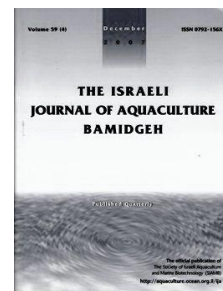




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## Free and Total Amino Acids in Eggs of Greasy Grouper, *Epinephelus tauvina*, during Embryogenesis

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### Abstract

Changes in the free and total amino acids in embryonic stages of eggs from captive bred greasy grouper, *Epinephelus tauvina* (Forsk.), were analyzed. The fertilized grouper eggs contained  $4.08 \pm 0.06$   $\mu\text{g/egg}$  free amino acids and  $23.22 \pm 0.31$   $\mu\text{g/egg}$  total amino acids. Indispensable leucine, lysine, valine, and dispensable alanine, glutamic acid, and proline were the most prevalent total amino acids while indispensable isoleucine, leucine, lysine, and dispensable alanine, glutamic acid, and serine dominated the free pool. Free amino acids constituted  $17.56 \pm 0.32\%$  of the total amino acids in the fertilized eggs, decreasing to  $14.75 \pm 0.25\%$  and  $7.48 \pm 0.24\%$  in the gastrula and pre-hatch stages, respectively, due to catabolic and anabolic utilization of the free amino acids in embryonic development. There were wide variations in the loss of individual free amino acids during embryonic development: losses of arginine, lysine, threonine, alanine, aspartic acid, glutamic acid, and glycine were very high ( $>70\%$ ) compared to losses of methionine, phenylalanine, valine, cystine, and proline ( $<40\%$ ). Approximately 50% of the disappeared free amino acids was used for anabolic purposes, i.e., protein synthesis, and the remaining 50% was used for catabolic energy. Retention of indispensable amino acids for protein synthesis was higher than retention of dispensable amino acids.

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## Introduction

Marine fish culture is an important contributor to the economy of coastal communities in the Asian-Pacific region. Aquaculture of highly valued species such as grouper, sea bass, and snapper provides greater economic benefits to farmers than that of lower valued species (Rimmer, 2004). Grouper is the most popular fish species in the Indo-Pacific and is extensively cultured in earthen ponds and cages (Taburan et al., 2001). Since live grouper commands a high price in southeast Asian countries, demand for seed is growing. Grouper aquaculture is heavily dependent on the capture and grow-out of wild-caught juvenile fish. Around 70-85% of cultured groupers are grown from wild-caught fry. Despite the continuing expansion of its aquaculture in the Asian-Pacific region, there are important constraints to the sustainable development of this species. Foremost is the limited availability of fingerlings (Rimmer, 2004; Gorshkov, 2010). Breeding has been induced in greasy grouper, *Epinephelus tauvina* (Forsk.) using human chorionic gonadotropic (HCG) and luteinizing hormone releasing (LHRHa) treatments (Watanabe et al., 1995a; Kailasam et al., 2007), but poor viability of eggs and larvae limits grouper breeding (Watanabe et al., 1995b; Kohno et al., 1997; Eusebio et al., 2010).

Sufficient nutrients are required for viability and proper growth of embryos. Larvae performance is dependent on egg quality. Thus, evaluation of egg quality is receiving much attention, especially because survival during embryonic stages is highly variable (Kjørsvik et al., 2003). Free amino acids are important fuel molecules in marine fish eggs and act as signaling factors, substrates for the synthesis of bioactive molecules and proteins, and energy for embryos (Finn and Fyhn, 2010). Total free amino acids, arginine, asparagine, glutamine, and glutamic acid correlates with viability in the common dentex, *Dentex dentex*, (Samaee et al., 2010). The utilization of amino acids was estimated by measuring aspartate aminotransferase in embryos of the spotted wolffish, *Anarhichas minor* (Desrosiers et al., 2008) and there has been considerable research into the sequence of substrate utilization in developing marine fish eggs and larvae (Rønnestad et al., 1992, 1994, 1998; Finn et al., 1995; Sivaloganathan et al., 1998; Desrosiers et al., 2008).

The proportions of free amino acids utilized for energy metabolism and protein synthesis during embryogenesis and early larval stages vary among fishes (Rønnestad and Fyhn, 1993; Sivaloganathan et al., 1998). In our earlier study with Asian sea bass, *Lates calcarifer*, the indispensable and dispensable amino acids of eggs and larvae dropped by 32.8% and 21.3%, respectively, in hatchlings and 50% and 12.4%, respectively, in 2-day post-hatchlings (Dayal et al., 2003). In the present study, changes in the content and concentrations of free and total amino acids during embryogenesis in eggs from induced greasy grouper were analyzed.

## Materials and Methods

**Sampling.** Fertilized and developing eggs of *E. tauvina* were collected from the fish hatchery of the Central Institute of Brackishwater Aquaculture, Chennai, India. The eggs were obtained from induced breeding trials with HCG at 1600 IU/kg body weight and LHRHa at 40 µg/kg body weight (Kailasam et al., 2007). Newly fertilized and developing eggs were collected on a 100-µm mesh sieve, rinsed with tap water followed by distilled water, and dried by placing absorbent paper on the bottom of the sieve.

**Amino acid analysis.** Total amino acids in the developing eggs were analyzed after sealed-tube hydrolysis with 6N HCl for 22 h at 110°C (Spackman et al., 1958; Finlayson, 1964). After hydrolysis, the acid was evaporated under a vacuum, and the residue was put into 1 ml sodium citrate-perchloric acid sample diluent (pH 2.20) and filtered through a 0.2-µm membrane filter. Free amino acids were measured by precipitating the protein with absolute ethanol (Lyndon et al., 1993). Amino acids were separated in a column (Shimpack ISC-07/S1504 Na) packed with a strongly acidic Na<sup>+</sup> type cation exchange resin (styrene-divinyl benzene copolymer with sulfinic group) under gradient elution at a flow rate of 0.3 ml/min using two buffers: (a) sodium citrate-perchloric acid (pH 3.2) and (b) boric acid sodium citrate-sodium hydroxide (pH 10.0). They were analyzed using a Shimadzu HPLC model LC-10A (Shimadzu Corp., Japan) and quantified using a

fluorescent detector (FLD-6A) after post column derivitization with O-phthalaldehyde and 2-mercaptoethanol (Dayal et al., 2003). A standard amino acid solution for fluorescent detection (Sigma-Aldrich Inc., USA) was used as an external standard; for every ten-sample injection, one standard run was carried out. Analyses were carried out in triplicate. Free amino acids are expressed as ng/egg while total amino acids are expressed as µg/egg.

**Statistical analysis.** Analysis of variance (ANOVA) was carried out by F-test using SPSS Version 17.0 statistical software package. Differences were considered significant when  $p < 0.05$ .

## Results

Isoleucine, leucine, lysine, alanine, glutamic acid, and serine constituted over 60% of the free amino acids in eggs (Table 1). Leucine, lysine, valine, alanine, glutamic acid, and proline constituted over half of the total amino acids in eggs. All free amino acids, except taurine, declined during embryonic development. Free amino acids constituted  $17.56 \pm 0.32\%$  of the total amino acids in the fertilized egg but dropped to  $14.75 \pm 0.25\%$  in the gastrula and  $7.48 \pm 0.24\%$  in the pre-hatch stages due to catabolic and anabolic utilization in the embryonic development.

The proportion of indispensable free amino acids ( $60.63 \pm 0.46\%$ ) was significantly higher than the proportion of dispensable free amino acids ( $39.37 \pm 0.46\%$ ) and remained almost constant throughout embryonic development (Fig.1). There were wide variations in the loss of free amino acids. More than 70% was lost in the free pools of arginine, lysine, threonine, alanine, aspartic acid, glutamic acid, and glycine, whereas less than 40% was lost in methionine, phenylalanine, valine, cystine, and proline. Losses of indispensable and dispensable free amino acids were  $58.06 \pm 1.4$  and  $61.84 \pm 0.31$ , respectively, and did not significantly differ; they remained relatively constant throughout embryonic development. Of the disappeared free amino acids,  $52.99 \pm 1.03\%$  were used for polymerization into body proteins and  $47.01 \pm 1.03\%$  was used for energy. Retention of indispensable amino acids (leucine, lysine, methionine, valine) for protein

Table 1. Free and total amino acid profiles of embryonic developmental stages of grouper, *Epinephelus tauvina* (mean±SD).

	Free amino acids (ng) <sup>1</sup>			Total amino acids (µg) <sup>2</sup>		
	Egg	Gastrula	Pre-hatch	Egg	Gastrula	Pre-hatch
<i>Indispensable amino acids</i>						
Arginine	203.81±7.98	166.36±1.29	53.22±11.92	1.457±0.023	1.424±0.031	1.379±0.021
Histidine	123.13±5.88	105.70±2.34	47.69±4.07	0.576±0.010	0.561±0.013	0.541±0.010
Isoleucine	385.29±14.63	308.14±11.63	169.11±13.18	1.355±0.031	1.296±0.031	1.250±0.023
Leucine	562.41±2.73	480.15±11.21	235.90±24.66	2.312±0.103	2.254±0.091	2.207±0.104
Lysine	454.68±18.32	371.71±22.81	129.18±14.17	1.815±0.033	1.758±0.015	1.695±0.033
Methionine	118.86±7.36	111.17±7.42	85.48±4.68	0.696±0.032	0.690±0.034	0.683±0.032
Phenylalanine	124.45±8.15	110.71±6.25	88.46±2.68	0.844±0.014	0.834±0.013	0.825±0.014
Threonine	201.68±8.78	159.76±18.92	26.90±6.01	1.085±0.019	1.053±0.016	0.994±0.018
Valine	298.21±12.41	263.07±14.62	200.02±5.60	1.685±0.076	1.654±0.075	1.645±0.072
<i>Dispensable amino acids</i>						
Alanine	320.39±22.65	216.73±23.89	88.62±12.84	1.824±0.094	1.731±0.097	1.707±0.084
Aspartic acid	46.59±3.52	35.66±4.19	13.02±1.81	1.333±0.021	1.323±0.020	1.315±0.020
Cystine	16.56±1.85	14.27±1.81	11.79±1.49	0.293±0.014	0.292±0.014	0.291±0.013
Glutamic acid	359.90±15.31	273.28±13.88	53.72±12.43	2.893±0.233	2.813±0.222	2.713±0.205
Glycine	80.83±9.68	51.84±8.54	14.25±2.49	0.784±0.045	0.757±0.043	0.743±0.041
Proline	72.51±3.05	64.94±2.39	50.13±1.19	1.583±0.028	1.576±0.028	1.574±0.028
Serine	416.20±10.35	341.80±26.87	180.47±28.15	1.526±0.065	1.455±0.058	1.415±0.063
Tyrosine	140.13±6.36	112.65±11.43	59.21±24.63	1.003±0.018	0.978±0.023	0.955±0.015
Taurine	152.27±4.40	144.34±5.85	141.39±2.86	0.152±0.004	0.144±0.006	0.145±0.006
Totals (µg)	4.08±0.06			23.22±0.31		

<sup>1</sup> Critical difference at 5%: embryonic stages 4.66, free amino acids 11.42, embryo × free amino acid 19.79

<sup>2</sup> Critical difference at 5%: embryonic stages 0.025, total amino acids 0.062, embryo × total amino acids 0.107

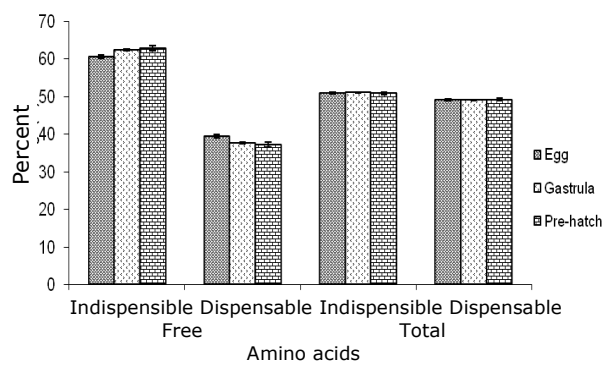


Fig. 1. Proportion (%) of indispensable and dispensable free and total amino acids in embryonic stages of the greasy grouper, *Epinephelus tauvina*

200 nmol/egg in Atlantic cod, *Godus morhua*, and 2300 nmol/egg in Atlantic halibut, *Hippoglossus hippoglossus* (Rønnestad and Fyhn, 1993). These large differences are attributed to egg size; the egg of the Atlantic cod was ~1 mg, while that of the Atlantic halibut was ~17 mg. Eggs of the turbot, *Scophthalmus maximus*, weigh ~0.6 mg and contain 55 nmol of free amino acids (Fyhn, 1990), while those of barfin flounder, *Verasper moseri*, weigh ~2.9 mg and contain 354 nmol (Matsubara and Koya, 1997) and those of walleye pollock, *Theragra chalcogramma*, weigh ~1.6 mg and contain 304 nmol (Ohkubo et al., 2006).

The amino acid profiles reported here for grouper are similar to those in other marine fish eggs (Table 2). Differences exist among species due to phylogeny as well as the biotic and abiotic conditions experienced by the adults (Finn et al., 1996; Dayal et al., 2003; Faulk and Holt, 2008). Leucine, lysine, valine, alanine, glutamic acid, and proline were the most prevalent total amino acids in the grouper eggs. Similarly high levels of leucine, lysine, and valine were reported in the polymerized amino acids of wild eggs of *Epinephelus coioides* (Alava et al., 2004).

Table 2. Comparison of dominant free amino acids in eggs of marine fish species.

Species	Dominant free amino acids	Source
Greasy grouper, <i>Epinephelus tauvina</i>	Isoleucine, leucine, lysine, alanine, glutamic acid, serine	This study
Turbot <i>Scophthalmus maximus</i>	Isoleucine, leucine, lysine, valine, alanine, serine	Finn et al., 1996
Seabass <i>Lates calcarifer</i>	Isoleucine, leucine, alanine, valine, glutamine, glutamic acid, serine	Sivaloganathan et al., 1998
Gilthead seabream <i>Sparus aurata</i>	Isoleucine, leucine, lysine, alanine, glutamic acid, serine, taurine	Rønnestad et al., 1994
Lemon sole <i>Microstomus kitt</i>	Isoleucine, leucine, lysine, alanine, glutamic acid, serine, taurine	Rønnestad et al., 1992
Dentex <i>Dentex dentex</i>	Isoleucine, leucine, lysine, alanine, glutamic acid, serine, taurine	Tulli and Tibaldi, 1997
Yellowtail kingfish <i>Seriola lalandi</i>	Isoleucine, leucine, valine, alanine, glycine, serine	Moran et al., 2007

No attempt was made in the current study to derive a stoichiometric energy budget during embryogenesis. However, the fate of the amino acids was revealed by quantitative analysis of the free and total amino acids during embryogenesis. The overall loss of free amino acids during embryogenesis was  $59.56 \pm 1.58\%$ . The loss did not significantly differ between indispensable and dispensable amino acids, but was not uniform for all amino acids. More than 70% of some free amino acids was lost during

synthesis was significantly higher than retention of indispensable amino acids (tyrosine, glycine, glutamic acid) for protein synthesis.

### Discussion

Grouper eggs contain  $32.27 \pm 0.398$  nmol free amino acids per egg (calculated by dividing the free amino acids by their molecular weights), comparable to 25.3 nmol/egg in *L. calcarifer* (Sivaloganathan et al., 1998), 21.7 nmol/egg in red snapper, *Lutjanus campechanus* (Hastey et al., 2010), and 30 nmol/egg in Atlantic mackerel, *Scomber scombrus* (Rønnestad and Fyhn, 1993), but much lower than

embryogenesis whereas taurine was maintained at an almost constant level. Similarly, no relative sparing of indispensable or dispensable amino acids was reported during embryonic development of *S. maximus* eggs (Finn et al. 1996) or *Dicentrarchus labrax* (Rønnestad et al., 1998).

Free amino acids were used for catabolic ( $47.01 \pm 1.03\%$ ) and anabolic ( $52.99 \pm 1.03\%$ ) purposes such as polymerization into body proteins. The relative proportions of free amino acids used for energy metabolism and protein synthesis vary widely among fish species: of the disappearing free amino acid pool, about 80% in *S. maximus*, 70% in *Microstomus kitt* (Rønnestad and Fyhn, 1993), and 80% in *Sparus aurata* (Rønnestad et al., 1994) was used for energy metabolism. However, in *L. calcarifer*, about 69% of the disappearing free acid pool was attributed to protein synthesis; the proportion available for energy metabolism was only 31% (Sivaloganathan et al., 1998). The embryonic deposition of proteins from free amino acids that seem to be removed for osmotic purposes can be regarded as temporary energy reserves in prefeeding larvae stages (Finn et al., 1995).

Based on our results, the proportionate loss of free dispensable and indispensable amino acids is uniform but the proportionate retention of the indispensable leucine, lysine, methionine, and valine for protein synthesis indicates their significance in diets for larvae of the grouper, *E. tauvina*. The correlation between the free amino acid content of eggs and viability of embryos and larvae should be further studied.

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