

Enzyme Activities Involved in Lipid Metabolism During Embryonic Development of *Macrobrachium borellii*

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ABSTRACT The activities of the enzymatic systems involved in the activation and degradation of fatty acids, and in the synthesis of triacylglycerols and phospholipids were studied in vitro using total cellular homogenate and subcellular fractions of eggs of the shrimp *Macrobrachium borellii* at different developing stages. Egg development was divided into seven stages based on morphological features of the embryo. Palmitoyl-CoA ligase activity increased as the embryo developed and showed its maximum at stage V. An increase in the synthesis of triacylglycerols and diacylglycerols was also observed at this stage. Diacylglycerylethers were synthesized more actively during the first stages of development. The higher specific activity observed in total homogenate than in microsomal fraction suggested that their synthesis was not exclusively microsomal. Phospholipid synthesis was very active all along development, reflecting active membrane biosynthesis. The highest activity of the cytosolic triacylglycerol lipase was observed at stage V. Fatty acid degradation, measured as mitochondrial β -oxidation activity, did not vary significantly during development. We conclude that both the anabolic and catabolic processes concerning lipid metabolism are very active, with values similar to those described for adult hepatopancreas, revealing the major role of lipids during shrimp embryogenesis energetics, and that the highest activities of lipid synthesis-hydrolysis take place at stage V when embryos are under active organogenesis. *J. Exp. Zool.* 286:231–237, 2000. © 2000 Wiley-Liss, Inc.

For several years, we have been studying the role of lipids in cell metabolism using adult specimens of the freshwater shrimp *Macrobrachium borellii*. When we studied the kinetic parameters of the enzymatic systems for activation and β -oxidation of fatty acids and for the synthesis of glycerolipids, we found that this shrimp has a very active lipid metabolism, with the hepatopancreas being the major site of lipid synthesis and degradation (González-Baró et al., '90; Irazú et al., '92; González-Baró and Pollero, '93).

In shrimps, female sexual maturation involves two main processes: vitellogenesis and final maturation of oocytes, followed by the fertilization of eggs (Yano, '95). The fertilized eggs are oviposited on the female's special pleopods and are brooded without any cytoplasmic connections (Claybrook, '83). The oviposited eggs have two main compartments, the ooplasm and the vitellus (yolk), whose major component is the conjugated protein lipovitellin (Harrison, '90). In decapod crustaceans, yolk proteins are produced in the ovaries, the hepatopancreas and perhaps some other tissues (Quackenbush, '89). The yolk

protein, called vitellin, is a high molecular weight complex that contains glucids, lipids and carotenoid moieties (Fyffe and O'Connor, '74; Anilkumar and Adiyodi, '85; Zagalsky et al., '90). Yolk provides the embryo with all the nutrients necessary for its growth. Embryonic development in *M. borellii* is accomplished after approximately 50 days from egg laying and during this time, the female carries the eggs within her pleopods (Boschi, '81). Lecithotrophy has evolved in the Decapoda and other aquatic invertebrates as an adaptation to nutritionally poor or unpredictable environments, allowing the embryos to develop independently from external food resources (Anger, '96). At hatching, the shrimps are born as 7-mm post-larvae. During their development, embryos are nourished exclusively by the vitellus

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present in the eggs, the triacylglycerols being their major lipid fuel (Heras et al., unpublished results).

The aim of our work is to determine the role of lipids in the embryonic development of these animals, assessing the activities of the main enzymes involved in their lipid metabolism.

MATERIALS AND METHODS

Samples

Samples were collected from a watercourse close to the Rio de la Plata, Argentina. Gravid females were collected weekly during springtime. They were taken to the laboratory and kept in glass aquaria with dechlorinated tap water at room temperature until the eggs were removed for the experiments. During the 7-week embryogenesis, seven developing stages were defined upon major development events. Stage I comprises embryos from 4-cell morula to gastrula with a highly viscous vitellus that takes up about 90% of the egg; stage II (10–18 days): vitellus fills up to 80% of the egg volume; stage III (18–26 days) embryos have incipient eyes visible and the vitellus fills up to 70%. In stage IV (26–35 days) the vitellus mass is divided into four lobules; stage V (36–40 days): the cuticle pigmentation becomes evident; In stage VI (40–46 days) blue from hemocyanin becomes visible and the vitellus is almost embodied in the embryo and represents about 20% of the egg volume; stage VII (46–50 days) comprises 5-mm length postlarvae fully developed and ready to hatch. In general, the vitellus decreases in viscosity during development.

Preparation of total cellular homogenate, microsomal, mitochondrial, and cytosolic fractions

Pools of three clutches belonging to each developmental stage were used for the experiments. They were removed from gravid females, immediately cooled on ice, and then homogenized in 0.25 M sucrose containing 0.15 M KCl, 62 mM potassium phosphate buffer, pH 7.4, 5 mM MgCl₂, and 1.4 mM N-acetyl-L-cysteine by using a glass-Teflon potter homogenizer (Thomas, Philadelphia, PA) as described in a previous work (González-Baró et al., '90). The crude homogenate was first centrifuged in a Sorvall RC-2 (Newtown, CT) refrigerated centrifuge at 400g at 4°C for 5 min using a SS-1 rotor to remove the egg envelopes and cellular debris. The supernatant was used directly for enzyme assays or further centrifuged to obtain subcellular fractions. To isolate the mitochon-

drial fraction, the eggs were homogenized and then centrifuged at 600g for 10 min and the supernatant was centrifuged at 10,000g for 20 min in the same refrigerated centrifuge. Other groups of eggs were similarly homogenized and then centrifuged sequentially at 12,000g for 20 min and at 110,000g for 60 min in a Beckman L8M ultracentrifuge (Beckman, Palo Alto, CA) using a Ti 70.1 rotor to obtain the microsomal pellet. The supernatant from the last centrifugation was used as cytosolic fraction. The total protein content was determined in all fractions by the method of Lowry et al. ('51).

Enzymatic assays

All enzymatic systems were assayed with the dosage of enzymatic protein and the incubation times within the linear range. They were also saturated by substrates, and assayed under optimal conditions of temperature and pH.

Long-chain fatty acyl-CoA ligase assays

The procedure used was basically a modification of that applied by Singh et al. ('88). The reaction mixture, in a total volume of 0.5 ml contained 0.18 µCi (6.4 µM) [1-¹⁴C] palmitic acid which was added dissolved in propylene glycol (10 µl/tube); 10 mM ATP; 50 mM CoASH; 5 mM MgCl₂; 0.5 mM dithiothreitol; 50 mM KCl; 0.05% (w/v) Triton X-100, and 30 mM MOPS [3-(N-morpholino) propane sulfonic] pH = 7.8. The reaction was started by the addition of either microsomes (60 µg protein/ml of reaction mixture) or total homogenate (2 mg protein/ml of reaction mixture), and was incubated at 32°C for 10 min. Afterwards, the reaction was stopped with a mixture of isopropyl alcohol/n-heptane/H₂SO₄ (200:50:5 v/v/v) (Dole, '56); the lower phase was washed with n-hexane to remove free fatty acids. Palmitoyl-CoA produced was quantified in the aqueous phase in a Pharmacia LKB Wallac 1219 Rackbeta liquid scintillation counter (Uppsala, Sweden).

Biosynthesis of acylglycerols

Ten nanomoles (0.57 µCi) of [1-¹⁴C] palmitic acid were incubated with either microsomes (714 µg protein/ml of reaction mixture) or total homogenate (8.57 mg protein/ml of reaction mixture) in a total volume of 0.35 ml at 32°C for 60 min as described in a previous paper (González-Baró and Pollero, '93). The reaction was stopped by the addition of 2 ml of chloroform-methanol (2:1, v/v); lipids were separated by high performance thin layer chromatography (HPTLC) on silica gel 60

plates (Merck, Darmstadt, Germany) with hexane-ethyl ether-acetic acid (80:20:1.5, v/v/v) and chloroform-methanol-acetic acid-water (65:25:4:4, v/v/v/v) for neutral and polar lipids, respectively. The radioactivity associated with each lipid class was quantified by proportional scanning counting in a Berthold LB2723 apparatus (Wildbad, Germany).

β -Oxidation assays

The procedure used followed that applied by Ruidera et al. ('88) and Hashmi et al. ('86), with modifications detailed in a previous work (Irazú et al., '92). [^{14} C] Palmitic acid (0.22 μ Ci, 7.72 μ M) was used as substrate and incubated with either total homogenate (6 mg protein/ml of reaction mixture) or mitochondrial fraction (160 μ g protein/ml of reaction mixture), in a total volume of 0.5 ml in the presence of 20 mM MOPS buffer pH = 7.8, 30 mM KCl, 5 mM $MgCl_2$, 10 mM ATP, 0.25 mM NAD^+ , 0.17 mM FAD, 2.5 mM L-carnitine, 0.43 mM L-malic acid and 0.08 mM CoASH at 32°C for 20 min. The reaction was stopped, samples were saponified, and the non-oxidized fatty acids removed by washing the samples with chloroform. The labeled acetate was quantified in the aqueous phase by liquid scintillation counting.

Triacylglycerol lipase assays

Lipase activity was assayed according to the method described by Arrese and Wells ('94) with some modifications as follows. A mixture of [carboxyl- ^{14} C] triolein (0.2 μ Ci/ml of reaction mixture, final concentration 1.78 μ M) and unlabeled triolein (final concentration 10 mM) was used as substrate. Purity of labeled and unlabeled triolein was confirmed by HPTLC and radio-TLC. In order to form a micellar triolein substrate, Triton X-100 (final concentration 0.03%) was added. After addition of 0.3 ml of reaction mixture (20 mM Tris-HCl buffer, pH = 7.5 containing 0.5 M NaCl and 0.1% (w/v) delipidated bovine serum albumin), substrate was dispersed by vortexing for 1 min. The reaction was initiated by adding 1 mg cytosolic protein/ml of reaction mixture and incubated at 30°C for 30 min. The reaction was stopped by the addition of 2 ml of chloroform-methanol (2:1, v/v). Lipids were separated by HPTLC, as described above, and the radioactivity associated with each lipid class was quantified by proportional scanning counting.

Statistical analysis

Data collected from all experiments were analyzed by ANOVA using Instat v. 2.0. Where sig-

nificant differences between samples occurred, a post-hoc Tukey's HSD test was performed to identify the differing means. The relation among the different enzymatic systems assayed was determined using Pearson's r correlation test. Results were considered significant at the 5% level.

RESULTS

Experimental results of enzymatic activities were obtained using either total homogenate or subcellular fractions. Total homogenate was assayed in order to test the enzymatic systems in the presence of the whole embryonic cell. Although changes observed among the embryonic stages followed a pattern similar to that obtained by assaying the purified subcellular fractions, the specific activities were very low. Thus the results from total homogenate are either not shown or described in text when necessary.

Fatty acid activation

The activity of palmitoyl-CoA ligase was determined using the microsomal fraction as enzyme source. This activity showed significant changes during development ($P < 10^{-4}$). Figure 1 shows that the maximum activating activity was found at stage V when the embryo is quite developed and the vitellus is still present in large quantities.

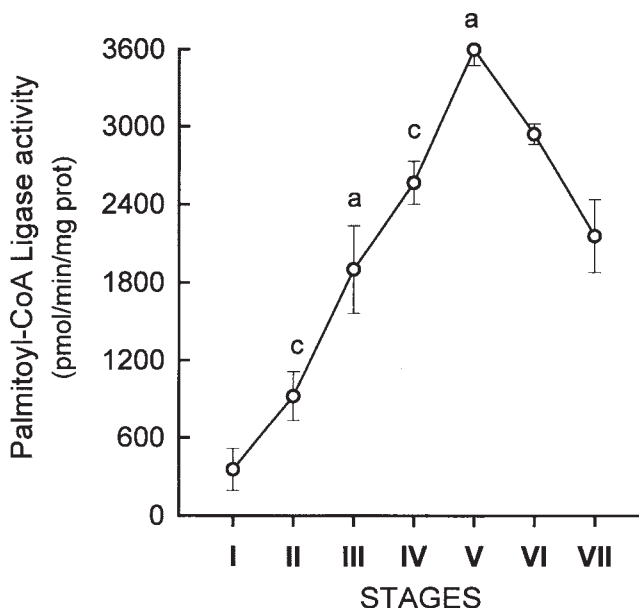


Fig. 1. Fatty acid activation in developing eggs of *M. borellii* measured as palmitoyl-S-CoA ligase activity in microsomal fraction. Values represent the average of three different pools of eggs $\pm$ 1 standard deviation. Letters above the symbols indicate that values are significantly different from those of the next stage. (a) $P < 0.05$; (b) $P < 0.01$; (c) $P < 0.001$. See Materials and Methods section for details.

Degradation of lipids and fatty acids

The fatty acid β -oxidation activity pathway was assayed in mitochondrial fraction (Fig. 2), and the differences that we observed among stages were not significant ($P = 0.35$). The activity of these enzymes showed a high correlation with palmitoyl-CoA ligase activity ($r = 0.99$).

The activity of TG-lipase during the embryonic development was measured in both the cytosolic fraction and microsomes of eggs, though cytosol was the only cellular fraction that showed measurable lipase activity (Table 1). Enzyme activity increased from 17 pmol/min/mg of protein at stage I to a maximum of 112 pmol/min/mg of protein at stage V, and then it declined to approximately 70 when embryos were ready to hatch. Variations among stages were highly significant ($P < 10^{-4}$). The maximum activity observed is coincident with the highest activities of fatty acid activation and degradation; Pearson's r correlation test revealed that the lipase activity correlated with palmitoyl-CoA ligase ($r = 0.97$) as well as β -oxidation activity ($r = 0.97$) during embryonic development.

Synthesis of glycerolipids

The activity of glycerolipid synthesis was observed during embryonic development (Fig. 3). When we studied the anabolic fate of palmitic acid, we observed that palmitic acid incorporation into

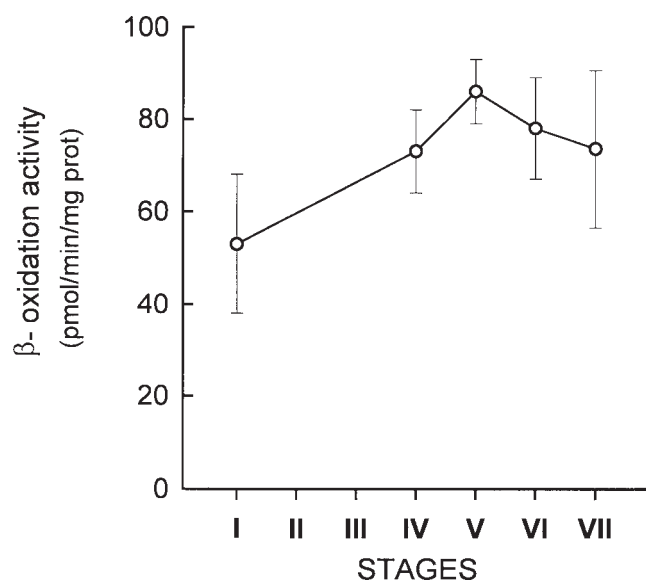


Fig. 2. Fatty acid β -oxidation activity measured in mitochondrial fraction of developing eggs of *M. borellii*. See Materials and Methods section for details. Values represent the average of three different pools of eggs ± 1 standard deviation.

TABLE 1. Triacylglycerol lipase activity measured in the cytosolic fraction of developing eggs¹

Stage	(pmol/min/mg protein) $\pm$ S.D.
I	17.4 $\pm$ 0.4
II	18.6 $\pm$ 0.4 ^b
III	49.9 $\pm$ 1.3 ^a
IV	62.0 $\pm$ 1.0 ^b
V	112.2 $\pm$ 5.1 ^b
VI	89.9 $\pm$ 2.5 ^b
VII	69.1 $\pm$ 3.7

¹Values represent the average of three different pools of eggs ± 1 standard deviation (S.D.). Superscript letters indicate that values are significantly different from those of the next stage.

^a $P < 0.01$.

^b $P < 0.001$.

the triacylglycerol (TG) fraction increased along with the stage of embryonic development (Fig. 3A). TG synthesis showed a significant correlation with palmitoyl-CoA ligase activity ($r = 0.94$) and TG lipase activity ($r = 0.90$). The activity of diacylglycerol synthesis, an intermediate in TG synthesis, reflected more clearly the increment of neutral glyceride synthesis (Fig. 3B). Another class of neutral glycerolipids whose synthesis was assayed is the diacylglycerylethers (DGE) (Fig. 3C). In this study, their biosynthetic activity was inverse to the other neutral glycerolipids analyzed, showing the highest values in the first stages. Nevertheless, this specific activity showed the lowest values among all glycerolipids assayed. Figure 3C also shows the biosynthetic activity of DGE in total homogenate. We could observe that, in this particular case, the location of the enzymes is not exclusively microsomal, because the specific activity in total cellular homogenate was higher than that in the microsomal fraction. Phospholipids (PL) (Fig. 3D) are synthesized during the whole embryo development, showing values higher than 3 nmole/hr/mg protein until hatching. The differences between stages were not significant. Again, we found that stage V was quite important since it showed the maximum synthesis of TG (Fig. 3A), and the minimum synthesis of phospholipids (Fig. 3D).

DISCUSSION

In the 2-mm-long eggs of *M. borellii* we could easily follow under the microscope the decrease of yolk as the embryo is growing.

Lipid metabolism during embryonic development was studied based on the activities of key enzymes or groups of enzymes. Previous data related to the function and variations of lipids in crustacean embryos were reported, but these studies were done by means of the analysis of the lipid

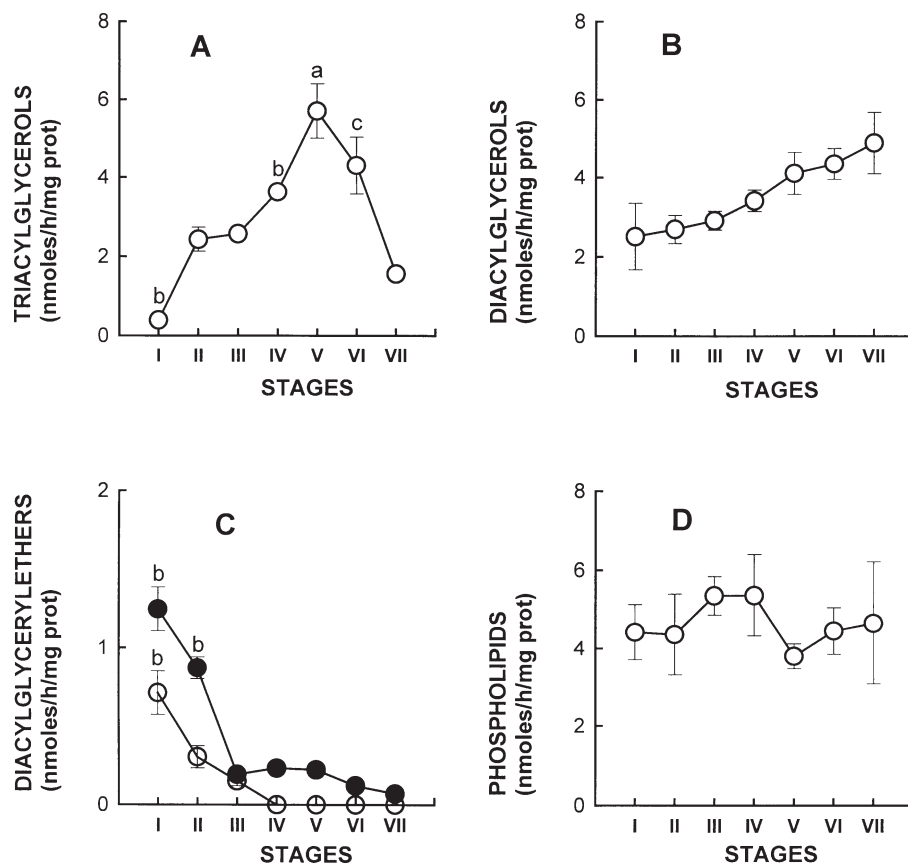


Fig 3. Synthesis of glycerolipids in developing eggs of *M. borellii* measured as specific activity using radioactive palmitic acid as substrate in total cellular homogenate (●) and microsomal fraction (○). Technical details are described in the Materials and Methods section. Values represent the av-

erage of three different pools of eggs ± 1 standard deviation. Letters above the symbols indicate that values are significantly different from those of the next stage. (a) $P < 0.05$; (b) $P < 0.01$; (c) $P < 0.001$.

contents and compositions (Anger, '96). A problem arising from the sole reliance on lipid levels (percentages) and contents (amounts) for evaluation of lipid dynamics is that many organisms have the ability to resorb body components, and thus they utilize lipids without affecting their levels (Lawrence, '76). This problem was overcome by assaying the activities of key enzymes in lipid metabolism.

Fatty acid activation in egg total homogenate increased during development, reaching the highest activity at stage VII. This would indicate that, as hatching is getting closer, the fatty acid metabolism becomes more dynamic. At the first stages of development, vitellin is quantitatively the major protein of total cellular homogenate and cytosolic fraction (González-Baró et al., unpublished results). Since vitellus showed no enzymatic activities regarding lipid metabolism, the specific activities of the enzymes assayed in total homogenate and cytosol were very low at early stages,

in which embryos represented less than 10% of the egg volume. On the other hand, the microsomal and mitochondrial fractions were exclusively obtained from embryonic cells, excluding any dilution by vitellus components. Surprisingly, in the microsomal fraction, the highest activities were found at stage V and then they dropped until hatching. This fact may be due to a greater metabolic effort associated with lipid hydrolysis/reesterification, which will clearly imply major demands on the energy metabolism of the embryonic cells. A similar decrease of activity in the lipid metabolism enzymes acylCoA:cholesterol acyltransferase and esterase at the end of the development was reported in avian embryos (Speake, '98), and in the crab *Emerita asiatica* (Subramonian, '91). At stage V, a sharp decrease in TG percentages was also observed (Heras, unpublished results).

Along embryonic development up to stage V, there is an increment in the TG-lipase activity

that may produce an increase in the available free fatty acids. As described for fish, the TG catabolism is delayed until later stages of development (Wiegand, '96). In *M. borellii* the maximum in TG and fatty acid degradation was observed at stage V, then it dropped until hatching even though cytosolic vitellin was steadily decreasing. This fact corroborates that lower values in TG-lipase specific activity at the end of the embryogenesis do not depend on the amount of vitellus present in the eggs. β -Oxidation is active along development showing similar values to the ones described in adult hepatopancreas in a previous work (Irazú et al., '92). TG synthesis increases together with the developing stage, as revealed by the analysis of acylglycerol synthesis. Probably, at the first stages the embryos do not need to synthesize TG; on one hand, the embryo body mass is negligible, and on the other hand, the vitellus TG is available. Yet, at early developmental stages, they do not need to incorporate hydrolyzed TG and resynthesize them. The results with total homogenate as enzyme source were similar to those obtained using microsomal fraction, except when the synthesis of DGE was assayed. They are diacylglyceroyl-alkylethers which are not quantitatively important in adult *M. borellii* (González-Baró and Pollero, '88) and their function in these shrimp is not well-established. Their maximum synthetic activity was observed at the beginning of the development and then it sharply decreased. We may state that at least one step in the DGE synthesis involved an enzyme or some other factor that is not located in the microsomal fraction, because the specific activity of DGE synthesis was higher in total homogenate than in the microsomal fraction. Further studies about this topic are needed to clarify this finding. PL need to be synthesized continuously because cell reproduction demands the formation of new membranes. We observed that PL synthesis was active during development, since at least 20% of the total radioactivity was found in this fraction at all stages.

The degradation of TG and their resynthesis in embryonic tissues increase steadily until stage V. This stage still has a high percentage of vitellus and the embryo is in the organogenic phase. At this particular stage, the highest activities of fatty acid activation, TG synthesis, TG-lipase, and fatty acid oxidation are found in subcellular fractions. On the other hand, DG synthesis increases steadily until hatching. Taking these facts into account, we can infer that at stage V the vitellus TG are actively consumed while the embryo biosyn-

thesizes body TG. A sharp decrease in the yolk TG content observed when the lipid composition was analyzed (Heras, unpublished results), corroborated this assumption. In total cellular homogenate the highest specific activities were found at stage VII, about 2–3 days before hatching, but at this stage the embryo occupies most of the egg volume giving rise to a higher amount of enzyme per mg of egg protein.

Lipid synthesis and catabolism undergo a characteristic pattern during embryonic development, where a positive correlation exists between the degradation and synthesis of TG and the activation and oxidation of fatty acids. This fact reflects the decrease of yolk components and the concomitant growth of the embryo.

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