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AMINO ACID PROFILES IN THE MIDGUT, OVARY, DEVELOPING EGGS AND ZOEA OF THE MUD CRAB, *SCYLLA SERRATA*

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Abstract

Culture of the mud crab, *Scylla serrata*, is beset by low and inconsistent survival of larvae in spite of the high fecundity of crab breeders. The nutrition of the embryo and pre-feeding zoea depends on what is stored in the egg. The protein and free amino acid contents of the midgut gland, ovary, eggs, pre-feeding zoea, live food and a maintenance diet for broodstock were analyzed by HPLC. The maintenance diet had lower arginine, histidine, methionine, threonine and tryptophan than the ovary and egg. The midgut had higher phenylalanine and valine and lower leucine, methionine and tryptophan than the ovary. Amino acid profiles in the ovary, egg and zoea showed that methionine was highest in the ovary and leucine was highest in the zoea. Low values were observed for isoleucine and valine in ovary, arginine in egg, methionine and phenylalanine in zoea. When live foods were compared to zoea, histidine in *Brachionus,* leucine and tryptophan in *Artemia,* and arginine, leucine and valine in *Acartia* were low. Essential free amino acids in fertilized eggs were 2.5 times higher than in unfertilized eggs. Arginine, histidine, lysine, methionine, tyrosine and threonine decreased with egg embryogenesis, suggesting that these are the major free amino acids utilized as the egg develops. Information on egg and zoea amino acids can be used to predict viable crab eggs while information on amino acid profiles in the ovary, egg and zoea can be used to develop broodstock diets. Identification of limiting amino acids in live foods can be used to develop larvae diets.

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Introduction

Mass production of the mud crab, *Scylla serrata*, is hampered by low and inconsistent survival of larvae in spite of the high fecundity of crab breeders. Poor nutrition during the zoea stage has been identified as a cause of mass mortality (Williams et al., 1999; Zeng and Li, 1999). In studies of fish (Brooks et al., 1997) and *P. monodon* (Millamena et al., 1999), the proper biochemical (specifically, amino acid) composition of broodstock diets has been shown to be a factor in successful larvae production, but such information is not available for the mud crab. In salmon broodstock, all essential amino acids must be present in the diet so they can be transported to developing oocytes (Srivastava et al., 1995). Similarly, the essential amino acid profile of the perch ovary was recommended as the basis of diets for perch broodstock (Ramseyer and Garling, 1994).

In decapod crustaceans, the general pattern is to store organic reserves from dietary sources in the midgut (Biesot and Perry, 1995). Mobilization to the ovary during maturation sometimes occurs (Kulkarni and Nagabhushanam, 1979; Castille and Lawrence, 1989), while some studies provided no evidence of such mobilization (Biesot and Perry, 1995). The embryonic period between spawning and hatching in brachyurans is supported by food reserves stored in the egg (Sulkin, 1978). Since the embryo depends on the egg for sustenance, a suitable feed for zoea should have a similar nutritional content as that of the egg or zoea (Fyhn and Serigstad, 1987; Waddy and Aiken, 1991).

Among the major components of an egg are free amino acids and crustaceans have a particularly high free amino acid pool (Awapara, 1962; Huggins and Munday, 1968; Claybrook, 1983; Rønnestad and Fyhn, 1993). Free amino acids are implicated as an energy source for fish eggs without oil globules (Rønnestad et al., 1994). Free amino acids, found in all tissues and body fluids, are utilized for synthesis of new proteins and other nitrogen-containing compounds or for catabolic reactions. Amino acids are utilized as a substrate for energy production by loosing its amino group by trans-amination, then forming α-ketoacid thru several enzymatic pathways and oxidation via the tricarboxylic acid cycle producing high-energy phosphates. Fyhn and Serigstad (1987) found that alanine, leucine, serine, isoleucine, lysine and valine were the six major catabolized free amino acids during embryogenesis in cod. They proposed that, for fish with large free amino acid pools, free amino acids could be used to estimate egg quality. Since crustaceans have a particularly high free amino acid titer, this might also be true for them.

To date there are few papers on amino acids of crustacean eggs and larvae (Costlow and Sastry, 1966; Huggins and Munday, 1968; Allen, 1971; Anger, 1989; Biesot and Perry, 1995) and the amino acid profile of the mud crab midgut, ovary, egg and zoea has not been studied. Thus, this study was carried out to (a) identify the major amino acids in the diet, midgut, ovary, fertilized eggs and zoea and use this information to improve existing broodstock diets, (b) evaluate the amino acid profile of day-old zoea and live foods to determine baseline data for amino acid supplementation that may improve survival and development and (c) compare the free amino acids in fertilized and unfertilized mud crab eggs and use the information for estimating egg quality.

Materials and Methods

Samples. Nine pond reared female mud crab broodstock with an average weight of 561±26.23 g and carapace width of 152.32±8.53 mm were used. Three brood crabs were sacrificed, then the midgut (hepatopancreas) and ovary were dissected out. Hepatosomatic (HSI) and gonadosomatic indices (GSI) were computed by dividing the midgut weight and ovary weight, respectively, by the total body weight. The remaining females were allowed to spawn according to the hatchery practices and maintenance protocols of Millamena and Quinitio (2000) using their broodstock diet for maintenance (Table 1). Spawning occurred 6-8 days after stocking and hatching 9-14 days after spawning.

Table 1. Composition of diet[1] fed to broodstock of mud crab, *S. serrata*[2].

Ingredient	*g/100 g diet*
Chilean fishmeal	20
Shrimp head meal	20
Squid meal	20
Wheat flour	17
Seaweed (*Gracilaria* sp.)	4
Cod liver oil	5
Lecithin	3
Cholesterol	1
Vitamin mix[3]	3
Mineral mix[3]	4
Dicalcium phosphate	3

[1] According to Millamena and Quinitio (2000)
[2] Crabs were fed at 2-3% of their body weight per day, distributed 40% at 08:00, 30% at 13:00 and 30% at 17:00.
[3] Vitamin and mineral mix after Kanazawa (1981).

With the use of tweezers, each batch of eggs was picked gently from different points of the egg mass until about 50 g of eggs was obtained. Collected eggs were quickly washed with a minimum amount of distilled water and blot dried with filter paper. Samples of about 0.1 or 1.0 g were taken in triplicate for counting or dry matter analysis. Samples for dry matter and amino acid analysis were freeze-dried and the latter kept frozen until analyzed. Samples were collected from six broods and batches of eggs were taken at two spawning stages (early and late). Newly hatched zoea were categorized into four groups: early fertilized eggs, sampled 1-3 days after spawning, the remaining portion of which later hatched; early unfertilized eggs, sampled 1-3 days after spawning, the remaining portion of which did not hatch; late fertilized eggs, sampled 7-8 days after spawning, the remaining portion of which later hatched; late unfertilized eggs, sampled 7-8 days after spawning, the remaining portion of which did not hatch. Zoea were day-old and pre-feeding.

Culture and harvesting of live foods. *Brachionus rotundiformis* were cultured in three replicates in 20-l outdoor tanks at 28-32°C provided with continuous aeration. *B. rotundiformis* at 15 individuals/ml were introduced to tanks that contained *Nannochlorum* (3-4M cells/ml as fed). After 3-4 days they were harvested with a 50-65 µ mesh plankton net. *Artemia* sp. cysts (1.5 g/l sea water) were disinfected with calcium hypochlorite (0.2 g/l) and washed thoroughly after 30 min. The cleaned cysts were then incubated in clean sea water at 30°C with continuous aeration to keep the cysts in suspension throughout the 24 h incubation. Nauplii (unfed) were separated from the cysts and harvested by siphoning. Three replicates were made. *Acartia* sp. were collected from three brackish-water ponds and washed with the least amount of distilled water to remove dirt and foreign particles. Samples were taken after passing them through a 120 µ mesh. After harvest, about 100 g from each tank/pond of these live foods were washed with a minimum amount of distilled water and freeze-dried prior to amino acid analysis.

Sample preparation for amino acid analysis. Duplicate 100-mg freeze-dried samples were homogenized at 4°C for 1 min after adding 1.5 ml chilled distilled deionized water. Cold 2 ml 10% trichloroacetic acid was added and the suspension was homogenized again for 2 min. The resulting slurry was allowed to stand for 18 h at 4°C (these conditions convert glutamine to glutamate acid, which contains glutamine), after which it was centrifuged (4°C, 30 min at 1925 g). The supernatant was made up to 5 ml with pH 2.2 lithium buffer, passed through a 0.45 mm filter and stored frozen until analyzed for free amino acids.

The resulting trichloroacetic acid pellet or residue was freeze dried for hydrolysis of protein bound amino acids (PAA). Approximately

3 mg trichloroacetic acid precipitable protein was hydrolyzed in duplicate with 1 ml 4N MSA (Simpson et al., 1976; Peñaflorida, 1989). Pierce reacti-therm heating module and hydrolysis tubes (10 x 150 mm) were used for hydrolysis. The hydrolysate was made up to 5 ml with pH 2.2 sodium buffer, passed thru a 0.45 mm filter and stored frozen until analyzed for protein amino acids.

Duplicate samples of each hydrolysate were analyzed using stepwise elution of either lithium or sodium buffers in a Shimadzu HPLC-10A post-column derivatization with fluorometric detection using o-phthalaldehyde/ N-acetylcystine reagent. Analysis conditions are shown in Table 2. Calibration was made daily at the start of analysis using Wako Chemical standard (Japan).

Data analysis. Total amino acids were calculated as the sum of free amino acids and protein amino acids and expressed as mg amino acids/g dry matter. Total amino acid profiles were compared with essential amino acids including cystine and tyrosine, rather than the amino acid content *per se* (Murai et al., 1984). Hence, the essential amino acid ratio was calculated as amino acids/total amino acids x 100 (Arai, 1981; Peñaflorida, 1989). The total amino acids of the diet, midgut, ovary, egg and day-old zoea were compared for use in formulating fortification schemes for existing broodstock diets. Live foods, namely *B. rotundiformis, Acartia* sp. and *Artemia* sp., were compared with pre-feeding zoea to identify possible amino acid deficiencies in live food.

The protocol of Millamena and Quinitio (2000) was followed, i.e., sampling for fertilization rate was made 6-8 days after transfer of berried females to hatching tanks. The number of eggs ranged 500-800. Fertilized eggs are pigmented, irregular in shape, and manifest eye formation while unfertilized eggs are unpigmented, spherical and uniformly dark or black. The fertilization rate was computed as (total fertilized eggs/fertilized + unfertilized eggs) x 100.

Eggs with a fertilization rate of 0-17% were classified as unfertilized eggs, indicating abnormal eggs. Those with a rate of 67-94% were considered fertilized eggs. For free amino acids, results were expressed as nmoles per egg or zoea based on count and dry matter. Free amino acids of fertilized and unfertilized eggs at two stages (early and late spawning) and pre-feeding zoea were compared.

Duncan's multiple range test ($p<0.05$) was used to detect differences in treatment means. All analyses were done using the SAS computer package.

Results

The mean HSI and GSI were 3.31±1.22 and 15.20±1.93, respectively, which are within the ranges obtained for mature crab broodstock at the SEAFDEC hatchery (E.T. Quinitio, pers. comm.). The maintenance diet had lower arginine, histidine, methionine, threonine and tryptophan levels than the ovary or egg (Table 3). Phenylalanine and valine were higher while leucine, methionine and tryptophan were lower in the midgut than in the ovary, with no difference in the rest of the essential amino acids. Most of the essential amino acids did not significantly differ ($p<0.05$) between the ovary, egg and zoea, except that methionine was highest in the ovary, leucine highest in the zoea, and there were low values of isoleucine and valine in the ovary, arginine in the egg, and methionine and phenylalanine in the zoea. Histidine in *Brachionus,* leucine and tryptophan in *Artemia*, and arginine, leucine and valine in *Acartia* were lower than in zoea (Table 4).

The mean egg size was 313±7 µm, similar to 315±9 µm for broodstock in Australia (Mann et al., 1999). Non-essential free amino acids were the major amino acids of mud crab eggs (Table 5). The fertilization rate ranged 0-94% and was grouped as unfertilized (0-17%) or fertilized (67-94%). Essential amino acids in fertilized eggs were 2.5 times higher than in unfertilized. The sulfur amino acids, methionine and cystine, were not detected in unfertilized eggs.

Table 6 shows more essential amino acids (2.53) in eggs about to hatch than in late unfertilized (1.03) eggs. The highest free amino acids in fertilized eggs was taurine but it was

Table 2. Analysis conditions using Shimadzu HPLC-10A.

Parameter	*Free amino acids*	*Protein amino acids*
Protocol	Shimadzu HPLC Amino Acid System, 1993, Protocol	
Method	Binary gradient elution; post-column derivatization with OPA/N-acetylcysteine	
Sample	Supernatant from trichloroacetic acid precipitation	Hydrolyzed trichloroacetic acid precipitate
Hydrolyzing agent	—	4N MSA
Standard, Wako Chemical, Japan	Physiological fluid	Protein hydrolyzate
Internal standard	nor-leucine	nor-leucine
Buffer system	Lithium	Sodium
Column type	Li sulfonic acid ion exchanger	Na sulfonic acid ion exchanger
Mobile phase	A: pH 2.6 B: pH 10 C: LiOH	A: pH 3.2 B: pH 10 C: NaOH
Mobile phase flow rate (ml/min)	0.4	0.3
Reaction solution flow rate (ml/min)	0.2	0.2
Pressure (kg/cm^2)	64-69	35-40
Column temperature (°C)	38-58 ascending temperature	55
Analysis time (min)	160	72
Injection volume (µl)	10	10
Concentration (nmole)	0.5-2.5	1
Detector	RF-535	RF-535
Ex (nm)	350	350
Em (nm)	450	450
Sensitivity (pmole)	10	10
Reproducibility (%CV)	0.7-4.0	0.9-1.6
Variability (%RSD)	around 1	around 1

CV= coefficient of variation
RSD = Relative standard deviation

Table 3. Essential amino acid content (%±SE)* in diet, midgut, ovary, egg and zoea of mud crab, n = 3.

	Diet	*Midgut*	*Ovary*	*Egg*	*Zoea*
Arginine	6.32±0.95^{c}	9.33±0.52^{ab}	9.67±0.20^{a}	8.63±0.00^{b}	10.17±0.24^{a}
Histidine	4.05±0.49^{b}	6.23±0.27^{a}	5.17±0.20^{a}	5.87±0.20^{a}	5.57±0.24^{a}
Isoleucine	15.55±0.69a	8.97±0.20^{bc}	8.63±0.15^{c}	10.53±0.24^{ab}	12.03±0.93^{a}
Leucine	21.69±0.69^{a}	16.60±0.15^{c}	18.63±0.64^{b}	18.07±0.23^{b}	21.70±1.10^{a}
Lysine	13.82±0.86^{a}	11.30±0.47^{a}	12.03±0.33^{a}	10.60±0.06^{a}	10.47±0.75^{a}
Methionine					
(+ cystine)	4.48±0.32^{c}	3.83±0.29^{c}	7.43±0.27^{a}	5.90±0.21^{b}	3.77±0.62^{c}
Phenylalanine					
(+ tyrosine)	15.82±0.60^{b}	20.13±0.08^{a}	18.03±0.57^{b}	17.13±0.43^{b}	14.50±0.26^{c}
Threonine	8.17±0.54^{b}	9.80±0.23^{a}	10.47±0.19^{a}	9.50±0.23^{a}	9.13±0.64^{a}
Tryptophan	0.0^{c}	0.0^{c}	1.13±0.22^{ab}	1.40±0.21^{a}	0.84±0.03^{b}
Valine	10.06±0.65^{c}	13.87±0.37^{a}	8.80±0.43^{c}	12.43±0.12^{ab}	12.13±0.69^{b}

Values in each row followed by the same letter are not significantly different (p<0.05).
* Essential amino acid content = (essential amino acid/total essential amino acids plus cystine and tyrosine) x 100

Table 4. Essential amino acid content (%±SE)* in mud crab zoea and its live foods, n = 3.

	Zoea	*Brachionus*	*Artemia*	*Acartia*
Arginine	10.17±0.24^{b}	12.07±0.32^{a}	12.69±0.48^{a}	8.65±0.66^{c}
Histidine	5.57±0.24^{a}	2.79±0.14^{b}	5.16±0.37^{a}	4.87±0.43^{a}
Isoleucine	12.03±0.93^{a}	10.57±0.28^{a}	10.35±0.39^{a}	11.82±0.47^{a}
Leucine	21.70±1.10^{a}	19.12±0.45^{ab}	14.95±0.59^{c}	18.70±1.07^{b}
Lysine	10.47±0.75^{b}	9.86±0.23^{b}	17.02±0.68^{a}	11.50±0.37^{b}
Methionine (+ cystine)	3.77±0.62^{a}	3.78±0.18^{a}	4.01±0.37^{a}	3.31±0.40^{a}
Phenylalanine (+ tyrosine)	14.50±0.26^{c}	17.25±0.09^{b}	16.00±0.47^{c}	19.54±0.53^{a}
Threonine	9.13±0.64^{a}	8.49±0.05^{a}	9.08±0.39^{a}	9.79±0.38^{a}
Tryptophan	0.84±0.03^{b}	0.93±0.20^{b}	0.00^{c}	2.69±0.24^{a}
Valine	12.13±0.69^{b}	15.14±0.23^{a}	10.72±0.43^{bc}	9.11±0.69^{c}

Values in each row followed by the same letter are not significantly different (p<0.05).
* Essential amino acid content = (essential amino acid/total essential amino acids plus cystine and tyrosine) x 100

low in non-fertilized eggs. In fertilized eggs, there was less taurine in late sampled eggs than in early, but the level rose again in day-old zoea. With egg development, arginine, histidine, lysine, methionine, tyrosine and threonine decreased while isoleucine, leucine, cystine, phenylalanine and valine were unchanged. Further development to pre-feeding zoea showed that, except for arginine and cystine that remained constant, the levels of the rest of the essential amino acids increased. The four highest free amino acids in day-old zoea were alanine, glutamic acid, glycine, and proline but these were low in eggs.

Discussion

Total amino acids. Essential amino acids, which are vital to protein quality, are more concentrated in crustacean tissues than in the free amino acid pool (Claybrook, 1983). Since protein amino acids and free amino acids are in dynamic equilibrium, total amino acids were used in this study. Whether some amino acids were mobilized from the midgut to the ovary is unclear from this study, since no samples for the different stages of ovary and midgut were collected. However, in *P. monodon* (Peñaflorida and Millamena, 1990) and *P. aztecus* (Kulkarni and Nagabhushanam, 1979), mobilization was not apparent although samples at different stages of maturity were studied.

In salmon broodstock, Srivastava et al. (1995) identified arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine as the essential amino acids that should be present for transport to developing oocytes. The amino acid profile of the maintenance diet used in our study showed that arginine, histidine, methionine, threonine and tryptophan were low compared to the levels in the ovary and egg. However, crabs used in this study were already mature and spawned after 6-8 days therefore the broods were maintained with these diets. It may be reasonable to assume

Table 5. Free amino acids in fertilized and unfertilized mud crab eggs (nmol±SE/egg), n = 3.

	Unfertilized eggs	*Fertilized eggs*
*Essential amino acids**		
Arginine	0.08±0.04[b]	0.25±0.02[a]
Histidine	0.14±0.06[b]	0.38±0.02[a]
Isoleucine	0.08±0.04[a]	0.16±0.06[a]
Leucine	0.12±0.01[b]	0.26±0.04[a]
Lysine	0.18±0.03[b]	0.37±0.03[a]
Methionine	0.00±0.00[b]	0.06±0.00[a]
Cystine	0.00±0.00[b]	0.09±0.01[a]
Phenylalanine	0.14±0.05[a]	0.25±0.03[a]
Tyrosine	0.32±0.06[b]	0.79±0.02[a]
Threonine	0.16±0.05[b]	0.62±0.11[a]
Valine	0.14±0.05[a]	0.24±0.04[a]
Total essential amino acids	1.36	3.47
Non-essential amino acids		
Alanine	0.74±0.17[a]	1.04±0.09[a]
Aspartic acid	0.23±0.11[a]	0.36±0.01[a]
Glutamic acid	1.20±0.25[b]	2.03±0.07[a]
Glycine	0.66±0.02[b]	3.01±0.37[a]
Proline	0.78±0.08[b]	4.01±0.32[a]
Serine	0.27±0.13[a]	0.51±0.11[a]
Taurine	3.85±1.17[b]	7.52±0.85[a]
β-alanine	0.04±0.01[a]	0.03±0.01[a]
γ-amino butyric acid	0.02±0.01[a]	0.01±0.00[a]
Total non-essential amino acids	7.79	18.52
Total amino acids	9.15	21.99

Values with the same superscript in each row are not significantly different (p<0.05).
* Including cystine and tyrosine

that the maintenance diet would be unable to contribute to the development of gonads in immature crabs.

To our knowledge, the time of spawning from maturity has not yet been established in mud crabs. Nevertheless, the amino acid profile of the ovary or egg would be a useful tool for refining the existing diet for broodstock of Millamena and Quinitio (2000) in order to improve broodstock performance and ultimate zoeal survival and development.

Total amino acids of the live foods were compared to that of pre-feeding zoea to identify possible deficiencies. *Brachionus* is the first feed given to zoea; *Artemia* and *Acartia* are given in a later stage (Quinitio et al., 1999).

Table 6. Free amino acids in mud crab, *Scylla serrata,* eggs at different stages and day-old zoea (nmol±SE/individual), n = 3.

	Unfertilized eggs		*Fertilized eggs*		*Zoea*
	Early post spawn	*Late post spawn*	*Early post spawn*	*About to hatch*	
Essential amino acids					
Arginine	0.08±0.04^{b}	0.08±0.01^{b}	0.25±0.02^{a}	0.13±0.01^{b}	0.12±0.00^{b}
Histidine	0.14±0.06^{bc}	0.08±0.00^{c}	0.38±0.02^{a}	0.24±0.03^{b}	0.35±0.03^{a}
Isoleucine	0.08±0.04^{c}	0.07±0.00^{c}	0.16±0.06^{bc}	0.19±0.01^{b}	0.49±0.01^{a}
Leucine	0.12±0.01^{c}	0.10±0.00^{c}	0.26±0.04^{b}	0.30±0.01^{b}	0.85±0.02^{a}
Lysine	0.18±0.03^{c}	0.23±0.01^{bc}	0.37±0.03^{a}	0.30±0.02^{b}	0.41±0.01^{a}
Methionine	0.00±0.00^{b}	0.00±0.00^{b}	0.06±0.00^{a}	0.00±0.00^{b}	0.00±0.00^{b}
Cystine	0.00±0.00^{b}	0.00±0.00^{b}	0.09±0.01^{a}	0.09±0.01^{a}	0.07±0.01^{a}
Phenylalanine	0.14±0.05^{cd}	0.10±0.01^{d}	0.25±0.03^{b}	0.22±0.01^{bc}	0.54±0.02^{a}
Tyrosine	0.32±0.06^{c}	0.19±0.01^{d}	0.79±0.02^{a}	0.37±0.00^{c}	0.50±0.02^{b}
Threonine	0.16±0.05^{c}	0.09±0.00^{c}	0.62±0.11^{a}	0.36±0.05^{b}	0.56±0.02^{a}
Valine	0.14±0.05^{c}	0.09±0.00^{c}	0.24±0.04^{b}	0.34±0.01^{b}	0.58±0.02^{a}
Total	1.36	1.03	3.47	2.53	4.47
Non-essential amino acids					
Alanine	0.74±0.17^{cd}	0.48±0.02^{d}	1.04±0.09^{c}	2.24±0.08^{b}	7.49±0.08^{a}
Aspartic acid	0.23±0.11^{a}	0.10±0.01^{a}	0.36±0.01^{a}	0.27±0.02^{a}	0.88±0.03^{a}
Glutamic acid	1.20±0.25^{d}	1.03±0.11^{d}	2.03±0.07^{c}	4.08±0.06^{b}	5.83±0.22^{a}
Glycine	0.66±0.02^{d}	0.60±0.02^{d}	3.01±0.37^{c}	4.50±0.09^{b}	10.14±0.23^{a}
Proline	0.78±0.08^{c}	0.61±0.03^{c}	4.01±0.32^{b}	4.02±0.10^{b}	9.31±0.10^{a}
Serine	0.27±0.12^{bc}	0.09±0.03^{c}	0.51±0.11^{b}	0.40±0.01^{b}	0.81±0.01^{a}
Taurine	3.85±1.17^{b}	1.48±0.09^{c}	7.52±0.85^{a}	4.63±0.33^{b}	7.84±0.20^{a}
β-alanine	0.03±0.01^{c}	0.04±0.00^{c}	0.03±0.01^{c}	3.05±2.97^{a}	0.50±0.03^{b}
γ-amino butyric acid	0.02±0.01^{c}	0.04±0.00^{c}	0.01±0.00^{c}	0.14±0.03^{b}	0.61±0.03^{a}
Total	7.79	4.49	18.52	23.33	47.88

Values with the same superscript in each row are not significantly different (p<0.05).

Among the three, only *Brachionus* had a leucine level comparable to that of zoea. *Brachionus* and *Artemia* can sustain all larval stages but survival rates were inconsistent, perhaps indicating a nutritional deficiency (Williams et al., 1999). The acquisition of the right amino acids early in larval development may be a key to survival. Thus, identification of limiting amino acids can help to formulate supplementation regimes for production of diets with optimal amino acid levels/ratios. However, the dynamics and regulation of amino acid metabolism must be studied as well.

Free amino acids. Free amino acids may not be the main cause of high fertilization but essential free amino acids were 2.5 times higher in fertilized than in unfertilized eggs. Whether the sulfur amino acids methionine and cystine (which were absent in unfertilized eggs), as well as the abundance of essential free amino acids in eggs about to hatch, contribute to egg viability needs further study. The role of free amino acids in hatching was hypothesized in egg development of the crab, *Hyas araneus,* where nitrogen remained constant in spite of decreasing protein (Petersen and Anger, 1997). Similarly, it was theorized that free amino acids have a role in ontogenesis and hatching in fish (Gunasekera et al., 1996). However, other factors (mechanical, chemical, biological and environmental) not covered in this study may also affect viability.

The decrease in arginine, histidine, lysine, methionine, tyrosine and threonine indicate their possible role in the embryogenesis of crab eggs. The decrease of these six essential free amino acids with egg development may indicate that these are the major free amino acids catabolized in crab eggs. In cod fish, since alanine, leucine, serine, isoleucine, lysine and valine are high at spawning and drop significantly as eggs develop, they are considered the major free amino acids catabolized for energy production (Fyhn and Serigstad, 1987).

The highest free amino acid in eggs and zoea is taurine. Constant taurine throughout embryo development suggests that all taurine is incorporated into the free amino acids of the egg before spawning (Rønnestad and Fyhn, 1993). Chesney et al. (1998) classified taurine as a “conditionally essential amino acid”, which means that deficiency results in clinical consequences that can be reversed by supplementation. They further found that, in human infants, taurine insufficiency results in impaired fat absorption, bile acid secretion, retinal function and hepatic function that can be reversed by taurine supplementation. Additional studies on cats, rats and dog kidney cells indicate the protective role of taurine in hyperosmolar stress. Taurine is a component of the emulsifying agent in crab and is vital in osmoregulation among marine crustaceans (Claybrook, 1983). In eggs, however, there is little evidence to implicate taurine as a balancing substance in maintaining osmotic pressure (Barnes and Blackstock, 1975). Pochon-Mason et al. (1983) indicated that, in crustaceans, taurine with β-alanine plays a major role in the brain as an inhibitory neurotransmitter and stabilizer of biological membranes. In humans, taurine acts as an anti-anxiety and anti-convulsant mechanism by regulating the sodium and potassium concentrations in the cells and the magnesium level between cells (Slagle, 2000).

Alanine, glutamic acid (+ glutamine), glycine and proline were the four highest free amino acids in zoea. Glycine is the most abundant in crab muscle (V. Peñaflorida, unpubl. data). Increases in alanine, glutamic acid and proline in zoea are expected since these amino acids are implicated as principal solutes in the osmoregulatory process of crustacean muscle (Claybrook, 1983). Proline is interrelated with glutamic acid while alanine and glutamic acid link the nitrogen-containing metabolites from these free amino acids to the citric acid cycle to supply energy (Barnes and Blackstock, 1975). A triple increase in proline and glycine in the cirripede *Balanus* embryo was observed (Barnes and Blackstock, 1975) indicating a partial similarity to mud crab. γ-amino butyric acid and β-alanine, which were almost absent in the egg, were high in zoea. The increase in γ-amino butyric acid may be related to the full development of the larval

nervous system (Barnes and Blackstock, 1975). β-alanine, which is not widely reported in invertebrate tissues (Barnes and Blackstock, 1975), is a component of carnosine, a muscle ATP (adenosine triphosphate) activator (Crush, 1970), hence may indirectly be involved with embryo movement within the egg before hatching. Low levels of γ-amino butyric acid and β-alanine were also observed in unfertilized eggs. This pattern has been found in unhatched *Balanus* embryos (Barnes and Blackstock, 1975).

In conclusion, information on amino acid profiles may be used to improve existing broodstock and larvae diets. Based on our findings, we suggest that the essential amino acids arginine, histidine, methionine, threonine and tryptophan in diets for broodstock closely match the respective levels in the ovary or egg. In addition, *B. rotundiformis* should be fortified with histidine, *Artemia* sp. with leucine and tryptophan and *Acartia* sp. with arginine, leucine and valine so that these foods attain a balanced amino acid composition for better larvae production. The absence of arginine, cystine, isoleucine, methionine and γ-amino butyric acid in unhatched eggs implies that these free amino acids may be related to the viability of the egg, or that they hydrolyzed, broke down, and were utilized by bacteria or were interconverted. Information on free amino acids may also be used to predict amounts of viable crab eggs.

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References

Allen J. and M.R. Garrett, 1971. Taurine in marine invertebrates. *Adv. Mar. Biol.*, 9:205-253.

Anger K., 1989. Growth and exuvial loss during larval and early juvenile development of the hermit crab *Pagurus bernhardus* reared in the laboratory. *Mar. Biol.*, 103(4):503-511.

Arai S., 1981. A purified test diet for coho salmon, *Oncorhynchus kisutch*, fry. *Bull. Jpn. Soc. Sci. Fish.*, 7:547-550.

Awapara J., 1962. Free amino acids in invertebrates: A comparative study of their distribution. pp. 158-175. In: J.T. Holden (ed.). *Amino Acid Pools.* Elsevier, Amsterdam.

Barnes H. and J. Blackstock, 1975. Studies in the biochemistry of cirripede eggs. IV. The free amino acid pool in the eggs of *Balanus balanoides* (L). and *B. Balanus* (L.) during development. *J. Exp. Mar. Biol. Ecol*., 19:59-79.

Biesot P.M. and H.M. Perry, 1995. Biochemical composition of the deep-sea red crab *Chaceon quinquedens* (Geryonidae): Organic reserves of developing embryos and adults. *Mar. Biol.,* 124:407-416.

Brooks S., Tyler C.R. and J.P. Sumpter, 1997. Egg quality in fish: what makes a good egg? *Rev. Fish Biol. Fish.,* 7:307-416.

Castille F.L. and A.L. Lawrence, 1989. Relationship between maturation and biochemical composition of the gonads and digestive glands of the shrimp, *Penaeus aztecus* Ives and *Penaeus setiferus* (L). *J. Crustacean Biol.*, 9:202-211.

Chesney R.W., Helmes R.A., Christensen M., Budreau A.M., Han X. and J.A. Sturman, 1998. The role of taurine in infant nutrition. *Adv. Exp. Med. Biol.*, 442:463-476.

Claybrook D.L., 1983. Nitrogen metabolism. pp. 162-213. In: D.E. Bliss (ed.). *The Biology of Crustacea. Vol 5: Internal Anatomy and Physiological Regulation*. Academic Press, New York.

Costlow J.D. and A.N. Sastry, 1966. Free amino acids in developing stages of two crabs, *Callinectes sapiclus* Rathburn and *Rhithropanopeus harrisii* Gould. *Acta Embryol. Morp. Exp.*, 9:44-55.

Crush K.G., 1970. Carnosine and related substances in animal tissues. *Comp. Biochem. Physiol*., 34:3-30.

Fyhn H.J. and B. Serigstad, 1987. Free amino acids as energy substrate in developing eggs and larvae of the cod *(Gadus morhua*). *Mar. Biol.*, 96:335-341.

Gunasekera R.M., Shim K.F. and T.J. Lam, 1996. Effect of dietary protein level on spawning performance and amino acid composition of eggs of Nile tilapia *Oreochromis niloticus*. *Aquaculture,* 146:121-134.

Huggins A.K. and K.A. Munday, 1968.

Crustacean metabolism. In: O. Lowestein (ed.). *Adv. in Comp. Physiol. Biochem.,* 3: 271-378.

Kulkarni G.K. and R. Nagabhushanam, 1979. Mobilization of organic reserves during ovarian development in a marine penaeid prawn, *Parapenaeopsis hardwickii* (Miers) (Crustacea, Decapoda, Penaeidae). *Aquaculture*, 18:373-377.

Mann D., Asakawa T. and A. Blackshaw, 1999. Performance of mud crab *Scylla serrata* broodstock held at Bribie Island Aquaculture Research Centre. pp. 101-105. In: C.P. Keenan, A. Blackshaw (eds.). *Mud Crab Aquaculture and Biology.* ACIAR Proc. no. 78, ACIAR, Canberra.

Millamena O.M. and E. Quinitio, 2000. The effects of diets on reproductive performance of eyestalk ablated and intact mud crab, *Scylla serrata*. *Aquaculture*, 181:81-90.

Millamena O.M., Teruel M.B., Kanazawa A. and S. Teshima, 1999. Quantitative dietary requirement of postlarval tiger shrimp, *Penaeus monodon*, for histidine, isoleucine, leucine, phenylalanine and tryptophan. *Aquaculture*, 179:169-179.

Murai T., Akiyama T. and T. Nose, 1984. Effect of amino acid balance on efficiency in utilization of diet by fingerling carp. *Bull. Jpn. Soc. Sci. Fish.*, 50:893-897.

Peñaflorida V. Dy, 1989. An evaluation of indigenous protein sources as potential component in the diet formulations for tiger prawn, *Penaeus monodon*, using essential amino acid index (EAAI). *Aquaculture,* 83:319-330.

Peñaflorida V. Dy and O.M. Millamena, 1990. Variation in the biochemical composition of *Penaeus monodon* tissues during the reproductive cycle. *Israeli J. Aquacult. - Bamidgeh,* 42(3):84-90.

Petersen S. and K. Anger, 1997. Chemical and physiological changes during the embryonic development of the spider crab, *Hyas araneus* L. (Decapoda: Majidae). *Comp. Biochem. Physiol*., 117B:299-306

Pochon-Mason J., Payen G.G., Portemer C. and F. Chatagner, 1983. Effect of experimental diets and of fasting on the concentration of taurine in various organs of the shore crab, *Carcinus maenas* (L) (Decapoda,: Brachura). *Comp. Biochem. Physiol*., 75A: 141-145.

Quinitio E.T., Estepa F.P. and V.R. Alava, 1999. Development of hatchery techniques for the mud crab, *Scylla serrata*. pp. 125-130. In: C.P. Keenan, A. Blackshaw (eds.). *Mud Crab Aquaculture and Biology.* ACIAR Proc. no. 78, ACIAR, Canberra.

Ramseyer L.J. and D.L. Garling Jr., 1994. Amino acid composition of the ovaries, muscle and whole body of yellow perch. *Prog. Fish. Cult.,* 56:175-179.

Rønnestad I., and H.J. Fyhn, 1993. Metabolic aspects of free amino acids in developing marine fish eggs and larvae. *Rev. Fish Sci.*, 1:239-259.

Rønnestad I., Koven W.M., Tandler A. and H.J. Fyhn, 1994. Energy metabolism during development of eggs and larvae of gilthead sea bream (*Sparus aurata*). *Mar. Biol*., 120:187-196.

Simpson R.J., Neuberger M.R. and T.Y. Liu, 1976. Complete amino acid analysis of proteins from single hydrolysate. *J. Biol. Chem.* 251:1936-1940.

Slagle P., 2000. Anxiety: Taurine. *The Way Up Newsletter*. 16:5.

Srivastava R.K., Brown J.A. and F. Shahidi, 1995. Changes in the amino acid pool during embryonic development of cultured and wild Atlantic salmon *(Salmo salar*). *Aquaculture,* 131:115-124.

Sulkin S.D., 1978. Nutritional requirements during larval development of the portunid crab*, Callinectes sapidus* Rathbun. *J. Exp. Mar. Biol. Ecol*., 34:29-41.

Waddy S.L. and D.E. Aiken, 1991. Egg production in the American lobster, *Homarus americanus*. In: A. Wenner, A. Kuris (eds.). *Crustacean Egg Production,* 7:267-290.

Williams G.R., Wood J., Dalliston B., Shelley C.C. and C. Kuo, 1999. Mud crab, *Scylla serrata* megalopa larvae exhibit high survival rates on *Artemia*-based diets. pp. 131-140. In: C.P. Keenan, A. Blackshaw (eds.). *Mud Crab Aquaculture and Biology.* ACIAR Proc. no. 78, ACIAR, Canberra.

Zeng C. and S. Li, 1999. Effects of density and different combinations of diets on survival, development, dry weight and chemical

composition of larvae of the mud crab, *Scylla paramamosain*. pp. 159-166. In: C.P. Keenan, A. Blackshaw (eds.). *Mud Crab Aquaculture and Biology.* ACIAR Proc. no. 78, ACIAR, Canberra.