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Changes in Biochemical Composition and Digestive Enzyme Activity During the Embryonic Development of the Marine Crab, *Charybdis japonica* (Crustadea: Decapoda)

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In this study, we investigated the biochemical composition and digestive enzyme activity during embryonic development in the marine crab, *Charybdis japonica.* Water, protein, and ash content increased, while levels of lipids and carbohydrates decreased significantly during embryonic development, and a marked correlation between water content and egg volume (r = 0.97; P < 0.01) was recorded. The utilization percentages of lipids and carbohydrates were 32.72% and 91.56%, respectively. The predominant essential amino acids (EAA) were lysine, leucine, arginine and valine, and the major nonessential amino acids (NEAA) were glutamic acid and aspartic acid. From the fertilized egg stage to the protozoea stage, total amino acid (TAA) concentration increased from 52.18% to 55.11% on a dry weight basis, but the ratio of EAA/TAA decreased from 52.57% to 48.90%. The quantitatively more important fatty acids were C16:0, C18:1n-9c, C16:1, C22:6n-3 (docosahexaenoic acid, DHA), and C22:2. Polyunsaturates (PUFA) and monounsaturated (MUFA) were consumed at similar rates (34.20% and 36.70%, respectively); both were consumed at higher rates than saturates (SFA) (26.56%). In particuler, n-3 fatty acids decreased significantly, with a high consumption rate of 43.74%. Activities of trypsin and pepsin increased during both the early and later embryonic stages, but decreased during the middle stages. Lipase activity increased gradually during embryonic development, except in the protozoea stage with a significant decrease, while activities of amylase and cellulase showed an ascending trend after an initial decline. The activity of all digestive enzymes increased, except for that of lipase, from the heartbeat stage to the protozoea stage.

Key words: *Charybdis japonica*, embryonic development, biochemical composition, metabolism, digestive enzyme activities

INTRODUCTION

In crustaceans, some females reproduce by producing eggs carried under the abdomen until hatching. The nutritional requirements of embryos and prefeeding larvae of crustaceans are dependent on the yolk reserve in the eggs (Harrison, 1990). The nutritional status of eggs, embryos, and larvae can be strongly related with their chances of survival and enhanced growth (Faleiro and Narciso, 2010). Thus, knowledge of nutritional composition and dynamics during embryonic development are essential for a better understanding of the nutritional requirements of brood stock and first-feeding larvae of crustaceans. Over the past decades, the biochemical metabolism during embryonic development of some crustaceans has been documented, relating to species such as *Homarus gammarus* (Rosa et al., 2005), *Macrobrachium borellii* (García et al., 2008), *Armases cinereum* and *Sesarma nr. reticulatum* (Hasek and Felder, 2005).

Charybdis japonica is an economically important crab, distributed widely along China's coast from the Bohai Sea to the South China Sea, as well as in Japan, Korea, Malaysia, and in the Red Sea area (Yu et al., 2005). The artificial breeding of *C. japonica* is attracting more and more attention, as natural resources have decreased significantly due to overfishing and environmental pollution (Liu, 2002). In one recent study, the sex gland development, reproductive cycle, breeding behavior and sexual ratio of *C. japonica* have been studied (Xu et al., 2010a), and the morphology and structure of sperm from *C. japonica* were observed in detail under light and electron microscope (Xu et al., 2010b). Moreover, the optimal induced conditions of acrosome reaction in vitro was obtained by orthogonal experiments (Xu et al., 2010c). However, little is known about how the yolk is utilized in the process of embryonic development of this species.

The purpose of this study is to present the first comprehensive study of variations in biochemical composition and digestive enzyme activity during embryonic development of *C. japonica*, in order to better understand the nutritional requirements of embryo and first-feeding larvae, and provide reliable protocols for the formula feed of parent crabs and larvae.

MATERIALS AND METHODS

Sampling

Spawning crabs of *C. japonica* were collected from the Ganyu

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Breeding Farm of Lianyungang, Jiangsu Province, China, in June 2010. The egg-bearing females were divided into three groups, and each group consisted of 10 crabs. These crabs were kept in a recirculatory system under husbandry conditions: salinity 25–26, water temperature 20–21°C, pH 8.0–8.1, natural lighting, and constant aeration. The crabs were fed with fresh clam meat twice a day. Eggs at different stages of embryonic development were collected from brooding females.

Embryonic development of *C. japonica* takes about 15 days. Embryonic developmental stages were identified according to Liu et al. (2002), by examining the eggs under a microscope (Nikon E1000) with a calibrated micrometer eyepiece. There are seven stages of embryonic development (Fig. 1): A. Fertilized egg stage (0–10 h): the embryo looks orange without cell division; B. cleavage stage (11 h–2 days) embryos from 2-cell to cellulous morula; C. blastula stage (3 days): the yolk occupies the center of the embryo and is surrounded with a layer of flattened cells; D. gastrula stage (4–8 days): a crescent-shaped bright zone appears on one side of the embryo; E. eyespot stage (9–10 days): two eyespots become clearly visible, with half the yolk consumed, and the embryo appears tawny with evident cuticle pigmentation; F. heartbeat stage (11–13 days): the embryo acquires a light grey color, and the heart begins to beat from feeble to strong; G. protozoea stage (14–15 days): the embryo presents as brownish-black, the heart beats 150–180 times/min, and the embryo wriggles in the egg membrane ready to hatch. Egg samples were removed from the 10 females of each group and pooled at the above-mentioned stages of development. The samples were stored in liquid nitrogen for further analysis.

Egg volume and water content

Egg volume was calculated using the formula: $V = 1/6\ (\pi W^2 L)$ (Turner and Lawrence, 1979). Water content was determined by measuring the dry weight of the egg samples after being freeze-dried in an FD-1D-50, and by relating it to the wet weight of the samples.

Biochemical analysis

Crude protein, fat, carbohydrate, and ash were determined using the following AOAC (1990) procedures: crude protein (N × 6.25) (%) was determined by the Kjeldahl method after acid digestion; crude fat was extracted using the Soxhlet extraction method with petroleum ether; the content of carbohydrate was measured using the DNS colorimetric methods; and crude ash by incineration in a muffle furnace at 550°C, for 8 h.

Amino acid analysis

The amino acid content of the samples was determined by high-performance liquid chromatography according to the GB/T14965-1994 in China: the sample was hydrolyzed for 22 h at 110°C with 6 M HCl, in nitrogen-filled sealed glass tubes. The amino acid concentration of the hydrolyzed samples was diluted to 50 mL with 0.2 N sodium citrate buffer, pH 2.2. The samples derived were analyzed in an LC98-I AAA Automatic Amino Acid Analyzer (BWAIC, China). The tryptophan content was determined by employing the colorimetric method of Landry et al. (1992) after alkaline hydrolysis of each sample. Quantification was obtained by using external standards (17 Amino acid Mix, Supelco; Sigma-Aldrich). All determinations were performed in triplicate.

Fatty acid analyses

Fatty acids were extracted, and fatty acid methyl esters (FAME) were prepared by the following steps, following the ISO5509 method (ISO 2000): first, Soxhlet extraction was carried out, next saponification, followed by esterification, and finally FAME extraction in hexane. FAMEs were analyzed using Shimadzu GC-2014 gas chromatography, equipped with a flame-ionization detector (FID). A Supelco column SPTM-2380 (30 m × 0.25 mm I.D., 0.2 μm film thickness) was used. Chromatography was performed under the following conditions: split injection; split ratio 50:1; injection volume 1 μL; carrier gas N_2 flowing at 35 cm/s; injector temperature 300°C; detector temperature 300°C; initial oven temperature was maintained at 140°C for 3 min, and then increased to 180°C at the rate of 5°C/min and held for 1 min, followed by raising the temperature to 220°C, while at the same time increasing the rate, and finally held for 8 min. Identification and quantification were performed based on retention times and areas of peaks in the standard mixture (37 FAME Mix, Supelco; Sigma-Aldrich) under the same chromatogram conditions.

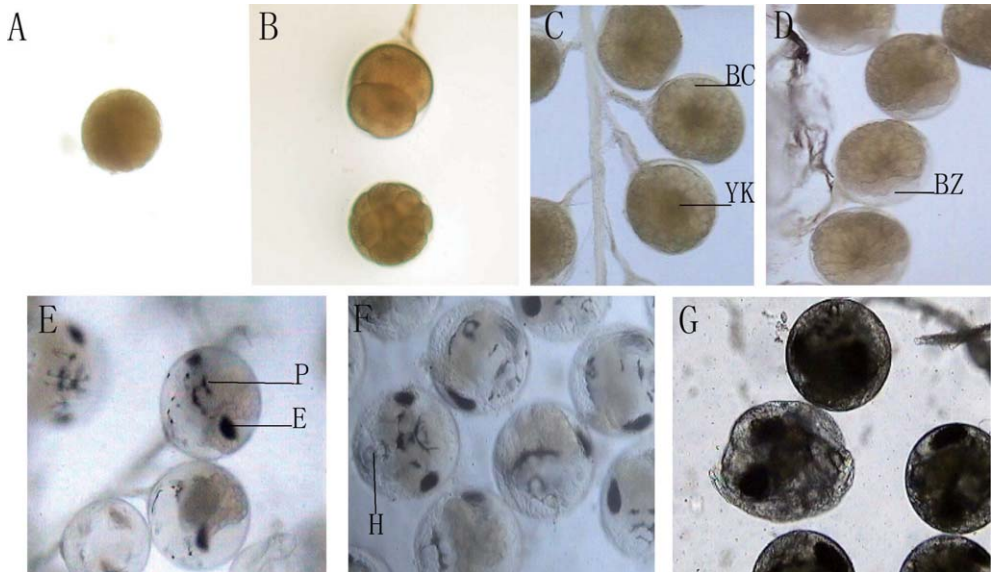

Fig. 1. Morphological characters of several stages in the embryonic development of *C. japonica*. **(A)** fertilized egg stage; **(B)** cleavage stage; **(C)** blastula stage; **(D)** gastrula stage; **(E)** eyespot stage; **(F)** heartbeat stage; **(G)** protozoea stage. (BC: blastodermal cells; YK: yolk; BZ: bright zone; P: pigmentation; E: eyespot; H: heart).

Digestive enzyme activity

Digestive enzyme activity was determined using the Pan Luqing method (Pan and Wang, 1997). The concentration of protein in enzyme solution was determined using Coomassie Brilliant Blue (Li et al., 1994).

Statistical analysis

The results of the analyses are presented as means ± standard deviation. Statistical analyses were conducted using the SPSS16.0 statistical software package. The analyzed concentrations were log-transformed, using a significance level of $P < 0.05$. Analysis of variance (ANOVA, Scheffe test) was used to cross-check if differences in the nutrient components were present among the groups analyzed.

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RESULTS

Egg volume and water content

Egg volume and water content increased during the embryonic development of *C. japonica.* A significant increase in egg volume was observed from 11.02×10^{-3} mm^3 in stage IV, the gastrula stage to 15.62×10^{-3} mm^3 in stage V, the eyespot stage (Fig. 2). A similar trend was noted for water content, increasing from 67.96% to 75.54%. There was a marked correlation between the egg volume and the water content ($r = 0.97$; $P < 0.01$).

Proximate composition dynamic during embryonic development

The content of lipids and carbohydrates decreased significantly, while the level of protein and ash increased as embryonic development progressed (Table 1).

The content of protein in the fertilized egg stage was 57.72% and decreased gradually to 56.42% in the blastula stage with no significant difference, on a dry weight basis, and then increased in the subsequent stage of development. A significant amplification was observed from the gastrula stage (57.52%) to the eyespot stage (59.53%).

From the fertilized egg stage to the protozoea stage, lipid concentration decreased from 30.20% to 20.32%, representing a utilization percentage of 32.72%. At the incipient

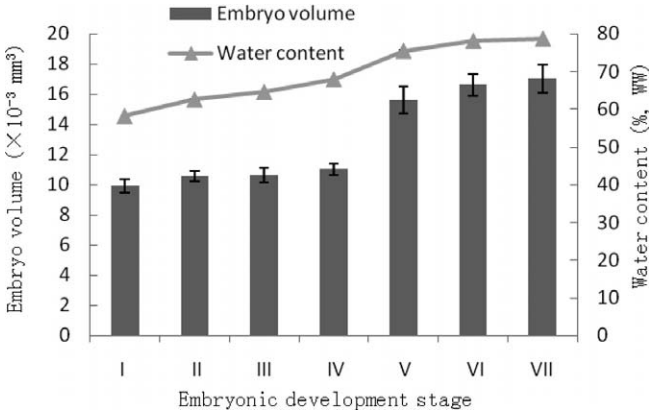

Fig. 2. Egg volume ($\times 10^{-3}$ mm^3) and water content (% of wet weight) of *C. japonica* eggs at different stages of embryonic development. (I. fertilized egg stage; II. cleavage stage; III. blastula stage; IV. gastrula stage; V. eyespot stage; VI. heartbeat stage; VII. protozoea stage).

Table 1. Proximate composition of embryo at different development stages of *C. japonica* (%, dry weight, mean value ± SD).

Component	Embryonic stages						
	I	II	III	IV	V	VI	VII
Protein	57.72 ± 0.59^{a}	57.61 ± 0.67^{a}	56.42 ± 0.82^{a}	57.52 ± 1.22^{a}	59.53 ± 0.84^{b}	60.60 ± 0.57^{b}	61.29 ± 0.95^{b}
Lipid	30.20 ± 1.02^{c}	29.99 ± 0.44^{c}	29.62 ± 0.97^{c}	29.34 ± 0.37^{c}	24.98 ± 0.65^{b}	20.41 ± 1.15^{a}	20.32 ± 0.38^{a}
Carbohydrates	7.23 ± 0.19^{f}	5.90 ± 0.21^{e}	6.07 ± 0.08^{e}	3.71 ± 0.15^{d}	2.78 ± 0.19^{c}	1.97 ± 0.08^{b}	0.61 ± 0.07^{a}
Ash	4.86 ± 0.19^{a}	6.49 ± 0.33^{b}	7.88 ± 0.15^{c}	9.43 ± 0.37^{d}	12.71 ± 0.53^{e}	17.02 ± 0.49^{f}	17.77 ± 0.47^{g}

The superscript notations ^{a,b,c,d,e,f,g} denote significant differences ($P < 0.05$) among all the groups. (I. fertilized egg stage; II. cleavage stage; III. blastula stage; IV. gastrula stage; V. eyespot stage; VI. heartbeat stage; VII. protozoea stage).

Table 2. Proportional composition of amino acids in the embryo at different development stages of *C. japonica* (%, dry weight).

Amino acids	Embryonic stages						
	I	II	III	IV	V	VI	VII
Threonine	2.82 ± 0.13^{ab}	2.72 ± 0.09^{a}	2.84 ± 0.12^{ab}	2.93 ± 0.07^{b}	3.11 ± 0.03^{c}	2.94 ± 0.07^{b}	3.13 ± 0.09^{c}
Valine	3.55 ± 0.13	3.43 ± 0.23	3.37 ± 0.12	3.41 ± 0.08	3.49 ± 0.11	3.33 ± 0.12	3.52 ± 0.12
Leucine	3.99 ± 0.16^{bc}	3.89 ± 0.10^{bc}	3.97 ± 0.06^{bc}	4.09 ± 0.21^{c}	4.07 ± 0.09^{c}	3.85 ± 0.05^{b}	3.48 ± 0.08^{a}
Lysine	4.67 ± 0.11^{e}	4.58 ± 0.08^{de}	4.51 ± 0.22^{cde}	4.39 ± 0.09^{bcd}	4.19 ± 0.07^{ab}	3.97 ± 0.10^{a}	4.28 ± 0.14^{bc}
Arginine	3.83 ± 0.09^{ab}	3.70 ± 0.13^{a}	3.93 ± 0.10^{b}	3.95 ± 0.17^{b}	3.78 ± 0.12^{ab}	3.85 ± 0.09^{ab}	3.96 ± 0.11^{b}
Methionine	1.32 ± 0.06^{b}	1.29 ± 0.06^{b}	1.11 ± 0.03^{a}	1.26 ± 0.09^{b}	1.29 ± 0.07^{b}	1.46 ± 0.05^{c}	1.58 ± 0.07^{d}
Isoleucine	2.01 ± 0.06^{ab}	1.93 ± 0.13^{a}	1.90 ± 0.03^{a}	2.01 ± 0.05^{ab}	1.92 ± 0.16^{a}	2.06 ± 0.08^{ab}	2.11 ± 0.04^{b}
Phenylalanine	2.40 ± 0.14^{c}	2.29 ± 0.17^{c}	1.84 ± 0.10^{b}	2.05 ± 0.14^{b}	1.86 ± 0.10^{b}	2.04 ± 0.05^{b}	1.47 ± 0.06^{a}
Tryptophan	1.25 ± 0.08^{a}	1.23 ± 0.04^{a}	1.16 ± 0.10^{a}	1.18 ± 0.06^{a}	1.21 ± 0.06^{a}	1.32 ± 0.08^{ab}	1.46 ± 0.13^{b}
Histidine	1.59 ± 0.14^{ab}	1.49 ± 0.09^{a}	1.40 ± 0.10^{a}	1.56 ± 0.08^{a}	1.76 ± 0.11^{bc}	1.79 ± 0.12^{c}	1.96 ± 0.07^{d}
ΣEAA	27.43 ± 0.16^{c}	26.55 ± 0.34^{ab}	26.03 ± 0.33^{a}	26.83 ± 0.43^{bc}	26.68 ± 0.68^{ab}	26.68 ± 0.24^{ab}	26.95 ± 0.26^{ab}
Glutamic acid	5.21 ± 0.09^{a}	5.15 ± 0.08^{a}	5.46 ± 0.13^{b}	5.33 ± 0.11^{ab}	5.92 ± 0.12^{c}	6.01 ± 0.12^{c}	5.97 ± 0.01^{c}
Aspartic acid	4.79 ± 0.10^{a}	5.02 ± 0.09^{b}	5.12 ± 0.16^{b}	5.23 ± 0.11^{b}	5.72 ± 0.16^{c}	5.95 ± 0.15^{d}	5.65 ± 0.11^{c}
Glycine	2.34 ± 0.12^{bc}	2.14 ± 0.08^{b}	1.59 ± 0.09^{a}	1.79 ± 0.08^{a}	2.18 ± 0.12^{bc}	2.37 ± 0.13^{c}	2.74 ± 0.16^{d}
Alanine	2.53 ± 0.08^{bc}	2.39 ± 0.06^{ab}	2.31 ± 0.15^{a}	2.38 ± 0.06^{ab}	2.47 ± 0.08^{abc}	2.65 ± 0.08^{cd}	2.78 ± 0.04^{d}
Cysteine	2.29 ± 0.12^{ab}	2.23 ± 0.10^{a}	2.20 ± 0.13^{a}	2.17 ± 0.08^{a}	2.47 ± 0.07^{bc}	2.59 ± 0.18^{cd}	2.68 ± 0.09^{d}
Proline	2.56 ± 0.07^{b}	2.38 ± 0.06^{a}	2.29 ± 0.04^{a}	2.57 ± 0.07^{b}	2.62 ± 0.06^{bc}	2.78 ± 0.09^{c}	2.67 ± 0.08^{bc}
Serine	3.07 ± 0.09^{a}	3.23 ± 0.12^{b}	3.48 ± 0.07^{cd}	3.57 ± 0.08^{de}	3.41 ± 0.08^{cd}	3.54 ± 0.09^{cde}	3.68 ± 0.08^{e}
Tyrosine	1.96 ± 0.11^{f}	1.88 ± 0.09	1.83 ± 0.12	1.88 ± 0.08	1.78 ± 0.05	1.94 ± 0.04	1.99 ± 0.11
TAA	52.18 ± 0.29^{cd}	50.97 ± 0.43^{ab}	50.31 ± 0.38^{a}	51.75 ± 0.64^{bc}	53.25 ± 0.73^{d}	54.44 ± 0.67^{e}	55.11 ± 0.84^{e}
EAA/TAA (%)	52.57 ± 0.39^{e}	52.09 ± 0.29^{de}	51.74 ± 0.29^{c}	51.85 ± 0.22^{de}	50.10 ± 0.59^{b}	48.88 ± 0.51^{a}	48.90 ± 0.33^{a}

The superscript notations ^{a,b,c,d,e,f} denote significant differences ($P < 0.05$) among all the groups. ΣEAA is the total essential amino acids (EAA); TAA is the total amino acids.

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and final development stages, the lipid content decreased slightly, whereas a marked decrease was recorded from the gastrula stage (29.34%) to the heartbeat stage (20.41%).

Carbohydrate content decreased sharply from the fertilized egg stage (7.23%) to the cleavage stage (5.90%), then increased slightly in the blastula stage (6.07%), and continued to decrease in the final embryonic developmental stage, particularly from the blastula stage (6.07%) to the gastrula stage (3.71%), and from the heartbeat stage (1.97%) to the protozoea stage (0.61%). Carbohydrate utilization percentages increased 91.56% during embryonic development.

Ash content increased constantly from the fertilized egg stage (4.86%) to the protozoea stage (17.77%). The rate of ash increase was 265.64% over the course of embryonic development of *C. japonica*.

Amino acid content

The amino acid composition of the embryo at different developmental stages of *C. japonica* is shown in Table 2. Eighteen amino acids, including 10 EAAs and 8 NEAAs, were tested at each stage. The predominant among the EAAs were lysine, leucine, arginine and valine, while those among the NEAAs were glutamic acid and aspartic acid.

The amino acid content in the fertilized egg stage was 52.18% of dry weight, and a significant decrease of TAA was measured from the cleavage stage (52.09%) to the blastula stage (51.74%), and then increased as development progressed. TAA reached the highest level of 55.11% in the protozoea stage.

The proportional amount of EAAs from the highest level of 27.43% in the fertilized egg stage decreased to 26.55% in the cleavage stage, and then showed a small-scope fluc-

Table 3. Fatty acid composition of the lipids extracted from embryos at different development stages of *C. japonic*a (μg mg^{-1} DW).

Fatty acids	Embryonic stages							Embryonic consumption(%)
	I	II	III	IV	V	VI	VII	
C10:0	0.08 ± 0.01^{b}	–	0.25 ± 0.03^{d}	–	–	0.18 ± 0.02^{c}	–	100
C11:0	0.17 ± 0.04^{ab}	0.77 ± 0.07^{d}	0.44 ± 0.07^{c}	–	1.18 ± 0.06^{a}	0.56 ± 0.07^{cd}	0.39 ± 0.03^{bc}	–129.41
C12:0	0.12 ± 0.01^{b}	0.13 ± 0.01^{b}	0.19 ± 0.02^{c}	0.21 ± 0.01^{c}	0.21 ± 0.01^{c}	0.10 ± 0.03^{c}	0.05 ± 0.01^{a}	58.33
C13:0	0.35 ± 0.06^{b}	0.92 ± 0.06^{d}	0.72 ± 0.06^{c}	–	1.57 ± 0.08^{e}	0.84 ± 0.06^{d}	0.31 ± 0.02^{b}	11.43
C14:0	3.46 ± 0.10^{de}	4.13 ± 0.12^{f}	3.24 ± 0.07^{d}	3.57 ± 0.12^{d}	2.24 ± 0.17^{b}	2.61 ± 0.09^{c}	1.72 ± 0.08^{a}	50.29
C15:0	1.01 ± 0.06^{b}	1.36 ± 0.08^{c}	0.93 ± 0.05^{b}	1.07 ± 0.09^{b}	2.43 ± 0.09^{d}	1.01 ± 0.03^{b}	0.70 ± 0.03^{a}	30.69
C16:0	33.92 ± 0.69^{d}	39.53 ± 0.75^{f}	36.97 ± 0.85^{e}	28.56 ± 0.80^{c}	25.33 ± 0.49^{b}	25.77 ± 0.49^{b}	24.17 ± 0.25^{a}	28.74
C17:0	1.97 ± 0.03^{c}	2.17 ± 0.06^{d}	2.18 ± 0.08^{de}	2.26 ± 0.09^{e}	1.66 ± 0.10^{b}	1.38 ± 0.05^{a}	1.70 ± 0.04^{b}	13.71
C18:0	9.15 ± 0.09^{d}	7.94 ± 0.11^{b}	8.55 ± 0.09^{c}	9.57 ± 0.06^{e}	8.67 ± 0.21^{c}	7.55 ± 0.27^{a}	8.80 ± 0.40^{cd}	3.83
C20:0	0.99 ± 0.07^{e}	0.44 ± 0.04^{ab}	0.32 ± 0.04^{a}	0.83 ± 0.07^{d}	0.66 ± 0.03^{c}	0.54 ± 0.06^{bc}	0.64 ± 0.06^{c}	35.35
C21:0	2.53 ± 0.05^{d}	0.63 ± 0.09^{a}	1.14 ± 0.11^{b}	1.79 ± 0.06^{c}	0.75 ± 0.02^{a}	1.03 ± 0.08^{a}	0.96 ± 0.06^{b}	62.06
C22:0	1.28 ± 0.12^{cd}	0.88 ± 0.09^{a}	1.14 ± 0.09^{b}	1.33 ± 0.07^{d}	0.94 ± 0.03^{a}	0.81 ± 0.03^{a}	1.17 ± 0.05^{bc}	8.59
C23:0	1.33 ± 0.05^{e}	0.08 ± 0.01^{a}	0.36 ± 0.05^{b}	1.75 ± 0.07^{f}	0.82 ± 0.10^{c}	1.09 ± 0.06^{d}	0.88 ± 0.08^{c}	33.83
C24:0	1.80 ± 0.04^{c}	1.65 ± 0.08^{c}	2.12 ± 0.16^{d}	2.43 ± 0.06^{e}	1.06 ± 0.12^{a}	0.85 ± 0.12^{a}	1.22 ± 0.03^{b}	32.22
ΣSFA	58.16 ± 1.03^{e}	60.03 ± 1.05^{f}	58.55 ± 0.92^{e}	53.37 ± 0.92^{d}	47.52 ± 0.25^{c}	44.32 ± 0.16^{b}	42.71 ± 0.95^{a}	26.56
C14:1	0.46 ± 0.04^{b}	1.92 ± 0.09^{d}	1.00 ± 0.04^{c}	0.24 ± 0.05^{a}	2.14 ± 0.10^{e}	1.13 ± 0.04^{c}	0.42 ± 0.09^{b}	2.4
C15:1	0.29 ± 0.05^{a}	2.32 ± 0.05^{d}	1.31 ± 0.07^{c}	0.30 ± 0.04^{a}	2.34 ± 0.09^{d}	1.26 ± 0.06^{c}	0.83 ± 0.04^{b}	–186.21
C16:1	17.62 ± 0.36^{d}	33.16 ± 0.48^{f}	20.67 ± 0.76^{e}	17.62 ± 0.31^{d}	10.14 ± 0.19^{c}	9.09 ± 0.20^{b}	18.33 ± 0.25^{a}	52.72
C17:1	2.32 ± 0.10^{b}	3.49 ± 0.08^{c}	3.52 ± 0.08^{c}	3.48 ± 0.11^{c}	2.30 ± 0.11^{b}	1.82 ± 0.13^{a}	2.33 ± 0.09^{b}	–0.43
C18:1n-9c	30.62 ± 0.44^{e}	24.91 ± 1.62^{d}	30.77 ± 0.86^{e}	21.51 ± 0.46^{c}	16.16 ± 0.42^{a}	18.43 ± 0.22^{b}	14.83 ± 0.64^{a}	51.57
C18:1n-9t	3.52 ± 0.15^{a}	11.69 ± 0.67^{e}	10.18 ± 0.22^{d}	9.70 ± 0.15^{d}	8.82 ± 0.44^{c}	3.92 ± 0.09^{a}	7.33 ± 0.16^{b}	–108.23
C20:1	1.01 ± 0.02^{c}	0.44 ± 0.04^{b}	0.45 ± 0.02^{b}	0.49 ± 0.04^{b}	0.43 ± 0.03^{b}	0.35 ± 0.02^{a}	0.34 ± 0.06^{a}	17.81
C22:1n-9	4.60 ± 0.04^{d}	4.01 ± 0.23^{c}	5.72 ± 0.13^{f}	4.94 ± 0.10^{e}	2.66 ± 0.07^{a}	3.20 ± 0.04^{b}	3.91 ± 0.11^{c}	15
C24:1	3.79 ± 0.02^{d}	6.03 ± 0.12^{f}	4.36 ± 0.11^{e}	4.55 ± 0.17^{e}	2.82 ± 0.11^{b}	3.17 ± 0.08^{c}	2.29 ± 0.05^{a}	44.25
ΣMUFA	64.23 ± 0.19^{c}	87.97 ± 1.52^{e}	77.98 ± 0.88^{d}	62.83 ± 0.58^{c}	47.81 ± 0.41^{b}	42.37 ± 0.20^{a}	40.66 ± 0.86^{a}	36.7
C18:2n-6c	1.53 ± 0.10^{d}	1.09 ± 0.08^{c}	1.50 ± 0.06^{d}	0.92 ± 0.09^{b}	1.09 ± 0.08^{c}	0.77 ± 0.07^{a}	0.98 ± 0.06^{bc}	35.95
C18:2n-6t	0.73 ± 0.07^{b}	0.92 ± 0.02^{d}	1.31 ± 0.12^{e}	1.37 ± 0.12^{e}	0.90 ± 0.02^{cd}	0.79 ± 0.05^{bc}	0.60 ± 0.04^{a}	17.81
C18:3n-6	0.70 ± 0.04^{d}	0.44 ± 0.04^{c}	0.49 ± 0.05^{c}	0.51 ± 0.07^{c}	0.21 ± 0.02^{a}	0.30 ± 0.02^{b}	0.22 ± 0.03^{a}	68.57
C18:3n-3	3.35 ± 0.12^{c}	2.42 ± 0.08^{b}	3.56 ± 0.22^{c}	4.64 ± 0.15^{d}	2.06 ± 0.18^{a}	2.37 ± 0.33^{ab}	2.04 ± 0.07^{a}	39.1
C20:2	0.33 ± 0.03^{a}	0.71 ± 0.03^{b}	0.72 ± 0.04^{b}	1.39 ± 0.09^{d}	1.15 ± 0.08^{c}	1.05 ± 0.12^{cd}	0.94 ± 0.08^{c}	–184.95
C20:3n-6	0.19 ± 0.03^{c}	0.15 ± 0.03^{bc}	0.30 ± 0.05^{d}	0.32 ± 0.03^{d}	0.29 ± 0.01^{d}	0.07 ± 0.02^{a}	0.13 ± 0.01^{b}	31.58
C20:3n-3	0.46 ± 0.05^{d}	0.06 ± 0.01^{a}	0.28 ± 0.05^{bc}	0.62 ± 0.04^{e}	0.22 ± 0.03^{b}	0.32 ± 0.04^{c}	0.02 ± 0.00^{a}	95.65
C20:4n-6	0.54 ± 0.08^{d}	0.44 ± 0.04^{c}	0.44 ± 0.01^{c}	0.45 ± 0.03^{c}	0.35 ± 0.03^{ab}	0.39 ± 0.04^{bc}	0.30 ± 0.06^{a}	44.44
C22:2	23.71 ± 0.49^{e}	20.11 ± 0.14^{c}	19.56 ± 0.70^{bc}	21.98 ± 0.88^{d}	25.46 ± 0.21^{f}	16.79 ± 0.33^{a}	19.20 ± 0.19^{b}	19.22
C20:5n-3	1.55 ± 0.11^{d}	1.40 ± 0.06^{c}	1.21 ± 0.12^{b}	1.65 ± 0.09^{d}	0.69 ± 0.04^{a}	0.56 ± 0.06^{a}	1.09 ± 0.06^{b}	29.68
C22:6n-3	37.85 ± 0.67^{e}	16.20 ± 0.11^{a}	23.59 ± 0.44^{c}	37.90 ± 0.71^{e}	32.24 ± 0.66^{d}	20.91 ± 0.21^{b}	21.16 ± 0.29^{b}	44.1
ΣPUFA	70.94 ± 0.77^{e}	43.94 ± 0.28^{a}	52.96 ± 1.12^{c}	71.15 ± 1.72^{e}	64.66 ± 0.51^{d}	44.32 ± 0.69^{a}	46.68 ± 0.43^{b}	34.2
Σn-3	43.21 ± 0.55^{e}	20.08 ± 0.10^{a}	28.64 ± 0.64^{c}	44.81 ± 0.56^{f}	35.21 ± 0.52^{d}	24.16 ± 0.18^{b}	24.31 ± 0.33^{b}	43.74
Σn-6	3.69 ± 0.10^{c}	3.04 ± 0.14^{b}	4.04 ± 0.23^{d}	3.57 ± 0.22^{c}	2.84 ± 0.11^{a}	2.32 ± 0.10^{a}	10.41 ± 0.41^{b}	39.57
n-3/n-6	11.71 ± 0.37^{cd}	6.61 ± 0.26^{a}	7.09 ± 0.25^{a}	12.55 ± 0.61^{e}	12.40 ± 0.53^{de}	10.41 ± 0.41^{b}	10.90 ± 0.61^{bc}	
TFA	193.33 ± 1.23^{d}	192.54 ± 2.71^{d}	189.49 ± 2.48^{cd}	187.95 ± 1.01^{c}	159.99 ± 0.37^{b}	131.01 ± 0.68^{a}	130.05 ± 1.05^{a}	32.73

The superscript notations ^{a,b,c,d,e,f} denote significant differences ($P < 0.05$) among all the groups. ΣSFA is the total saturated fatty acids (SFA); ΣMUFA is the total mono-unsaturated fatty acids (MUFA); ΣPUFA is the total polyunsaturated fatty acids (PUFA); TFA is the total FAME. "–" indicates unfound.

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Table 4. Variations of digestive activities during embryonic development of *C. japonica* (U mg protein^{-1}).

Enzyme	Embryonic stages						
	I	II	III	IV	V	VI	VII
Pepsin	4.78 ± 0.11^{b}	5.24 ± 0.31^{c}	7.85 ± 0.27^{e}	3.27 ± 0.12^{a}	5.62 ± 0.26^{d}	7.74 ± 0.16^{e}	9.93 ± 0.17^{f}
Trypsin	3.16 ± 0.24^{c}	3.35 ± 0.12^{c}	3.28 ± 0.27^{c}	2.19 ± 0.10^{a}	2.44 ± 0.09^{b}	3.12 ± 0.05^{c}	4.78 ± 0.10^{d}
Lipase	0.42 ± 0.02^{a}	0.47 ± 0.02^{ab}	0.49 ± 0.04^{abc}	0.55 ± 0.04^{bc}	0.98 ± 0.06^{d}	1.16 ± 0.08^{e}	0.58 ± 0.07^{c}
Amylase	19.18 ± 0.43^{d}	17.83 ± 0.20^{ab}	17.04 ± 0.43^{a}	17.69 ± 0.70^{ab}	18.17 ± 0.36^{bc}	18.24 ± 0.45^{bc}	18.88 ± 0.48^{cd}
Cellulase	0.075 ± 0.007^{d}	0.058 ± 0.006^{bc}	0.052 ± 0.007^{ab}	0.046 ± 0.005^{a}	0.049 ± 0.003^{ab}	0.052 ± 0.004^{ab}	0.063 ± 0.006^{c}

The superscript notations ^{a,b,c,d,e,f} denote significant differences ($P < 0.05$) among all the groups.

tuation with embryonic development. The ratio of EAA/TAA decreased markedly from the fertilized egg stage (52.57%) to the protozoea stage (48.90%).

Among EAAs, the content of lysine, leucine, and phenylalanine dropped noticeably, while the content of threonine, methionine, tryptophan and histidine rose. There was no evident change in the content of valine, arginine, and isoleucine.

Changes in the content of most NEAAs appeared to have a rising trend after an initial decline, except aspartic acid and serine, which showed contrary trends. The content of tyrosine and proline changed slightly in the whole embryonic process.

Fatty acid content

The fatty acid composition (μg mg^{-1} dry weight) of the different embryonic developmental stages of *C. japonic*a is shown in Table 3. The quantitatively more important fatty acids were the SFAs C16:0 and C18:0, the MUFAs C18:1n-9c and C16:1, and the PUFAs DHA, and C22:2.

A significant decrease was noted in TFA concentrations during embryonic development (32.73%), from 193.33 μg mg^{-1} DW in the fertilized egg to 130.05 μg mg^{-1} DW in the protozoea stage. Compared with the fertilized egg, most fatty acids decreased during embryonic development, which resulted in lower quantities of TFA, SFA, MUFA, and PUFA in the postembryonic stage. PUFA and MUFA were consumed at a similar rate (34.20% and 36.70%, respectively), and both were consumed at higher rates than SFA (26.56%). In particular, n-3 fatty acids decreased significantly with the high consumption rate of 43.74%. During embryonic development, SFA and MUFA both appeared to exhibit a declining trend after an initial ascent, but PUFA dropped noticeably in the cleavage stage, and then rose gradually to the maximum in the gastrula stage, subsequently decreasing after this stage.

In terms of the utilization of individual fatty acids, there was preference for catabolization of C14:0, C16:0, C16:1, C18:1n-9c, C18:3n-3, C21:0, C20:5 (EPA; eicosapentaenoic) and DHA. The content and consumption rate of DHA were both higher than those of EPA. A slight increase in fatty acid content was measured only for the C11:0, C15:1, C17:1 and C20:2, and their contents were all reduced.

Digestive enzyme activity

Changes in the activity of five digestive enzymes showed different trends during the embryonic development of *C. japonica* (Table 4). Activities of pepsin and trypsin increased during both the early and later embryonic stages, but decreased during the middle stages. From the fertilized egg stage to the blastula stage, the activity of pepsin increased significantly from 4.78 U/mg to 7.85 U/mg, but decreased obviously to 3.27 U/mg in the gastrula stage, and then rose gradually in the last developmental phase. Trypsin in the cleavage stage has a slightly higher activity (3.35 U/mg) than that in the fertilized egg (3.16 U/mg). From the blastula stage to the gastrula stage, the activity of trypsin continues to decrease, and then increased to 4.78 U/mg in the protozoea stage.

Activity of lipase increased gradually during the embryonic development, except in the protozoea stage, in which it decreased significantly. The rising extent of lipase activity in the eyespot stage and heartbeat stage was more obvious than in the other stages. In contrast, the activity of amylase and cellulase showed an ascending trend after an initial decline. The activity of amylase and cellulase decreased to the lowest level in the blastula stage and in the gastrula stage, respectively. Amylase activity was 19.18 U/mg in the fertilized egg stage, and decreased to a minimum of 17.04 U/mg in the blastula stage, and then increased gradually to 18.88 U/mg in the protozoea stage. Cellulase showed lower activity than other digestive enzymes.

During the embryonic development, activities of trypsin, pepsin, and cellulase dropped to the minimum value in the gastrula stage, and then rose rapidly in the following eyespot stage. From the fertilized egg stage to the cleavage stage, the activity of trypsin, pepsin and lipase increased, while that of amylase and cellulose decreased. The activity of digestive enzymes, with the exception of lipase, increased from the heartbeat stage to the protozoea stage.

DISCUSSION

The egg volume of *C. japonica* increased continuously throughout embryonic development, which is consistent with other crustacea, e.g., *Macrobrachium rosenbergii* (Yao et al., 2006) and *Homarus gammarus* (Rosa et al., 2005). Egg size increased slightly in the early embryonic stage of *C. japonica*, whereas it dramatically increased from the gastrula stage to the eyespot stage. Most animal species have a short cell cycle, without gap 1 and gap 2 phases, in the cleavage stage, such that although cell numbers increase, single blastomere size is reduced, and the volume of the whole embryo does not increase apparently with the development of the cleavage stage (Müller, 1998). In the gastrula stage, qualitative changes occur; zygotic genes begin to be expressed, and the embryonic cells migrate to a particular position (Townes and Holtfreter, 1955). Meanwhile the embryo absorbs a large amount water to meet the requirements of vigorous metabolism, which also leads to a great

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increase in volume of the embryo.

A decrease in proteins was measured during embryogenesis of *Pseudicarcinus gigas* (Gardner, 2001) and *Homarus americanus* (Sibert et al., 2004), and the yolk proteins were considered as being used as energy resources, as well as being reincorporated into the embryo's tissue. In the present study, the content of protein and TAA appeared to have an ascending trend after an initial decline during the embryonic development of *C. japonica*. In the early embryonic stage, the substance for cell division is of egg origin stored during the egg development, so the protein concentration of the embryo shows a reduction (Kane and Kimmel, 1993). The increase in protein and TAA after the gastrula stage suggests that protein in the yolk acts as the main structural substance of embryonic development of *C. japonica*. Similar observations have been made for *M. rosenbergii* (Yao et al., 2006) and *Nephrops norvegicus* (Rosa et al., 2003).

The percentage of utilization of carbohydrates and lipids were 91.56% and 32.72%, respectively, during the embryonic development of *C. japonica*, which suggests that carbohydrates and lipids serve as energy sources, as reported in a previous investigation (Yao et al., 2006). The utilization of these two nutrient substances in the embryo of *C. japonica* shows a regular pattern: the carbohydrates are the main source of energy in the incipient and final developmental stages, while the lipids serve as an energy source in the mid-term stage of embryonic development. Lipids are not only an important source of energy during embryonic development of most marine animals, but are also the structural components of biomembranes, pigments of compound eyes, and precursors of some hormones (Sargent et al., 2002).

Crustaceans have a chitinous exoskeleton, which becomes larger with development, accounting for the clear increase in the ash concentration with the embryonic development of *C. japonica*.

Wang et al. (2005) have documented that the contents of water, protein and lipid in the muscle of adult *C. japonica* were 78.02%, 85.99% and 2.75%, respectively. In contrast with the adult, the embryo of *C. japonica* before hatching contained less protein and more lipid, which suggests that sufficient protein is extremely important for postembryonic development.

In crustaceans, the major functions of amino acids are associated with the processes of metamorphosis, anisosmotic regulation, molting, and growth (Anger, 1998). Among the EAAs of the embryo in *C. japonica*, the content of lysine, leucine, and phenylalanine dropped significantly over the course of embryonic development, while the content of threonine, methionine, tryptophan, and histidine increased. Lysine is an important precursor for the *de novo* synthesis of glutamate (Papes et al., 2001). Leucine can be transformed into acetyl-Co A and acetyl-acetic acid, which are important intermediates in carbohydrate and lipid metabolism (Shen and Wang, 1990). A decrease in these amino acids is closely correlated with their utilization in the embryo.

Contents of C16:0, C18:0, C16:1, C18:1n-9c, C22:2 and DHA were abundant in embryos of *C. japonica*. This is similar to the results of a previous investigation in *Plesionika martia martia* and *Palaemon serratus* (Morais et al., 2002). SFA was consumed less than PUFA and MUFA in the present experiment. SFAs are nonessential and can be synthesized de novo, or obtained by desaturation of MUFA and PUFA (Sargent, 1999). PUFA decreased obviously in the cleavage stage, which indicates that these fatty acids were participating in some important metabolic functions in this stage, and probably act as a main source of energy during embryogenesis. The n-3 fatty acids, especially DHA, dropped significantly with the development of embryo. In the past few decades, n-3 fatty acids have been identified as essential nutrients for marine animals (Rainuzzo et al., 1997). EPA is important as structural components of cell membranes (Bell and Sargent, 2003), and DHA plays an important role in the development of the central nervous system of crustaceans (Bell and Dick, 1990). According to the reports of Cavalli et al. (2000), the high dietary levels of EPA and DHA improve not only the hatchability, but also the quality, of decapod and crustacean larvae. The content and consumption rate of DHA were both higher than those of EPA, which suggests that DHA is more important than EPA in the embryonic development of *C. japonica*.

Digestive enzymes play an important role in hydrolyzing the yolk to provide energy and materials for embryonic development (Biesiot, 1986). Activity of pepsin, trypsin, lipase, amylase, and cellulase have been tested at a certain level in the fertilized egg stage of *C. japonica*, and that activities of amylase and cellulase were higher than other embryonic developmental stages in connection with the energy supplied with carbohydrates in the early developmental stage. In the cleavage stage, the activity of pepsin, trypsin, and lipase, which are necessary for utilizing higher proteins and lipids in the embryo of *C. japonica*, increased.

During the embryonic development of *C. japonica*, the activity of trypsin, pepsin, and cellulase dropped to a minimum value in the gastrula stage, which may be due to the consumption of ovum-originated enzymes. The gastrula stage is a special phase of animal embryonic development, with cell differentiation, genes of zygote expression, and the appearance of organ anlage. In this stage, more digestive enzymes were synthesized to ensure the utilization of yolk (Yao et al., 2006). All digestive enzymes tested show higher activity in the eyespot stage than in the gastrula stage. The pepsin activities measured were relatively higher than trypsin throughout the embryonic process, indicating that pepsin might possibly play more roles in utilizing yolk protein. The activity of all digestive enzymes increased, except for lipase, in the protozoea stage, suggesting that the embryos were prepared for hatching and were ingesting external food.

In conclusion, proteins in yolk were mainly used as structural components during the embryonic development of *C. japonica.* The energy for embryonic development was mainly supplied by carbohydrates and lipids, and carbohydrates are the main source of energy in the incipient and final developmental stages, while lipids serve as an energy source in the middle stages of embryonic development. The major EAA were lysine, leucine, arginine and valine, and the major fatty acids were C16:0, C18:0, C16:1, C18:1n-9c, C22:2, and DHA. N-3 fatty acids, especially, DHA, decreased significantly with a higher consumption rate. The activity of pepsin, trypsin, lipase, amylase, and cellulase

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have been tested at a certain level in the fertilized egg stage of *C. japonica*, and play important roles in hydrolyzing the yolk, and providing energy and materials for embryonic development. Higher pepsin, trypsin, and amylase activity, and lower lipase activity, were detected in the protozoea stage before hatching.

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Erratum

In the article "Changes in Biochemical Composition and Digestive Enzyme Activity During the Embryonic Development of the Marine Crab, *Charybdis japonica* (Crustadea: Decapoda)" by Xinghong Xu, Xiang Liu, and Jianjun Tao, which appeared on pages 160–166 of Volume 30, No. 3 (March 2013), there was an error in the title. The word "Crustadea" should be replaced with "Crustacea".

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