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VARIATION IN LIPID, PROTEIN, AND CARBOHYDRATE CONTENT DURING THE EMBRYONIC DEVELOPMENT OF THE CRAYFISH *CHERAX QUADRICARINATUS* (DECAPODA: PARASTACIDAE)

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A B S T R A C T

Eggs of 20 females were sampled to analyze lipid, protein, and carbohydrate content during the embryonic development of *Cherax quadricarinatus*. Sampling was performed on recently spawned eggs to first juvenile stage. Ten eggs were sampled every 48 h during the first two weeks and every 72 h thereafter for biochemical analysis. Total proteins, lipids, and carbohydrates were measured. Proteins were the most abundant egg component (63.2%), followed by lipids (32.3%), and carbohydrates (4.4%). A steady decrease of lipid content was observed ($y = 895.38 - 9.93x$, $r^2 = 0.94$; y = lipid concentration, x = days). For proteins, two different depletion rates were observed, with the initial rate maintained for 30 days ($y_1 = 1{,}443.0 - 4.46x_1$, $r^2 = 0.90$) and an increased depletion rate after hatching ($y_2 = 1{,}936.60 - 21.10x_2$, $r^2 = 0.96$). Carbohydrates were always present as a minor constituent, and its concentration did not change significantly. Water content increased from 52% in recently spawned egg to 85% in juveniles. Energy equivalents were calculated from each component, and the overall energy expenditure is represented by the equation $y = 13.86 - 0.11x$ ($r^2 = 0.95$). Relevant morphological features for each sample day are indicated.

The crayfish *Cherax quadricarinatus* (Von Martens) is a valuable species in freshwater aquaculture. A current limitation for commercial production is the insufficient supply of juveniles for pond stocking (Villarreal and Peláez, 2000). Studies on crayfish reproduction and development are essential to optimize production. However, available information of nutritional requirements and adequate culture techniques is scarce (Villarreal and Peláez, 2000). Some studies have analyzed the embryonic development of *Cherax* species based on morphological changes (Sandeman and Sandeman, 1991; Yeh and Rouse, 1994). However, no information exists about the changes in biochemical composition that occur during development. Biochemical composition of eggs depends on the reproductive strategy and nutritional status of the female and determines the success of the embryonic or larval development and the transition from yolk-dependent (lecitotrophy) to free-feeding organisms (Holland, 1978; Rønnestad *et al.*, 1998). It is generally considered that the most appropriate way to evaluate the nutritional requirements of lecitotrophic larvae is to measure changes in the biochemical composition of yolk during embryonic or larval development (Fraser, 1989).

Many authors have analyzed the biochemical changes in different organisms, such as fish (Watanabe *et al.*, 1984; Petersen and Anger, 1997; De Silva *et al.*, 1998; Rønnestad *et al.*, 1998; Mookerji and Ramakrisha, 1999); crustaceans (Pandian, 1970; Clarke *et al.*, 1990; Chu and Ovsianico, 1994; Lovrich and Ouellet, 1994; Rodríguez *et al.*, 1994; Biesiot and Perry, 1995; Mourente *et al.*, 1995; Petersen and Anger, 1997; Palacios *et al.*, 1998; Lemos and Rodríguez, 1997; Cavalli *et al.*, 2000; Roustaian and Kamarudin, 2001); and mollusks (Fraser, 1989; Whyte *et al.*, 1990; Heras *et al.*, 1998; Laing and Earl, 1998). From these studies, it is generally accepted that lipids, particularly tryglycerides, are the main energy supplier during embryonic development. Lipids are, in general, essential as structural components of cell membranes and as an energy source, and they provide essential fatty acids (Coutteau *et al.*, 1997). On the other hand, the fundamental role of proteins is as structural components of embryonic tissue, and, under certain conditions, as fuel (Stryer, 1995; Lemos and Rodríguez, 1997).

In the present study, lipid, protein, and carbohydrate variations during embryonic development were evaluated in order to quantify the relative importance of each component during the ontogeny of *C. quadricarinatus*.

Materials and Methods

Fifty *C. quadricarinatus* females weighing 50 ± 7.5 g, were placed in two 1,500-l fiberglass tanks at 28 ± 0.5°C, with continuous aeration, and fed with a commercial shrimp pellet (32% crude protein, 8% lipid; PIASA®). Tanks were provided with PVC tubes (15 cm diameter, 25 cm long) as shelter. Once mature, five males were placed in each tank. After two weeks, 20 ovigerous females were obtained and placed individually in a 40-l tank with water at 26 ± 0.5°C, continuous aeration, and PVC shelters. The first sample, which corresponds to recently spawned eggs, was taken just before the female transfer. Thereafter, samples of 15 eggs from each sampled female, were taken every 48 h for the first two weeks and every 72 h to the end of the experiment. Ten eggs from each sample were used for biochemical analyses and five for observation of morphological features.

For biochemical analyses, the eggs were homogenized in saline solution (1.2% NaCl). To quantify proteins, the homogenate was first digested with NaOH 0.5N. The concentration was determined by the Bradford method (1976), using albumin as standard, and absorbance was read at 595 nm using a Spectron Genesys Spectophotometer. For carbohydrates, proteins were precipitated with 20% trichloroacetic acid and centrifuged at 3,000 rpm for 10 min at 4°C. Carbohydrates were then quantified from the supernatant by the Anthrone method (Van Handel, 1965), using glucose as standard, and absorbance was read at 620 nm using the same spectrophotometer. For total lipids, an adaptation of the method of Barnes and Blackstock (1973) was used. An aliquot of the homogenate was mixed with pure H_2SO_4 and incubated at 80°C for 10 min. The acid solution obtained was mixed with the fosfovanillin reagent and the absorbance was recorded using a Biorad 560 microplate reader at 560 nm, using a mixture of tryglycerides (12 mg/ml) and cholesterol (8 mg/ml) as standard. Ten eggs from two other females were used to determine moisture content. Dry weight was obtained by keeping samples in an oven at 70°C for 48 h.

Eggs used for morphological analysis were fixed in 16% Formalin. Morphological changes were observed under a stereoscopic microscope at 8× by observing the appearance of new embryonic structures, according to the guidelines described in Sandeman and Sandeman (1991).

Differences in concentration of proteins, lipids, and carbohydrates through time were established by analysis of variance, followed by the Newman-Keuls *post hoc* test (Sokal and Rohlf, 1995). Variations of nutrient concentration and energy expenditure over time were obtained by regression analysis. For proteins, an inflection point was detected with the stepwise lineal regression analysis described by Sokal and Rohlf (1995), and the adjustment is based on the r^2 function.

To establish the values for energy consumption, the conversion factors suggested by Heras *et al.* (1998) were applied: 4.1 cal/mg (17.2 joules/mg) for carbohydrates; 4.3 cal/mg (17.9 joules/mg) for proteins; and 7.9 cal/mg (33.0 joules/mg) for lipids.

Results

Main morphological changes for each sampled day are summarized in Table 1. The development of *C. quadricarinatus* at 26.0 ± 0.5°C from recently spawned eggs to juvenile lasted 45 days. The lipid, protein, and carbohydrate concentrations of *C. quadricarinatus* eggs and embryos through time are shown in Table 1. Proteins were the most abundant component, followed by lipids and carbohydrates. Lipid concentration decreased significantly during the embryonic development ($F = 9.22$, $P < 0.05$). For proteins, there was a significant variation in content ($F = 2.33$, $P < 0.05$), although significantly lower levels were observed only at day 45. For carbohydrates, significant differences through time were not detected. Figure 1 shows that the lipid concentration declined over time, represented by the equation $y = 895.38 - 9.93x$, $r^2 = 0.94$ ($P < 0.001$), (y = lipid concentration, x = days). The decrease in the concentration of proteins (Fig. 2) is represented by the equations $y_1 = 1{,}443.0 - 4.46\,x_1$, $r^2 = 0.90$ ($P < 0.001$) for the first 30 days and $y_2 = 1{,}936.60 - 21.10x_2$, $r^2 = 0.96$ ($P < 0.001$) for the last 15 days ($y_{1,2}$ = protein concentration; x_1: day 1 to 31; x_2: day 31 to 45). Relative composition of lipids, proteins, and carbohydrates expressed as percentage organic matter, are shown in Fig. 3. Lipids decreased significantly, from 37.10% to 27.02% of total composition while proteins increased from 58.97% to 67.11% and carbohydrates from 3.70% to 5.87%. On the other hand, total average weight of spawned eggs was 5.1 ± 0.4 mg, which increased to 11.5 ± 1.23 mg for the juvenile stage (Fig. 4). This increase in weight was due to water accumulation, which represented 52% and 85% of total weight for eggs and juveniles, respectively. Individual dry weight decreased from 2.5 mg to 1.9 mg during development from recently spawned eggs to juveniles.

To evaluate changes in total energy in the embryo, caloric equivalents were calculated from biochemical composition (Fig. 5). Although lipids were not the most abundant component, they contributed more than 50% of the total energy content in recently spawned eggs. The change in total energy through time during the embryonic development of *C. quadricarinatus* is represented by the equation $y = 13.86 - 0.11x$, $r^2 = 0.95$ ($P < 0.001$, y = energy content, x = days). The average relative

Table 1. Mean biochemical composition (± SD) and water content during embryonic development of *Cherax quadricarinatus* from ovoposition to juvenile stage.

Day	Development stage (*)	Lipid (µg/ind)	Protein (µg/ind)	Carbohydrate (µg/ind)	Moisture (%)
1	Yolk evenly distributed	901.3 ± 127.2 a	1,424.8 ± 322.7 a	89.3 ± 26.1 a	50.8
3	Yolk break up in droplets	883.1 ± 91.4 a	1,473.6 ± 342.9 a	83.7 ± 21.6 a	55.5
5	Gastrulation is taking place	775.5 ± 141.6 ab	1,428.6 ± 377.9 a	87.7 ± 28.1 a	60.9
7	Cephalic primordia begin to bud	870.7 ± 126.6 a	—	90.9 ± 35.5 a	63.5
9	Post naupliar segments rises as buds	820.1 ± 159.2 a	1,384.0 ± 343.7 ab	86.0 ± 25.2 a	63.9
11	Caudal papilla takes horseshoe shape	809.7 ± 195.6 a	1,406.6 ± 393.4 ab	89.9 ± 29.3 a	66.2
14	Eye lobe outlines become visible	750.0 ± 183.3 abc	—	95.7 ± 37.8 a	68.4
18	The heart is beating regularly	726.7 ± 189.2 abc	1,401.5 ± 345.9 ab	89.1 ± 31.1 a	73.1
21	Eyes have grown and become spherical	599.3 ± 206.0 bcd	—	86.6 ± 25.1 a	74.7
24	Chelae and legs cover the embryo front	564.2 ± 210.1 bcd	1,329.2 ± 347.7 ab	92.1 ± 27.1 a	74.9
27	Eyes and thoracic limbs are fully developed	667.4 ± 234.6 abc	1,372.4 ± 464.3 ab	89.5 ± 24.7 a	77.7
30	Abdomen is fully developed	689.1 ± 186.5 abc	1,302.5 ± 407.1 ab	97.1 ± 34.9 a	78.9
33	Embryo hatches and releases appendices	543.6 ± 176.5 cd	1,228.4 ± 385.1 ab	92.7 ± 27.2 a	80.1
36	Postembryo I (very big yolk hunchback)	578.2 ± 267.7 bcd	1,167.3 ± 372.7 ab	92.1 ± 26.5 a	80.7
39	Postembryo II (medium yolk hunchback)	551.7 ± 250.7 cd	1,152.8 ± 316.5 ab	97.6 ± 32.4 a	82.8
42	Postembryo II (small yolk hunchback)	465.0 ± 171.2 d	1,068.8 ± 319.9 ab	96.9 ± 30.4 a	82.8
45	Juvenile (yolk completely depleted)	409.0 ± 162.3 d	958.3 ± 296.6 b	86.0 ± 37.8 a	84.1

Within each column, means with the same letter are not significantly different at $P > 0.05$.
* According to Sandeman and Sandeman (1991).

contribution of lipids and proteins to the energy consumed was, respectively, equivalent to 4 and 2 cal/day.

Discussion

A high variability in the concentration of the different proximal components exists among eggs obtained from different females. This can be due to differences in spawn quality, which reflects the nutritional status of the female and its general condition, as well as genetic differences among individuals (Holland, 1978; Clarke *et al*., 1990).

Proteins were the most abundant component in eggs followed by lipids. This proportion is very common among eggs of aquatic invertebrates, especially crustaceans (Holland, 1978; Whyte *et al*., 1990; Clarke, 1992). It is generally accepted that protein is the major component

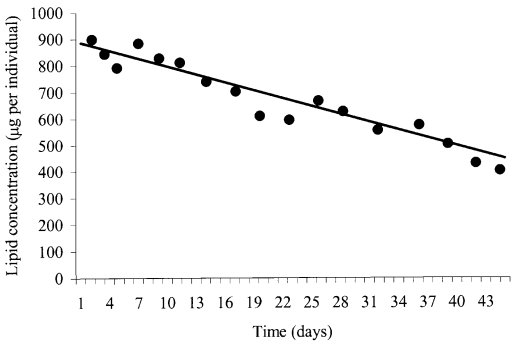

Fig. 1. Lipid concentration change through time in *C. quadricarinatus* eggs. The regression line is represented by the equation $y = 895.38 - 9.93x$, $r^2 = 0.94$.

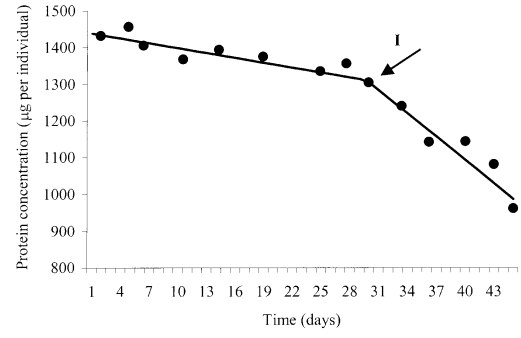

Fig. 2. Protein concentration change through time in *C. quadricarinatus* eggs. The regression line is represented by the equations $y_1 = 1{,}443.0 - 4.46x_1$, $r^2 = 0.90$ and $y_2 = 1{,}936.60 - 21.10x_2$, $r^2 = 0.96$. I = inflection point.

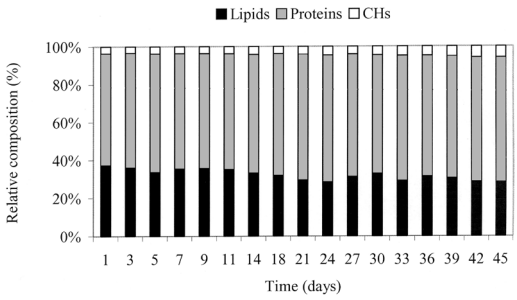

Fig. 3. Relative composition of lipid, protein, and carbohydrate (percent of organic matter per day) during embryonic development of *C. quadricarinatus*.

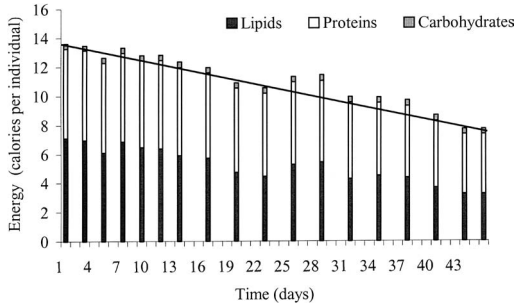

Fig. 5. Energetic equivalent for proteins, lipids, and carbohydrates in the biochemical composition per day on the embryonic development of *C. quadricarinatus*. The energy expenditure regression line is given by the equation $y = 13.86 - 0.11x$, $r^2 = 0.95$.

and lipid is the main energy source for embryonic development (Holland, 1978). Most studies made on crustacean eggs show that lipids are the major energy reserve (Pandian, 1970; Holland, 1978; Clarke *et al.*, 1990; Ouellet and Taggart, 1992; Chu and Ovsianico, 1994; Xu *et al.*, 1994; Petersen and Anger, 1997; Cavalli *et al.*, 2000; Heras *et al.*, 2000; Roustaian and Kamarudin, 2001). The continuous depletion of lipids shown by the regression line in the present study suggest that they are the main energy source in *C. quadricarinatus* eggs. Proteins were also used during the first 30 days but at a lower rate than lipids. After 30 days of development, the measured consumption of protein suggests an increase in the need of this substrate even if lipids are still used at the same rate. This increase in the use corresponds to hatching and could be explained by a higher energy demand for the differentiation and growth processes occurring in postembryo stages. Similar phenomena for protein depletion have been established for various crustacean species (Holland, 1978; Murugadass and Pandian, 1991; Clarke, 1992; Chu and Ovsianico, 1994; Biesiot and Perry, 1995; Roustaian and Kamarudin, 2001), for mollusks (Fraser, 1989; Whyte *et al.*, 1990; Videla *et al.*, 1998), and for fish (Rønnestad *et al.*, 1998; Sargent *et al.*, 1999). However, the strategies for energy use are varied. In the spider crab *Hyas arenus* total protein amount increases during zoeal development but decreases in the megalopa (Petersen and Anger, 1997). In penaeid shrimp, while no significant differences in protein consumption were observed between eggs and nauplii (Chu and Ovsianico, 1994; Hernández-Herrera, 2001), the relation between oxygen consumption and nitrogen excretion decreased in advanced nauplii stages, indicating an increase in protein use. In addition to protein use for energetic purposes, a decrease of protein after hatching could be explained by chorion loss (70% protein; Pandian, 1970).

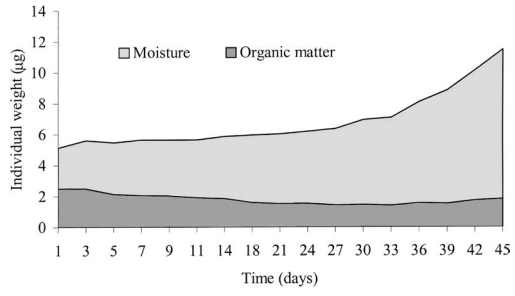

Fig. 4. Time course of wet and dry weight during embryonic development of *C. quadricarinatus*.

It should be noted that differences between yolk and structural proteins were not defined. Sample digestion with NaOH dissolves tissue protein, allowing them to react when adding the Bradford solution, providing total protein concentration in the egg.

The low carbohydrates concentrations found in eggs and embryos of *C. quadricarinatus* suggest that their use is not for energy production. Similar observations have been reported for other decapods (Clarke, 1992; Biesiot and Perry, 1995; Petersen and Anger, 1997; Roustaian and Kamarudin, 2001) and fish larvae (Sargent *et al.*, 1999). It is well known that carbohydrates are not the main energy source in aquatic eggs (Holland, 1978; Sargent *et al.*, 1999). However, carbohydrates are essential during embryogenesis, for the synthesis of specific compounds, such as chitin for the exosqueleton (Holland, 1978; Stryer, 1995).

It is possible to suggest that lipids provide, on average, a half of the available energy in the egg during embryonic development (Fig. 5). The equation obtained for energy shows that embryogenesis consumes half of the total energy contained in the egg (6.01 cal per organism).

The increase of water in the eggs is directly related to water uptake during new cell formation in the embryo, as high water content is characteristic of aquatic invertebrate tissue (Fig. 4). When hatching occurs, additional water enter in the egg as a way to facilitate chorion breakage by increasing the internal pressure (Petersen and Anger, 1997). After this, a continuous growth of the embryo is observed, and water content increases because of the high water content of living tissue compared with the lower water content in yolk.

The biochemical composition of the egg is frequently presented as a function of dry weight. This is because important changes of composition could be masked by changes in water content (Clarke *et al*., 1990). When data are expressed as dry weight or organic matter basis, the internal dynamics of the egg are better represented (Roustaian and Kamarudin, 2001). For the present work, relative composition of proteins, lipids, and carbohydrates in the egg were also calculated as a proportion of organic matter and this confirms a higher use of lipids than of protein and carbohydrates.

During development, there is ion uptake from the environment, because calcium salts and minerals dissolved in water are needed for exoskeleton building. However, it was not possible to determine variations for specific concentrations in ash due to the small sample size. As shown for other crustaceans (i.e., Holland, 1978; Roustaian and Kamarudin, 2001), ash content increased during development, probably because of mineralization of the exoskeleton. Crayfish eggs behave like a closed system in terms of nutrients; that is, all the energy- and tissue-building requirements are covered by the yolk. However, there is ion uptake, gas exchange, and matter loss during excretion (Petersen and Anger, 1997). These processes were not specifically considered because the purpose of the present work was not to determine dynamics of specific molecules but the variations in prime yolk components, to determine gross nutrient requirements for egg development.

In summary, the present study shows that the biochemical composition of *C. quadricarinatus* eggs is similar to other crustaceans, with proteins as the major component in the egg, followed by lipids and carbohydrates. Lipids are the main energy source during the embryonic development, but they are also required for tissue synthesis and for other metabolic tasks. Proteins are used primarily for tissue structure formation, but can be also used as fuel in the final stages of development. *Cherax quadricarinatus* maintains a yolk fraction after hatching because the postembryo is not able to feed itself from the environment. Future research should focus on quantifying specific lipids and proteins through the developmental stages.

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