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Original Article

Fatty acid profiles and carotenoids accumulation in hepatopancreas and ovary of wild female mud crab (*Scylla paramamosain*, Estampador, 1949)

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Abstract

Fatty acid profiles and carotenoid concentrations in the hepatopancreas and ovary of wild female mud crab, *Scylla paramamosain,* collected from Surat Thani Province, Thailand were investigated for better understanding of nutritional status influencing the reproductive performance of mud crab broodstock. The results showed that palmitic acid (16:0), stearic acid (18:0) and oleic acid (18:1n-9) were the major fatty acids in both hepatopancreas and ovary. The concentrations of poly-unsaturated fatty acid (PUFA) accumulated in ovary were about 2.31-4.40 times greater than those in hepatopancreas. Whereas linoleic acid (LA, 18:2n-6), arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosaenoic acid (DHA, 22:6n-3) were the dominating FA in ovary, only ARA and EPA were abundant in hepatopancreas. The levels of carotenoids in both tissues increased with ovarian maturation particularly in the ovary in which levels were 2.34-6.68 times greater than those in hepatopancreas. β-carotene was the only form found in hepatopancreas while five forms of carotenoids, β-carotene, echinenone, canthaxanthin ester, cryptoxanthin and zeaxanthin, were found in the ovary. However, β-carotene and zeaxanthin were the main groups that showed increased levels with gonad maturation. This study demonstrated that PUFA, particularly 18:2n-6, 20:4n-6, 20:5n-3, and 22:6n-3 in association with carotenoids, mainly β-carotene and zeaxanthin, possibly play an important role in gonad maturation of female mud crab *S. paramamosain*.

Keywords: female mud crab, fatty acid and carotenoid levels, ovary maturation

1. Introduction

Mud Crab, *Scylla paramamosain*, Estampador, 1949 (Keenan, 1999; Sodsuk *et al*., 2007), commonly found in the Gulf of Thailand, is a very important species for local fisheries communities in Thailand as it provides a basic source of income. However, the mud crab production in Thailand is mostly from wild catches that do not meet consumer demand. Although mud crab larvae can be produced in hatcheries by the Department of Fisheries, Ministry of Agriculture and Co-operatives (Srimukda, 1995), this is only for stock enhancement. Practically, the artificial propagation relies on wild broodstock which are not consistent or adequate for mass production. Alternatively, mud crab broodstock could be obtained from cultivation in captivity (Kaonoona, 2000) and fed normally with fresh food such as trash fish, cockle, and green mussel. However, production of berried female mud crab is often low due to insufficient nutrients from fresh food which might have deteriorated from improper handling and storage (Kaonoona, 2000; Srimukda and Yosyingbunlue, 2002).

Carotenoids are an essential dietary supplement for improving reproductive performance of crustacean broodstock (Pangantihon-Kuhlmann *et al.*, 1998; Kalinina *et al.*,

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2009). Essential fatty acids are also important in reproduction of crustacean, particularly linoleic acid (n-6) and linolenic acid (n-3), which cannot be synthesized de novo by crustaceans (D'Abramo, 1997). Thus, the biochemical changes of carotenoids and fatty acid in the hepatopancreas and ovary during ovarian development of wild female mud crab will provide useful knowledge of nutrient reserves that can be applied for diet formulation for mud crab broodstock.

2. Materials and Methods

2.1 Animals

Mature female mud crab (*Scylla paramamosain*), wild caught in Ban Don Bay, Surat Thani Province, Thailand were collected and classified into 4 groups according to ovarian stages. Three crabs from each group were sampled for biological and histological determination and biochemical analysis.

2.2 Sample preparation and biological characteristic determination

The crab was anaesthetized in cold water (15°C) for approximately 10 minutes, then the carapace width (the distance of the internal groove of the 9th lateral spines described by Robertson and Kruger, 1984) and body weight were measured using vernier caliper and electric balance (AND, FX-2000i). Subsequently, the carapace was dissected for removal of hepatopancreas and ovary. Gonado-somatic index (GSI) was determined according to Barber and Blake (2006) by the following equation.

GSI = Gonad weight/Body weight*100

A part of the ovary approximately 10 mm long was immediately preserved in 10% formalin for histological section. The rest of ovary and whole hepatopancreas were dehydrated by lyophilization (EYELA FDU-1 100) and stored at -20°C for biochemical analysis.

2.3 Histological sample preparation

After 2 days in 10% formalin, ovary tissue was transferred to 70% alcohol. The tissue was prepared for histological examination by dehydration through a series of ethyl alcohol baths and de-alcoholization through a series of xylene baths. After that, the tissue was embedded in paraffin (mp. 56-60°C), sectioned into ribbon about 5 μm thickness with microtome and stained by haematoxylene and eosin technique (Humason, 1972).

2.4 Lipid extraction

The analysis of fatty acid composition was undertaken in two steps: the extraction of crude lipid (Kates, 1986) and fatty acid separation following the method described by Gandhi and Weete (1991) using gas chromatography (Shimadzu 14 A). Briefly, isopropanol (2 mL) was added to 2.0 g dry weight of samples and well mixed using a vortex for 30 minutes. The mixed sample was warmed in a water bath at 60-70°C for 5 minutes, then vortexed for 1 minute and centrifuged at 1,287 xg for 5 minutes. The supernatant was removed into a vial and the residue was repeatedly extracted as mentioned above. The 2nd extract was collected into the same vial and the residue was repeatedly extracted with chloroform: methanol: water (1:2:0.8) and vortexed for 1 minute, then centrifuged at 1,287 xg for 5 minutes. The 3rd extract was again removed into the same vial and the residue was discarded. The whole solution was finally extracted with chloroform: methanol (1:1) and vortexed for 1 minute, then centrifuged (1,287 xg) for 5 minutes. The extract was dried in N_2, then crude lipid was obtained.

The crude lipid was dissolved in 1 mL chloroform. The 0.2 mL of solution was taken out into a vial and 2 mL sodium methoxide (0.5 N) in dry methanol and 1 mL benzene added, then mixed well using vortex for 1 minute. Subsequently, the solution was heated at 70-75°C for 20 minutes. After cooling, 2 mL of hexane and 2 mL of water was added and vortexed for 1 minute, then centrifuged at 1,287 xg for 5 minutes. The supernatant (the 1st extract) was removed into vial. The residue was repeatedly extracted by the same protocol, the 2nd extract was removed into the same vial and the residue was discarded. N_2 was applied to condense the extractant containing fatty acid ester. The extractant of 0.5 μL was then injected into GC.

2.5 Carotenoid extraction

Carotenoid concentrations in hepatopancreas and ovary of the crab samples were determined by thin layer chromatography as described by Britton *et al*. (1995). Approximately, 2-5 g of samples were moistened with water and homogenized in 20 mL acetone by high speed homogenizer (5,000-10,000 rpm), and repeatedly extracted until the residue and the filtrate were colorless. The solution was then extracted in a separating funnel with diethyl ether (1:1 v/v). Deionized water was added to the extracted samples (1:1 v/v) and shaken cautiously until two phases separated. Repeated extractions were done until there was no colored residue in the hypophase, and the saturated Na_2SO_4 solution was applied into the extractant for removing the emulsion. The extract was evenly divided for the determination of total carotenoids and carotenoid derivatives analysis. For total carotenoids determination of both ovary and hepatopancreas, the extract was kept in -20°C for 12 hours before examination by spectrophotometer (Thermo Nicolet Evolution 300) at 400- 600 nm. Before the extraction of carotenoid derivatives from hepatopancreas, the samples were saponified with 4% KOH (4 g of KOH dissolved in water and absolute alcohol added to 100 mL) at 1:1 (v/v) and kept in a dark chamber for 12 hours. Then the extract of both hepato-

pancreas and ovary was warmed in a water bath at 40°C and condensed using N_2 before spotting onto the layer plate (silica gel 60 F254). After that, the plate was put into mobile phase (diethyl ether: petroleum = 1:1) until bands clearly appeared. Each band was scraped out and eluted with diethyl ether. The solution was eluted through a cotton column until colorless; carotenoid components were then determined by spectrophotometer at 400-600 nm.

2.6 Statistical analysis

Levels of fatty acid and carotenoids in hepatopancreas and ovary related to ovarian stage were subjected to ANOVA at the significance level of 5 % followed by Duncan's New Multiple Range Test using SPSS 12.0 for Windows. The linear regression of GSI and concentrations of fatty acid and caroteniods was also conducted.

3. Results and Discussion

3.1 Some biological characteristics of female mud crab

The average body weight and carapace width of the crab were not significantly different among samples. However, GSI and the oocyte diameter were significantly ($p<0.05$) increased with the progress of ovarian development (Table 1).

3.2 Morphological and histological characteristics of female mud crab ovary

The morphological and histological characteristics of female mud crab ovary are illustrated in Table 2 and Figure 1. The yolk globules clearly appeared at the developing stage (stage 2) and generally apparent in entire cytoplasm at the late maturing stage. The ovary visibly occupied the digestive gland at the maturing stage.

Histological section of female mud crab ovary showed that oocyte diameter and GSI increased significantly with ovarian growth ($R^2 = 0.899$). The results were similar to other studies (Prajakvimol, 2001; Quinitio *et al*., 2007; Islam *et al*., 2010; Namkul, 2011). Thus, this indicated that GSI was applicable for the determination of the ovary growth of mud crab.

3.3 Lipid accumulation in hepatopancreas and ovary of female mud crab

In the hepatopancreas, the level of total lipid at stage 2 was significantly greater than those at stage 3, 4 and 5 ($p<0.05$). The results showed 21 fatty acids which were dominated by 16:0, 18:0 and 18:1n-9 (Table 3). Saturated fatty acids were the main groups while monounsaturated fatty acids and poly-unsaturated fatty acids were the second and the third groups, respectively. The concentrations of 20:4n-6

Table 1. Mean body weight, carapace width, GSI, and oocytes diameter of female mud crab

Ovarian stage	Weight (g)		Carapace width (mm)	GSI (%)	Oocyte diameter (μm)
	Body	Ovary			
2 (developing)	333.79±17.43	2.54±0.38	122.33±3.06	0.76±0.10^a	82.67±1.15^a
3 (early maturing)	345.93±15.44	4.85±0.16	121.00±3.00	1.40±0.07^b	102.67±2.52^b
4 (late maturing)	338.13±14.20	21.40±0.83	115.53±8.04	6.34±0.38^c	133.00±2.00^c
5 (maturing)	347.58±19.05	48.02±2.94	119.27±6.01	13.81±0.10^d	206.33±2.31^d

Data are mean±SEM (n=3). Means with different superscripts in the same column are significantly different ($p<0.05$) by Duncan's New Multiple Range Test.

Table 2. Morphological and histological characteristics of ovary of mud crab

Ovarian stage	Morphological characteristics	Histological characteristics
2 (developing)	Yellow to orange ovary, occupies 10% of the cavity.	Oocyte diameter 70-100 (82.67±1.15) μm. Yolk globules appeared.
3 (early maturing)	Yellow to orange ovary, occupies 20% of the cavity.	Oocyte diameter 90-120 (102.67±2.52) μm. Yolk globules appeared.
4 (late maturing)	Yellow to orange ovary, occupies 40-50% of the cavity.	Oocyte diameter 110-150 (133.00±2.00) μm. Yolk globules occupied the entire cytoplasm. Nucleolus was hardly recognized.
5 (maturing)	Yellow to orange ovary, occupies >75% of the cavity. Individual eggs were visible.	Oocyte diameter 190-220 (206.33±2.31) μm. Follicle and nucleolus were barely visible.

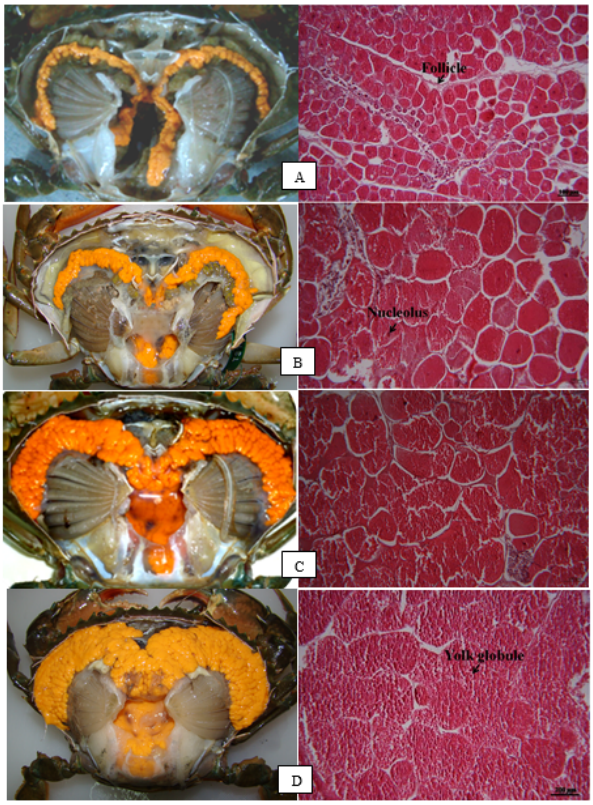

Figure 1. Morphological and histological characteristics of ovary of *S. paramamosain*: A, developing; B, early maturing; C, late maturing and D, maturing.

and 20: 5n-3 were predominant and were not significantly different between groups ($p>0.05$).

In the ovary, the concentrations of total lipid did not differ significantly with stage of maturation ($p>0.05$). There were 19 fatty acids and 16:0, 18:0 and 18:1n-9 were dominant (Table 4). Saturated fatty acid was the main group, while polyunsaturated fatty acid and monounsaturated fatty acid were the second and the third group, respectively. The main classes of PUFA were 20:4n-6, 20:5n-3 and 22:6n-3. The levels of 18:2n-6 and 20:5n-3 at stage 5 were significantly greater than those of stage 2, 3 and 4 ($p<0.05$). The concentrations of 18:2n-6 was also related to GSI ($R^2 = 0.709$). The concentrations of 22:4n-6 at stage 4 and 5 were greater than and significantly different from those of stage 2 and 3 ($p<0.05$).

The concentrations of total lipid in hepatopancreas were significantly ($p<0.05$) decreased over the maturing stages of ovary (from 57.53+6.88% at stage 2 to 41.24+3.21% at stage 5), while those of the ovary were slightly increased (from 20.97+2.51% at stage 2 to -27.56+0.16% at stage 5). The results were similar to those of Lin *et al.* (1994, cited by Zhong *et al*., 2005 and Alava *et al*., 2007) who reported that the total lipid levels in ovary of *Scylla serrata* increased from 6.97% at stage 2 to 25.2% at stage 4, and those of hepatopancreas decreased from 17.1% to 13.8% during stage 1 to stage 5. In addition, the results of the present study showed that the total lipid levels in hepatopancreas were 1.5-2.76 times greater than those of the ovary. This indicated that lipid was mobilized from hepatopancreas to ovary during ovarian development of mud crab and confirmed that hepatopancreas is a storage organ of lipid serving for ovarian growth (Kanazawa *et al*., 1985 cited by Ying *et al*., 2006).

The 16:0 18:0 and 18:1n-9, were the main groups of fatty acids found in hepatopancreas and ovary of female mud crab. These fatty acids were apparently the main source of energy of female mud crab (*S. paramamosain*) during maturation (Ying *et al*., 2006). Poly-unsaturated fatty acid accumulation in the ovary was about 2.30-4.41 times greater than that of the hepatopancreas, which was similar to the studies by Ying *et al*. (2006) and Alava *et al*. (2007). This indicated that poly-unsaturated fatty acids are very important in ovary maturation. The concentrations of essential fatty acids, 18:2n-6, 18:3n-3, and 22:6-3 in the ovary increased as the maturation progressed. These fatty acids are important components of phospholipids, which are the major constituents of cell membranes and transport lipoproteins (D'Abramo, 1997) and are believed to be important in shrimp reproduction, fertilization and hatching (Middleditch *et al.*, 1980; Lytle *et al.*, 1990). The level of 18: 2n-6 was also related to GSI (R^2=0.709), this may suggest that the fatty acids were reserved for embryo development of mud crab.

3.4 Carotenoid accumulation in hepatopancreas and ovary of female mud crab

The main form of carotenoids found in the hepatopancreas (Table 5) was β-carotene; however, the concentrations were not significantly different between groups ($p< 0.05$). There were five forms of carotenoids, β-carotene, echinenone, canthaxanthin ester, cryptoxanthin and zeaxanthin, found in the ovary with β-carotene and zeaxanthin being the main forms (Table 5). The ovary levels of the total carotenoids significantly increased with the ovary stage ($p< 0.05$). The level of zeaxanthin at stage 5 was significantly higher than those of stage 2, 3 and 4 ($p<0.05$). The results also showed that GSI was related to the concentrations of the total carotenoids ($R^2 = 0.864$) and zeaxanthin ($R^2 = 0.769$) as shown in Figure 2. The similar results were reported in crayfish (Sagi *et al*.,1995) and mitten crab (Kalinina *et al*., 2009). Since carotenoids act in embryonic development as an antioxidant to protect the developing tissue from peroxidative damage by free radicals (Yamada *et al*., 1990; Miki, 1991, cited by Sagi *et al*., 1995; Surai and Speake, 1998), β-carotene and zeaxanthin mainly found in the ovaries of mud crabs may probably play an important role in protection of the developing oocytes of mud crab. In addition, they may also play an important role in providing the necessary reserves in embryos and pre-feeding larvae for the development of chromatophores and eyes, and protection of eggs against radiation (Dall *et al.*, 1995). It could be concluded from the results of this study that essential PUFA, 18:2n-6,

Table 3. Levels of fatty acid accumulated in hepatopancreas of female mud crab.

Total lipid (%)	Ovarian stage			
	2	3	4	5
	57.83±6.88[a]	39.03±9.85[b]	44.05±3.61[b]	41.24±3.21[b]
Saturated fatty acid (% of total lipid)				
Total	61.70±7.09	54.77±5.61	55.97±5.75	53.10±4.83
14:00	5.53±3.14	3.37±3.09	4.40±1.35	3.60±1.28
15:00	3.40±1.31	3.50±0.66	2.60±0.36	2.07±0.31
16:00	36.33±4.22	31.50±5.57	34.33±2.38	33.23±5.30
17:00	1.50±1.35	3.30±1.06	2.03±1.82	1.53±1.34
18:00	14.93±6.48	12.73±3.00	12.60±1.40	12.67±2.25
Monousaturated fatty acid (% of total lipid)				
Total	22.23±5.18	22.33±1.27	21.20±6.70	23.40±7.81
16:01	9.00±2.67	8.10±3.65	8.27±2.66	5.00±1.73
18:1n9	8.87±8.35	10.87±3.95	9.67±5.25	15.73±10.65
20:1n9	4.37±0.76	3.37±0.50	3.27±0.99	2.67±1.38
Polyusaturated fatty acid (% of total lipid)				
Total	7.37±3.87	13.43±5.23	13.87±7.39	13.37±10.25
n-6 PUFA (% of total lipid)				
Total	5.40±2.38	6.37±0.99	7.33±4.82	5.03±3.97
16:2n6	2.10±0.10	0.80±0.92	0.00	0.00
18:2n6	0.83±0.55	1.53±1.19	3.87±2.61	0.87±1.50
18:3n6	0.00	0.23±0.21	0.80±0.40	0.00
20:2n6	0.60±0.26	0.67±0.47	0.57±0.38	0.57±0.29
20:3n6	0.27±0.25	0.03±0.06	0.00	0.00
20:4n6	1.33±1.72	2.63±2.18	1.77±1.70	2.87±2.23
22:4n6	0.27±0.23	0.47±0.45	0.33±0.29	0.73±0.75
n-3 PUFA, (% of total lipid)				
Total	1.97±1.48	7.07±4.24	6.53±2.57	8.33±6.28
18:3n3	0.30±0.10[a]	0.57±0.35[a]	0.60±0.10[a]	1.97±0.50[b]
18:4n3	0.50±0.26	0.87±0.45	0.53±0.32	0.90±0.36
20:5n3	0.43±0.51	1.70±1.32	1.47±1.10	1.57±1.66
21:5n3	0.17±0.06	1.97±1.06	2.57±0.83	1.13±1.40
22:5n3	0.20±0.35	0.50±0.44	0.30±0.30	0.80±0.70
22:6n3	0.37±0.46	1.47±1.19	1.07±0.86	1.97±2.17

Data are mean±SEM (n=3). Means with different superscripts in the same row are significantly different ($p<0.05$) by Duncan's New Multiple Range Test.

18:3n-3, 20:4n-6, 20:5n-3, and 22:6n-3 in association with carotenoids, mainly β-carotene and zeaxanthin, played an important role in gonad maturation of female mud crab. This information could be applied for diet formulation for mud crab broodstock.

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Table 4. Levels of fatty acid accumulated in ovary of female mud crab.

Total lipid (%)	Ovarian stage			
	2	3	4	5
	20.97±2.51	21.33±7.19	26.68±0.24	27.56±0.16
Saturated fatty acid (% of total lipid)				
Total	35.64±1.93	37.79±0.16	33.92±2.73	36.62±1.13
14:00	2.22±0.83	2.03±0.52	1.97±0.28	2.56±0.40
15:00	1.91±0.70	1.60±0.11	1.40±0.27	1.23±0.28
16:00	22.92±1.20[a]	24.10±0.47[b]	20.85±1.86[a]	23.62±0.59[b]
18:00	8.58±0.61	10.06±0.62	9.70±0.83	9.21±0.21
Monousaturated fatty acid (% of total lipid)				
Total	22.86±4.82	20.96±4.73	24.35±5.36	24.76±2.48
16:01	5.74±1.28	7.18±0.72	8.30±1.25	6.99±2.70
18:1n9	16.63±5.62	13.03±5.45	14.53±4.39	16.32±5.78
20:1n9	0.49±0.12[a]	0.76±0.06[a]	1.52±0.12[b]	1.44±0.65[b]
Polyusaturated fatty acid (% of total lipid)				
Total	32.51±2.36	30.84±5.9	32.14±1.59	30.91±3.23
n-6 PUFA (% of total lipid)				
Total	10.87±0.91	9.92±0.99	11.37±0.86	12.79±2.23
18:2n6	1.77±0.61[a]	1.34±0.21[a]	2.33±0.69[a]	4.35±1.26[b]
20:2n6	0.94±0.20	0.89±0.16	0.92±0.31	0.82±0.32
20:3n6	0.00	0.00	0.08±0.14	0.00
20:4n6	7.35±1.06	6.67±1.51	6.50±1.62	6.00±1.09
22:4n6	0.82±0.43[a]	1.02±0.23[a]	1.55±0.05[b]	1.62±0.39[b]
n-3 PUFA, (% of total lipid)				
Total	21.64±1.44	20.92±4.91	20.76±0.73	18.12±1.00
18:3n3	1.00±0.71	1.21±0.60	1.13±0.62	1.49±0.60
18:4n3	0.94±0.47	0.80±0.31	0.55±0.15	0.38±0.11
20:5n3	9.83±1.67[a]	8.82±3.36[a]	7.62±0.98[a]	4.46±1.15[b]
21:5n3	0.00	0.46±0.13	0.93±0.13	0.39±0.67
22:4n3	1.40±0.59	1.67±0.19	1.51±0.50	1.81±0.50
22:5n3	2.55±0.10	2.73±0.31	2.51±0.67	2.25±0.75
22:6n3	5.93±1.73	5.23±1.50	6.51±0.42	7.32±1.06

Data are mean±SEM (n=3). Means with different superscripts in the same row are significantly different ($p<0.05$) by Duncan's New Multiple Range Test.

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Table 5. Levels of caroteniods (μg g^{-1}) in hepatopancreas and ovary of female mud crab

Carotenoids	Ovarian stage			
	2	3	4	5
Hepatopancreas				
Total	32.02±7.20	28.00±13.12	43.83±33.92	67.80±56.20
β-Carotene	2.72±2.19	3.32±1.97	19.3±23.17	9.10±12.58
Ovary				
Total	75.02±27.27[a]	156.05±13.04[b]	269.61±20.75[c]	453.44±114.5[d]
β-Carotene	44.48± 23.30	61.82±6.81	86.87±29.11	106.05±5.81
Echinenone	0.77±0.68	9.85±7.75	11.46±8.57	13.45±7.48
Canthaxanthin ester	0.00	5.82±4.81	28.40	3.00±5.20
Cryptoxanthin	0.52±0.9	1.36	7.88±3.33	5.01±5.89
Zeaxanthin	14.70±9.36[a]	48.73±7.36[a]	88.78±51.14[a]	229.05±89.81[b]

Data are mean±SEM (n=3). Means with different superscripts in the same row are significantly different ($p<0.05$) by Duncan's New Multiple Range Test.

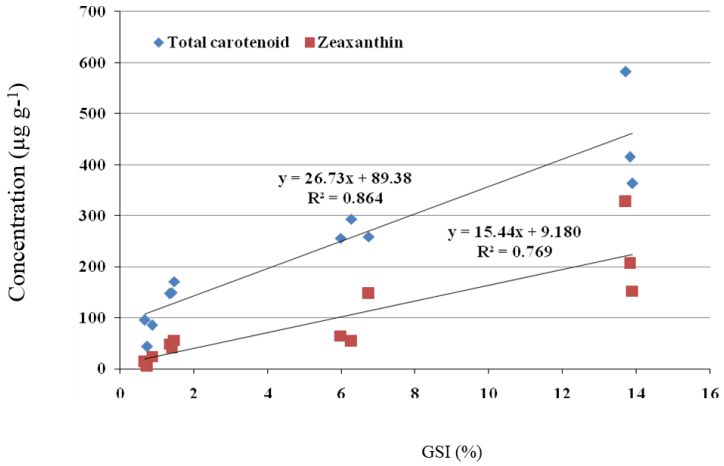

Figure 2. Linear regression of GSI (%) and levels of total carotenoids and zeaxanthin in ovary of female mud crab.

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