

Asian Fisheries Society, Manila, Philippines  
Available online at [www.asianfisheriessociety.org](http://www.asianfisheriessociety.org)

## **Dietary influence on the egg production and larval viability in True Sebae Clownfish *Amphiprion sebae* Bleeker 1853**

**BINU VARGHESE<sup>\*</sup>, R. PAULRAJ, G. GOPAKUMAR and K. CHAKRABORTY**

Central Marine Fisheries Research Institute  
Post-graduate Programme in Mariculture  
Kochi - 682 018, India

### **Abstract**

Broodstock nutrition is one of the most important research areas in aquaculture. In this study, sebae clownfish was used to find out the influence of diet on reproductive performance parameters like egg production, fertilization rate, hatchability, and larval quality. The feeds used were of marine origin such as squid, cuttlefish, deep sea prawn, immature and mature mussel. The diets were analyzed for their proximate composition, amino acids profile, fatty acids profile and astaxanthin. The sub-adult fishes were collected from wild and conditioned prior to experiment. Data were collected after initial three spawning to achieve stability in egg production and quality. The egg production was found to be significantly influenced by diet and those fed cuttlefish meat gave the highest number of eggs per clutch (1520±260 eggs). The fertilization rate and hatchability were found to be unaffected by the tested diets. The highest larval survival (62.3±7%) after 12 days post hatching was obtained for fish groups fed deep sea prawn. The dietary carotenoid content was also found to influence the egg and larval pigmentation. The result also indicates the importance of dispensable amino acids in egg production. The role of protein, lipids, and essential fatty acids in the broodstock diets for sebae clownfish are also discussed.

### **Introduction**

Broodstock diet has considerable influence on the success of hatchery operations directly by affecting the fecundity, fertilization rate, egg quality, embryo development, and larval quality (Bromage 1998; Furuita 2000; Izquierdo et al. 2001). Nutrition of brood fishes critically influences the breeding success and is vital during early larval stages, especially during yolk nourishment stage. Despite the remarkable advances in the field of fish larval and juvenile nutrition, new frontiers have to be explored regarding the nutritional requirements of broodfish. This dearth in information is affecting the

---

\*Corresponding author. Tel.: +91 9447501644  
Email address: [bvargheseb@gmail.com](mailto:bvargheseb@gmail.com)

diversification and development of potential mariculture technologies.

In fishes with short vitellogenic period, the gonadal development and fecundity can be manipulated by giving diets shortly before or during spawning (Izquierdo et al. 2000). In fishes like sea breams, broods continue to feed during spawning and the nutrient composition of egg and the larvae are greatly influenced by the diet within this short duration (Watanabe et al., 1985; Tandler et al., 1995). However, in salmonids the process of reproduction generally involves a dramatic decrease in food intake and a substantial mobilization of nutrients from various body stores into the developing oocytes (Aksnes et al. 1986), with vitellogenic period extending upto six months (Fremont et al. 1984).

The necessity of suitable indoor or outdoor culture facilities for maintaining large groups of adult fishes and the consequent high cost of running and conducting extended trials are critical factors hindering the progress in broodstock nutrition (Izquierdo et al. 2001). Most of these difficulties can be effectively tackled by using smaller test specimens with minimal space and culture requirement. Clownfishes hold several vital characteristics required to be a test specimen for broodstock studies like their continuous spawning nature, (it spawns two to three times a month), early maturity, easy to maintain and manage fairly large groups of individuals in small area, acceptance of formulated diets etc. These fishes expressed the influence of changed dietary carotenoids within a short span of 48 hrs (Binu Varghese 2004).

The objective of the present study is to suggest small continuous spawners with predictable reproductive performance as test specimens for marine fish broodstock nutrition studies. Further studies in similar species can provide the importance of various nutrients in broodstock development and spawning much faster than the conventional aquacultured species.

### Materials and Methods

The fishes (60 - 70 mm TL) were collected from the Gulf of Mannar (Lat. 79<sup>o</sup> 09'E and Long. 9<sup>o</sup> 16'N) using hand nets. The host anemone, *Stichodactyla haddoni* was also collected from the same location. The fishes were packed individually in oxygenated polythene bags with filtered seawater and transported to the Marine Aquarium at the Vizhinjam Research Centre of Central Marine Fisheries Research Institute.

Broodstock experiments were carried out for six consecutive spawning by maintaining three replicates for each treatment. As the numbers of eggs spawned were considerably low in the initial spawning, data were collected after three successive spawning. The FRP tanks (2.0 m x 1.0 m x 0.5 m) were equipped with a sub-gravel filter system. An air-water lift system was established by erecting PVC tubes at the corners of filter plate. Clay pots were provided as spawning substrate and the continuous aeration

supplied from air blowers. Apart from indirect sunlight, tanks were individually illuminated with fluorescent tubes (40 W) hung 50 cm above the water surface to provide a constant photoperiod of 12L: 12D. Each tank was provided with two individuals having 5- 8 mm length variation to hasten pair formation; two sea anemones were also included in each of the tank. Filtered seawater was supplied and the water quality in the experimental system was regularly monitored. The mean seawater parameters observed during the experimental period were: salinity ( $33.8 \pm 1.6$ ), temperature ( $27.9 \pm 0.9^{\circ}\text{C}$ ), pH ( $8.1 \pm 0.1$ ), ammonia ( $0.005 \pm 0.002$  mg L<sup>-1</sup>) and nitrite ( $0.008 \pm 0.004$  mg L<sup>-1</sup>).

The fishes were fed *ad libitum* twice daily, at 10.00 hrs and 15.30 hrs. The natural diets used were the brown mussel, *Perna indica* (MSM), brown mussel with ripe gonads (MGD), squid *Doryteuthis* sp. meat (SQD), cuttlefish *Sepiella inermis* meat (CUT) and deep sea prawn *Heterocarpus* sp. meat (DSP). The brown mussels were steamed for 2 hr and the shucked meat was used. The proximate composition, amino acid profiles and fatty acid profiles of natural diets were determined.

### **Larval rearing**

Rearing was done in triplicate, each glass aquarium (3' x 2' x 2') was stocked with 250 newly hatched larvae and the experiment lasted for 12 days. Larvae were fed with rotifers as first feed, followed by a combination of rotifers and *Artemia* from 4 dph (days post hatching) to 12 dph. No water exchange was done during the first three days and later 25% daily exchange was done.

### **Biochemical analysis**

The feed moisture, crude protein and crude fibre were determined following AOAC (1990). Crude lipid was estimated by soxhlet extraction with petroleum ether and the ash content was determined from the residue remaining after incineration of the samples at 550°C in a muffle furnace. The nitrogen free extracts were computed by difference.

The extraction of carotenoids was carried out by using Weber and Davoli (2003) method. The extracted polar carotenoids was dissolved in 0.5 ml ethyl acetate and used for astaxanthin estimation. Astaxanthin was estimated using spectrophotometer at 480 nm, the absorption peak of astaxanthin in visible light. The amount of astaxanthin was estimated by the following equation.

$$x = (A_{480} \times Y \times 1000) / (A^{1\%}_{1\text{cm}} \times 100)$$

Where x = astaxanthin (mg),  $A_{480}$  = absorbance at 480 nm, Y = volume of sample (2 ml + crude extract added), and  $A^{1\%}_{1\text{cm}}$  = specific absorption coefficient of astaxanthin for a 1% solution in a 1 cm cell, in methanol it is 2100. The x value obtained was multiplied with total volume of crude extract for further calculations.

Amino acid was analysed by using Waters reversed-phase PICO.TAG amino acid analysis system. Samples were injected in triplicates and the output was analyzed using Breeze software. Tryptophan was estimated by the spectrophotometric method as described by [Sastry and Tummuru \(1985\)](#) after alkali hydrolysis of the sample. Lipid extraction was carried out by [Folch et al. \(1957\)](#) method and the fatty acid analysis was performed on a Perkin Elmer AutoSystem XL, Gas chromatograph. A secondary reference standard of cod-liver oil FAME was used to confirm the peaks. Samples were injected in triplicate and the data acquisition was done with TotalChrome 6.X.X software.

### ***Data analysis***

Data are presented as mean  $\pm$  standard deviation and analysed using one way ANOVA. When a significant difference was found, the mean differences between treatments were tested for significance ( $p < 0.05$ ) by Duncan's multiple range tests. Statistical analyses were performed using the SPSS 7.5 version for WINDOWS and results were treated statistically significant at the 5% level.

## **Results**

**Pair formation and spawning:** The establishment of breeding pair took place within two months after introduction into the experimental system and spawning started about one to two months after pairing. A cascade of events like clearing of nesting site, biting the substratum and parallel swimming by the pairs occur during spawning. The study reveals that the spawning took place usually between 09.00 hrs and 13.30 hrs. Pairs exhibited parental care by guarding and fanning the eggs and by removing unfertilized and unhealthy eggs. The spawning interval remained unaffected between the various diets tested.

**Nutrient profiles:** The proximate compositions of the tested natural diets are given in Table 1. The crude protein varied from 50 - 70% and the lipid 5 - 16%. The cuttlefish meat with about 60% protein and 11% lipid gave highest egg production; followed by the deep sea prawn with 56% protein and 9.5% lipid. Amino acid profile showed significant difference between the diets (Table 2). Among the essential amino acid lysine content in mussel meat was found to be significantly higher whereas, the arginine content was high in cuttlefish meat. The total essential amino acids content was high in mussel meat and the least value was recorded in the deep-sea prawn (Table II). However, the ratio of essential to non essential amino acid showed a significant relation to the egg production.

Fatty acid profile showed significant difference between the diets (Table 3). The total n-3 and n-6 fatty acid levels also significantly varied among the diets, with higher

Table 1. Proximate composition (% dry matter) of diets fed to broodstock

Diet	Crude protein (%)	Crude lipid (%)	NFE (%)	Crude ash (%)
Deep sea prawn meat (DSP)	56.03	9.45	9.23	22.59
Mature mussel meat (MGD)	55.96	16.71	15.60	8.86
Cuttlefish meat (CUT)	59.50	10.70	9.26	19.54
Squid meat (SQD)	70.06	5.30	5.58	15.99
Mussel meat (MSM)	49.54	11.28	23.82	13.32

levels of n-3 in mussel meat, whereas the n-6 content was high in deep-sea prawn. The polyunsaturated fatty acid levels also showed significant variation between diets with the highest level in mussel meat (41.12%) and relatively low levels in deep sea prawn and cuttlefish, wherein it accounted for less than 30%. The docosahexaenoic acid level was high in squid and cuttlefish and low in mussel diets; however, eicosapentaenoic acid was significantly higher in mussel diets.

Astaxanthin concentration was found to be high ( $42.77 \mu\text{g g}^{-1}$  wet weight) in the deep-sea prawn. The mature mussel meat (MGD) had  $23.25 \mu\text{g g}^{-1}$  of astaxanthin followed by the mussel meat (MSM) with  $17.42 \mu\text{g g}^{-1}$  wet weight. Cuttlefish gave the least value of  $5.13 \mu\text{g g}^{-1}$  wet weight astaxanthin. The mussel diets had the predominance of carotenoids other than astaxanthin as the peak absorption was observed well below 480 nm during survey scan.

**Influence on egg production and quality:** The number of eggs spawned by the fish was found to be significantly influenced by the diets. The results showed that the cuttlefish meat fed group gave the best performance with an average clutch size of  $1521 \pm 264$  eggs followed by those fed with the deep sea prawn ( $1300 \pm 445$ ), mature mussel gonad ( $1150 \pm 141$ ), and squid meat ( $1025 \pm 232$ ). Those fed mussel meat yielded the least with an average clutch size of  $885 \pm 55$  eggs. The clutch colouration distinctly varied from pale yellow (MSM), pale pink (SQD and CUT), orange (MGD) to pinkish red (DSP).

The egg capsule length did not vary significantly between the tested diets (Table 4).

Table 2. Amino acid profile (g/100g protein) of diets used for the broodstock experiments

Amino acid	DSP	MGD	MSM	SQD	CUT
ARG	5.48 <sup>a</sup>	8.18 <sup>b</sup>	5.29 <sup>a</sup>	4.69 <sup>c</sup>	10.16 <sup>d</sup>
HIS	2.08 <sup>ac</sup>	1.84 <sup>ab</sup>	1.73 <sup>b</sup>	2.30 <sup>cd</sup>	2.62 <sup>d</sup>
ILE	4.14 <sup>a</sup>	4.14 <sup>a</sup>	4.12 <sup>a</sup>	4.65 <sup>b</sup>	4.80 <sup>b</sup>
LEU	8.24 <sup>a</sup>	7.84 <sup>a</sup>	7.56 <sup>a</sup>	9.02 <sup>b</sup>	8.90 <sup>b</sup>
LYS	9.88 <sup>a</sup>	8.67 <sup>a</sup>	15.79 <sup>b</sup>	8.93 <sup>a</sup>	4.48 <sup>a</sup>
MET	2.50 <sup>a</sup>	2.54 <sup>a</sup>	2.55 <sup>a</sup>	3.26 <sup>b</sup>	2.93 <sup>c</sup>
THR	4.23 <sup>a</sup>	5.47 <sup>c</sup>	5.39 <sup>c</sup>	4.72 <sup>b</sup>	4.42 <sup>a</sup>
TRY	1.25 <sup>a</sup>	1.74 <sup>b</sup>	1.67 <sup>b</sup>	1.26 <sup>a</sup>	1.77 <sup>b</sup>
PHE	4.22 <sup>ab</sup>	4.40 <sup>bc</sup>	4.50 <sup>c</sup>	3.94 <sup>a</sup>	3.56 <sup>d</sup>
VAL	4.69 <sup>a</sup>	5.04 <sup>b</sup>	5.23 <sup>b</sup>	4.67 <sup>a</sup>	4.62 <sup>a</sup>
ALA	8.28 <sup>b</sup>	6.24 <sup>a</sup>	5.80 <sup>a</sup>	9.18 <sup>c</sup>	8.13 <sup>b</sup>
ASP	7.11 <sup>a</sup>	9.17 <sup>b</sup>	9.95 <sup>b</sup>	7.86 <sup>a</sup>	7.42 <sup>a</sup>
CYS	0.47 <sup>a</sup>	0.78 <sup>c</sup>	0.64 <sup>bc</sup>	0.71 <sup>bc</sup>	0.60 <sup>b</sup>
GLU	10.43 <sup>a</sup>	9.93 <sup>ab</sup>	9.74 <sup>b</sup>	11.24 <sup>c</sup>	10.45 <sup>ab</sup>
GLY	11.85 <sup>a</sup>	9.98 <sup>c</sup>	7.46 <sup>d</sup>	10.47 <sup>bc</sup>	12.09 <sup>ab</sup>
PRO	5.38 <sup>a</sup>	4.92 <sup>b</sup>	4.23 <sup>c</sup>	5.50 <sup>a</sup>	5.64 <sup>a</sup>
SER	6.48 <sup>a</sup>	5.68 <sup>b</sup>	5.06 <sup>bc</sup>	4.83 <sup>c</sup>	4.70 <sup>c</sup>

Values with same superscript in the row are not significantly different from each other ( $P < 0.05$ )  
 DSP- deep sea prawn, MGD- mature mussel meat, MSM- mussel meat, SQD- squid, CUT-cuttlefish

ARG- Arginine, HIS- Histidine, ILE- Isoleucine, LEU- Leucine, Lys- Lysine, MET- Methionine, THR- Threonine, TRY- Tryptophan, PHE- Phenylalanine, VAL- Valine, ALA- Alanine, ASP- Aspartic acid, CYS- Cysteine, GLU- Glutamic acid, GLY- Glycine, PRO- Proline, SER- Serine, TYR- Tyrosine

<sup>a</sup>EAA- Total essential amino acids

<sup>b</sup>NEAA- Total non-essential amino acids

However, the capsule width exhibited significant variations among the treatments. The capsule width was high when fed with mature mussel's meat (914  $\mu\text{m}$ ) and was least when fed with immature mussel's meat (845  $\mu\text{m}$ ).

Fertilization rate and hatchability: The test diets have no significant effect on the fertilization rate and hatchability of the eggs in the present study.

Table 3. Amino acid profile (g/100g protein) of diets used for the broodstock experiments

Fatty Acids	DSP	CUT	SQD	MGD	MSM
C14:0	3.62 <sup>b</sup>	2.24 <sup>a</sup>	2.25 <sup>a</sup>	6.65 <sup>c</sup>	6.9 <sup>c</sup>
C16:0	19.4 <sup>b</sup>	17.01 <sup>a</sup>	18.95 <sup>ab</sup>	29.18 <sup>c</sup>	26.77 <sup>c</sup>
C16:1 n7	2.52 <sup>b</sup>	2.85 <sup>b</sup>	0.32 <sup>a</sup>	2.89 <sup>b</sup>	3.97 <sup>c</sup>
C18:0	n.d	14.95 <sup>b</sup>	3.31 <sup>a</sup>	n.d	2.97 <sup>a</sup>
C18:1 n9	21.86 <sup>d</sup>	0.19 <sup>b</sup>	10.12 <sup>a</sup>	14.47 <sup>c</sup>	9.96 <sup>a</sup>
C18:1 n7	4.33 <sup>a</sup>	0.22 <sup>b</sup>	n.d	n.d	n.d
C18:2 n6	1.91 <sup>b</sup>	0.27 <sup>a</sup>	0.58 <sup>a</sup>	2.4 <sup>b</sup>	1.95 <sup>b</sup>
C18:3 n3	0.35 <sup>a</sup>	0.54 <sup>a</sup>	0.22 <sup>a</sup>	1.74 <sup>b</sup>	2.73 <sup>c</sup>
C18:4 n3	0.41 <sup>a</sup>	n.d	n.d	n.d	2.31 <sup>b</sup>
C20:1	1.18 <sup>a</sup>	13.71 <sup>b</sup>	12.32 <sup>c</sup>	1.57 <sup>a</sup>	1.33 <sup>a</sup>
C20:4 n6	7.14 <sup>a</sup>	3.35 <sup>b</sup>	2.41 <sup>c</sup>	n.d	n.d
C20:5 n3	3.57 <sup>a</sup>	3.58 <sup>a</sup>	3.68 <sup>a</sup>	16.1 <sup>c</sup>	21.15 <sup>b</sup>
C22:1	0.98	0.51	n.d	2.42	2.19
C22:5 n3	0.48 <sup>a</sup>	n.d	0.48 <sup>a</sup>	0.93 <sup>b</sup>	0.97 <sup>b</sup>
C22:6 n3	13.64 <sup>b</sup>	21.39 <sup>c</sup>	28.74 <sup>d</sup>	12.64 <sup>a</sup>	12.01 <sup>a</sup>
C24:1 n9	0.73 <sup>a</sup>	n.d	0.77 <sup>a</sup>	0.47 <sup>c</sup>	0.51 <sup>b</sup>
Σ Saturated	23.02 <sup>a</sup>	34.2 <sup>b</sup>	24.51 <sup>a</sup>	35.83 <sup>b</sup>	36.64 <sup>c</sup>
Σ Unsaturated	62.33 <sup>a</sup>	46.61 <sup>b</sup>	60.86 <sup>a</sup>	55.63 <sup>d</sup>	59.08 <sup>c</sup>
Σ MUFA	31.6 <sup>c</sup>	17.48 <sup>a</sup>	23.53 <sup>b</sup>	21.82 <sup>b</sup>	17.96 <sup>a</sup>
Σ PUFA	28.21 <sup>a</sup>	29.13 <sup>a</sup>	36.11 <sup>b</sup>	33.81 <sup>d</sup>	41.12 <sup>c</sup>
Σ n-3	18.45 <sup>a</sup>	25.51 <sup>b</sup>	33.12 <sup>c</sup>	31.41 <sup>e</sup>	39.17 <sup>d</sup>
Σ n-6 <sup>1</sup>	9.05 <sup>b</sup>	3.62 <sup>c</sup>	2.99 <sup>d</sup>	2.4 <sup>a</sup>	1.95 <sup>a</sup>
Σ n-3 HUFA	17.69 <sup>a</sup>	24.97 <sup>b</sup>	33.05 <sup>c</sup>	29.67 <sup>d</sup>	34.13 <sup>e</sup>
Saturated/ Unsaturated	0.37 <sup>a</sup>	0.73 <sup>b</sup>	0.40 <sup>a</sup>	0.64 <sup>d</sup>	0.60 <sup>c</sup>
n3/n6	2.04 <sup>b</sup>	7.05 <sup>c</sup>	11.08 <sup>a</sup>	13.09 <sup>a</sup>	20.09 <sup>d</sup>
DHA/EPA	3.82 <sup>b</sup>	5.97 <sup>c</sup>	7.81 <sup>d</sup>	0.79 <sup>a</sup>	0.57 <sup>a</sup>
EPA/AA	0.50	1.07	1.53	-	-

Means having same superscript in the row are not significantly different from each other (<0.05) n.d not detected

MUFA Mono Unsaturated Fatty Acids EPA Eicosa Pentaenoic Acid  
 PUFA Poly Unsaturated Fatty Acids AA Arachidonic Acid  
 DHA Docosa Hexaenoic Acid

Table 4. Influence of diets on the egg dimensions of sebae clownfish (mean  $\pm$  SD)

Diet	Egg capsule length (mm)	Egg capsule width ( $\mu$ m)	Larval length (mm)
DSP	2.21 $\pm$ 0.04	851 $\pm$ 37 <sup>a</sup>	4.18 $\pm$ 0.15
MGD	2.19 $\pm$ 0.05	914 $\pm$ 36 <sup>b</sup>	4.05 $\pm$ 0.19
CUT	2.21 $\pm$ 0.02	855 $\pm$ 26 <sup>a</sup>	4.09 $\pm$ 0.12
SQD	2.23 $\pm$ 0.03	910 $\pm$ 10 <sup>b</sup>	4.31 $\pm$ 0.09
MSM	2.19 $\pm$ 0.01	845 $\pm$ 20 <sup>a</sup>	3.96 $\pm$ 0.10

Values with same superscript in the column are not significantly different from each other ( $P < 0.05$ )

DSP - Deep sea prawn meat

MGD - Mature brown mussel

CUT - Cuttlefish meat

SQD - Squid meat

MSM - Brown mussel meat

Larval quality: The total length of newly hatched larvae from the different treatments was not statistically significant (Table IV). Among the tested diets, total length of larvae was found higher with squid meat fed group (4.31 mm) and lower in immature mussel meat (3.96 mm). Another significant finding was that the larvae from the pairs fed with deep-sea prawn or mature mussel exhibited brighter pigmentation.

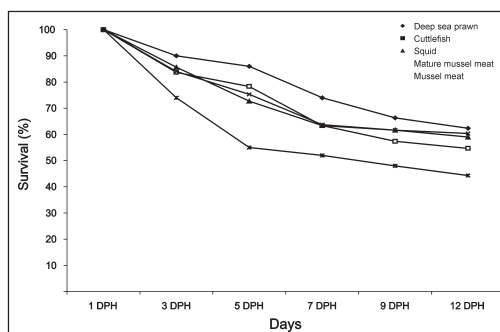


Figure 1. Survival graph of *Amphiprion sebae* larvae

The influence of natural diets on the larval survival is depicted in Fig. 1. The larval survival has no significant difference at 3 dph, wherein the broodstock diet was expected to play a major role. At the end of experiment, i.e. 12 dph, the deep-sea prawn provided highest survival (62.3  $\pm$  6.7%) and was followed by mature mussel (60.3  $\pm$  2.1%), squid (59  $\pm$  5.3%) and cuttlefish (54.7  $\pm$  11.2%). The least larval survival was observed with immature mussel (44.3  $\pm$  5.7%).

## Discussion

The energy requirements of anemone fishes are higher during spawning, because of their protracted spawning nature, continuous growth even after sexual maturity, and



extensive parental care behaviours. All these energy-demanding processes need to be catered through adequate diet to maintain consistent spawn quality. Fresh natural diets are traditionally used to feed broodstock and are the most effective way of meeting the nutritional needs of fish and ensuring good quality eggs (Bruce et al. 1999). The diets fed to brood fish are known to influence the larval quality in fishes (Duray et al. 1994; Fernandez-Palacios et al. 1995; Tandler et al. 1995).

Among the nutrients dietary protein is known to significantly influence the process of reproduction in fishes (Watanabe et al. 1984a; Harel et al. 1994; Tandler et al. 1995). In sea breams reduction in dietary protein significantly affected the broodstock performance (Cerdeira et al. 1994; Watanabe et al. 1984b). Present study on clown fishes revealed the importance of protein and amino acids as the major nutrients influencing the egg production, provided fatty acids are fairly balanced. In general diets with 55 to 60% protein and 9 to 12% lipid positively influenced the broodstock performance in sebae anemonefish. The superior egg production and egg quality observed with the cuttlefish fed groups are in conformity to the observations as in other marine fishes (Watanabe et al. 1985, 1991; Harel et al. 1994). This superiority of cuttlefish diet in enhancing the egg production and viability are attributed to its fat insoluble portion (Watanabe et al. 1991).

Vitellogenin is the main yolk precursor in teleosts, the role of amino acids and fatty acids in vitellogenin synthesis is well described (Specker & Sullivan 1994; Sargent 1995). In the case of protracted spawners like sebae anemonefish (2-3 spawning per month round the year), the diet forms a major source of amino acids required for vitellogenesis than the muscle proteins. Further studies on the process of vitellogenesis in protracted spawners are essential to establish the role of dietary nutrients and the regulatory factors. The significant variations observed in egg production between the treatments may be due to the difference in the rate of vitellogenin synthesis and uptake.

Vitellogenin is characterised by high content of alanine, glutamic acid and leucine and with a lower concentration of serine in gilthead sea bream (Fernandez-Palacios et al. 1997). The vitelline envelope proteins of gilthead sea bream have high content of proline and glutamic acid and a relatively low content of cysteine (Hyllner et al. 1995). This indicates the role of non-essential amino acids in the vitellogenin synthesis and thus in egg formation.

The essential to non-essential amino acid ratio in the cuttlefish meat, which gave the highest egg production, was found to be 0.93:1. Similarly, the egg production was found to be lower in pairs fed with diets which have higher level of essential amino acid (Table II). These findings are reconfirmed in further studies using formulated moist diets (Binu Varghese 2004). Though, fishes can synthesise dispensable amino

acids adequately for normal protein synthesis, dietary supplementation may help to improve the egg production in continuous spawners where its requirement may be high during the process of egg production as a source of energy and also a egg constituent. Further studies are also needed to confirm the essentiality and role of dispensable amino acids to broodstock.

The poor response obtained with mussel meat in the present study might have been due to the significantly higher level of lysine (15.8%), and consequently the amino acid imbalance. However, the cuttlefish which gave superior egg production had lower lysine (4.5%) and higher arginine content. The role of lysine and arginine and their possible interactions in egg production may give further insights in broodstock nutrition.

The dietary lipids are also known to influence the egg and larval quality (Watanabe et al. 1984a; Duray et al. 1994). The qualitative and quantitative lipid content in the diets as well as the feeding regime during gonadogenesis was found to influence the spawning and egg quality (Watanabe et al. 1984a; Harel et al. 1994) and the egg fatty acid profiles in fishes (Mourente & Odriozola 1990).

In fishes optimum n-3 HUFA requirement was suggested to be approximately 20% of total fatty acids for higher egg quality (Fernandez-Palacios et al. 1995; Furuita et al. 2002). The upper level depends on the composition of HUFA, since ratios of EPA, DHA and AA in broodstock diets are important for high quality eggs (Bruce et al. 1999). Deficiency of n-3 HUFA in the broodstock diet was reported to critically affect the fecundity, hatchability and viability (Mourente & Odriozola 1990; Fernandez-Palacios et al. 1995; Rodriguez et al. 1998). Fernandez-Palacios et al. (1995) found that excess n-3 HUFA in the diet causes decreased fecundity and yolk-sac hypertrophy in newly hatched *Sparus aurata* larvae. In the present study dietary n-3 HUFA level between 17 to 25% resulted in better performance and higher levels (>30%) significantly decreased the egg production.

Delbare et al. (1995) reported that the fatty acid profile of larvae from bad quality eggs of tomato clownfish had higher EPA: DHA ratio (7.3:1). In the present study, the cuttlefish with DHA: EPA ratio of about 6:1 gave the higher egg production followed by the deep-sea prawn with a ratio of 3.8:1. However, squid meat with a higher ratio (7.8:1) and mussel meat with a lower ratio (0.57:1) resulted in significantly lower egg production. Thus the ratio of DHA: EPA plays an important role in the egg production.

The fertilization rate and hatchability are often regarded as indices of spawning success, and considered as major factors influenced by broodstock diets in marine fishes (Fernandez-Palacios et al. 1995; Rodriguez et al. 1998). Unlike other fishes the fertilization rate and hatchability in sebae anemonefish were unaffected by the tested diets. The extensive parental care exhibited by the clownfishes makes these parameters

insignificant as determinants of egg quality. The studies have to be further conducted to check the effects of these parameters on the diets with inferior nutrient profiles. The hatchability will be affected only when the eggs were artificially incubated and in the present study the eggs were hatched in the broodstock tank itself.

Though, sebae clownfish larvae have the propensity to initiate feeding immediately after hatching the yolk reserves continue to play a major role in larval development as the availability and assimilability of exogenous diets vary drastically in early stages. The survivability of larvae was found generally high in all treatments at 3 dph, wherein higher influence of broodstock diet is expected. However, those from the pairs fed mussel meat (MSM) resulted in least survival (<70%), perhaps due to the imbalance caused by excess dietary n-3 HUFA levels, which is known to affect larval survival (Furuita et al. 2000).

The exact role played by carotenoids in eggs and larval quality is still a debated issue. Hoff (1996) attributed hatching success and viability in clownfish larvae to the egg pigmentation. The carotenoids are also known to influence immune system in fishes (Thompson et al. 1994; Paripatananont et al. 1999). The improved larval survival may also be due to the vitamin A activity of the carotenoids, as they form precursor to vitamin A, which in turn can increase the visual capabilities of the larvae and thereby resulting in increased prey strike success. The mobilization of carotenoids to the ovaries and then to larvae was reported (Torrissen & Christiansen 1995; Choubert et al. 1998).

Among carotenoids, astaxanthin is known to influence the egg quality in many fishes (Watanabe & Miki 1993). Active mobilization of dietary carotenoids to developing eggs within a short span (< 48 hrs) indicates their importance in the egg and larval quality in sebae anemonefish (Binu Varghese 2004). In the present study, deep-sea prawn and mature mussel fed pairs showed higher larval survival and this may be attributed to the dietary astaxanthin content.

### **Conclusion**

The broodstock nutrition is an interdisciplinary research area involving nutrition, reproductive endocrinology and physiology. The various factors influencing initial recruitment of oocytes into the pool of maturing eggs is still in its infancy. From this study it can be speculated that the egg production in continuous spawners like sebae anemonefish is controlled largely by the quality of broodstock diet. Among the nutrients amino acids (both essential and non-essential) with adequate level of essential fatty acids in the diet was found to facilitate superior egg production. The better understanding

of nutritional factors involved in maturation and spawning processes and their interactions with other factors are critical to meet the future aquaculture demand.

### Acknowledgements

The authors are thankful to Director, Central Marine Fisheries Research Institute and those supported the research work at Vizhinjam Marine Aquarium and CMFRI, Kochi. Help rendered by Shylaja in amino acid analysis is specially acknowledged. The research fellowship provided by the CIFE/ICAR to the first author is gratefully acknowledged.

### Reference

- Aksnes, A., B. Gjerds and S. O. Roland. 1986. Biological, chemical and organoleptic changes during maturation of farmed Atlantic salmon, *Salmo salar*. *Aquaculture* 53: 7-20.
- AOAC. 1990. Official methods of analysis of the association of official analytical chemists. (ed. Helrich, K.). Association of Official Analytical Chemists, Arlington, VA, USA, 1298pp.
- Binu Varghese, 2004. Nutritional studies on seabae anemonefish, *Amphiprion sebae* Bleeker 1853, with special reference to protein and lipid requirements. Ph. D. Thesis, Central Institute of Fisheries Education, Mumbai, 192 pp.
- Bromage, N. R. 1998. Broodstock management and the optimization of seed supplies. *Suisanzoshoku* 46: 395-401.
- Bruce, M., F. Oyen, G. Bell, J. F. Asturiano, B. Farndale, M. Carrillo, S. Zanuy, J. Ramos and N. Bromage. 1999. Development of broodstock diets for the European seabass (*Dicentrarchus labrax*) with special emphasis on the importance of n-3 and n-6 highly unsaturated fatty acids to reproductive performance. *Aquaculture* 177: 85-97.
- Cerda, J, M. Carrillo, S. Zanuy, J. Ramos and M. de la Higuera. 1994. Influence of nutritional composition of diet on seabass, *Dicentrarchus labrax* L., reproductive performance and egg and larval quality. *Aquaculture* 128: 345-361.
- Choubert, G., J-M. Blanc and H. Poisson. 1998. Effects of dietary keto-carotenoids (canthaxanthin and astaxanthin) on the reproductive performance of female rainbow trout *Onchorhynchus mykiss* (Walbaum). *Aquaculture Nutrition* 4: 249-254.
- Delbare, D., P. Lavens and P. Sorgeloos. 1995. Clownfish as a reference model for nutritional experiments and determination of egg/larval quality. In: Fish and Shellfish Larviculture Symposium 1995, (ed. Lavens, P., E. Jaspers and I. Roelants), European Aquaculture Society, Special Publication 24:22-25.
- Duray, M., H. Kohno and F. Pascual. 1994. The effect of lipid enriched broodstock diets on spawning and on egg and larval quality of hatchery-bred rabbitfish (*Siganus guttatus*). *Philippines Science* 31: 42-57.
- Fernandez-Palacios, H., M. S. Izquierdo, L. Robaina, A. Valencia, M. Salhi and J. M. Vergara. 1995. Effect of n-3 HUFA level in broodstock diets on egg quality of gilthead sea bream (*Sparus aurata*). *Aquaculture* 132: 325-337.
- Fernandez-Palacios, H., M. S. Izquierdo, L. Robaina, A. Valencia, M. Salhi and D. Montero. 1997. The effect of dietary protein and lipid from squid and fish meals on egg quality of broodstock for gilthead seabream (*Sparus aurata*). *Aquaculture* 148: 233-246.
- Folch, I., M. Lass and G. H. S. Standy. 1957. A simple method for extraction and purification of total lipids from animal tissue. *Journal of Biological Chemistry* 226: 497-509.
- Fremont, L., C. Leger, B. Petridou and M. T. Gozzelino. 1984. Effects of a polyunsaturated fatty acid deficient diet on profiles of serum vitellogenin and lipoprotein in vitellogenic trout (*Salmo gairdneri*). *Lipids* 19:522-528.
- Furuuta, H. 2000. Nutritional requirements in broodstock of marine fishes. In: Proceedings of the twenty-eighth

- UJNR Aquaculture Panel Symposium, Kihei, Hawaii, November 10-12, 1999. UJNR Technical Report 28: 53-60.
- Furuita, H., H. Tanaka, T. Yamamoto, M. Shiraishi and T. Takeuchi. 2000. Effects of n-3 HUFA levels in broodstock diet on the reproductive performance and egg and larval quality of the Japanese flounder, *Paralichthys olivaceus*. Aquaculture 187: 387-398.
- Furuita, H., H. Tanaka, T. Yamamoto, N. Suzuki and T. Takeuchi. 2002. Effects of high levels of n-3 HUFA in broodstock diet on egg quality and egg fatty acid composition of Japanese flounder, *Paralichthys olivaceus*. Aquaculture 210: 323-333.
- Harel, M., A. Tandler, G. W. Kissil and S. W. Applebaum. 1994. The kinetics of nutrient incorporation into body tissues of gilt-head seabream, *Sparus aurata* females and subsequent effects on egg composition and egg quality. British Journal of Nutrition 72: 45-58.
- Hoff, F.A. 1996. Conditioning, spawning and rearing of fish with emphasis on marine clownfish. Aquaculture Consultants Inc. USA. 212 pp.
- Hyllner, S. J., H. Fernandez-Palacios, D. G. J. Larson and C. Haux 1995. Amino acid composition and endocrine control of vitelline envelope proteins in European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*). Molecular Reproductive Development 41: 339-347.
- Izquierdo, M. S., J. Socorro, L. Arantzamendi and C. Hernandez-Cruz. 2000. Recent advances in lipid nutrition in fish larvae. Fish Physiology and Biochemistry 22: 97-107
- Izquierdo, M. S., H. Fernandez-Palacios and A. G. J. Tacon. 2001. Effect of broodstock nutrition on reproductive performance of fish. Aquaculture 197:25-42.
- Mourente, G and J. M. Odriozola. 1990. Effect of broodstock diets on lipid classes and their fatty acid composition in eggs of gilthead sea bream (*Sparus aurata* L.). Fish Physiology and Biochemistry 8: 93-101.
- Pariwatanont, T., J. Tangtrongpaioj, A. Sailasuta and N. Chansue. 1999. Effect of astaxanthin on the pigmentation of goldfish *Carassius auratus*. Journal of World Aquaculture Society 30: 454-460.
- Rodriguez, C., J. R. Cejas, M. V. Martin, P. Badia, M. Samper and A. Lorenzo. 1998. Influence of n-3 highly unsaturated fatty acid deficiency on the lipid composition of broodstock gilthead seabream (*Sparus aurata*) and on egg quality. Fish Physiology and Biochemistry 18: 177-187.
- Sargent, J. R. 1995. Origin and functions of egg lipids: nutritional implications. In: Broodstock Management and Egg and Larval Quality (ed. Bromage N. R, Roberts, R. J). Blackwell, Oxford, pp. 353-372.
- Sastry, C. S. P. And M. K. Tammura. 1985. Spectrophotometer determination of tryptophan in protein. Food Science and Technology 22: 146-147.
- Specker, J. L and C. V. Sullivan. 1994. Vitellogenesis in fishes: Status and perspectives. In: Perspectives in comparative endocrinology. (ed. Davey, K. C., Peter, R.G. and Tobess). National Research Council of Canada, Ottawa. pp. 304-315.
- Tandler, A., M. Harel, W. M. Koven and S. Kolkovski. 1995. Broodstock and larvae nutrition in gilthead seabream *Sparus aurata* - new findings on its mode of involvement in improving growth, survival and swimbladder inflation. Israeli Journal of Aquaculture Bamidgah 47: 95-111.
- Thompson, I., T. C. Fletcher, D. F. Houlihan and C. J. Secombes. 1994. Effect of dietary vitamin A intake on the immunocompetence of Atlantic salmon (*Salmo salar*). Fish Physiology and Biochemistry 12: 513-523.
- Torrissen, O. J and R. Christiansen. 1995. Requirements for carotenoids in fish diets. Journal of Applied Ichthyology 11: 225-230.
- Watanabe, T., T. Arakawa, C. Kitajima and S. Fujita. 1984a. Effect of nutritional quality of broodstock diets on reproduction of red seabream. Bulletin Japanese Society of Science Fisheries 50: 495-501.
- Watanabe, T., A. Itoh, C. Kitajima and S. Fujita. 1984b. Effect of dietary protein levels on reproduction of red seabream. Bulletin Japanese Society of Science Fisheries 50: 1015-1022.
- Watanabe, T., T. Koizumi, H. Suzuki, S. Satoh, T. Takeuchi, N. Yoshida, T. Kitada and Y. Tsukashima. 1985. Improvement of quality of red sea bream eggs by feeding broodstock on a diet containing cuttlefish meal or on raw krill shortly before spawning. Nippon Suisan Gakkaishi 51:1511-1521.

- Watanabe, T., M.-J Lee, J. Mizutani, T. Yamada, S. Satoh, T. T. Takeuchi, N. Yoshida, T. Kitada and T. Arakawa. 1991. Effective components in cuttlefish meal and raw krill for improvement of quality of red seabream *Pagrus major* eggs. *Nippon Suisan Gakkaishi* 57: 681-694.
- Watanabe, T and W. Miki. 1993. Astaxanthin; An effective dietary component for red sea bream broodstock. *In: Fish Nutrition in Practice* (ed. Kaushik, S.J. and Luquet), PINRA, Paris, pp 27-36.
- Weber, R. W. S and P. Davoli. 2003. Teaching techniques for mycology:20. Astaxanthin, a carotenoid of biotechnological importance from yeast and salmonid fish. *Mycology* 17:30-34.