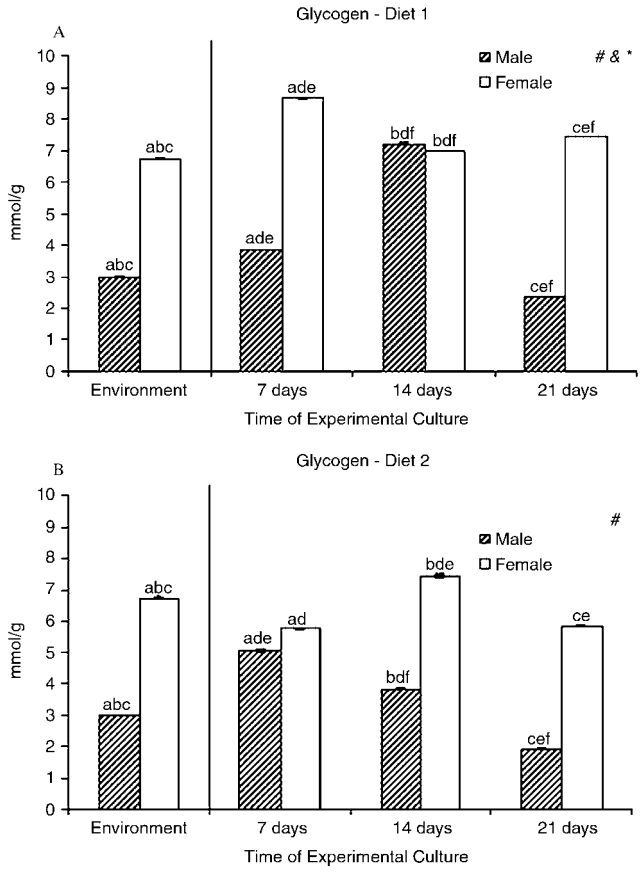

Fig. 1. Concentrations of glycogen of *Hyalella castroi* in animals from the natural environment and maintained in experimental culture. Columns represent the mean and bars represent the standard error of the mean. Results are expressed in $mg\,g^{-1}$. The same letter indicates a significant difference between the days of culture or environment. ♯ indicates a significant difference between sexes maintained on the same diets. & indicates a significant difference between males fed with different diets. ∗ indicates a significant difference between females fed with different diets.

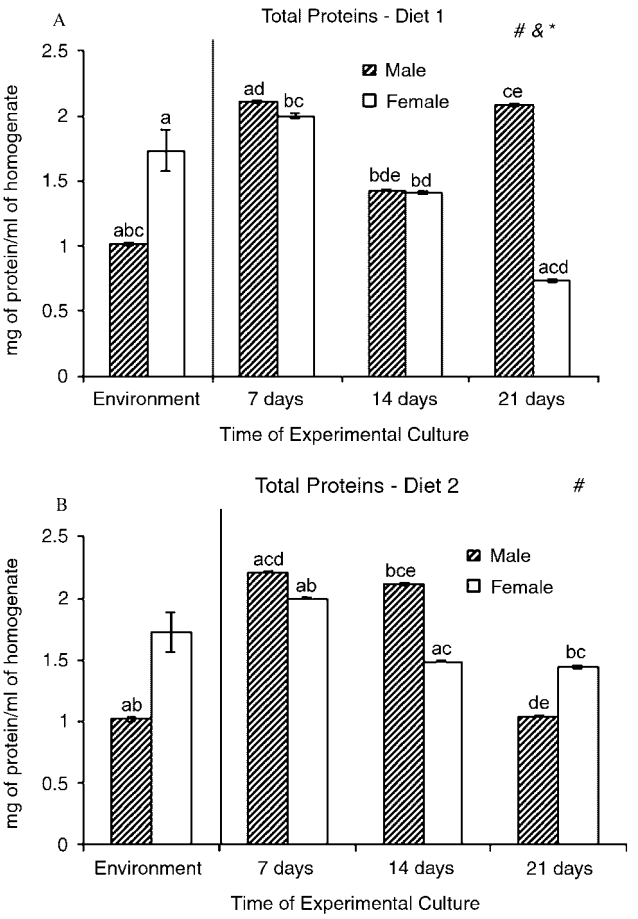

Fig. 2. Concentration of total proteins of *Hyalella castroi* in animals from the natural environment and maintained in experimental culture. Columns represent the mean and bars represent the standard error of the mean. Results are expressed in $mg\,mL^{-1}$. The same letter indicates a significant difference between the days of culture or environment. ♯ indicates a significant difference between sexes maintained with the same diets. & indicates a significant difference between males fed with different diets. ∗ indicates a significant difference between females fed with different diets.

until day 14 of culture, and at day 21 were lower than that at day 14 and in animals from the environment. In females, glycogen increased to day 7 and remained high until the end of the experiment. There was a significant difference in the behavior of glycogen levels during the culture period between males and females fed with Diet 1 ($P<0.05$).

The glycogen contents in males and females of *H. castroi* in the natural environment and maintained on Diet 2 are shown in Figure 1B. Males fed with Diet 2 showed a peak (1.7-fold) of glycogen on day 7, and glycogen levels then decreased gradually until day 21. In females, at day 7, glycogen had decreased by 20%, after 14 days it increased by 30% and at the end of the experiment the levels had returned to those seen at day 7. There was a significant difference in the behavior of glycogen between males and females fed with Diet 2 ($P<0.05$).

There were significant differences in glycogen levels during the 21 days of culture between both males and females on the different diets ($P<0.05$).

Proteins

Protein concentrations in males and females from the natural environment and fed with Diet 1 are shown in Figure 2A. After 7 days, males showed a two-fold increase in protein levels, which at day 14 had decreased by 40%, and at day 21 had returned to the levels seen at 7 days. In females,

proteins were higher after 7 days, and gradually decreased until day 21, reaching levels lower than those in the animals from the environment and after 7 days of culture. Total protein contents differed significantly in both females and males on Diet 1 ($P<0.05$).

The levels of total protein in males and females from the natural environment and fed with Diet 2 are shown in Figure 2B. Males fed with Diet 2 at 7 days showed total protein levels 2.2 times higher than those males from the natural environment; these levels remained constant until day 14, and at day 21 had decreased again to the levels of the animals in natural environment. In females, the levels of total proteins after 7 days on Diet 2 were similar to the amphipods collected in the natural environment, although these levels decreased significantly during the culture period (14 and 21 days). Total protein content differed significantly between males and females maintained on Diet 2 ($P<0.05$).

Comparing the different diets, we observed a significant difference in the behavior of the total proteins during the 21 days of the experiment. The same response was observed in females ($P<0.05$).

Total lipids

The concentrations of total lipids in males and females in the environment and fed with Diet 1 are shown in Figure 3A. Lipid levels were lower ($P<0.05$) after 7 days of culture and remained stable until day 14; after 21 days of feeding, lipid levels had decreased approximately 2.2 times. Females showed lower lipid levels than the animals in the environment after 7 days; at day 14 lipids increased, and decreased again by day 21. There was a significant difference between total lipid contents of males and females maintained in the laboratory and fed with Diet 1.

Total lipid contents in males and females of *H. castroi* in the environment and maintained on Diet 2 are shown in Figure 3B. The males from the natural environment showed total lipid levels approximately 2.6 times higher ($P<0.05$) than the animals fed with Diet 2 by day 7; females maintained for the same period showed no significant difference ($P>0.05$). In males, lipid levels decreased gradually to minimum values on day 21. Females showed a peak after 14 days of experiment, with levels 3.7 times higher than the females cultured for 7 days; total lipid levels decreased (2.2 times) by day 21, although they were higher than those at the beginning of the

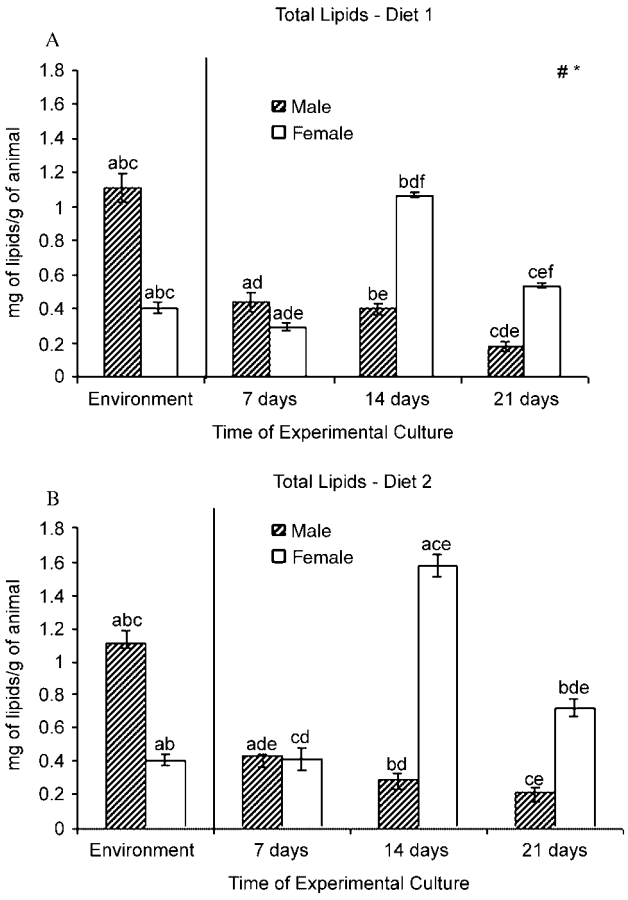

Fig. 3. Concentration of total lipids of *Hyalella castroi* in animals from the natural environment and maintained in experimental culture. Columns represent the mean and bars represent the standard error of the mean. Results are expressed in $mg\,g^{-1}$. The same letter indicates a significant difference between the days of culture or environment. ♯ indicates a significant difference between sexes maintained with the same diets. ∗ indicates a significant difference between females fed with different diets.

experiment. Total lipid levels differed significantly between males and females fed with Diet 2 ($P<0.05$). There was no significant difference in the levels of total lipids in males submitted to the different diets ($P>0.05$), but females showed a significant difference between the groups that received Diets 1 and 2 ($P<0.05$).

Cholesterol

The concentrations of cholesterol in males and females in the environment and fed with Diet 1 are shown in Figure 4A. The males and females from the environment showed total cholesterol contents 12.7 and 3.7 times, respectively, higher than the animals fed with Diet 1 for 7 days. Comparing the levels of this metabolite between

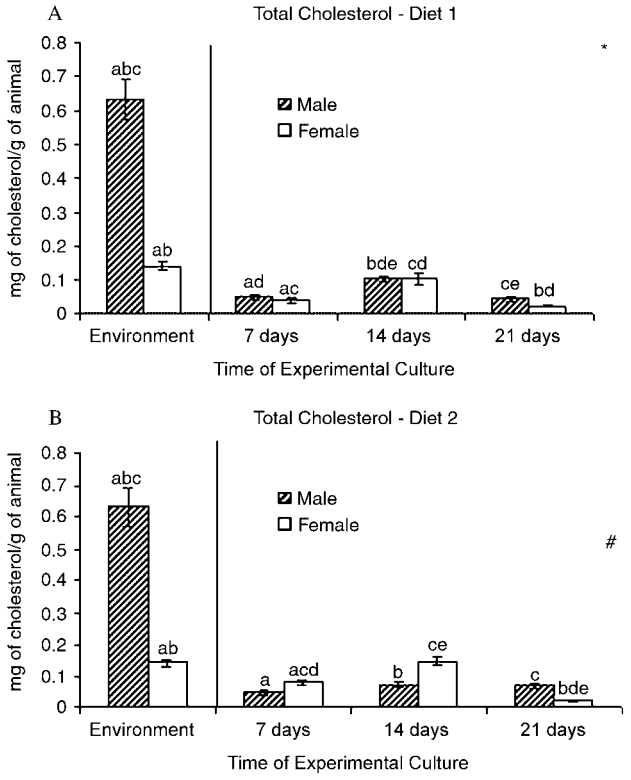

Fig. 4. Concentration of cholesterol of *Hyalella castroi* in animals from the natural environment and maintained in experimental culture. Columns represent the mean and bars represent the standard error of the mean. Results are expressed in $mg\,g^{-1}$. The same letter indicates a significant difference between the days of culture or environment. ♯ indicates a significant difference between sexes maintained with the same diets. ∗ indicates a significant difference between females fed with different diets.

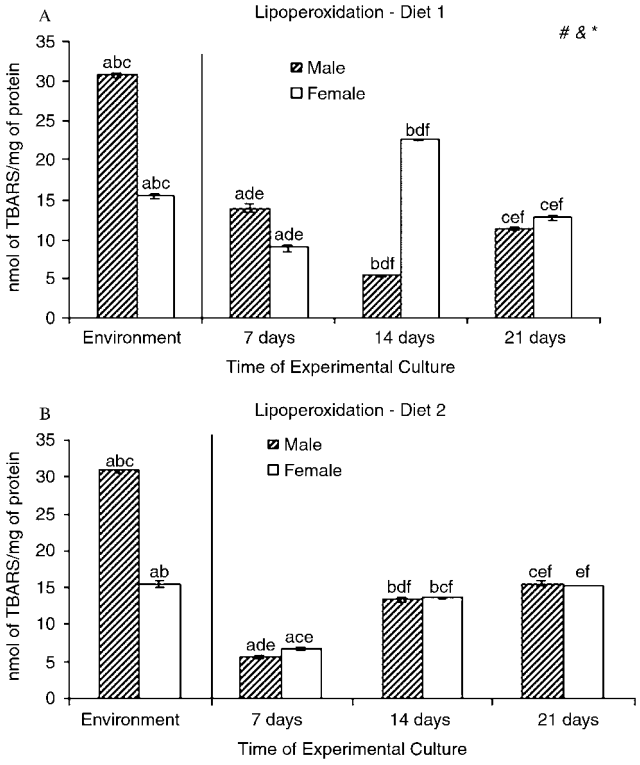

Fig. 5. Levels of lipoperoxidation of *Hyalella castroi* in animals from the natural environment and maintained in experimental culture. Columns represent the mean and bars represent the standard error of the mean. Results are expressed in nmol of TBA-RS mg^{-1} of protein. The same letter indicates a significant difference between the days of culture or environment. ♯ indicates a significant difference between sexes maintained on the same diets. & indicates a significant difference between males fed with different diets. ∗ indicates a significant difference between females fed with different diets.

the experimental periods, we observed that in males the level was the highest after 14 days of experiment, but after 21 days of feeding they returned to the levels shown after 7 days. The same response was observed in females. There was no significant difference in the total cholesterol content between males and females fed with Diet 1 ($P > 0.05$).

The cholesterol contents in males and females of *H. castroi* in the environment and maintained with Diet 2 are shown in Figure 4B. Males and females collected in the natural environment showed cholesterol levels approximately 13.3 and 1.8 times higher than the animals fed with Diet 2 at 7 days ($P < 0.05$). Males showed no significant differences in cholesterol levels during the culture period ($P > 0.05$). In females, after 14 days in experimental culture we observed levels 1.8 times higher than those at 7 days, and cholesterol decreased 7.0 times by 21 days. There was a significant difference in the behavior of cholesterol levels between males and females fed with Diet 2 ($P < 0.05$).

There was no significant difference in the levels of cholesterol in males on the different diets ($P > 0.05$), but the females showed a significant difference between the groups that received Diets 1 and 2 ($P < 0.05$).

Levels of lipoperoxidation

Figure 5A shows the levels of lipoperoxidation in males and females in the natural environment and those maintained on Diet 1. Males and females fed for 7 days showed lower values of lipoperoxidation than did animals in the natural environment. After 14 days of experiment, lipoperoxidation levels decreased in males and increased in females. Over the 21 days of culture, this response was inverted: in males the levels increased and in

females the levels decreased. There was a significant difference in the behavior of lipoperoxidation levels between males and females fed with Diet 1 ($P<0.05$).

The levels of lipoperoxidation in males and females of *H. castroi* in the environment and maintained on Diet 2 are shown in Figure 5B. The males and females collected in the natural environment showed lipoperoxidation levels approximately 5.5 and 2.3 times higher, respectively, than the animals fed with Diet 2 for 7 days. Comparing the levels of TBA-RS between the experimental periods, we observed that after 14 days, in males and females the levels had increased 2.4 and 2.0 times, respectively, and increased again after 21 days in both sexes. There was no significant difference in the levels of lipoperoxidation between males and females given Diet 2, in the different times of culture ($P>0.05$). There was a significant difference in the levels of lipoperoxidation in both males and females on the different diets ($P<0.05$).

Na^+/K^+ATPase activity

The Na^+/K^+ATPase activity in males and females in the environment and fed with Diet 1 is shown in Figure 6A. After 7 days on Diet 1, males showed a lower level of Na^+/K^+ATPase activity (1.7 times) than the animals collected in the natural environment. After 14 days, the levels increased, and returned to the initial values at day 21. In females, Na^+/K^+ATPase activity increased after 7 days of culture with Diet 1, the values were similar at 14 days and the activity had increased by the end of the experiment (21 days).

Figure 6B shows the levels of activity of Na^+/K^+ ATPase in males and females in the environment and fed with Diet 2. Males collected in the environment showed Na^+/K^+ATPase activity 1.3 times higher than the males fed with Diet 2 at day 7. After 14 days of experimental culture, activity increased 2.4 times, and on day 21 the Na^+/K^+ATPase remained higher than that on day 7. In females, activity increased 8.1 times by day 7 and gradually decreased until day 21 although remaining higher than that at day 7 and in animals from the natural environment. There was a significant difference between the Na^+/K^+ ATPase activity levels of males and females maintained with Diet 2 ($P<0.05$). There was a significant difference in Na^+/K^+ATPase activity between both males and females maintained with the different diets ($P<0.05$).

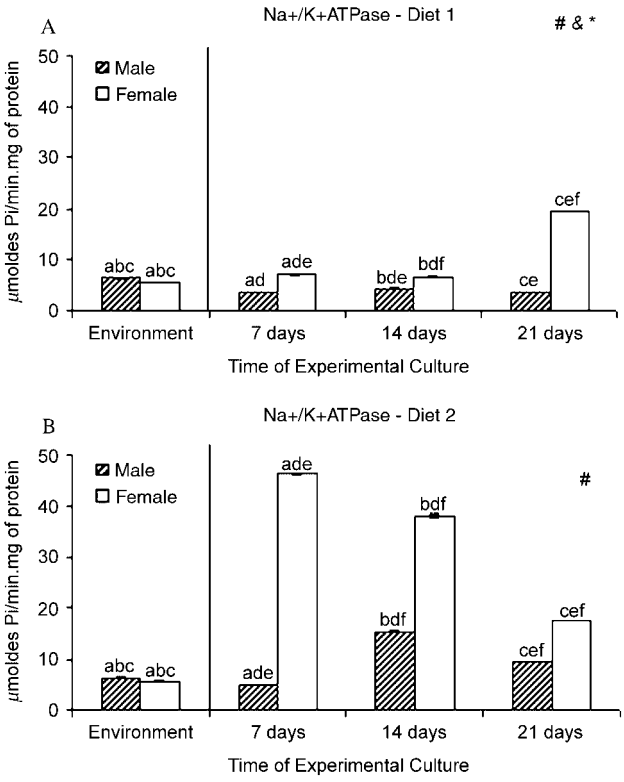

Fig. 6. Activity of Na^+/K^+ATPase of *Hyalella castroi* in animals from the natural environment and maintained in experimental culture. Columns represent the mean and bars represent the standard error of the mean. Results are expressed in µmol of Pi min^{-1} mg^{-1} of protein. The same letter indicates a significant difference between the days of culture or environment. ♯ indicates a significant difference between sexes maintained on the same diets. & indicates a significant difference between males fed with different diets. ∗ indicates a significant difference between females fed with different diets.

DISCUSSION

In this study, Diet 1 or 2 elicited different responses of glycogen, total proteins, lipoperoxidation levels and Na^+/K^+ATPase activity in both sexes of these amphipods, but of total lipids and cholesterol levels only in females. The levels of glycogen and total lipids observed in these amphipods on both diets were similar to the levels observed in other crustaceans fed protein-rich diets (Kucharski and Da Silva, '91a; Ferreira et al., 2005). The results may suggest that amphipods have a higher protein content in their natural diets, and that this diet is more generalized.

Dutra et al. (2007) reported that *H. castroi* mostly exploits the sediment, where it finds more organic matter of animal origin, although its precise feeding habits are unknown. For *H. azteca*, Hargrave ('70) reported that it is an omnivorous

deposit feeder, primarily feeding on algae and bacteria associated with the sediments and aquatic macrophytes. Casset et al. (2001), studying *H. curvispina* in a river in Argentina, suggested that this amphipod is herbivorous, feeding mainly on the phytobenthos and occasionally on sediment. Byrén et al. (2002) showed for the amphipods *M. affinis* and *P. femorata* that sedimented phytoplankton and organic detritus are their main food sources, but bacteria, meiofauna and temporary meiofauna are also included in the diet. Cruz-Rivera and Hay (2000) observed that *G. mucronatus* is often assumed to be an omnivore feeding on plants, animals and detritus.

Most studies on nutrition of freshwater crustaceans are focused on proteins (Cortés-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 (Shiau and Peng, '92). The accumulation of energy reserves in species of crustaceans dependent upon unstable food resources has been reported by several authors (Lee et al., '71; Griffiths, '77; Oliveira et al., 2003; Rosa and Nunes, 2003b).

In this study, Diet 1 contained less protein (30.88%) and more carbohydrate (43.19%) than Diet 2 (protein 39.78% and carbohydrate 28.99%). Animals that received Diet 1 showed a higher survival rate and number of reproductive mating pairs. However, we observed that in the groups maintained on both diets, fewer ovigerous females appeared compared with animals from the natural environment. In the animals on the artificial diets, we did not observe juveniles liberated by ovigerous females (fertility). These differences may be related to the significant decrease in protein reserves, which was observed in females on both diets, and/or the quantity of protein in both diets and/or the higher temperature of the water in the aquariums. In the natural environment, Castiglioni and Bond-Buckup (2007) found this species in high densities, which may improve the reproductive success.

Castiglioni and Bond-Buckup (2007), studying the reproductive strategies of *H. castroi* in laboratory conditions (19°C and 12/12-hr light/dark), estimated a fecundity of 7–42 eggs and a fertility of 16–36 juveniles per female. Castiglioni and Bond-Buckup (2007) fed the animals with macrophytes plus fish food containing 43% protein. Cruz-Rivera and Hay (2000) observed that *G. mucronatus* is likely to be a more generalized feeder that exploits detrital, animal and plant matter, although it is capable of surviving and maturing when feeding only on certain seaweeds. On the latter diet, it achieves lower fecundity than on a mixed diet or a diet of animal matter.

The yolk protein, vitellin, is a glycolipoprotein found in many crustaceans (Riley and Tsukimura, '98; 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 Garcia-Guerrero et al. (2003), who reported that proteins are the important components of the eggs. According to Sastry ('83), oogenesis involves an intense biochemical synthesis, with the mobilization of lipids and proteins for egg development.

In general, the lower levels of glycogen in females and males fed with Diet 2 in relation to animals fed with Diet 1 can be explained by the lower percentage of carbohydrates in Diet 2. Diet 1 contained 43.19 g/100 g of carbohydrates and Diet 2 contained 28.99 g/100 g. Studies on other crustaceans, such as *C. granulata* (Kucharski and Da Silva, '91a), *A. platensis* (Ferreira et al., 2005) and *P. brasiliensis* (Dutra et al., 2008d), have shown that increased carbohydrate in the diet changes the homeostasis of glycogen compared with animals that receive a high-protein diet, causing an increase in the levels of this polysaccharide.

In this study, although the animals were given free access to food, males and females fed with Diet 2 and males fed with Diet 1 did not maintain their glycogen and lipid reserves during the culture period. The males and females were kept in aquariums separated with a nylon mesh, and remained in chemical contact because the water passed through both parts of the aquarium, and the ovaries of the females matured. According to Dutra et al. (2007), winter is the reproductive peak of this species and autumn is the season when it prepares for reproduction (synthesis of gametes and vitellin and reproductive behavior). In our study, the animals were collected in autumn.

The importance of the quantity and quality of the food provided can be evaluated through the offspring produced in the cultures, because diet can directly influence the reproductive capacity of individuals. Herbert ('78), studying the genus *Daphnia*, observed that the number of neonates produced by ovigerous females depends directly on the food ingested. The number of juveniles, together with the sensitivity of the organism to some reference substance and the course of accumulation

of lipids, can be adopted as the criteria to evaluate the quality of the cultures of organisms used in ecotoxicological bioassays (Zagatto, '88).

In this study, males fed with both diets maintained protein reserves similar to those of animals from the natural environment after 21 days of culture. In contrast, females fed with both diets showed decreased protein reserves. A major cost of energy for the reproductive activity, principally the synthesis of vitellin in females, may explain the lower quantity of protein, especially in Diet 1, which contained less protein than Diet 2.

The levels of proteins present in Diet 1 (30.88 g/100 g) and Diet 2 (39.78 g/100 g) may have caused the low number of ovigerous females and low fertility. Broodstock nutrition is important for reproductive success, because production of eggs and larvae is strongly dependent on the diets offered (Bromage, '95; Harrison, '97; García-Ulloa, 2000; Rodríguez-González, 2001; Wouters et al., 2001). According to Harrison ('97), 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 (Harrison, '90; Abdu et al., 2000; Wouters et al., 2001; Garcia-Guerrero et al., 2003).

In this study, the levels of total lipids and cholesterol of the animals fed with Diet 1 tended to decrease in the beginning of the experiment and then remain stable until the end of the culture, in both males and females. In both sexes fed with Diet 2, the response was biphasic: in the first period these reserves decreased, but with the continuation of the culture the levels of these metabolites increased.

Dutra et al. (2007) reported that in *H. castroi*, proteins, lipids and cholesterol were depleted during precopula and copula (winter) because *H. castroi*, similar to other crustaceans, produces large eggs. Egg size is related to maternal investment, mainly lipid metabolism (Rosa and Nunes, 2003a,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, '97; Rainuzzo et al., '97).

Maternal care in amphipod crustaceans involves the passive carriage of embryos in an external broodpouch, from which fully developed young emerge. However, more active brood-care behaviors have recently been identified in this group (Dick et al., '98; Thiel, '99). Post-emergence care involves, for example, defense of juveniles from cannibals and predators (Aoki, '97; Thiel, '97, '98, '99), whereas pre-emergence care, identified in *Crangonyx pseudogracilis*, involves brood ventilation, egg cycling and ejection of nonviable eggs (Dick et al., '98). Such pre-emergence brood-care activities may be a feature of amphipods living in harsh environments (Dick et al., '98). 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 (Castiglioni and Bond-Buckup, 2007).

In both diets, the females always showed higher rates of Na^+/K^+ATPase activity than males, which may be related to more intense degradation of proteins and total lipids. The lower levels of Na^+/K^+ATPase activity in animals fed with Diet 1 can perhaps be explained by the higher levels of lipoperoxidation. 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 function (Halliwell and Gutteridge, '86; Vercesi et al., '97; Milatovic et al., 2005). These degenerative changes can affect the dynamic properties of the membranes such as fluidity and permeability, and consequently the activity of various membrane-associated enzymes (Mecocci et al., '97). 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., '97; Mark et al., '97).

Dutra et al. (2007) showed a peak of lipoperoxidation in autumn in females of *H. castroi*. In this season, the period of precopulation and copulation prior to the peak of reproduction in winter (Castiglioni and Bond-Buckup, 2007), the males also showed a peak of lipoperoxidation, because they consume more energy for precopulatory mate guarding and carrying females during this period. In this study, the animals were collected in autumn and the males and females utilized in the metabolic study were kept separate, but females showed mature ovaries. We presume that they continued preparation for the reproductive period.

In conclusion, our results showed that these diets changed the biochemical patterns of the animals taken from the natural environment, and could not improve reproduction (number of ovigerous females and fertility). These points should be further investigated.

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