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Variations in biochemical composition and lipoperoxidation levels of *Hyalella bonariensis* maintained in laboratory with different diets

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Abstract

We compared the effect of different diets in the biochemical composition, levels of lipoperoxidation and survival rates of males and females of *Hyalella bonariensis*. These crustaceans live in limnetic environments and different kinds of food habits are present in the genus *Hyalella*. Adult animals were collected in the spring (September) in a stream near to Salto city, Buenos Aires. In the laboratory, the animals were kept submerged in aquariums, separated by sex, under controlled conditions and survive rates were observed. They were fed *ad libitum* for 45 days with four different diets, and after this period the animals were used for biochemical determinations. Statistical analysis revealed significant differences in the responses to glycogen, proteins, lipids, triglycerides and lipoperoxidation levels in both sexes of these amphipods taken from the natural environment. Animals that received macrophytes associated a ration with high content of proteins (43%) during 45 days presented the major survival rates (males= 86.6% and females= 96.6%). These responses can be revealed that this specie primarily feeding macrophytes associated with deposit feeder, and that this diet is more generalized. This study showed the importance of the proteins in diet of this amphipod, were the females increased the reserves of proteins and triglycerides with different diets probably for reproductive events and male increased the reserves of glycogen and triglycerides for reproductive events and growth.

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Introduction

Members of the genus *Hyalella* are common in the Nearctic and Neotropical regions, with 45 described species (González and 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 for their role in trophic wiles (Kruschwitz, 1978; Wellborn, 1995; Grosso and Peralta, 1999). Several kinds of food habits (herbivores, detritivores and planktotrophic) are present in the genus *Hyalella* (González et al., 2006). In the other hand, they are also important as food for other organisms, such as birds, fishes and macroinvertebrates, allowing the transfer of energy from plants to higher trophic levels (Muskó, 1993; González et al., 2006).

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).

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, 1979; Cruz-Rivera and Hay, 2000; Poltermann, 2001).

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 are strongly dependent on the diets offered (Bromage, 1995; Harrison, 1997; 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 (1997), 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).

Dutra et al. (2009) compared the effect of different diets on the biochemical composition, levels of lipoperoxidation, survival rate, and reproductive aspects of *Hyalella pleoacuta* and *H. curvispina*. The animals fed with macrophytes only present a response of the metabolic parameters similar the animals were maintained in 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 associate with a commercial ration these responses were reversed because the energy reserves were maintained and the levels of lipoperoxidation were higher than in those on diet with macrophytes. The authors observed that animals fed with associate diet

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 associate diet, which provided more carbohydrates, proteins and lipids. This diet was also important for adequate maintenance in the toxicology experiments.

Malondialdehyde, a breakdown product of lipid endoperoxides, is an expression of lipoperoxidation, 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 *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., 1991; Correia, 2002; Correia et al., 2003).

The present study had the aim of evaluating the effect of different diets on the biochemical composition, lipoperoxidation and survival rate of the freshwater amphipod *Hyalella bonarienses* (Santos et al., 2008) maintained in laboratory conditions. The purpose was to obtain basic physiological data to support future studies in the development of toxicology tests in this specie.

Material and methods

Adult animals were collected in spring, during September of 2005, in a stream near to Salto city, Buenos Aires Province (34°18'15.6"S – 60°20'0.0"W). Animals and macrophytes (*Ceratophyllum demersum*) were collected by means of fish traps and bottom grabs.

Part of the animals were immediately frozen in natural environment (Control Group) for analyzing of the different metabolites, and other part were transported in cold water (5°C) in insulated containers to the Laboratório de Fisiologia Animal Comparada of Universidad de Buenos Aires, where they were separated by sex and placed in aerated aquariums for 24 hours without food (period 1).

Experimental procedure

After the period 1, the animals were kept submerged in aerated aquariums, in density of 1 animal per liter of water, with a photoperiod of 14:10 hours light/dark and constant temperature (20°C). The amphipods were divided into four groups, which were fed *ad libitum* in each two days, in late afternoon when most of the animals were active, for a period of 45 days. The animals were monitories daily, water and macrophytes were replaced in each two days.

Group 1: animals received only macrophytes during 30 days and by more 15 days these animals received macrophytes associated a ration with high content of proteins (43%);

Group 2: animals received macrophytes associated a ration with high content of proteins (43%) during 45 days;
Group 3: animals received macrophytes associated a ration with lower content of proteins (30%) during 45 days;
Group 4: animals received macrophytes only during 45 days.

The macrophytes were not washed and were maintained in aquariums aerated, hence periphyton community was maintained. The ration was used have proteins of animal origin. After 45 days of experimental culture the animals of each group diet and of 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.

Biochemical Analyses

Metabolites. Metabolic determination for *H. bonariensis* Santos, Araújo and Bond-Buckup (2008) was done in total homogenates of three pools of five males and five females. One pool of each sex 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 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 in 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 mg/ml 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. (1957). After two wash with physiological solution and centrifugation (2000rpm) total lipids were determined by the sulfophosphovanillin method (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 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 TBARS/mg of protein.

Statistical Analysis. Results are expressed as mean ± standard error, and for statistical analysis of the different diets and sexes was used Student 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 (Zar, 1996).

Results

During the samplings in natural environment the authors observed a high number of ovigerous females and precopulatory mating pairs. This fact permit to suggest that spring is the principal period of the reproduction in this amphipod specie.

Table 1 shows the glycogen concentration in males and females in the natural environmental and cultivation with different diets. The glycogen levels were significantly lower in the males of the control group (natural environment) and of the group 4 (fed with macrophytes) when compared with others groups (1, 2 and 3). Already, in the females the glycogen levels were higher ($p< 0.05$) in animals of the group 2 (only macrophytes for 30 days and more 15 days with macrophytes and ration with high protein content) when compared with others experimental groups (control, 1, 3 and 4) ($p<0.05$). There were significant differences in glycogen levels after 45 days of culture between males and females of the group 1, 2 and 3 ($p<0.05$).

Protein concentrations in males and females in environment and feeding with different diets were showed in table 2. The protein levels showed no significant difference in all the experimental groups of males ($p>0.05$). Already, in females was observed an increased of the levels of proteins in group 2, 3 and 4 when compared with control group ($p<0.05$). The lowest protein levels in females were verified in females collected in natural environmental (control group). The protein levels were significantly different between males and females in control group, group 1, 3 and 4 ($p<0.05$).

The concentrations of total lipids in males and females in the environment and feeding with different diets are showed in table 3. The levels of total lipids were significantly lower in males of the group 1 (macrophytes during 30 days and by more 15 days these animals received macrophytes associated a ration with high content of proteins (43%))

Table 1.
Level of glycogen in males and females of *Hyalella bonariensis* in different experimental groups. The data represent the mean ± standard error. Results are expressed in mg/g. The different letter indicates a significant difference between the experimental groups. * indicates a significant difference between males and females.

	Males	Females
Control	0.349 ± 0.111 ^{c}	0.294 ± 0.067 ^{b}
Group 1	0.981 ± 0.028 ^{a}	0.161 ± 0.027 ^{b} *
Group 2	0.832 ± 0.036 $^{a\,b}$	1.574 ± 0.031 ^{a} *
Group 3	0.727 ± 0.015 ^{b}	0.140 ± 0.046 ^{b} *
Group 4	0.336 ± 0.024 ^{c}	0.156 ± 0.014 ^{b}

Table 2.
Level of total proteins in males and females of *Hyalella bonariensis* in different experimental groups. The data represent the mean ± standard error. Results are expressed in mg/g. The different letter indicates a significant difference between the experimental groups. * indicates a significant difference between males and females.

	Males	Females
Control	9.245 ± 0.254 [a]	3.612 ± 0.193 [c]*
Group 1	8.283 ± 0.547 [a]	4.067 ± 0.149 [c]*
Group 2	9.971 ± 0.571 [a]	9.385 ± 0.679 [a]
Group 3	10.006 ± 0.235 [a]	6.376 ± 0.231 [b]*
Group 4	8.038 ± 0.332 [a]	5.974 ± 0.201 [b]*

Table 3.
Level of total lipids in males and females of *Hyalella bonariensis* in different experimental groups. The data represent the mean ± standard error. Results are expressed in mg/g. The different letter indicates a significant difference between the experimental groups. * indicates a significant difference between males and females.

	Males	Females
Control	1.039 ± 0.195 [a]	2.096 ± 0.084 [a]*
Group 1	0.691 ± 0.027 [b]	1.100 ± 0.088 [b]*
Group 2	0.889 ± 0.030 [a]	0.832 ± 0.063 [c]
Group 3	0.933 ± 0.029 [a]	0.728 ± 0.033 [c]
Group 4	0.273 ± 0.005 [c]	0.934 ± 0.103 [bc]*

and group 4 (macrophytes only during 45 days of culture) when compared with the others experimental groups (control, 2 and 3), and the highest levels were observed in animals in natural environment ($p<0.05$). In females, the levels of total lipids were lower in all experimental groups (1, 2, 3 and 4) ($p>0.05$). When we compared the levels of total lipids between males and females was observed significant differences in the levels of this metabolite in the control group, group 1 and 4 ($p<0.05$), being more raised in females of that in males.

The concentrations of triglycerides in males and females in the environment and feeding with different diets are showed in table 4. In the analysis of the triglycerides levels was observed that the males of group 1, 2 and 3 presented the highest levels of this metabolite ($p<0.05$) and animal fed with only macrophytes showed the lowest triglycerides levels. In females, the highest triglycerides levels was observed only in animals of the group 3 (macrophytes and ration with low protein content during 45 days of culture) ($p<0.05$). The triglycerides levels were similar between males and females in all experimental groups ($p>0.05$).

Table 5 shows the levels of lipoperoxidation in males and females in the natural environment and different groups of the diets. The lipoperoxidation levels were lower values in males and females of the group 2 and 3 ($p<0.05$). The lipoperoxidation levels

Table 4.
Level of triglycerides in males and females of *Hyalella bonariensis* in different experimental groups. The data represent the mean ± standard error. Results are expressed in mg/g. The different letter indicates a significant difference between the experimental groups. * indicates a significant difference between males and females.

	Males	Females
Control	0.198 ± 0.042 [c]	0.226 ± 0.030 [b]
Group 1	0.319 ± 0.052 [a b]	0.278 ± 0.074 [b]
Group 2	0.309 ± 0.035 [b c]	0.379 ± 0.037 [b]
Group 3	0.449 ± 0.076 [a b]	0.625 ± 0.068 [a]
Group 4	0.152 ± 0.044 [c]	0.376 ± 0.054 [b]

Table 5.
Level of lipoperoxidation in males and females of *Hyalella bonariensis* in different experimental groups. The data represent the mean ± standard error. Results are expressed in mg/g. The different letter indicates a significant difference between the experimental groups. * indicates a significant difference between males and females.

	Males	Females
Control	9.035 ± 0.744 [a c]	6.862 ± 0.704 [a *]
Group 1	6.080 ± 1.017 [a b c]	5.888 ± 0.350 [a b]
Group 2	3.241 ± 0.508 [b]	2.700 ± 0.464 [c]
Group 3	4.557 ± 0.608 [b c]	3.555 ± 0.604 [b c]
Group 4	9.134 ± 1.257 [a]	4.006 ± 0.350 [b c *]

Table 6.
Survival indices (%) of males and females of *Hyalella bonariensis* maintained in experimental culture for 45 days with different diets.

Group	Male	Female
01	70.0	66.6
02	86.6	96.6
03	70.0	76.6
04	76.6	86.6

showed significant difference between males and females only in animals fed with macrophytes only during the culture, being raised in males ($p<0.05$).

The survival rates of males and females fed with different diets during culture period (45 days) are given in table 6. Males fed with diet 2 (macrophytes and ration with 43% of proteins) showed survival rates of 10% until 16.6% higher than the animals fed others diets (1, 3 and 4). The same pattern of response was observed in females, where animals fed with diet 2 showed survival rates of 10% until 30% higher animals fed diets 1, 3 and 4.

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 and activity of different crustacean species under food-limited conditions (Aldrich, 1975; Regnault, 1981; Du Preez, 1983; Hiller-Adams and Childress, 1983; Oliveira et al., 2004).

For *Hyalella azteca*, Hargrave (1970) 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 *Hyalella curvispina* in a river in Argentina, suggest that this amphipod is herbivorous, feeding mainly on the phytobenthos and occasionally on sediment. 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. Cruz-Rivera and Hay (2000) observed that *Gammarus mucranatus* is often assumed to be an omnivore feeding or plants, animals and detritus. In this work we verified that when the animals were feeding in laboratory with only macrophytes the glycogen, proteins, triglycerides and lipoperoxidation levels in males and glycogen and triglycerides in females showed no significant difference in relation of the animals collected in natural environment and animals presented high levels of survival (76.6% in males and 86.6 in females). These responses can be revealed that this specie, *H. bonarienses*, primarily feeding on macrophytes and occasionally macrophytes associated with deposit feeder, and that this diet is more generalized. This profile associated with an increased of some metabolites observed in animals of the groups 1, 2 and 3 (animals that received diet with more contents of proteins) reinforced this suggestion.

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, 1992). The accumulation of energy reserves in species of crustaceans dependent upon unstable food resources has been reported by several authors (Lee et al., 1971; Griffiths, 1977; Oliveira et al., 2003; Rosa and Nunes, 2003).

In males that received macrophytes associated with proteins (diet 2: 43% of the proteins and diet 3: 30% of the proteins) during all time of cultivation showed the same pattern of response with levels of glycogen and triglycerides were increased, proteins and total lipids were stables and lipoperoxidation levels were reduced. In females the same response was observed too in diets 2 and 3, where levels of total proteins and triglycerides were increased and total lipids and lipoperoxidation levels were reduced.

These responses showed the importance of the proteins in diet of this amphipod, were the females increased the reserves of proteins and triglycerides and decreased in the levels of total lipids probably for reproductive events (oogenesis, vitellogenesis and maternal care) and male increased the reserves of glycogen and triglycerides and

decreased the concentration of lipids for reproductive events (spermatogenesis and copulatory behaviors) and growth. This hypothesis is reinforced for higher levels of survival rates observed in both sexes (86.6% in males and 96.6% in females) that received diet 2 (macrophytes and ration with 43% of proteins).

Castiglioni and Bond-Buckup (2007), studying the reproductive strategies of *H. castroi* in laboratory conditions (19°C and 12/12hours light/dark) where animals were fed with macrophytes plus fish food containing 43% protein, estimated a fecundity of 7 to 42 eggs and a fertility of 16 to 36 juveniles per female. These results were similar to dates obtained in natural environment for *Hyalella castroi*. However, for *Hyalella bonariensis* dates of fecundity and fertility were not know in natural environment.

In animals that received diet 2 (macrophytes and ration with 43% of proteins) we observed the higher values of the survival rates (86.6% in males and 96.6% in females) this response can be explained for a decreased of 2.8 and 2.5 time in males and female respectively, in lipoperoxidation levels.

In this study, males fed with different diets maintained protein reserves similar to those of animals from the natural environment after 45 days of culture. In contrast, females fed with different diets showed increased of the protein reserves. All diets studies showed a significant reduced in levels of the total lipids in female, and in males only diet 2 (macrophytes and ration with 43% of proteins) and 3 (macrophytes and ration with 30% of proteins) the animals presented this reduction. A major cost of energy for reproductive activity, principally the synthesis of vitellin in females, may explain the increment of proteins and lower quantity of the lipids. Lehtonen (2004) showed that lipids act as fuel biosynthesis in the build-up of reproductive products in amphipods.

The yolk protein, vitellin, is a glycolipoprotein found in many crustaceans (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. (2003), who reported that proteins are the important components of the eggs. According to Sastry (1983), oogenesis involves an intense biochemical synthesis, with the mobilization of lipids and proteins for egg development.

In conclusion, our results showed that these diets changed the biochemical patterns of the animals taken from the natural environment, where diets that contain proteins increased the energetic reserves and survival rates in males and females can be improved condition of cultivation and probably reproduction in laboratory. These points should be further investigated.

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