NUTRITIONAL STUDIES ON SEBAE ANEMONEFISH, *AMPHIPRION SEBAE* BLEEKER 1853, WITH SPECIAL REFERENCE TO PROTEIN AND LIPID REQUIREMENTS

THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

Fish and Fisheries Science (Mariculture)

OF THE

CENTRAL INSTITUTE OF FISHERIES EDUCATION
(DEEMED UNIVERSITY)
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AUGUST 2004

Dedicated to

Clownfishes and their host anemones

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ICAR

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Certified that the thesis entitled **"NUTRITIONAL STUDIES IN SEBAE ANEMONEFISH, *AMPHIPRION SEBAE* BLEEKER 1853, WITH SPECIAL REFERENCE TO PROTEIN AND LIPID REQUIREMENTS"** is a record of independent bonafide research work carried out by **Mr. Binu Varghese** during the period of study from September 1999 to August 2004 under our supervision for the degree of **Doctor of Philosophy in Fish and Fisheries Science (Mariculture)** and the thesis has not previously formed the basis for award of any degree, diploma, associateship, fellowship or any other similar title.

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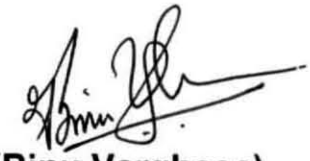

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ACKNOWLEDGEMENTS

I take this opportunity to express my deep sense of gratitude to my major advisor Dr. R. Paulraj, Principal Scientist and Head Division of Physiology Nutrition and Pathology & SIC, PGPM, for his inspiring guidance and immense encouragement throughout the period of study. I thank my co-advisor Dr. G. Gopakumar, Principal Scientist and Head Mariculture Division for his timely advice and keen interest, during my work at Marine Aquarium and Hatchery Complex, Vizhinjam. I also thank Shri. D. C. V. Easterson, Principal Scientist, Tuticorin, Dr. N. Kaliaperumal, Principal Scientist Mandapam, and Dr. M. Srinath, Principal Scientist & Head FRAD, for forming a supportive advisory committee.

I am grateful to Dr. Mohan Joseph Modayil, Director CMFRI for providing me necessary facilities for the smooth conduct of experiments. I thank Dr. A. P. Lipton, SIC and Dr. P.P. Pillai former SIC, Vizhinjam research centre of CMFRI for their timely help and support.

I am happy to express my sincere thanks to Dr. Manpal Sridhar, Senior Scientist Shri. P. Vijayagopal Scientist (SG), Dr. J. P. George Principal Scientist, Dr. M. K. Anil Scientist (SS), Shri. Bobby Ignatius Scientist, Dr. D. Noble Senior Scientist and Mrs. Mini Scientist for their kind interest and timely help.

I honestly thank Dr. Kajal Chakraborthy Scientist, for the help rendered in the biochemical and fatty acid analyses. Thanks are also due to Smt. Shylaja Senior Technical Assistant, for the amino acid analysis and Shri. Nandakumar Rao Technical Assistant, for his help in proximate analyses.

The help rendered by the staff of PGPM and PNPD is highly acknowledged. My sincere thanks are due to Mr. Chandrasekharan, Mr. Anil, Mrs. Vineetha and Mr. Joy.

I sincerely thank the Staff and contract labourers of Marine Aquarium and Hatchery Complex at Vizhinjam Research centre of CMFRI for their relentless support and co-operation.

I wish to express my gratitude to Dr. Horst Kaiser, Rhodes University, South Africa for his keen interest and timely help and Dr. Gerald Allen, Australia for confirming the species identity. My thanks are also due to researchers who supported the study by providing reprints and valuable suggestions to make this work a reality. Thanks are always due to Rosalie Shaffer, NMFS and Jean Collins, FAO for their assistance in getting important research articles. Facilities provided by the libraries of CMFRI at Kochi and Vizhinjam are greatly acknowledged.

My thanks are due to all my colleagues and friends, especially Unnikrishnan, Dr. Abraham, Dr. Sreeraj, Dr. Ajith Kumar, Krishnapriyan, Raja, Gopakumar, Dr. Satheesh, Joice, Anikuttan, Ramalinga, Balu and Radhika for their help and companionship.

I acknowledge the ICAR for awarding me with fellowship for my research programme. Finally I would like to thank all my well wishers especially my parents and sister for their constant encouragement and whole-hearted support.

सारांश

समुद्री जलजीवशाला मछली व्यापार में एनीमोन मछलियों या क्लाउन मछलियों को उच्चतम मूल्य मिल जाता है. इन प्रवाल मछलियों का वाणिज्यिक तौर से उत्पादन किए जाने से देश केलिए अत्यधिक विदेशी मुद्रा कमाया जा सकता है. सीबे एनीमोन मछली *एम्फीप्रिओन सीबे* ब्लीकर 1853 एक लोकप्रिय समुद्री जलजीवशाला मछली है. भारत में इस मछली का सफल रूप से पालतूकरण किया गया है. हाल ही में मछली पालन मुख्यतः जीवंत और ताज़ा आहार पर निर्भर पडता है. कभी कभी मछली के जीवन चक्र के विभिन्न स्तरों की पौष्टिक आवश्यकता के बारे में पर्याप्त जानकारी के बिना रूपाइत खाद्य भी उपयुक्त किया जाता है. वर्तमान अध्ययन में, मछली की प्रग्रहण स्थिति में ब्रूडस्टॉक, डिंभक और किशोर अवस्थाओं की पौष्टिकता पर ध्यान दिया जाता है. अंड मछलियों केलिए अनुयोज्य खाद्य पहचानने के उद्देश्य से छह प्राकृतिक और पांच रूपाइत खाद्यों का प्रभाव निर्धारित किया गया. परीक्षण किए गए प्राकृतिक आहार गभीर सागर चिंगट, कटल फिश, स्क्विड, प्रौढ़ शंबु, शंबु मांस तथा पोलीकीट कृमि और शंबु मांस का मिश्रण (1:1) थे. प्राकृतिक आहार में कटल फिश का मांस दिए जाने पर अंडजनन में अधिकतम अंडों की प्राप्ति हुई (1521±264 अंड), जिसके बाद गभीर सागर चिंगट देने पर भी अधिक अंड प्राप्ति (1300±445 अंड) हुई है. उपयुक्त किए गए पांच रूपाइत अर्ध नम आहारों में CBD_s आहार में 50% प्रोटीन और 10% लिपिड मौजूद है और यह आहार देने पर प्रति अंडजनन में उच्चतम अंड (2137±110) प्राप्ति हुई. इसके बाद CBD_2 आहार में 40% प्रोटीन और 20% लिपिड मौजूद है और यह आहार देने पर भी प्रति अंडजनन में अधिकाधिक अंड प्राप्त हुए (1683±436 अंड). अंड मछली को, दिन में एक बार यथेष्ट आहार देने पर अंडजनन की संख्या (416±28 अंड) दिन में दो बार यथेष्ट आहार देने पर प्राप्त अंडों (885±55 अंड) से कम देखा गया है. स्फुटनशाला में पालन किए गए डिंभक जिन को तीसरे हफ्ते से लेकर सूखा आहार दिया जाता है, 15 वां महीने (मछली की आयु) से लेकर जलजीवशाला में परिपक्व होकर अंड छोड देते हैं. खाद्यों पर किए गए परीक्षणों से व्यक्त हो गया कि सूक्ष्म शैवाल और रोटिफर तथा *आर्टीमिया* से खिलाए गए डिंभकों ने प्रत्येक खाद्य या रोटिफर, *आर्टीमीया* और मोइना का मिश्रण से खिलाए गए डिंभकों से भी अधिक अतिजीविततता दिखाई. खाद्य बदलने पर किए गए अध्ययनों से यह मालूम पडा कि डिंभकों को 3 हफ्तों के बाद जीवंत खाद्य से सूखे माइक्रोबाउन्ड पर्टिकुलेट आहार देने पर उनकी अतिजीविततता पर प्रतिकूल प्रभाव नहीं पडता है क्योंकि स्फुटन के बाद 30 दिन खाद्य बदलते का उचित समय है. 50% प्रोटीन और 6% लिपिड होने वाले परिशोधित और अर्ध-परिशोधित खाद्यों से किए गए परीक्षणों से व्यक्त हो गया कि किशोरों केलिए परिशोधित खाद्य की अपेक्षा अर्ध-परिशोधित खाद्य बेहत्तर है. अर्ध-परिशोधित आइसोकलोरिक खाद्यों को उपयुक्त करके किए गए प्रोटीन आवश्यकता परीक्षणों के आंकडों का विश्लेषण करने पर यह दिखाया पडा कि किशोरों को अधिकतम बढती केलिए आवश्यक प्रोटीन की दर 46.2% है और विशेष बढ़ती दर 44.9% है. पांच आइसोकलोरिक, आइसोप्रोटीक अर्ध - परिशोधित आहारों को उपयुक्त करके किशोरों में लिपिड आवश्यकता पर किए गए परीक्षणों से व्यक्त हो गया कि अधिकतम वृद्धि केलिए आवश्यक लिपिक 10.96% है बाल्कि विशेष बढ़ती दर 11.05% है. वर्तमान अध्ययन से क्लाउन मछली के डिंभक पालन, किशोर और अंडावस्था में रूपाइत खाद्यों की प्रभावकारिता साबित हुई है. अध्ययन के परिणाम इन उच्च मूल्य वाली मछलियों के वाणिज्यिक तौर के उत्पादन और समुद्री जलजीवशाला केलिए देशीय तल में खाद्य के निर्माण केलिए उपयुक्त करने लायक हैं.

ABSTRACT

Anemonefishes or clownfishes command higher price than most other pomacentrids in the marine aquarium fish trade. Commercial production of these reef fishes can generate valuable foreign exchange for the country. The sebae anemonefish, *Amphiprion sebae* Bleeker 1853, is one of the popular marine aquarium fish. At present rearing of the fish relies heavily on live and fresh diets, and formulated diets are seldom used due to inadequate knowledge of the nutritional needs of the different life-history stages. The present study focuses on the nutrition of broodstock, larvae and juvenile stages of the fish in captivity. In order to identify suitable diets for brood fishes the efficacy of six natural and five formulated diets was determined. The natural diets tested were deep sea shrimp, cuttlefish, squid, mature mussel, mussel meat, and a mixed diet of polychaete worm and mussel meat (1:1). Among the natural diets cuttlefish meat gave significantly higher number of eggs per spawning (1521±264 eggs; mean ± SD, n=9) followed by the deep sea shrimp (1300±445 eggs). Among the five formulated semi-moist diets used, diet CBD_5 with about 50% protein and 10% lipid, gave significantly higher number (2137±110) of eggs per spawning followed by diet CBD_2 (1683±436 eggs) which had 40% protein and 20% lipid. The number of eggs per spawning was found to decrease significantly when broodfish were fed *ad libitum* once a day (416±28 eggs) compared to those fed *ad libitum* twice daily (885±55 eggs). Hatchery reared larvae weaned to a dry diet from 3^{rd} week onwards matured and the broodfish spawned successfully in aquaria from 15^{th} month (age of fish) onwards. Feeding experiments using exclusive or combinations of rotifers, *Artemia*, and *Moina* showed better survival when the larvae were reared with micro algae and fed rotifers and *Artemia*. Weaning studies revealed that weaning from livefeed to a dry microbound particulate diet after 3 weeks (21dph) did not significantly affect survival, though the best age of weaning was found to be 30 days post hatching. Experiments with a purified and semi-purified diet having 50% protein and 6% lipid showed semi-purified diets to be better than purified diets. Analysis of data from protein requirement experiments using semi-purified isocaloric diets showed 46.2% as protein requirement for maximum weight gain in juveniles and 44.9% in terms of SGR. Lipid requirement experiment on juveniles using five isoproteic semi-purified diets showed 10.96% as lipid requirement for maximum weight gain and 11.05% in terms of SGR. The present study proved the effectiveness of formulated diets in rearing larvae, juveniles and broodstock of the clownfish. The results are significant with enormous application in commercial production of these high value fishes and in promoting indigenous aquarium feed manufacture.

ABBREVATIONS

AA	-	Arachidonic Acid
CUT	-	Cuttlefish meat
DHA	-	Docosahexaenoic Acid
DSP	-	Deep-sea prawn
dph	-	Days Post Hatching
EAA	-	Essential Amino Acids
EFA	-	Essential Fatty Acids
EPA	-	Eicosapentaenoic Acid
FAA	-	Free Amino Acids
FFA	-	Free Fatty Acids
HUFA	-	Highly Unsaturated Fatty Acids
MGD	-	Mature mussel meat with gonad
MSM	-	Mussel meat
MUFA	-	Monounsaturated Fatty Acids
NFE	-	Nitrogen Free Extracts
NEAA	-	Non-Essential Amino Acids
PC	-	phosphatidyl choline
PE	-	phosphatidyl ethanolamine
PUFA	-	Polyunsaturated Fatty Acids
PWM	-	Mussel meat : Polychaete worm (1:1)
SQD	-	Squid meat
TG	-	Triglycerides
Vg	-	Vitellogenin

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INTRODUCTION

1. INTRODUCTION

Aquarium keeping is an exciting, challenging and an important scientific hobby in the world. The trade of ornamental fishes and aquarium accessories is a multi-billion dollar international business that caters the requirements of hobbyists worldwide. The total wholesale value of ornamental aquatic trade is estimated to be around US$ 1 billion, and retail trade about US$ 3 billion (FAO, 2002). The international import of marine ornamental species is estimated to be between US$ 28 and 44 million and its retail trade between US$ 90-300 million (Wood, 2001). Since 1985 the aquarium fish trade showed an average growth rate of about 14 % per annum in terms of value (FAO, 1999).

More than 95 % of the marine aquarium trade depends upon wild caught fish from the coral reef ecosystems of the developing countries, especially those of the Southeast Asian countries like Indonesia and the Philippines. The trade began as early as in the 1930's and it is now well established with the spectacular advances in aquarium technologies and development of efficient methods of transportation. The consistent increase in demand for marine ornamentals in the international markets is leading to indiscriminate exploitation of the wild populations and the destruction of coral reef habitat. The destructive collection methods like dynamite fishing, cyanide fishing, coral notching, and the use of destructive gears have caused significant coral reef destruction and overexploitation of reef resources in many regions.

Governmental and non-governmental organisations worldwide are now actively involved in formulating and legalizing policies to protect the coral reef ecosystem, besides campaigning for sustainable and ecofriendly collection methods and healthy trade practices. These initiatives are already showing impact in the control of cyanide fishing in many countries, especially in the Philippines. Many nations have imposed regulations to restrict or ban completely the collection, importation, exportation, and sale of selected marine species (Tellock, 1996). Thus, hatchery seed production and farming are the only ecofriendly and sustainable alternatives to meet the demand of the marine

aquarium trade and replenishment of the wild populations, thereby to safeguard the fragile coral reef ecosystem.

At present, of the more than a thousand species involved in the global marine ornamental fish trade around hundred species are bred in captivity with a quarter in commercial levels. The commercial propagation of marine ornamentals began in the early 70's, and is now well established in the United States and certain nations of the European Union, as small and medium scale ventures. Despite this, the production of marine ornamental fishes through captive breeding and culture is negligible compared to the freshwater ornamentals, wherein more than 90% are commercially reared in captivity.

The ever increasing popularity of marine aquarium keeping has led to the active involvement of institutional research agencies and commercial entrepreneurs. At present much research attention is focussed on breeding and rearing of highly valued species. These increased efforts have resulted in experimental and commercial level production of many hitherto considered difficult species.

Anemonefish, popularly known as clownfish, belonging to the family Pomacentridae, is one of the most popular tropical marine ornamental fish in the global trade (Hoff, 1996), due to its small size, attractive colours, peculiar behaviour patterns, and the symbiotic association with sea anemones. There are about 28 valid species of anemonefishes, which are distributed around the coral seas of the tropical western, central, and south Pacific Oceans, the Indian Ocean, and the Red Sea (Allen, 1991). They are highly territorial with distinct social hierarchy and are associated with specific host anemones. Anemonefishes generally are protandrous hermaphrodites and monogamous. They are continuous spawners, and lay demersal capsular eggs attached to hard substratum. They also exhibit vigorous parental care till hatching.

Clownfishes are the first popular marine ornamental species successfully bred and reared in captivity (Wilkerson, 1998). They are considered to be one of the 'easiest' to breed among marine ornamentals and many captive-bred species are commercially available. Captive-bred clownfishes apart from

their ecological significance are generally hardier, disease resistant, easily adapt to captivity and readily accept prepared diets. The tank raised clownfishes are sold at a young age (5-7 months) thus increasing their life expectancy in the aquaria. On the other hand the wild collected ones are of unknown age because of their social hierarchy.

Clownfishes are usually reared using live and fresh diets, either alone or in conjunction with prepared diets. The larvae of clownfishes have been successfully reared by using livefeed such as rotifers, *Artemia*, copepods, cladocerans and wild zooplankton (Allen, 1972; Frakes and Hoff, 1983; Hoff, 1996; Johnston, 1997). The feeding behaviour (Coughlin, 1993, 1994), the influence of feeding on body condition (Green and McCormick, 1999), and the ontology of digestive and feeding system (Green and McCormick, 2001; Gordon and Hecht, 2002) are all important in clownfish larval nutrition. The weaning of fishes from livefeed to formulated diets (Gordon *et al.*, 1998) has great importance in the commercial hatchery production.

Like any farmed fish species, ornamental fishes do have typical nutritional requirements (Earle, 1995) which need to be effectively satisfied by formulating and developing cost effective feeds. However, there is no comprehensive information on the dietary requirements of marine ornamental fishes (Tacon and Haring, 1999). Eventhough meagre scientific information is available on their nutrition the industry is flooded with a wide variety of feeds, even specialized feeds for particular species. The prepared feeds available in the market are generally made by extrapolating the formulae of established marine fish feeds or even freshwater ornamental fish feeds and are not designed targeting the candidate species due to the paucity of nutritional information.

The feed formulation concepts for marine ornamental fishes are significantly different from those of farmed food fishes. Their food and feeding habits and their habitat must be accounted before formulating a feed. As they inhabit reef areas, with one of the richest biodiversity, the feed variety and feeding options are enormous and it makes the formulation rather difficult. The limitations imposed by the captive environment also must be carefully considered in the case of aquarium fish nutrition. The major focus is on maintaining fishes in

their natural brilliance in smaller confinements. Thus, formulation of cost effective feeds is central to the sustainable and ecofriendly development of marine ornamental fish industry.

The inshore areas of peninsular mainland and the islands of India are endowed with vast stretches of biodiversity rich coral reefs and rocky areas, that offer great potential for developing a sustainable and ecofriendly capture and culture industry. Our contribution towards the aquarium fish trade is negligible with an estimated export value of about 3 crores in 2001 (MPEDA, 2003). The remarkable progress in commercial exploitation made by our neighbouring countries, Srilanka and Maldives, with similar resources indicates at our huge potential. Developing an organised marine ornamental fish industry in India is one of the means to diversify the marine culture fisheries output and to stimulate the stagnant coastal economy and livelihood of coastal fisherfolk in reef-dominated areas. It can also provide a part time employment option to the fisherfolk and a lucrative avenue for the unemployed and underemployed people in both rural and urban coastal areas.

In India the mariculture of ornamental fishes got research attention only by the late 90's, with the successful breeding of clownfish, and the development of hatchery technology at the Central Marine Fisheries Research Institute (Gopakumar *et al.*, 1999, 2001; Ignatius *et al.*, 2001). Though research on marine ornamental fishes had made rapid strides in recent years, a great deal of refinement is still required to make this a viable industry. The development of exclusive and nutritionally complete feeds is of utmost importance in evolving farming and aquarium keeping technology packages of these fishes. Once we ensure the availability of suitable feeds and feeding regimes, particularly for broodfish and newly hatched larvae the industry will progress rapidly. Recognising these aspects, the present study was taken up on the nutrition of sebae anemonefish, *Amphiprion sebae* Bleeker 1853, with the following objectives:

- To determine the efficacy of selected broodstock diets and their influence on eggs and larvae.

- To determine the efficacy of selected larval diets on their performance.
- To determine the optimum weaning age of larvae from livefeed to formulated diets.
- To determine the optimum protein and lipid requirements of juveniles.

The thesis is organised in to six consecutive chapters as follows; Introduction, Review of Literature, Materials and Methods, Results and Discussion. A Summary of the thesis is presented after the Discussion, followed by the list of References cited.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. Broodstock Nutrition

In marine fish hatcheries unpredictable reproductive performance is the major limiting factor hindering production of seed at required levels. Broodstock performance is determined by biological factors such as age and size of the fish, diet, physiological status, genetic makeup, health *etc.* and abiotic factors such as water quality, photoperiod, season, rearing system *etc.* The diet provided to the broods considerably influences 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).

2.1.1. Diet and broodstock performance

The impact of broodstock diets on reproductive performance of various fish species has been well documented (Luquet and Watanabe, 1986; Bromage and Roberts, 1995; Bromage, 1998). The nutrition of broodstock directly influences the fecundity, nutrient reserves of the eggs and larvae, larval development and pose potential larval rearing problems like shock syndrome and early mortality (Davis, 2002). The research on broodstock nutrition is aimed at increasing the fecundity, and fortifying the eggs with essential nutrients which ultimately decides larval viability.

In fishes like 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; Nassour and Leger, 1989), with a vitellogenic period extending upto six months (Fremont *et al.*, 1984). Therefore, the broodstock needs to be well fed from several months before spawning to achieve better reproductive performance (Watanabe *et al.*, 1984c; Corraze *et al.*, 1993). However, in continuous spawners 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 seabreams, broods continue to feed during spawning and the nutrient

composition of egg and the larvae are greatly influenced by the diet within a short duration (Watanabe *et al.*, 1985; Tandler *et al.*, 1995).

2.1.2. Feed availability

The availability of adequate quantity of feed significantly influences the reproductive performance of fishes (Scott, 1962; MacKay and Mann, 1969; Kuznetzov and Khalitov, 1978). Feed availability reportedly affects the process of maturation in rainbow trout (Scott, 1962), goldfish (Sasayama and Takahashi, 1972), European seabass (Cerda *et al.*, 1994a), and Atlantic salmon (Berglund, 1995). Restricted feeding has been shown to affect the maturation and fecundity in fish (Scott, 1962; Springate *et al.*, 1985). Food shortage resulted in lowered fecundity through follicular atresia in the rainbow trout, *Salmo gairdneri* (Scott, 1962). Cerda *et al.* (1994a) observed that feeding the European seabass broodstock with half ration decreased the growth and spawning, and also produced smaller eggs and larvae compared to those fed full ration.

2.1.3. Influence of dietary protein and amino acids

The quantity and quality of dietary protein in broodstock diets have been reported to affect the reproductive output. In rainbow trout, broodstock fed high protein diets (48-49% protein and 16-17% lipid) produced more eggs with larger size than those fed diets with lower levels (36 or 42% protein, and 6 or 9% lipids) of protein (Smith *et al.*, 1979). Maturation and spawning in milkfish were achieved by feeding pellets with 42% protein (Lacanilao and Marte, 1980). An optimum protein level of 45% in the diet has been suggested for red seabream broodstock (Watanabe *et al*, 1984b). In European seabass a reduction of dietary protein from 51% to 34% resulted in a significant reduction in the broodstock performance (Cerda *et al.*, 1994); a similar trend was also observed in red seabream (Watanabe *et al.*, 1984a). In ayu, dietary tryptophan, precursor to serotonin, positively affected the maturation and increased the level of serum testosterone and spermiation (Akiyama *et al.*, 1996).

Broodstock diets formulated with cuttlefish meal and squid meal were found to be superior to fishmeal as protein source in seabreams (Watanabe *et al.*, 1984a; Harel *et al.*, 1992; Tandler *et al.*, 1995; Fernandez-Palacios *et al.*,

1997). Watanabe *et al.* (1991a) reported that the effective component of cuttlefish meal contained a non-fat soluble fraction. The superior performance of squid protein was related to its essential amino acids (EAA) composition, which resembles that of seabream egg protein (Harel *et al.*, 1995; Tandler *et al.*, 1995).

In teleosts, vitellogenin is secreted from the liver and gets incorporated into the growing oocytes, which then proteolytically cleaved into smaller yolk proteins (Specker and Sullivan, 1994). Harel *et al.* (1995) found that the essential amino acid (EAA) composition of broodstock diet significantly affects the plasma vitellogenin (Vg) level and its binding capacity to the oocyte membrane. In gilthead seabream vitellogenin binding capacity of the oocytes was affected by the diet, with low level of Vg-binding when fed a wheat gluten based diet and high level in those fed a squid meal based diets (Harel *et al.*, 1995). They attributed the positive effect of squid protein fraction to its balanced composition of EAA, which was similar to that of seabream egg protein, and suggested that dietary EAA may affect egg quality through the control of Vg synthesis and its uptake, without any apparent effect on egg EAA composition. Dietary essential fatty acids (EFA) on the other hand affect egg quality mainly through changing the egg EFA composition without any apparent effect on Vg synthesis.

2.1.4. Influence of dietary lipid and fatty acids

Lipids are the main constituents of broodstock diet that directly influence the composition of eggs and larvae (Watanabe, 1985). Lipids form an important membrane constituent and energy reserve in fish eggs. The fatty acid composition of eggs is affected by the fatty acid composition of diet (Mourente and Odriozola, 1990). Studies on egg lipid contents of yellowtail (Verakunpiriya *et al.*, 1996) and striped jack (Vassallo-Agius *et al.*, 1998) showed that a low lipid diet results in decreased egg lipids. In rabbitfish, *Siganus guttatus*, an increase in dietary lipid from 12% to 18% improved the fecundity and hatchability (Duray *et al.*, 1994). In contrast, higher lipid content in the eggs of turbot and European seabass caused a decrease in hatching rate (Devauchelle *et al.*, 1982).

Fatty acids are mobilized from the neutral lipid reserves of fish adipose tissue during gonadogenesis and bound to egg specific lipoprotein and transferred *via* serum to the eggs (Sargent, 1995). However, the mobilization of body stores of essential fatty acids (EFA) during spawning can probably compensate only minor deficiencies in the diet. The influence of fatty acids on egg quality was observed in Japanese flounder *Paralichthys olivaceus* and Atlantic halibut *Hippoglossus hippoglossus* (Parrish *et al.*, 1994). Marine fish oils rich in long chain highly unsaturated fatty acids (HUFA) were found essential for improving the egg quality in the European sea bass *Dicentrarchus labrax* (Bell *et al.*, 1997; Navas *et al.*, 1998; Bruce *et al.*, 1999). When *Pagrus major* was fed a diet with high percentage of EFA deficient corn oil the egg viability, hatchability and normal larvae were found significantly lower than the control (Watanabe *et al.* 1984a). Poor egg and larval quality of red seabream (Watanabe and Kiron, 1995) and gilthead seabream (Zohar *et al.*, 1995) were traced back to low levels of PUFA, phospholipids and carotenoids in the diet.

The n-3 HUFA are the major constituent of egg fatty acids and account for 30 - 40% in many marine species (Harel and Place, 1998). The n-3 HUFA, especially the docosahexaenoic acid (DHA), plays an important role in the development of larvae (Watanabe, 1993; Furuita *et al.*, 1996).

Tandler *et al.* (1995) found 15 mg of n-3 HUFA in the diet with 50-60% DHA as optimum for larval survival, growth and swim bladder inflation in gilthead seabream. Higher levels of n-3 HUFA (especially DHA) in broodstock diets were reported to increase the weight and osmotic shock resistance in fish larvae (Abi-ayad *et al.*, 1997). The quality of gilthead seabream eggs could be improved by increasing the dietary n-3 HUFA level to 1.6%; but higher HUFA concentrations may have a negative effect on larval survival (Fernandez-Palacios *et al.*, 1995). A low n-3 HUFA diet fed to *Sparus aurata* resulted in decreased fecundity, hatching rate and egg viability (Fernandez-Palacios *et al.*, 1995; Rodriguez *et al.*, 1998; Almansa *et al.*, 1999); and induced a 34% decrease in larval growth and a reduction in swim bladder inflation rate from 85% to 55% (Tandler *et al.*, 1995). However, excessive levels of n-3 HUFA in the broodstock diet (31.5 g kg^{-1} DW) resulted in lower fecundity and yolk-sac hypertrophy in

newly hatched *S. aurata* larvae and a level of 16 g n-3 HUFA kg^{-1} diet was recommended for improved spawning performance (Fernandez-Palacios *et al.*, 1995).

Selective retention of DHA has been observed during embryogenesis (Izquierdo, 1996) and starvation (Tandler *et al.*, 1989). This kind of preferential retention of DHA probably occurs mainly in the phosphatidylethanolamine fraction as is observed in the case of developing yolk-sac stage of Atlantic halibut (Ronnestad *et al.*, 1995). A remarkable decrease in DHA after hatching has been reported in red seabream, yellowtail, striped jack, and cod (Takeuchi, 1991; Watanabe and Kiron, 1995).

Dhert *et al.* (1995) did not observe any significant difference in reproductive output of *Scophthalmus maximus* when fed diets supplemented with n-3 HUFA. In red seabream (Watanabe *et al.*, 1984a) and gilthead seabream EFA deficient diets showed increase in number of lipid droplets in the eggs (Fernandez-Palacios *et al.*, 1997).

Diets containing 2% arachidonic acid (AA) significantly improved the egg and larval quality in Atlantic halibut as compared to those containing 0.5% or 1.0% AA (Bromage *et al.*, 2001). The n-3 to n-6 ratio and the optimum ratio between DHA:EPA:AA in the broodstock diet of fish needs further elucidation (Bell *et al.*, 1997).

2.1.5. Egg composition

Newly spawned marine fish eggs have a total amino acid content of 40 - 60% in their dry mass (Fyhn, 1989; Ronnestad and Fyhn, 1993; Thorsen *et al.*, 1993), which includes amino acids polymerised in the protein and other macromolecules and those of the free pool. In marine pelagic eggs, free amino acids (FAA) may constitute upto 50% of the total amino acids in contrast to the marine demersal and freshwater fish eggs with only 2 to 5% (Dabrowski *et al.*, 1985; Thorsen *et al.*, 1993). In demersal eggs the FAA pool is dominated by taurine, whereas in pelagic eggs it is dominated by leucine, valine, isoleucine, alanine and serine (Ronnestad *et al.*, 1999).

The FAA are utilized as energy reserves in many fish eggs (Fyhn, 1989, 1990), mostly in eggs that lack or have insignificant oil globules. Finn (1994) suggested that eggs with or without oil globules seem to differ in the catabolic substrate oxidation sequence with amino acids as the main source of energy for the former group and amino acids and neutral lipids equally in the latter group. Fish eggs with oil globules seem to use lipid as the major energy source while amino acids play a major role in species without oil globules (Ronnestad *et al.*, 1994).

The lipid composition of egg generally is related to the egg incubation period or the larval first feeding (Blaxter, 1969; Kaitaranta and Ackman, 1981). Leger *et al.* (1981) reported that lipoproteins (composed of lipovitellin and phosvitin) are directed into the yolk globule, while vitellogenin enters preferentially the oil globule.

The pattern of lipid classes and their composition in eggs were similar in seabream, cod, herring, yellowtail and haddock. The eggs contained more than 80% neutral lipids (Watanabe *et al.*, 1984d; Devauchelle *et al.*, 1988; Verakunpiriya *et al.*, 1996), with major components being sterol esters (SE), triglycerides (TG) and free fatty acids (FFA). The neutral lipids especially TG in the form of oil-globules is generally the most important energy reserve for the developing marine teleost eggs (Blaxter, 1969).

The polar lipids formed about 20% of total lipids with phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) being dominant components. The major fatty acids in the polar lipid fraction of eggs are the DHA and palmitic acid; followed by EPA and oleic acid. Phosphatidylcholine is the major phospholipid in fish eggs, and the fishes with short and long egg incubation period have low and high levels of triacylglycerols respectively (Sargent, 1995). PC acts as a source of metabolic energy and essential fatty acids for marine fish during oogenesis (Watanabe and Kiron, 1995; Watanabe, 1984d; Watanabe, 1982). Phospholipids and triacylglycerols in eggs have n-3 PUFA of 50% and 30% respectively and composed mainly of DHA and EPA in an approximate ratio of 2:1 (Sargent, 1995). In salmon both triacylglycerol and phosphatidylcholine were utilized in the embryo development up to swim up fry (Cowey *et al.*, 1985).

The fatty acids in both fractions are utilized as energy substrate by the eggs and larvae. PUFA plays an important role in the formation of cellular membranes. Arachidonic acid in small quantities occurs in the phosphatidyl inositol and is involved in the eicosanoid formation (Henderson and Sargent, 1985; Bell and Dick, 1990; Sargent *et al.*, 1994). Saturated and monounsaturated fatty acid levels were high in yellowtail eggs (Verakunpiriya *et al.*, 1996). In gilthead seabream EPA level in the egg was more sensitive to a dietary change in n-3 HUFA than DHA, and the EPA and arachidonic acid (AA) influenced the fertilization rate (Fernandez-Palacios *et al.*, 1995). According to Mourente and Odriozola (1990) the fatty acids composition of polar lipids was less affected by the broodstock diets.

DHA was typically reported to be high in marine fish eggs (Watanabe, 1993; Watanabe and Kiron, 1995), and it accumulates faster than EPA in the lipids of fish eggs. In sea bream egg lipids DHA accumulates over 70% faster than EPA or linolenic acid (LA) in response to a dietary increase (Harel *et al.*, 1994). The DHA serves as a metabolic energy reserve during the development of eggs; it is also found in the neural cell membranes and forms an integral part of brain and eye formation (Mourente *et al.*, 1991; Bell *et al.*, 1995). Therefore, the DHA/EPA ratio of eggs is extremely important in determining the egg and larval viability (Sargent *et al.*, 1997).

2.1.6. Influence of dietary carotenoids

Carotenoids are reported to influence the reproductive performance of marine fishes. Studies relating to the role of carotenoids on the reproductive performance in fish are limited to salmon, trout, seabream and yellowtail. In many fishes pigments accumulate in the oocyte during maturation and are often the direct reflection of broodstock diet (Craik, 1985). The active transfer of carotenoids from diet in continuous spawners or from the body of females in batch spawners to gonads and gametes suggests their importance in egg and larval development. Role of carotenoids in egg quality may be due to its strong scavenging actions on singlet oxygen and other free radicals, thus preventing reactive damage to other molecules, particularly PUFA (Watanabe and Kiron, 1995). The larval quality improvement may be due to Vitamin A activity

(increased visual acuity), photosensitization or enhanced immunity (Thompson *et al.*, 1994; Paripatananont *et al.*, 1999).

The significance of pigments in eggs of salmonids has been a topic of discussion for long (Craik, 1985). These pigments are mainly astaxanthin and lutein (Longinova, 1967; Kitahara, 1983). Salmonids absorb and deposit carotenoids in the muscle, and selective mobilisation and transfer of carotenoids from the flesh to skin and gonads were observed during maturation (Crozier, 1970; Sivtseva and Dubrovin, 1981; Kitahara, 1983). The mobilization and transportation of the accumulated astaxanthin or canthaxanthin is by the very high density lipoproteins (*e.g.* vitellogenin) or high density lipoproteins to the ovaries and finally to the larvae (Torrissen and Christiansen, 1995). During maturation about 18% of the total body carotenoids are mobilized to eggs in rainbow trout, and the composition of egg carotenoids is similar to that of flesh (Sivtseva, 1982). In farmed trout, Choubert *et al.* (1998) observed that dietary canthaxanthin is transferred to eggs and larvae. In chum salmon astaxanthin is transported by vitellogenin from the muscle or gastro-intestinal tract to the ovaries (Ando *et al.*, 1986). In eggs of chum salmon carotenoids are associated with the egg yolk protein, lipovitellin (Ando and Hatano, 1986).

Astaxanthin was also reported from the sperm of rainbow trout (Czeczuga, 1975). In Atlantic salmon the carotenoids, especially astaxanthin, are essential for broodstock diets. The fry from broods fed astaxanthin deficient diet exhibited high mortality and this can be avoided by feeding broods or fry with astaxanthin enriched diets (Christiansen *et al.*, 1995).

An improved reproductive performance and egg quality was observed in red sea bream fed diets supplemented with synthetic astaxanthin (Watanabe and Miki, 1993; Watanabe and Kiron, 1995). The recommended astaxanthin level in red sea bream was 20mg/kg diet (Watanabe and Miki, 1993). The inclusion of krill meal at 20% and above decreased the reproductive performance in yellowtails and it was suspected as due to astaxanthin overdose (Verakunpiriya *et al.*, 1997a). By supplementing astaxanthin in the diet Verakunpiriya *et al.* (1997b) found that the egg and larval quality were superior at 30mg astaxanthin, and that the egg quality decreased above this level. The

supplementation of pure astaxanthin (30 ppm) or paprika xanthophylls (30 ppm) supplementation improved the spawning performance in yellowtails (Verakunpiriya *et al.*, 1997b).

The carotenoids in yellowtail were metabolized to zeaxanthin or lutein and deposited in the eggs (Matsuno *et al.*, 1985; Verakunpiriya *et al.*, 1997b). Strong yellow coloured yellowtail eggs denoted the presence of high content of zeaxanthin and lutein (Verakunpiriya *et al.*, 1997a). They are incorporated into the eggs through dietary supplementation with astaxanthin (Verakunpiriya *et al.*, 1996). Zeaxanthin is reported to be absorbed in yellowtail at higher rates than the astaxanthin in goldfish (Hata and Hata, 1973). The intensity of egg pigmentation in yellowtail fed astaxanthin supplemented diet peaked at 30 ppm and above which it did not give better results. The final seed production was also found to be higher with diets supplemented with astaxanthin (Verakunpiriya *et al.*, 1997b).

In striped jack, *Psuedocaranx dentex* the broodstock fed with raw fish mix produced colourless eggs and carotenoids were not detected though the feed contained upto 7.7ppm astaxanthin (Vassallo-Agius *et al.*, 1998). Though the striped jack did not incorporate astaxanthin in eggs the supplementation of astaxanthin improved the egg production (Vassallo-Agius *et al.*, 2001a). The total egg production was higher in raw fish diet than the prepared diets, and among prepared diets the astaxanthin supplemented diets gave better result (Vassallo-Agius *et al.*, 1998; 2001a). Inclusion of spirulina did not enhance spawning performance, though it improved pigmentation and flesh quality (Okada *et al.*, 1991; Vassallo-Agius *et al.*, 1999). The β-carotene incorporation in egg was found to be a slow process in seabream (Watanabe *et al.*, 1984e) and yellowtail (Verakunpiriya *et al.*, 1996).

2.1.6. Importance of vitamins

The vitamins that are known to play an important role in fish reproduction are vitamin E, C and A. Vitamin E was found essential in the reproduction of ayu, common carp and rainbow trout (Watanabe, 1985). Supplementation of vitamin E in the broodstock diet improved egg viability and

hatchability in red seabream (Watanabe *et al.* 1991b), gilthead seabream (Fernandez-Palacios *et al.*, 1996) and salmonids (Watanabe, 1990). In Japanese flounder it increased the egg production, but the rate of fertilization and hatching were unaffected (Takeuchi, 1997). In Atlantic salmon the survival of eggs and fry were not observed with the α-tocopherol levels up to 370mg/kg (Eskelinen, 1989), while Waagbo *et al.* (1991) found reduced hatching success and survival of the fry with dietary levels below 50mg/kg.

Ascorbic acid also is important in fish reproduction (Dabrowski and Ciereszko, 2001), as it is involved in the biosynthesis of gonadal steroid hormones (Sandnes, 1984). According to Halver (1989), vitamin C acts synergistically with vitamin E and selenium to maintain activity of glutathione peroxidase and superoxide dismutase. Its influence on the systemic level of 17β - estradiol and thereby on the vitellogenin level was also suggested (Waagbo *et al.*, 1989). It also plays an important role in the biosynthesis of collagen in connective tissue. The ascorbic acid level in eggs before spawning is crucial for normal development of newly hatched larvae (Ikeda, 1985). Insufficient dietary levels have negative impacts on Japanese parrotfish *Oplegnathus fasciatus* (Ishibashi *et al.*, 1994) and sardine *Sardinops melanosticta* (Akiyama *et al.*, 1990). The gonadosomatic index of female parrotfish was correlated with dietary vitamin C level (Ishibashi *et al.*, 1994). Sandnes and Braekkan (1981) found increased ascorbic acid content in gonads of cod during maturation. In cod the ascorbic acid supplementation affected the free amino acid profile, egg strength, and neutral buoyancy but had no effect on the fertilization and survival rate (Mangor-Jensen *et al.*, 1994). Dhert *et al.* (1995) using ascorbyl palmitate observed the incorporation of vitamin C in the eggs of turbot, *Scophthalmus maximus*.

Vitamin A received little attention in broodstock nutrition when compared to vitamin C and E. Vitamin A deficiency, in the broodstock diet decreased the relative fecundity in bighead carp (Santiago and Gonzal, 2000). Supplementation of astaxanthin in the diet increased the vitamin A level in rainbow trout ovary (Guillou *et al.*, 1989) and gave better reproductive performance in Japanese flounder (Furuita *et al.*, 2003).

2.1.7. Dietary influence on male fish

Male sexual maturation, sperm production and its quality must also be optimised by the broodstock diets as it can be highly variable and is partly dependent on nutrition (Billard *et al.*, 1995). Data on sperm motility of marine species indicated a variation between 2 and 20 minutes of motility among the species studied (Billard, 1978; Suquet *et al.*, 1992). The fatty acid profiles of semen showed dependence on dietary fatty acid profiles in trout (Labbe *et al.*, 1993; Watanabe *et al.*, 1984c), yellowtail (Verakunpiriya *et al.*, 1996), and in European sea bass (Bell *et al.*, 1996); and are likely to play an important role for optimal sperm motility and duration. Vassallo-Agius *et al.* (2001d) investigated the effects of an n-3 HUFA deficient diet on eggs and sperm of rainbow trout and reported lower spawning quality from males fed a n-3 HUFA deficient diet. The importance of dietary ascorbic acid (Vitamin C) on male fish fertility has been demonstrated in rainbow trout (Dabrowski and Ciereszko, 1996).

2.1.8. Broodstock dietary sources

In general, commercial marine fish hatcheries are heavily dependent on fresh diets of marine origin. Fresh marine products along with commercial maturation diets are also used in certain hatcheries. The fresh diets commonly used are squid, cuttlefish, bivalve meat, krill and other small crustaceans, fish gonads, polychaete and other worms, and enriched products. Formulated commercial maturation diets are available only for a few species. Most of these diets are prepared by using high quality nutrient sources to meet the requirements of essential fatty acids and amino acids and the additives which are expected to boost the broodstock performance. The major constraints with these fresh diets are disease transmission, nutrient imbalance, higher cost and the difficulties in production and storage.

The ultimate goal of the broodstock nutrition studies is to optimise dietary nutrients to maximize egg production and quality. To achieve this it is essential to understand the factors influencing initial recruitment of oocytes into the pool of maturing eggs. A properly formulated broodstock diet must satisfy requirements for higher fecundity as well as egg quality for an optimum spawning

performance. 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.

2.2. Larval Nutrition

Larval feeding is a major challenge in marine fish hatcheries and the lack of suitable livefeed as first feed has hindered the development of many potential aquaculture operations. The quantification of larval nutritional requirements is difficult, and generally, the larvae are assumed to have requirements similar to the composition of yolk (Heming and Buddington, 1988). Most larvae hatch with considerable endogenous nutrient reserves in their yolk-sac; which significantly influence initial survival. In larvae with larger yolk-sac (salmonids and halibut), the period of endogenous nourishment lasts several weeks. But, in most species yolk resorption occurs quite rapidly and their further survival depends on availability of suitable prey. The onset of exogenous feeding is critical to most marine fish larvae where transitional period between absorption of endogenous reserves and first feeding is short (Bagarinao, 1986; Houde and Zastrow, 1993). This shift in early stages is often characterized by higher mortalities in fish larvae (Balon, 1986; Sarasquete *et al.*, 1995). Deficiencies in the supply and assimilation of nutrients at this stage affect the growth and development irreversibly. The development of exclusive diets for rearing marine fish larvae from initial feeding is currently an intensely researched area in mariculture.

The growth rate of fish larvae is the highest among all vertebrates reported (Weiser, 1994), and this faster growth rate is suggested to be attained by higher protein resynthesis at minimal energy cost (Weiser, 1994; Weiser and Medgyesy, 1990). In newly hatched larvae, proteins form the major yolk constituents used for energy and development of body tissues (Cowey and Walton, 1989). In fish eggs and larvae, depletion rates of both essential and non-essential amino acids do not exhibit any sparing or selectivity (Ronnestad and Fyhn, 1993), and therefore EAA needs to be supplied in larval diets to compensate the metabolic loss. Finn (1994) suggested that eggs with or without oil globules seem to differ in catabolic substrate oxidation sequence, with amino

acids as the main source of energy in the former group and amino acids and lipids in equal proportion in the latter group.

Marine fish larvae are usually small, fragile and physiologically underdeveloped. The developmental status of larval digestive system dictates the ability of larvae to digest and assimilate different types of feeds. In many cases the mouth opens few days post hatching; moreover, the mouth gape restricts the size of ingestion. The digestive system in most larvae is underdeveloped on hatching and is expected to be fully functional only after metamorphosis (Kolkovski *et al.*, 1993; Kolkovski, 2001). The system primarily consists of a tube like alimentary canal, liver and pancreas. Digestion occurs mostly in the midgut and hindgut regions. The first feeding larvae of many species lack functional stomach, acid secretion, and peptic enzyme activity until metamorphosis (Munilla-Moran and Stark, 1989; Miwa *et al.*, 1992; Bisbal and Bengston, 1995).

The exogenous enzymes of livefeed and their contribution to larval digestion process are reported (Lauff and Hofer, 1984; Munilla-Moran *et al.*, 1990). Kolkovski *et al.* (1993) are of the opinion that these enzymes play an important role in activating the endogenous enzymes by cleaving the zymogens. Lazo (1999) observed that the exogenous enzymes play a minor role in digestion in red drum, which has a functional digestive system at first feeding and attributed the low survival and growth to failure of microdiets in stimulating ingestion, digestion and assimilation of nutrients as required.

At present, successful hatchery production of marine fish seed relies almost exclusively on livefeed. Rotifer and *Artemia* are the most commonly used livefeed; copepods are also gaining importance in marine hatcheries. Nutrient imbalance or deficiency is the major difficulty and so no single livefeed is nutritionally complete for larvae. This adds to the cost of production by requiring enrichment with various nutrient sources. As the fish larvae have higher metabolic and gut evacuation rates a continuous feed supply is needed for the normal growth and survival. The production costs of livefeed even reaches up to 50% of the operating costs of hatcheries. The nutritional profile of the livefeed varies with source, age and culture techniques (Sorgeloos *et al.*, 1986; Leger *et al.*, 1986).

The addition of microalgae to the larval rearing tank (green-water technique) is widely practised in marine fish hatcheries (Næss *et al.*, 1990; Reitan *et al.*, 1993; Oie *et al.*, 1997). This technique is assumed to have several advantages like, light attenuation or shading, provide contrast for feeding, maintenance of nutritional quality of prey, may increase appetite and have growth promoting effects (Næss *et al.*, 1990; Reitan *et al.*, 1993; Oie *et al.*, 1997). It has also been proposed that bacteria controlling function of the microalgae are more important than their nutritional effects (Stottrup *et al.*, 1995). Maintenance of nitrogen balance and oxygen are also proposed (Tamaru *et al.*, 1994). The microalgae widely used for this purpose are *Chlorella*, *Nannochloropsis* and *Tetraselmis*.

The success of marine fish hatcheries involving smaller larvae is credited to rotifers which serve as prey at first feeding. The small, slow moving and nutritious rotifers provide excellent feed for the small fish larvae with poor visual acuity and movement. The nutritional profile, especially the fatty acids, of rotifers can be easily manipulated before feeding (Lubzens, 1987). *Artemia* feeding follows the rotifer feeding stage and is generally continued till the larvae attain weaning stage.

Nutritional enrichment of livefeed to make its nutrient profiles similar to the requirements of the species concerned is the prime research area in larval nutrition. The filter-feeders like rotifers and *Artemia* are used for bioenrichment because of easiness to maintain and enrich. Microalgae, lipid emulsions, fish oils, microparticles, microcapsules with lipids and vitamins are generally used for enrichment. There are two kinds of enrichments, the long term technique combines growth and n-3 HUFA enrichment during production of livefeed, and in short term the exposure to high concentration of nutrient for short duration (<24 h) after harvest (Oslen *et al.*, 1993). The nutritional value of *Artemia* is greatly affected by the utilization of metabolic reserves during the non-feeding nauplii (instar I) stage. So the feeding is done immediately after hatching. The instars II onwards are generally enriched to maintain high nutritional quality before feeding to larvae.

The dietary n-3 HUFA influence on coral reef damselfish, *Acanthochromis polyacanthus*, was studied using *Artemia* enriched with squid or cod-liver oil and the enrichment yielded higher larval survival than unenriched nauplii (Southgate and Kavanagh, 1999). When seahorse larvae were fed n-3 HUFA enriched *Artemia* significant correlation was found between survival of fry and the dietary EPA and DHA contents (Chang and Southgate, 2001). They also observed a significant relation between weight gain and DHA content and indicated the possibility of limited biosynthesis of DHA from a dietary precursor, as reported for turbot (Linares and Henderson, 1991). The co-feeding of live diet in conjunction with dry diet yielded improved growth in *Lates calcarifer* (Walford and Lam, 1993), turbot (Munilla-Moran *et al.*, 1990) and in red drum (Holt, 1993).

The concept of absolute requirement for livefeed to rear marine fish larvae has been changing with the advances in the field of larval nutrition. In seabass, significant growth and survival was obtained by feeding larvae with compounded diet from 20 dph (days post hatching) onwards (Zambonino-Infante *et al.*, 1997). Cahu *et al.* (1998) reported 35% survival for the seabass larvae fed exclusively compounded diet from first feeding to 28 dph. Similar studies were also reported in *Sparus aurata* (Fernandez-Diaz and Yufera, 1997) and *Pagrus major* (Takeuchi *et al.*, 1998).

The microparticulate diets used for larvae are microencapsulated diets (MED), microbound diets (MBD) and microcoated diets (Kanazawa, 1986; Paulraj, 1993). They have been tested with varying levels of success on fish larvae (Teshima *et al.*, 1982; Walford *et al.*, 1991; Lopez-Alvarado *et al.*, 1994) for nutrition studies and for commercial production, mostly as weaning diets. The efficiency of MED is affected by the capsule wall which impairs proper digestion in fish larvae (Southgate and Lee, 1993) resulting in poor growth and survival (Teshima *et al.*, 1982; Walford *et al.*, 1991). In MBD the nutrients are held within a gelled matrix or using a binder (Lopez-Alvarado *et al.*, 1994). The binders commonly used are agar, carrageenan, alginate, zein and gelatin.

Studies with microparticulate diets in marine fish larvae often resulted in poor growth and survival, and lead to increased deformities (Le Ruyet *et al.*, 1993). Formulating complete diets for larvae remains still unresolved. Even

partial replacement of livefeed or early weaning can result in considerable cost saving in hatchery production (Jones *et al.*, 1993; Lavens *et al.*, 1995). Inclusion of digestive enzymes in the diet has significantly improved the nutrient utilization and performance of larvae. Kolkovski *et al.* (1991) reported 30% increase in assimilation by *S. aurata* larvae when fed microbound diet incorporated with commercially available pancreatic enzymes. Inclusion of pre-hydrolysed protein in formulated diets has given better results. Co-feeding is generally practised in commercial marine fish hatcheries with considerable reduction in cost of seed production. The actual nutrient requirements can only be worked out after the development of satisfactory standard microdiets.

Particle size is one important factor determining ingestion of microdiets. Small feed particles are difficult to detect and often cause pollution to the rearing system and larger particles cause blockage of the digestive tract (Walford *et al.*, 1991). The capability of larvae to distinguish microdiets of different sizes has been observed in seabream (Fernandez-Diaz *et al.*, 1994). A diet formulated with fishmeal, shrimp meal, squid meal, and lactic yeast was used for 24 dph seabass larvae (Zambonino Infante and Cahu, 1994).

Protein requirement studies on the larvae are scarce. Peres *et al.* (1996) using diets with graded levels of protein found that 50% dietary protein produces best growth for seabass larvae from 15 dph to 35 dph. Cuzon *et al.* (1989) reported 50% dietary protein as optimum level for *Lates calcarifer*. The higher protein requirements of larvae compared to juveniles was attributed to higher growth rate and high utilization of protein as energy source in larvae (Dabrowski, 1986).

Lipid levels in larval diets are generally high and are in the range of 25-37% when *Artemia* is used as diet (Koven *et al.*, 1992; Furuita *et al.*, 1998) and was only 18% in compounded diet used for *S. aurata* (Salhi *et al.*, 1999). Brinkmeyer and Holt (1995) using graded levels of menhaden oil obtained best growth in red drum larvae with 18% lipid in the diet. In European seabass larvae growth and survival were directly related to the dietary lipid content and the best result was obtained with a diet containing 30% lipid (Zambonino Infante and Cahu, 1999).

Phosphatidylcholine (PC) catabolism has been observed in the larvae of halibut, plaice and cod, whereas phosphatidylethanolamine (PE) was synthesized (Rainuzzo *et al.*, 1992). PC was reported to be used as a source of metabolic energy at the developmental stage (Fraser *et al.*, 1988). PC also might have been used as a source of inorganic phosphate in the synthesis of nucleic acids and choline in neurotransmission (Tocher *et al.*, 1985). The synthesis of PE is supposed to be from the fatty acids released during PC catabolism (Fraser *et al.*, 1988; Rainuzzo *et al.*, 1992).

It has been established that levels of n-3 HUFA in the livefeed can affect growth and survival of fish larvae in a number of species (Izquierdo *et al.*, 1989; Takeuchi *et al.*, 1990; Rodriguez *et al.*, 1993). The n-3 HUFA requirement of marine fish larvae ranges from 0.3 to 39 g kg^{-1} feed on dry basis. Many researchers have observed a decrease in swimming and feeding activities in EFA deficient larvae, which usually are seen floating on the water surface. In gilthead seabream, n-3 HUFA deficient rotifers affected the swimbladder inflation rate (Koven, 1991).

The lack of information on the optimum EFA requirement of larval stages is one of the major constraints in the rearing of marine fish larvae. The EPA:DHA ratio and their individual contributions are important in larval nutrition. The larvae of marine fishes are known to require HUFA of n-3 series such as EPA and DHA (Owen *et al.*, 1975; Watanabe, 1982; Sargent *et al.*, 1989). The essentiality of DHA over EPA has been suggested for the marine fish larvae (Watanabe, 1993). Rodriguez (1994) reported improved growth and survival of gilthead seabream larvae fed rotifers enriched with higher levels of DHA than EPA.

The functions of EPA and DHA during the early stages of marine fish larvae are different (Watanabe, 1993). An excess in EPA over DHA causes an imbalance in the structural composition of phospholipids and affect the normal growth and larval quality. The specific role of DHA in the development of neural tissues in brain and retina has been well documented (Mourente *et al.*, 1991: Bell *et al.*, 1995). The higher content of DHA in developing larvae is obvious since head forms the significant part of the body mass (Rainuzzo *et al.*, 1997).

In *Scophthalmus maximus*, larval pigmentation was affected by lower DHA:EPA ratio in the total lipid fraction of the larvae (Rainuzzo *et al.*, 1994; Reitan *et al.*, 1994). In gilthead seabream larvae, dietary arachidonic acid induced better survival and growth (Bessonart *et al.*, 1999). Sargent *et al.* (1999) suggested that both the concentration and ratio of all the three essential HUFA are important in larval marine fish nutrition and, while the optimum ratio would be species specific, the range would probably be around 10:5:1, for DHA/EPA/AA, respectively.

Lipid depletion occurs rapidly in starved larvae after exhaustion of the endogenous supply. This has been established in plaice (Ehrlich, 1974; Rainuzzo, 1993), gilthead seabream (Koven *et al.*, 1989) and turbot (Rainuzzo *et al.*, 1994), where rapid decrease in lipid and dry weights were observed. According to Sargent *et al.* (1989), regardless of tissue or species the triglycerides are the prominent form of reserve lipid which is mobilized before phospholipids during starvation. In starved larvae of *Sparus aurata* fatty acids were lost according to the pattern n-6 > n-9 > n-3 (Koven *et al.*, 1989).

2.3. Juvenile Nutrition

2.3.1. Protein requirement

The success of commercial aquaculture relies heavily on development of cost-effective and eco-friendly diets. As the feed cost contributes about half of total operational expenditure in many cases (DeSilva and Anderson, 1995), a marginal improvement in feed and feeding can provide great returns to the entrepreneur. The physiological nutrient requirements can be defined as the lowest dietary level resulting in a satisfactory physiological response (Barrows and Hardy, 2001). The available information on the nutritional requirements indicates the essentiality of forty-odd nutrients (Wilson, 1991; NRC, 1993).

Protein is required in the diet to provide amino acids that are needed for maintenance, growth, reproduction and repletion of tissues. For many species protein fraction of the diet approximates 40 to 50% and accounts for about half of the total feed cost. Protein being the most expensive component of feed must therefore be optimally incorporated to give maximum growth and profit.

Protein requirements of fishes are higher than the terrestrial animals because of their ability to eliminate nitrogenous wastes through the gills (Cowey and Walton, 1989). The dietary protein in excess to that of growth is often utilized for energy in fishes (Cowey, 1979).

The protein requirement of fishes is affected by various biotic and abiotic factors. Biotic factors like species, age, size, protein quality, feeding rate, stocking density, natural productivity, dietary energy, hereditary characteristics, health and abiotic factors like temperature and salinity are found to affect the requirement (DeLong *et al.*, 1958; Zeitoun *et al.*, 1973; NRC, 1973, 1993; Dias *et al.*, 1998).

Information on the protein requirement is mostly available for the carnivorous species, as they constitute the majority among species under mariculture. The dietary protein requirements for salmonids were the most researched area during the initial phase of fish nutrition studies. The protein requirement was first investigated in chinook salmon (DeLong *et al.*, 1958). The dietary requirement for the Atlantic salmon *Salmo salar* was found to be around 44% (Austreng, 1977).

Dietary protein requirement varied from 45 to 55% for the seabreams (Sabaut and Luquet, 1973; Yone, 1976; Santinha *et al.*, 1996; Vergara *et al.*, 1996), 40 to 50% for European and Asian seabass (Sakaras *et al.*, 1989; Wong and Chou, 1989; Catacutan and Coloso, 1995; Perez *et al.*, 1997; Dias *et al.*, 1998), 35 to 45% for red drum (Daniels and Robinson, 1986; Serrano *et al.*, 1992), and 40 to 50% for groupers (Teng *et al.*, 1977,1978). Dietary protein requirements of flatfishes were estimated to be higher than other fishes in culture as it ranged from 56 to 74% (Aksnes *et al.*, 1996; Daniels and Gallagher, 2000).

Contrary to our expectations, the dietary protein requirement studies in marine fishes show that omnivorous and herbivorous fish species also have relatively higher protein requirement. The dietary requirement for milkfish juveniles is estimated to be around 40 - 43% (Lim *et al.*, 1979; Coloso *et al.*, 1988), and rabbit fish (*Siganus guttatus*) larvae 53 - 59% (Hara *et al.*, 1986), and juveniles 33 and 46% (Soletchnik, 1984; Juario *et al.* 1985; Parazo, 1990).

Though many studies have been conducted to optimize the dietary protein requirement of fishes, uncertainties are still prevailing due to the differences in values reported for the same species. These variations are mainly due to the differences in the experimental conditions and methodologies involved.

2.3.2. Lipid requirement

Lipids are added as a source of essential fatty acids, phospholipids and energy in aquafeed formulations. Dietary lipid partly spares the expensive protein as an energy source without affecting growth, and functions as an important structural component in the cellular and sub-cellular membranes and help maintain their flexibility and permeability. Lipids also serve as precursor for eicosanoids and improve the flavour and texture of diets.

Lipid requirement of fishes have been extensively reviewed and studied (Cowey and Sargent, 1977; Halver, 1975; Hashimoto, 1975; Watanabe, 1982; Sargent *et al.*, 1997; Rainuzzo *et al.*, 1997). The incorporation of lipids in feed can spare protein for growth in fishes (Takeuchi, 1991 Dias *et al*, 1998; Lanari *et al.*, 1999). The dietary lipid requirement of juvenile marine fishes was estimated to be between 10 and 18% (Yone, 1976; Sakaras, 1989; Tucker *et al.*, 1988; Lin and Shiau, 2003). Even dietary level up to 30% improved feed and protein utilization in salmon (Johnsen and Wandsvik, 1990; Hardy, 2000) and levels as high as 35% were also reported (New, 1996).

Fishes, such as rainbow trout, ayu, eel and tilapia, can convert linolenic acid to EPA and DHA (Owen *et al*, 1975; Kanazawa *et al.*, 1980). Chain elongation of short chain fatty acids to PUFA takes place in the endoplasmic reticulum and desaturation in cell microsomes (Harel and Place, 1998). However, marine fishes have limited ability to chain elongate and desaturate shorter chain fatty acids (Owen *et al.*, 1975; Sargent *et al.*, 1993). The marine fish studied to date are barely able to convert 18:3n-3 to EPA and DHA (Sargent *et al.*, 1989, Sargent, 1995). This metabolic insufficiency has been identified as a relative deficiency in one or two enzymes in the conversion pathway from 18:3n-3 to EPA, *i.e.* the C_{18} to C_{20} elongase multienzyme complex (Ghioni *et al.*, 1999) or the Δ5- fatty acid desaturase (Tocher and Ghioni, 1999). However, either or both

of these enzyme deficiencies mean that in addition to a block in the conversion of 18:3n-3 to EPA, there will be a similar inability to convert 18:2n-6 to arachidonic acid (Bell and Sargent, 2003). Though, the bioconversion of EPA to DHA has been established in many marine species in small quantities, DHA needs to be supplemented in the diets of early stages (Sargent *et al.*, 1993).

Lipid requirements in fishes were found to vary with dietary source (Alava and de la Cruz, 1983; Camacho and Bien, 1983; Paulraj and Thirunavukarasu, 1987). In general, coldwater fishes require more n-3 HUFA while the warm water fishes require n-3 or n-6 or both highly unsaturated fatty acids (HUFA). Fishes contain high levels of HUFA in their body tissues and consequently have high nutritional requirements for these fatty acids (Sargent *et al.*, 1993, 1997).

Essential fatty acid requirement of all the fishes studied so far falls within the range of 1 - 2% of diet (Castell, 1972; Watanabe *et al.*, 1974; Deshimaru *et al.*, 1982; Wanakowat *et al.*, 1993; Sargent *et al.*, 1997; Takeuchi, 1997; Hasan, 2001). According to Takeuchi and Watanabe (1976) the influence of n-3 HUFA varies with the overall lipid content in the diet and must be expressed as percent of lipid.

An imbalance in EFA results in poor viability and higher mortality. Atlantic salmon fed 2% methyl esters of n-3 fatty acids had lower weight gain than those fed 1% showing a suppression of weight gain by excess n-3 PUFA (Ruyter *et al.*, 2000), similarly growth reduction was also reported in red drum (Lochmann and Gatlin, 1993). The higher dietary HUFA also affects fish growth as they are readily oxidized by reactive oxygen species to lipid peroxides (Porter *et al.*, 1995) and tend to be less available for energy (Murata, 1983).

In juvenile *Sparus aurata*, the EPA:DHA ratio of 1:2 promoted better growth (Ibeas, 1996). The EPA and DHA requirement of seabream juveniles were 1 and 0.5% respectively (Takeuchi *et al.*, 1990). The superiority of DHA to EPA in early stages of marine fishes is well-documented (Watanabe *et al.*, 1989 a, b).

Studies on the requirement for arachidonic acid (AA) in juvenile turbot fed diets containing 15% lipid, comprising a mixture of hydrogenated coconut oil and oleic acid and 1% pure AA, DHA or mixtures of these two HUFA showed that turbot fed AA as the only HUFA (0.78% of diet dry weight) showed higher growth and survival (Castell *et al.*, 1994). This evidenced the arachidonic acid as an essential fatty acid for the growth and development of juvenile marine fish. In gilthead seabream inclusion of 1% AA in larval microdiet resulted in improved growth and survival (Bessonart *et al.*, 1999). Koven *et al.* (2001) showed that gilthead seabream larvae fed rotifers enriched with AA showed significantly lower mortality when subjected to handling stress compared to those without an AA supplement.

2.4. Aquarium fish nutrition

2.4.1. Freshwater ornamental fish

Even though aquarium keeping has a long history nutritional studies on aquarium species are sparse (Shim and Chua, 1986; Shim and Ng, 1988). Recently, Sales and Janssens (2003) compiled most of the information on the nutrient needs of these fishes. The traditional feeds of ornamental fishes, such as the livefeeds and fresh diets, are often nutritionally deficient. Feeding of aquarium fishes at present is mostly based on the feeds developed for food fishes. The concept of aquarium fish nutrition is rather limited to maintaining colour and maturation in captivity.

In guppy, *Poecilia reticulata*, the dietary protein level for sustaining maximum growth and best feed conversion was found to be 30% with a diet composed of fishmeal and casein as protein source and the reproductive performance was significantly high at 30% and 40% protein (Shim and Chua, 1986). In dwarf gourami, *Colisa lalia* the maximum growth and fecundity were reported with a 45% protein diet (Landesman, 1988). In pearl gourami, *Trichogaster leeri*, the optimum growth was achieved with diets having protein in the range 26 to 36% (Degani and Gur, 1992) and in tin foil barb a diet with 41.7% protein produced better growth performance (Elangovan and Shim, 1997). The optimum protein requirement reported for juvenile goldfish was 29% (Lochmann and Phillips, 1994), and for larvae a much higher level of 53% (Fiogbe and

Kestemont, 1995). In redhead cichlid, *Cichlasoma synspilum*, Olvera-Novoa *et al.* (1996) estimated 40.81% as dietary protein requirement. Chong *et al.* (2000) reported 45 to 50% as optimum dietary protein requirement in Discus, *Symphysodon* sp. In the juvenile swordtail, *Xiphophorus helleri*, diet with 45% protein and 6% lipid provided better growth and FCR (Kruger *et al.*, 2001).

The optimal dietary protein to energy ratio for golden shiners and goldfish is estimated to be 103 mg protein $kcal^{-1}$ (Lochmann and Phillips, 1994). Pannevis and Earle (1994) observed that the energy and protein need for maintenance of goldfish decrease with increasing fish size. Goldfish showed higher survival rate when fed with diets containing 7 to 13% lipid (SRAC, 1998).

Vitamin nutrition is another area where little work has been carried out. In Oscar, *Astronotus ocellatus*, ascorbic acid concentration of 25 mg kg^{-1} diet was found to prevent growth reduction and deficiency signs in juveniles (Fracalossi *et al.*, 1998). A 360mg kg^{-1} ascorbic acid supplemented diet was found to improve the tissue storage of ascorbic acid in angel fish juveniles (Blom *et al.*, 2000).

Carotenoids attracted much research attention in ornamental fishes due to their importance in skin pigmentation and appearance. Carotenoids and their complexes with proteins and lipids (carotenoproteins and carotenolipoproteins) are responsible for the wide range of colours in fishes. The existence of ornamental fish industry owes greatly to the hues and patterns of fish skin provided by pigments. Animals, including fishes, are unable to synthesize carotenoids *de novo* (Goodwin, 1984). Fishes get these pigments through their diet and deposit in their skin, flesh, gonads *etc.* with or without transformation. The concentration and nature of carotenoids in fishes were also reported to vary with geographical and environmental conditions (Gilchrist and Lee, 1972).

Earlier, the bright colouration of fishes was considered as a genetic trait and physical cell structure, however now it is well established that diet plays the most important role in maintaining it. This fact is evidenced by the loss of intense colouration within few days in captivity. In order to avoid this problem now

feeds are produced by incorporating pigments or pigment rich ingredients for the aquarium fish feeding. The pigment concentrations are also affected by factors like the duration of dietary treatment, pigment concentration in diet, lipid level (Torrissen, 1985), genetic background, the stage of sexual maturation (Torrissen and Naevdal, 1988) *etc.*

Dietary supply of carotenoids can improve the skin pigmentation and market value of ornamental fishes. The pigmentation of goldfish and koi is improved by the addition of carotenoids and these fishes are found to be capable of metabolising zeaxanthin to astaxanthin (Hata and Hata, 1972). However, goldfish lacks the ability to metabolise lutein, and have limited ability to convert β-carotene to astaxanthin (Hata and Hata, 1972). Choubert (1979) used blue green algae as a source of pigmentation for Koi carp. Boonyaratpalin (1975) reported increased skin pigmentation in tiger barbs, *Barbus tetrazona* when fed diets containing carotenoids from shrimp meal, marigold petal meal, and annatto seed extract. The effect of various pigment sources on the colouration of pearl gourami, *Trichogaster leeri* were studied (Fey and Meyers, 1980; Meyers and Thibodeaux, 1983). In pearl gourami the pigmentation of integuments and fins were found to improve by the addition of crawfish meal or extracted astaxanthin in vegetable oil carrier in the diet (Meyers and Thibodeaux, 1983).

In goldfish, *Carassius auratus*, the optimum level of astaxanthin for intense colouration was found to be 36-37mg/kg diet and the supplementation significantly improved the survival rate (Paripatananont *et al.*, 1999). In red velvet sword tail (*Xiphophorus helleri*) rainbow fish (*Pseudomugil furcatus*) and topaz cichlids (*Cichlasoma myrnae*), the intensity of colouration significantly improved when fed a diet containing 1.5-2 % of a carotenoid rich strain of *Spirulina platensis* and 1% of *Haematococcus pluvialis* for 3 weeks (Ako *et al.*, 2000).

2.4.2. Clownfish and other marine ornamental fishes

In spite of the economic importance of clownfish in marine ornamental fish trade, there has been no research effort for the development of cost-effective feeds. There is no published information yet on the nutritional requirements of clownfishes. Presently, culturists and hobbyists heavily rely on

the live and freshly prepared diets. Compared to live and fresh diets dry feeds are easier to produce and friendlier to the user and the used, when properly formulated.

When compared to most other marine fish larvae, clownfish larvae hatch out in an advanced stage. The larvae hatch out with an opened mouth, and start feeding from the first day. The yolk and oil globule reserves are rather limited, and complete absorption of yolk-sac was observed 3 dph (days post hatching) in *Amphiprion melanopus* and *A. percula* (Green and McCormick, 2001; Gordon and Hecht, 2002). The clownfish larvae have well developed eyes and visual acuity upon hatching. The feeding and search behaviours of clownfish larvae (Coughlin *et al.*, 1992; Coughlin, 1993; 1994) and the suction mode of prey capture have been reported in *A. perideraion* (Coughling, 1994). Coughling (1994) observed 100% prey strike success by the 3 dph larvae of *A. perideraion* while 94% strike success has been reported in *Premna biaculeatus* (Job and Bellwood, 1996).

Green and McCormick (2001) studying the digestive system of clownfish, *A. melanopus*, reported on the differentiation of gut into foregut, midgut and hindgut on the day of hatching, though the muscular sphincter dividing foregut from midgut was not observed. Apart from the single looped gut, kidney, liver, pancreas *etc.* were also present on hatching (Green and McCormick, 2001). In *A. percula*, Gordon and Hecht (2002) observed mouth opening and alimentary canal differentiation into oesophagus, rudimentary stomach, intestine and rectum even before hatching, and suggested pinocytotic digestion on 5 dph and extracellular digestion and absorption across lumen on 9 dph (Gordon and Hecht, 2002). Thus they concluded that *A. percula* can effectively utilize prepared diets from 9dph.

After conducting a series of weaning and growth studies on *A. percula*, Gordon *et al.* (1998) found that dry feed did not affect the survival when fed 7 dph, but the survival was low (20%) when 4 dph larvae were fed dry diet indicating the inability of early larvae to properly utilize the feed. However, based on the higher growth rate they have suggested 15 to 20 dph as the optimal weaning age. Johnston *et al.* (2003) studied the influence of feeding frequencies

and feed rations on the growth of juvenile *A. percula*, and suggested a ration of 10.2% at three feedings per day as optimum.

Delbare *et al.* (1995) observed that in *A. ephippium* the larvae from bad quality eggs (BQE; <50% survival on first day) were highly susceptible for mortal shock syndrome. This phenomenon of sudden mortality due to stressors (handling, light intensity and disturbance in rearing media) was attributed to EFA deficiency. They also observed that upon enrichment with cod-liver oil larvae from the same brood did not exhibit the syndrome. The fatty acid profile of larvae from BQE showed a high DHA:EPA ratio of 7.3:1. Therefore they concluded that rather than deficiency an imbalance in the EFA may probably be responsible for the mortality.

On analysing the eggs (2hr and 9th day after spawning) of clownfish *Amphiprion clarkii* Dominguez *et al.* (2001) reported that the major phospholipids were PC and PE, and that the development of eggs caused a decrease in the total concentration of phospholipids with an overall decrease in PC but increases in PE and lysophosphatidyl choline levels. Saturated fatty acids were the major fatty acids in the eggs. The concentration of mono- unsaturated fatty acids decreased and PUFA increased with incubation. DHA showed the greatest increase from about 10 to 18% indicating its significance for the development of eggs, and larval viability (Dominguez *et al.*, 2001).

The importance of EFA has been reported in other marine ornamental fishes also. DHA was found to improve the larval survival in coral reef damsel fish, *Acanthochromis polyacanthus* (Southgate and Kavanagh, 1999), and newborn seahorses *Hippocampus* sp. (Chang and Southgate, 2001). The dietary DHA above 9.3 mg g^{-1} dry weight was found to improve survival and growth in seahorses fed enriched *Artemia* nauplii (Chang and Southgate, 2001).

The pigmentation of tropical marine ornamental fishes is poorly understood. Most clownfishes are brightly coloured, generally with combinations of orange, red, yellow, black, brown or white. Different phenotypes are also common within the same species (e.g. *Amphiprion clarkii*). In wild anemonefishes (*Amphiprion ocellaris*, *A. biaculeatus, A. frenatus* and *A. clarkii*) zeaxanthin was

the dominant pigment followed by astaxanthin (Tanaka *et al.*, 1992). This was further confirmed by feeding studies on *A. ocellaris* wherein dietary zeaxanthin and astaxanthin were incorporated into the integument. The addition of astaxanthin in the diet of cultured anemonefishes (*A. ocellaris* and *Premna biaculeatus*), significantly improved the intensity of colouration (Ako and Tamaru, 1999).

In general, the ornamental fish producers do not have the same restrictions as that of the producers of food fish with respect to body composition. The nutritional needs of multi-species aquarium systems are very difficult to satisfy as the feeding patterns and dietary preferences differ greatly among the fish species. Therefore, the critical requirements of a satisfactory diet for aquarium fish are different, and actually more demanding, than those for commercial food fish.

The review of literature revealed that information on nutrition of marine ornamental fishes is meager and that on their nutrient requirements are still lacking. In marine finfish hatcheries the production depends heavily on the proper (qualitative and quantitative) nutrition of broodstock and larvae. However, studies are mostly focused on the importance of essential fatty acids like n-3 HUFA. The role played by other important nutrients like amino acids, vitamins, minerals, carotenoids *etc* are yet to be properly acknowledged. In juvenile nutrition much work has been done especially on their protein, amino acid and fatty acid requirements. In order to have a better understanding of the nutritional needs of sebae clownfish the present investigation was designed and it involved broodstock, larval and juvenile stages of the fish. More studies in similar line using clownfish as a model for broodstock and larval nutrition studies can yield valuable information which can be applied to other marine species.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

Experiments were conducted at the Marine Aquarium and Hatchery Complex of Vizhinjam Research Centre of the Central Marine Fisheries Research Institute (CMFRI). The formulation and preparation of feeds, and biochemical analyses of the feed ingredients and feeds, were carried out in the nutrition laboratory at CMFRI, Kochi. Experiments were carried out on adults, larvae and juveniles of the sebae anemonefish. Studies on broodstock nutrition were focussed on evaluation of selected natural and formulated diets. Larval nutrition studies were done for assessing the nutritional value of selected livefeeds, and to determine optimum weaning age. Juveniles were used for the experiments to arrive at optimum protein and lipid levels in the diet.

3.1. Experimental Animals

3.1.1. Fish

The sebae anemonefish, *Amphiprion sebae* Bleeker 1853, was used for the study (Plate I). Fishes (60 - 70 mm) for broodstock nutrition experiments were collected from Rameswaram area of the Gulf of Mannar (Lat. 79°9'E and Long. 9°16'N). The fishes were caught from the coral reefs, 12 - 15 ft depth, by engaging traditional skin divers using scoop nets, and immediately transferred to aerated seawater holding tanks of 100 ℓ capacity. The fishes were packed individually in oxygenated polythene bags of two litres capacity with filtered seawater. The bags were packed inside cardboard cartons and transported to the experimental facility at Vizhinjam.

Larvae for each of the experiments were obtained from the same broodstock pair maintained at the hatchery. Juveniles for the protein and lipid requirement studies were also obtained from the same brood pair maintained at the hatchery.

3.1.2. Anemone

The Haddon's sea anemone, *Stichodactyla haddoni* (Saville-Kent, 1893), one of the natural hosts of *A. sebae*, with a disc diameter of 15 - 25 cm

Plate I. Sebae anemonefish with the host anemone

was collected from the same area as that of the fish by hand picking. The sebae anemonefish exhibit symbiotic association with this anemone and with this association fish gets protection from the predators. The packaging and transportation procedure followed was similar to that of the fishes.

3.2. Water Quality Parameters

Seawater used for all the experiments was collected from the Vizhinjam Bay and transported regularly to the Marine Aquarium and Hatchery facility at Vizhinjam. The water was initially stored in a settling sump and later pumped into two storage tanks, each of 10 tons capacity. The water used for larval and juvenile studies were pre-chlorinated with liquid sodium hypochlorite (200 ppm) and vigorously aerated for two days in a one ton tank exposed to sunlight.

Salinity, pH, temperature, dissolved oxygen, ammonia, nitrate and nitrite levels in the seawater were determined. Salinity was measured using a refractometer (ERMA, Japan), pH using a digital pH meter, and temperature using a mercury thermometer. UV Spectrophotometer (Genesys 10UV, Thermospectronic) was used for ammonia, nitrate and nitrite determination. Dissolved oxygen was determined by adopting Winkler's procedure (Strickland and Parsons, 1972). Ammonia was estimated by the phenol-hypochlorite method as per Strickland and Parsons (1972) and the intensity of colour developed was measured at 640 nm and values were computed from standard graph. The nitrite and nitrate levels were determined as per APHA (1980) by measuring the intensity of colour developed at 545 nm. Temperature measurement was taken twice daily (0930 hrs and 1530 hrs). Salinity and pH were taken at three days interval, and ammonia, nitrite and nitrate were estimated once a week. The water from the storage tanks were also analysed frequently.

3.3. Broodstock Nutrition Experiments

3.3.1. Acclimation and pairing

Transportation bags containing the fishes were kept afloat in FRP tank of one ton capacity for 30 minutes on their arrival to the wet lab. They were

then gently taken out using a small hand net and released into the tank. The fishes were not fed on the first day. Two fishes were released into each of the experimental tanks. Two sea anemones were also introduced into each of the experimental tanks by adopting the same procedure. The fishes were observed for compatibility and pairing.

3.3.2. Experimental design and setup

Broodstock nutrition experiments were done maintaining three replicates for each treatment and the duration of the study was extended to three consecutive spawnings after the initial three spawnings, as the number of eggs spawned was considerably low in the initial spawning and was found to stabilise after the first two to three spawnings.

The fishes were maintained in rectangular FRP tanks of 2.0 m length X 1.0 m width X 0.5 m depth (Plate II). Each tank was provided with two fishes and two anemones. The tanks were equipped with an under gravel bio-filter system by spreading coral sand over an acrylic filter plate. An air-water lift system was established by erecting PVC tubes on the corners of filter plate. Continuous aeration was provided with blowers and a filtration rate of one cycle per 1½ hr was maintained. All the tanks were individually illuminated with fluorescent tubes (40 W) fixed in a tube assembly 50 cm above the water surface to provide a constant photoperiod of 12L:12D. Water was exchanged twice daily (0900 hrs and 1630 hrs) after waste removal.

3.3.3. Broodstock feeds and feeding

The fishes were fed *ad libitum* twice daily, at 1000 hrs and 1530 hrs. After initiation of spawning, the feeding time on the day of spawning was adjusted to avoid distraction during spawning. Fishes were observed to be hesitant to feed from the bottom, especially in substrate laid tanks. Hence, the feed dispersal was modified such that the feed, which was not immediately consumed, falls within the expanded disc surface of anemone 'Disc-feeding' (Plate III).

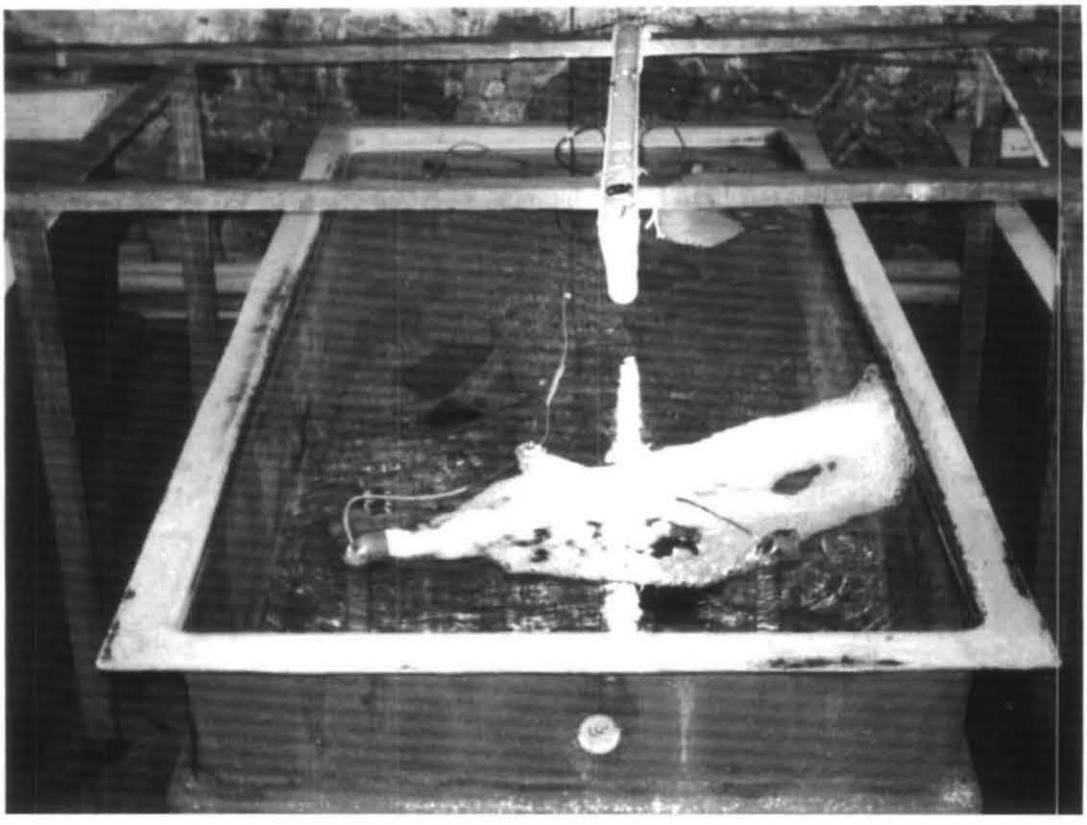


Plate II. Experimental system for broodstock diet evaluation

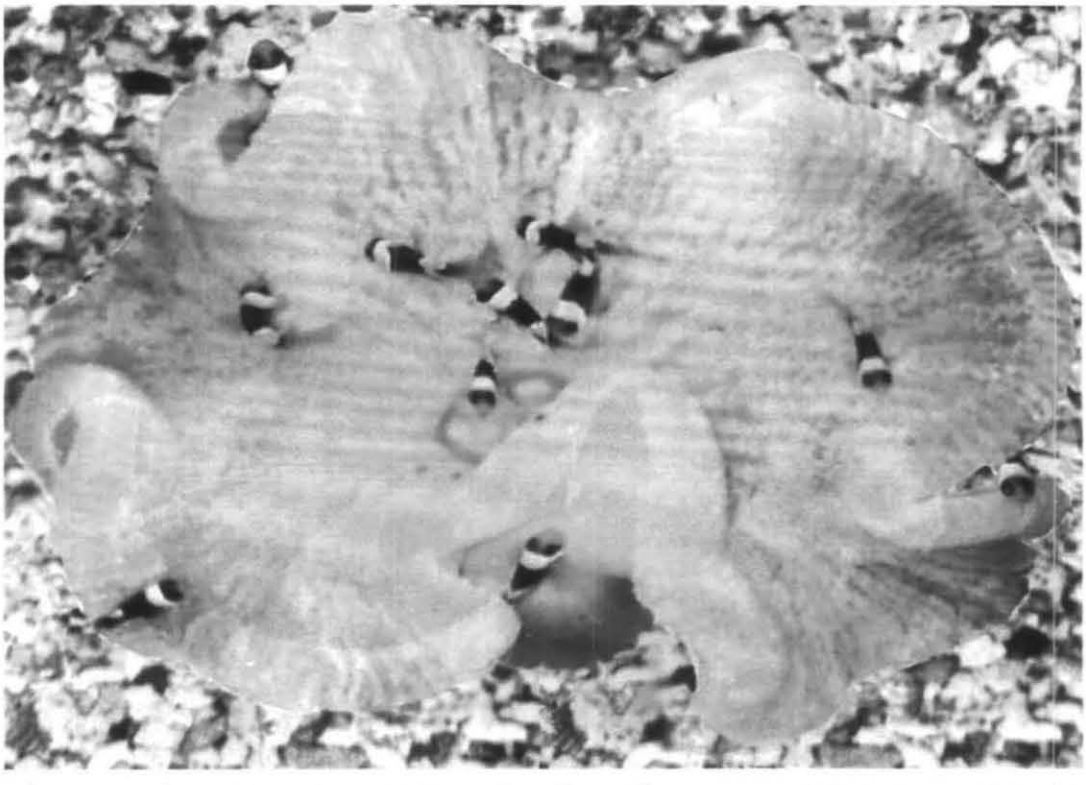


Plate III. Juvenile clownfishes feeding dietary particles from the disc of anemone

3.3.4. Evaluation of natural diets

The natural diets used were the brown mussel, *Perna indica* (MSM), mature brown mussel with ripe gonads (MGD), squid (*Doryteuthis* sp.) meat (SQD), cuttlefish (*Sepiella inermis*) meat (CUT), deep sea prawn (*Heterocarpus* sp.) meat (DSP), and polychaete worm (*Nereis* sp.) and brown mussel meat (PWM) 1:1 ratio (Plate IVa & IVb). The mussels were steam boiled for ½ hr and the shucked meat was used. The diets, except polychaete worms, were cut into suitable size before feeding. All fresh diets were sourced from the Vizhinjam harbour. The diets were kept under refrigeration for a maximum period of three days.

The proximate composition, amino acid profiles, fatty acid profiles and astaxanthin levels of natural diets were determined.

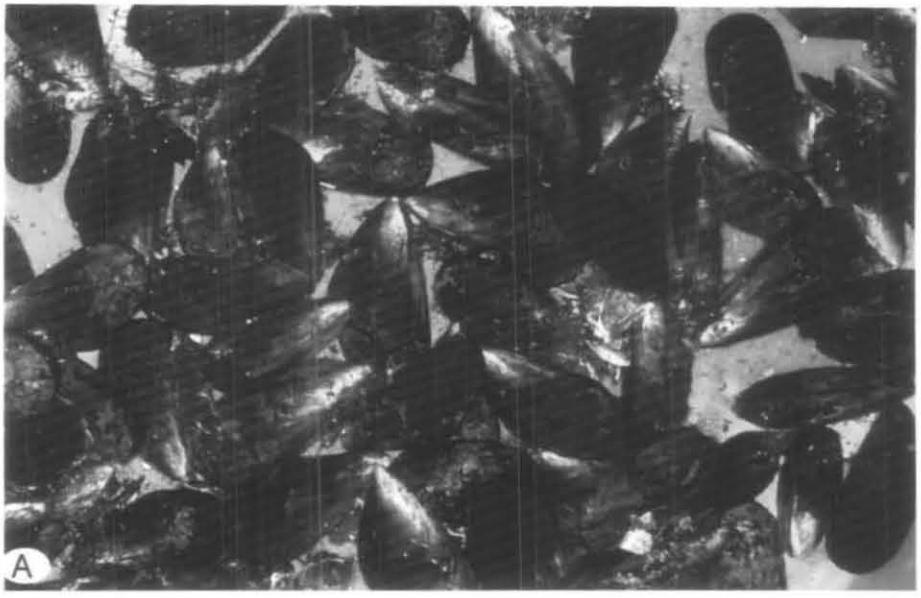
3.3.5. Evaluation of compounded diets

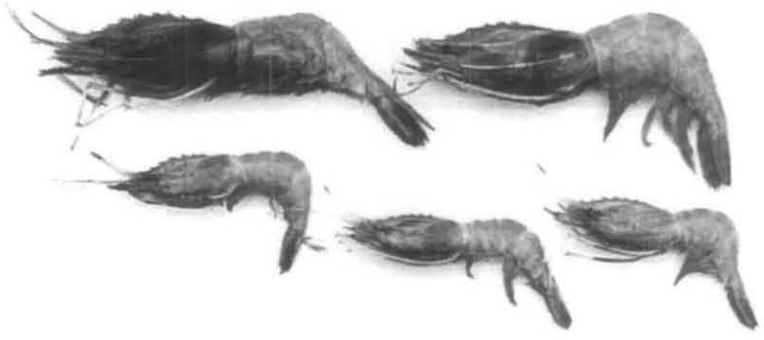
Moist diets were used for this experiment (Plate IVb). Five diets (CBD_1 to CBD_5) were formulated with selected ingredients and nutrient levels (Table I). The diets prepared were CBD_1 without spirulina supplementation and CBD_2 to CBD_5, with spirulina having different protein (40% and 50%) and lipid (10% and 20%) levels. The flowchart for the feed preparation is given in Fig. 1. The feeds were stored under refrigeration and used within three days after preparation.

3.3.6. Influence of broodstock diet

Influence of the broodstock diets on the larval survival was studied by collecting larvae from the respective broodstock dietary treatments. The newly hatched larvae were stocked @ 250 per tank and the experiment was conducted for 12 days. Glass aquaria (90 cm x 60 cm x 60 cm) were used for assessing the influence of broodstock diet on larvae. All the experiments were carried out in triplicates.

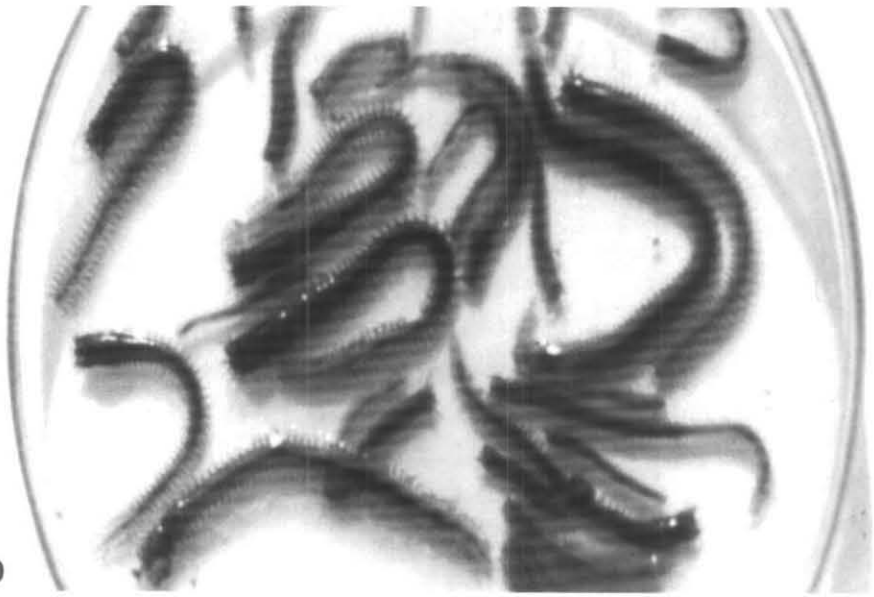
Larvae obtained from each dietary treatment were fed with rotifers as first feed, followed by a combination of rotifers and *Artemia* from 4 dph to 12





B

Plate IVa. Diets used for broodstock nutrition experiments:-
A) Brown mussel B) Deep-sea prawn C) Squid



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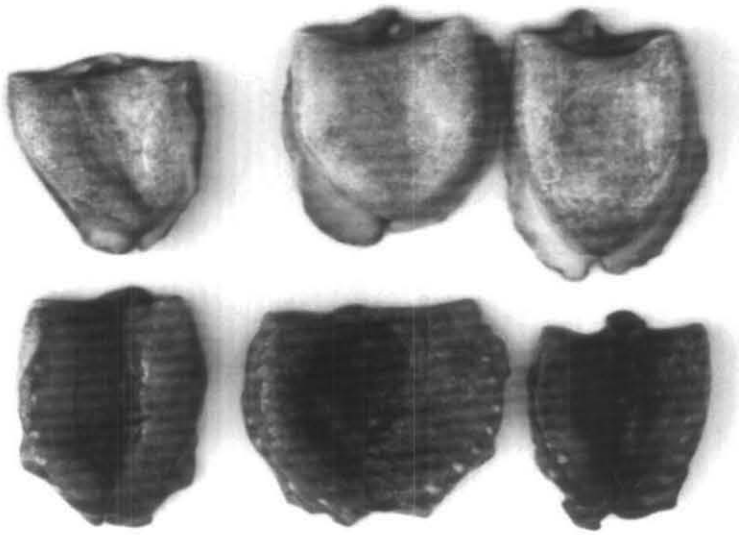

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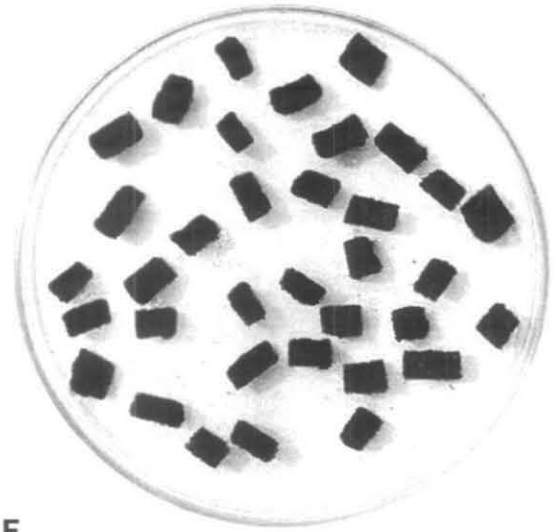


F

Plate IVb. Diets used for broodstock nutrition experiments:-
D) Polychaete worm E) Cuttlefish meat F) Compounded moist diets

Table I. Ingredient levels, proximate composition and energy levels of the moist broodstock diets

	Diets				
	CBD_1	CBD_2	CBD_3	CBD_4	CBD_5
Ingredients (g Kg^{-1})					
Basal mix[¥]	755.0	700.0	700.0	450.0	[$]700.0
Spirulina	0.0	50.0	50.0	450.0	195.0
Starch	25.0	30.0	135.0	0.0	0.0
Fish oil	160.0	160.0	55.0	50.0	50.0
Binder[‡]	0.0	0.0	0.0	10.0	15.0
Vitamin mix[§]	20.0	20.0	20.0	20.0	20.0
Mineral mix [Ψ]	40.0	40.0	40.0	20.0	20.0
Proximate composition (% dry matter)					
Crude protein	38.33	38.64	40.26	49.62	48.85
Crude lipid	20.09	19.92	9.84	8.56	9.16
Crude ash	20.17	19.17	19.32	15.13	19.57
N-free extract	2.90	3.92	11.38	7.42	4.66
Gross energy[•]	17.48	17.66	15.35	16.37	15.95
P/E ratio[¶]	21.93	21.88	26.23	30.31	30.63

¥ Contains fish meal, shrimp meal and squid meal (14:3:3 and [$]4:3:3).

Ψ Mineral mix (g/kg mix): $MgSO_4.7H_2O$, 90.0; $MnSO_4.H_2O$, 1.3; KI, 0.3; $NaH_2PO_4.2H_2O$, 80; $NaHSeO_3$, 0.01; $CoCl_2.6H_2O$, 0.8; $ZnSO_4.7H_2O$, 3.0; NaCl, 25; $CuSO_4.5H_2O$, 0.5; $KH_2PO_4.2H_2O$, 100; $FeSO_4.7H_2O$, 12.5; $CaHPO_4$, 50 (cellulose filler).

§ Vitamin (mg or IU kg^{-1} in diet): Vit.A, 5000 IU; cholecalciferol, 3000 IU; tocopherol acetate, 350 IU; menadione, 10; ascorbic acid, 300; thiamine hydrochloride, 25; riboflavin, 15; calcium pantothenate, 50; pyridoxine hydrochloride, 20; folic acid, 6; nicotinic acid, 60; biotin, 0.5; cyanocobalamin, 0.04; choline chloride, 900; inositol, 200.

‡ CMC and Agar.

•Gross energy (KJ g^{-1}) was calculated based on 23.6 kJ g^{-1} for protein, 39.5 kJ g^{-1} for lipid and 17.2 kJ g^{-1} for carbohydrate.

¶ mg protein kJ^{-1} of gross energy.

Preparation of Moist Diet (100 g) for Broodstock Nutrition Experiments

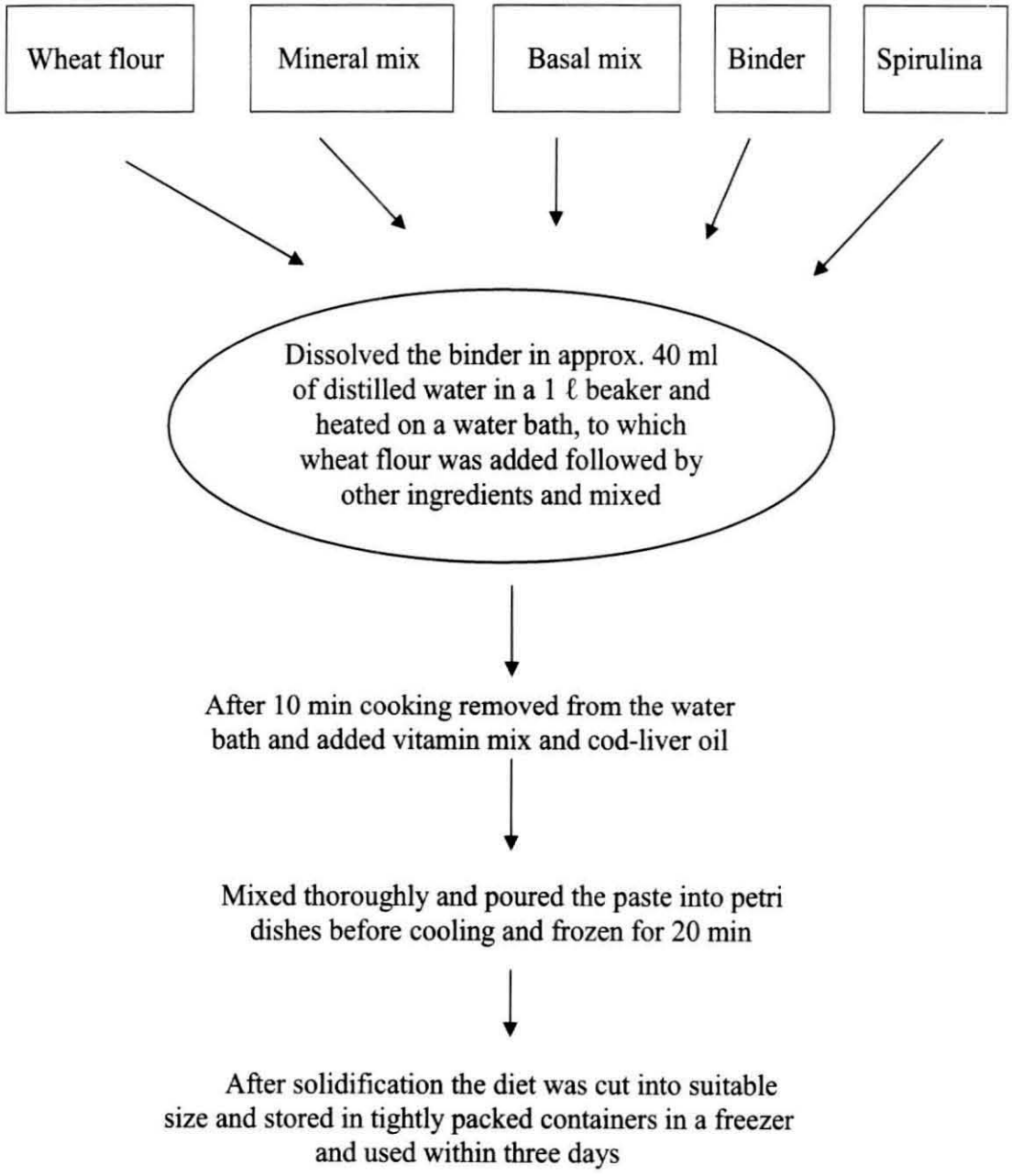


Fig. 1. Flow chart for the preparation of moist diets

dph. Aeration was provided and water exchange was kept low during the initial larval rearing period. During the first three days only the dead larvae and debris at the tank bottom were cleaned without replenishment. From the 4th day onwards water volume equal to that from the tank was replaced. The water exchange was kept at 25% from first week till metamorphosis and further increased to 50% after metamorphosis.

3.3.7. Influence of level of feeding

In order to study the effect of daily feed allowance on broodstock performance an experiment was conducted by feeding the fish *ad libitum* either once or twice daily. Mussel meat was used as the diet and the control was fed *ad libitum* twice daily and the treatment *ad libitum* once a day. The response parameters considered were number of eggs spawned, egg dimensions and hatchability.

3.3.8. Broodstock development from juveniles using dry diets

The larvae from the same brood and batch were weaned to dry diets by 21 dph. After weaning they were shifted to a 250 ℓ capacity FRP tank and reared there for two months before transferring into experimental tanks. The tanks used for the broodstock development experiments were glass tanks (90 cm X 45 cm X 45 cm) (Plate V). The experiment was done in triplicate aquaria, each with two juveniles of 28-30 mm TL (Plate VI). The filtration rate was kept @ one cycle/hr with two air-water lift tubes. The biological filter system used was similar to that of broodstock diet evaluation studies. Each tank was provided with one sea anemone.

Water exchange (50%) was carried out twice daily in the morning (0900 hrs) and evening (1630 hrs) along with waste removal. Three feeds with different protein and lipid levels (45% CP and 9% CF for feed A, 42% CP and 6% CF for feed B, 45% CP and 12% CF for feed C) were used for the study; the ingredient level and proximate composition are given in Table II. The feeds and feeding schedule used for the broodstock development study are given in Table III.

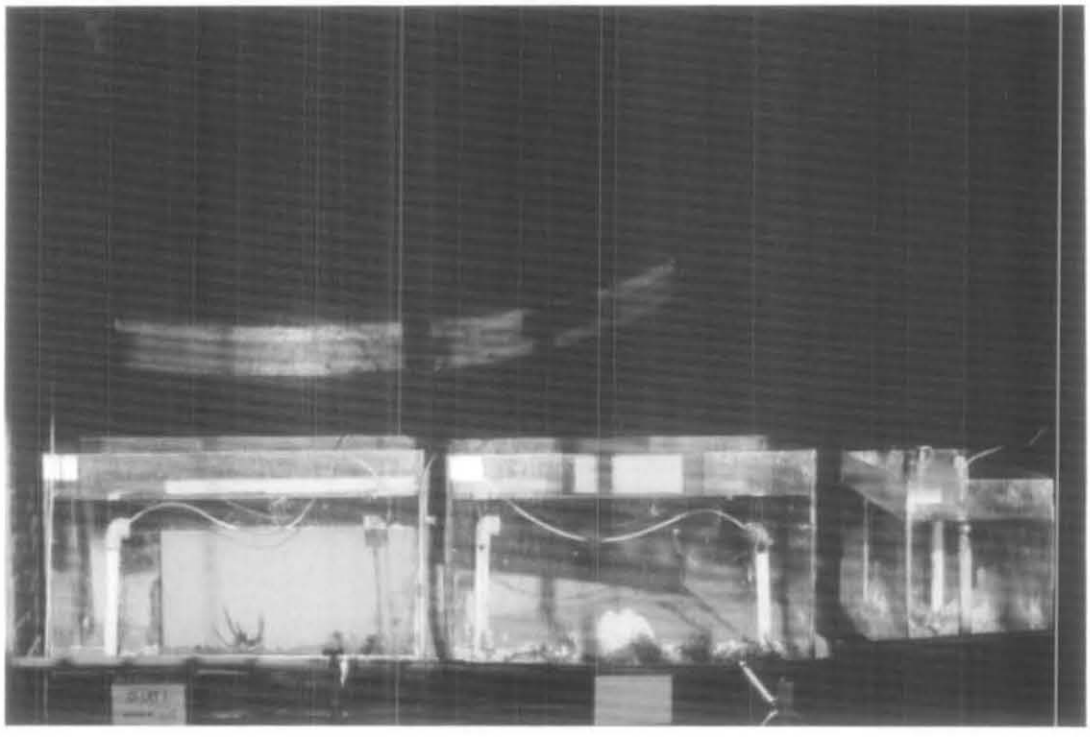


Plate V. Experimental setup for broodstock development from juveniles

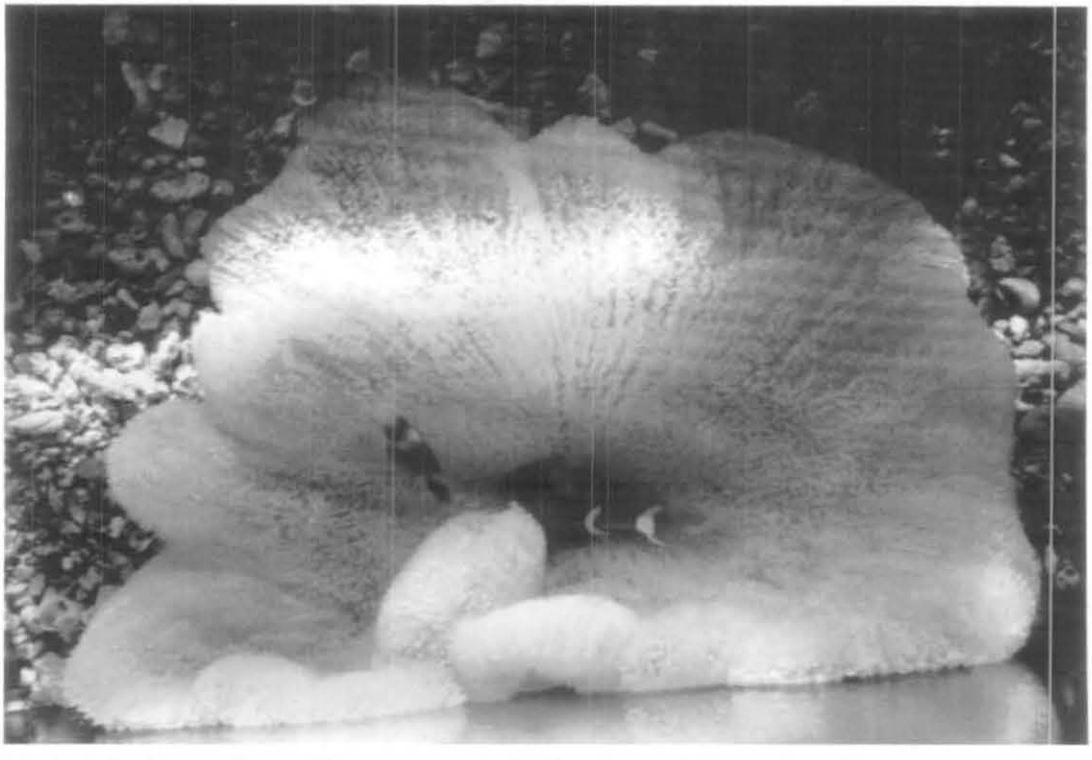


Plate VI. Juveniles with anemone in the broodstock development tank

Table II. Ingredient levels, proximate composition and energy levels of the diets used in broodstock development experiment

	Lipid levels (%)		
	A	B	C
Ingredients (g Kg^{-1})			
Basal mix¥	745	580	745
Starch	5	150	5
Dextrin	107	93	57
Cod-liver oil	63	17	93
Vitamin mix§	20	20	20
Mineral mixΨ	40	40	40
CMC‡	10	50	20
Cellulose	10	50	20
Proximate composition (% dry matter)			
Crude protein (CP)	44.93	41.88	45.12
Crude lipid (CF)	9.05	6.03	12.08
Crude ash	22.41	13.51	22.47
N-free extracts	12.03	21.23	7.09
Gross energy*	16.25	15.92	16.64
P/E ratio¶	27.65	26.31	27.12

¥ Contains fish meal, shrimp meal and squid meal (15:4:1).

Ψ Mineral mix (g/kg mix): $MgSO_4.7H_2O$, 90.0; $MnSO_4.H_2O$, 1.3; KI, 0.3; $NaH_2PO_4.2H_2O$, 80; $NaHSeO_3$, 0.01; $CoCl_2.6H_2O$, 0.8; $ZnSO_4.7H_2O$, 3.0; NaCl, 25; $CuSO_4.5H_2O$, 0.5; KH_2PO_4. $2H_2O$, 100; $FeSO_4.7H_2O$, 12.5; $CaHPO_4$, 50 (cellulose filler).

§ Vitamin(mg or IU kg^{-1} in diet): Vit.A, 5000 IU; cholecalciferol, 3000 IU; tocopherol acetate, 250 IU; menadione, 10; ascorbic acid, 250; thiamine hydrochloride, 25; riboflavin, 15; calcium pantothenate, 50; pyridoxine hydrochloride, 20; folic acid, 6; nicotinic acid, 60; biotin, 0.5; cyanocobalamin, 0.04; choline chloride, 900; inositol, 200.

‡ Carboxymethylcellulose.

*Gross energy (KJ g^{-1}) was calculated based on 23.6 kJ g^{-1} for protein, 39.5 kJ g^{-1} for lipid and 17.2 kJ g^{-1} for carbohydrate.

¶ mg protein kJ^{-1} of gross energy.

Table III. Feeds and feeding schedule used for the development of *Amphiprion sebae* broodstock using compounded dry diets

Duration (days post hatching)	Feed	Feeding frequency (day^{-1})	Particle size
21dph to 2months	45% CP & 9% CF (A)	3	<500 µm
2-3 months	45% CP & 9% CF (A)	2	500 µm - 1mm
3-6 months	42% CP & 6% CF (B)	2	1 mm-2mm
6 months to first spawning	42% CP & 6% CF (B)	2	2mm
After first spawning	45% CP & 12%CF (C)	2	2mm

3 times (0900 hrs, 1300 hrs and 1700 hrs)
2 times (0900 hrs and 1600 hrs)

3.3.9. Data collection

The broodstock tanks were tagged for proper record maintenance, and each of the tanks was provided with a clipboard indicating the date of spawning and the experimental diet given. The data collected included the spawning time and date, duration of spawning, number of eggs spawned, compactness of clutch (egg mass), colour of eggs or yolk, date and time of hatching. Egg samples (n=12) were randomly taken for micrometer measurements of egg dimensions (Fig. 2). The newly hatched larvae (n=12) were also collected for measurements (Fig. 3)

3.4. Larval Nutrition Experiments

3.4.1. Experimental set up and design

Larval nutrition experiments were carried out in rectangular FRP tanks (250 ℓ) with recirculation. Recirculation system was used for the evaluation of livefeeds. The recirculation system (Fig. 4) comprised of a storage tank of one ton capacity. This was connected to the larval rearing tanks (LRT) by pipes provided with control valves. The receiver tank was of half ton capacity with biological filter and water was pumped back to the main tank. Daily 10-20% water exchange was done from the receiver tank. Both the storage and the receiver tanks were given vigorous aeration. In this system, filtered seawater entered the LRT through a centrally placed PVC pipe with a wider mouth (3/4") fixed to a 1/2" pipe at the receiving area, and a narrow distal part (1/4") with numerous minute perforations around it an inch above the closed distal end. The flow rate was maintained at 15 - 20 $\ell\ hr^{-1}$. The outlet valve was provided with a small extension inside the tank. The inlet and outlet were covered with 40 µm and 100 µm mesh bolting silk respectively. Mild aeration was given continuously through a centrally placed air stone. A constant photoperiod (12L: 12D) was maintained with the help of fluorescent tubes (40W).

3.4.2. Livefeed evaluation experiment

Larvae produced from domesticated broodstock, maintained at the hatchery, were used for the livefeed evaluation study. Each of the replicate tanks

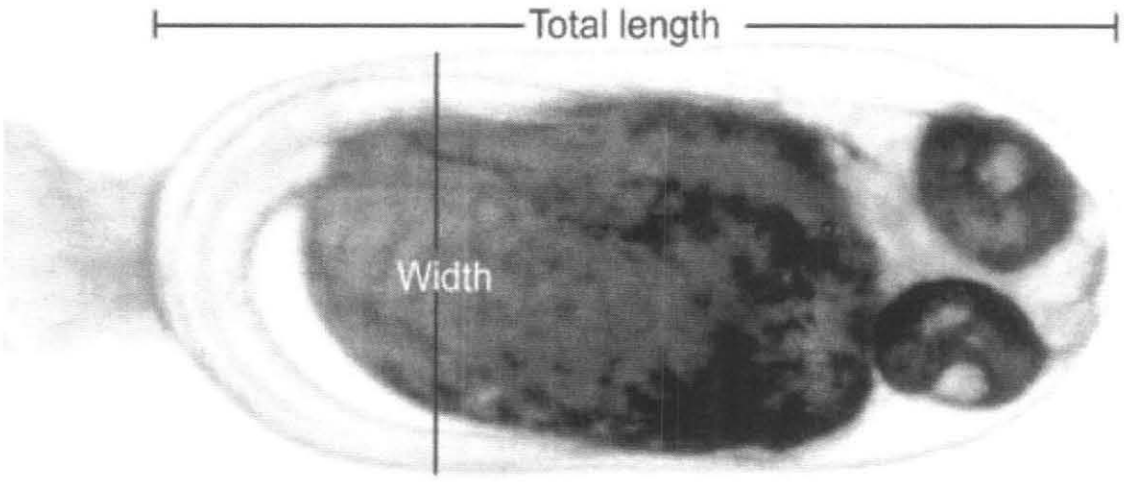


Fig. 2. Egg capsule measurements

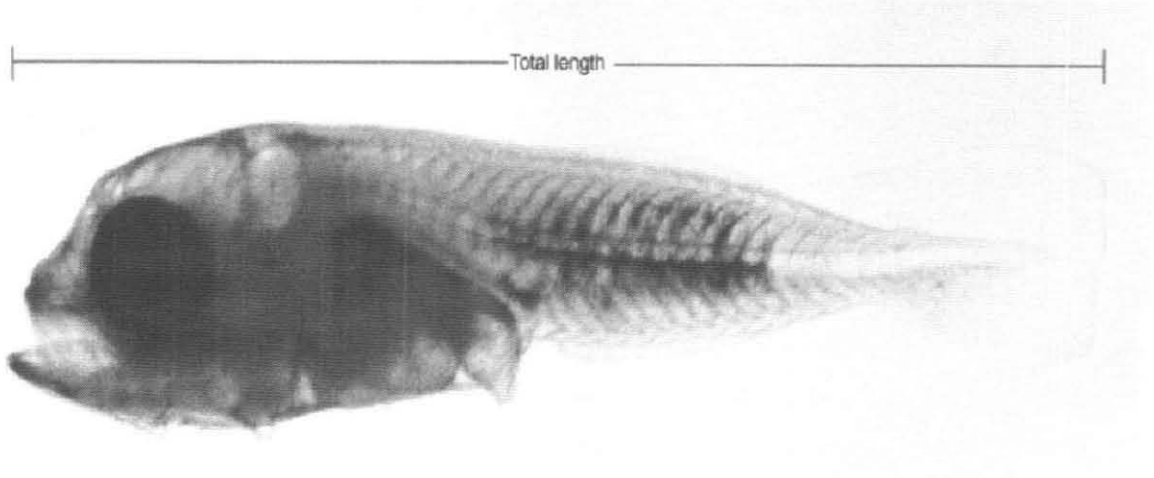
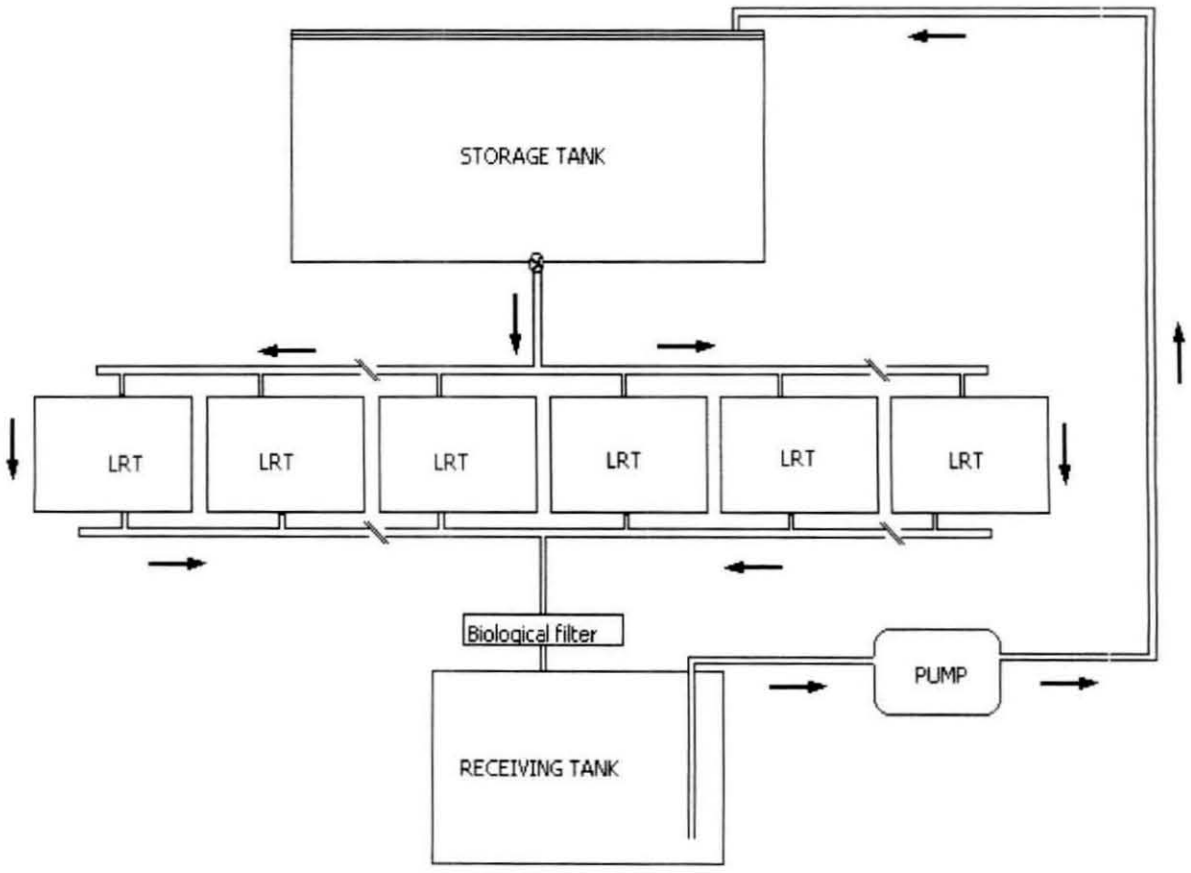


Fig. 3. Larval measurement



LRT – Larval rearing tank

Fig. 4. Recirculatory system used for larval rearing

was stocked with 300 freshly hatched larvae, for the experimental duration of one month.

The livefeeds used were the rotifer (*Brachionus rotundiformis*), *Artemia* nauplii (O.S.I. PRO 80™) and moina (*Moina micrura*). The treatments were LF_1 with rotifer alone, LF_2 with mixture of rotifers and *Artemia*, LF_3 with mixture of rotifers, *Artemia* and *Moina* and LF_4 in which green algae were used in the rearing medium along with rotifers and *Artemia*. In LF_1 rotifers alone were used throughout the rearing period. In LF_2 *Artemia* was given from 4 dph (days post hatching) onwards along with rotifers. In LF_3 after the commencement of *Artemia* feeding, rotifers were stopped and after metamorphosis *Moina* was given together with *Artemia*. In LF_4 rotifers and *Artemia* were given and *Chlorella* was provided to retain the greenish appearance and for the continuous enrichment of livefeeds. The larval feeding schedule is presented in Table IV.

3.4.3. Data collection

Total length of larvae, gut anatomy and pigmentation patterns of larvae were noted. Dead larvae, if any, were removed and counted in the morning and evening to assess the survival.

3.4.4. Micro-dissection of larvae

The larvae were dissected using surgical needles under a stereozoom binocular microscope (Carl-Zeiss, Stemi 2000C) to study the alimentary system. The mouth gape and the type of dentition were noted. The acceptance and ingestion of tested larval feeds were confirmed by gut dissection.

3.4.5. Weaning studies with dry micro-diets

Weaning studies were carried out to find out the optimum weaning age. A micro-diet with 45% protein and 12% lipid (Diet C of Table II) with a particle size of <500 µm was used. The weaning studies were carried out from 4 dph, 14 dph, 21 dph and 30 dph to a period of two weeks. Experiments were carried out in triplicate using glass aquaria. The number of individuals stocked in

Table IV. Feeds and feeding schedule used for livefeed evaluation experiment

Treatment	Period	Feed and concentration (ml^{-1})	Feeding level (No of times)
LF_1 (rotifer alone)	1-3 dph	Rotifers (4-6 ml^{-1})	6
	4 -10 dph	Rotifers (6-8 ml^{-1})	5
	10-20 dph	Rotifers (6-8 ml^{-1})	5
	20-30 dph	Rotifers (8-10 ml^{-1})	5
LF_2 (rotifer and artemia)	1-3 dph	Rotifers (4-6 ml^{-1})	6
	4 -10 dph	Rotifers (4-6 ml^{-1})	3
		Artemia (0.5-1 ml^{-1})	3
	10-20 dph	Rotifers (4-6 ml^{-1})	2
		Artemia (0.5-1.5 ml^{-1})	3
	20-30 dph	Rotifers (4-6 ml^{-1})	2
		Artemia (1-2 ml^{-1})	2
LF_3 (rotifer, *Artemia* and *Moina* with rotifer till 3 dph and *Moina* after 20 dph)	1-3 dph	Rotifers (4-6 ml^{-1})	6
	4 -10 dph	Artemia (0.5-1 ml^{-1})	5
	10-20 dph	Artemia (0.5-1.5 ml^{-1})	5
	20-30 dph	Artemia (0.5-1 ml^{-1})	2
		Moina (0.5 ml^{-1})	2
LF_4 (Green algae along with rotifer and *Artemia*)	1-3 dph	Rotifers (4-6 ml^{-1})	6
	4 -10 dph	Rotifers (4-6 ml^{-1})	3
		Artemia (0.5-1 ml^{-1})	3
	10-20 dph	Rotifers (4-6 ml^{-1})	2
		Artemia (0.5-1.5 ml^{-1})	3
	20-30 dph	Rotifers (4-6 ml^{-1})	2
		Artemia (1-2 ml^{-1})	2

dph - days post hatching

each replicate of the treatments was 200 (4 dph), 150 (14 dph), 100 (21 dph) and 75 (30 dph).

Weaning protocol from livefeed to dry micro-diet was extended for a period of three days (Gordon *et al.*, 1998). *Artemia* nauplii were used as live diet in all the treatments. On the first day of weaning 25% of dry diet and 75% live diet were given and the dry diet was increased to 50% on second day. On the third day of weaning 75% dry diet and 25% live diet were served. The tanks were observed for mortalities daily morning and evening.

3.5. Experiments on Juveniles

3.5.1. Feed formulation and preparation

The ingredients used were fish meal, shrimp meal and squid meal as protein sources, fish oil (cod-liver oil, Seven seas) as the lipid source, dextrin and wheat flour as carbohydrate source, and the energy levels of diets were adjusted with cellulose as inert filler.

The ingredients for the basal feed mix were processed from raw materials in the laboratory. Fishmeal was prepared by procuring dry fishes (Anchovies) and pulverised in a hammer mill (<250 µm) after oven drying (50° C) for 24 hrs. Shrimp meal was also prepared from dried shrimp (*Metapenaeus* and *Penaeus* sp.) as stated above. For squid meal fresh squids (*Loligo* sp.) were procured and cut to small pieces after removing the ink glands, dried in hot air oven (55° C) and powdered. The feed ingredients were stored in tightly packed containers.

The basal mix was formulated and prepared using the marine protein sources to achieve the required nutrient levels after proximate analyses. The basal mix was prepared with 15:4:1 ratio of fishmeal, shrimp meal and squid meal respectively. The feed formulation was done by using Microsoft Excel® software. The proximate analyses of raw materials and prepared feeds were done (refer 3.6.1).

Vitamin (Sigma) and mineral mixtures were prepared using a blender with cellulose as filler to achieve uniform distribution. Vitamin mix was

prepared without incorporating fat soluble vitamins and the hygroscopic choline chloride. Fat soluble vitamins were mixed with fish oil before feed preparation. Choline chloride was dissolved in distilled water and mixed at the time of dough preparation.

All the ingredients, except vitamin mix and cod-liver oil, were accurately weighed and mixed in a blender. The ingredients were steam cooked and the oil and vitamins were added during the dough preparation. A porous aluminium scrape plate with 2 mm diameter perforations was used to prepare the feed manually. By applying pressure the dough was scraped through the aluminium plate with a foil below and the collected granules were oven dried at 45° C overnight.

The dried feeds were sieved through standard sieves to different particle sizes (Plate VII). They were graded into three different size ranges for weaning stage (below 500 µm), early juveniles (500 to 1000 µm) and juveniles (below 2 mm), and packed in air tight containers and stored in desiccators. Feeds were analysed for their proximate composition.

3.5.2. Evaluation of semi-purified and purified diets

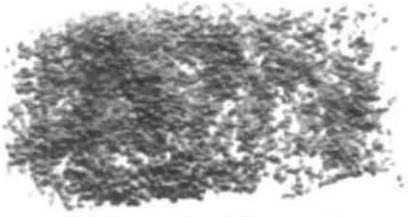
This experiment was conducted for 45 days with two replicates per treatment, each with eight juveniles to ascertain the acceptability of semi-purified and purified diets by the clownfish juveniles. Blue plastic basins 70 ℓ capacity provided with a biological filter (Fig. 5) were used for the study. The formulations used were purified diet and semi-purified diet (Plate VIII). The ingredient levels and the proximate composition of the feed are given in Table V. The flow charts of feed preparation are given in Fig. 6a - 6b.

3.5.3. Protein and lipid requirement studies

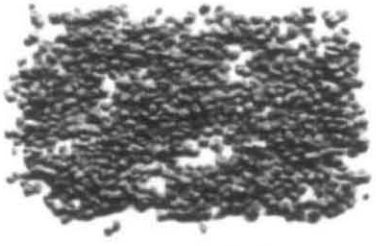
3.5.3.1. Experimental setup and design

Feeding trials to determine the protein and lipid requirement of juveniles were carried out for 9 weeks each. The completely randomized design with three replicates per treatment was used for the requirement studies (Plates IX and X). The containers used for rearing were 70 ℓ circular blue plastic tubs with

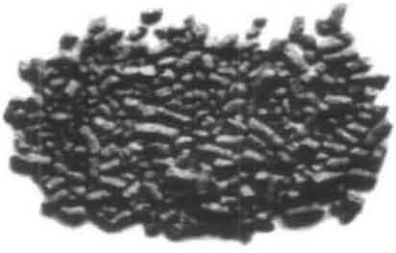
200 μ –800 μ



800 μ-1.40 mm

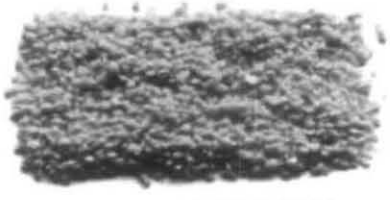


1.40 mm-2.00 mm

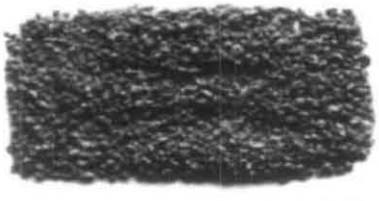


2.00 mm-5.00 mm

Plate VII. Particle sizes of diets used in different experiments



PURIFIED DIET



SEMI-PURIFIED DIET

Plate VIII. Purified and semi-purified diets

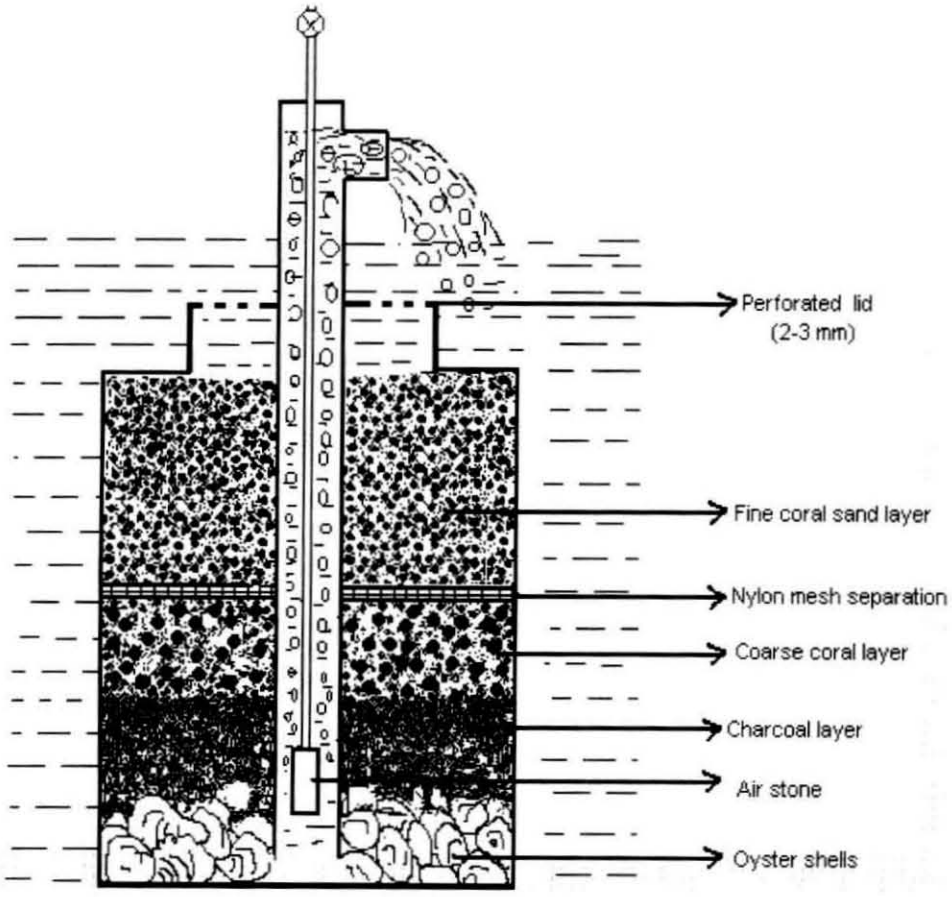


Fig. 5. Diagrammatic cross-section of the biofilter

Table V. Ingredient levels, proximate composition and energy levels of purified and semi-purified diets

Purified Diet		**Semi-purified Diet**	
Ingredients		Ingredients	
Casein-Gelatin (38:12)	50	Casein-Gelatin (38: 12)	40
Dextrin	32	Basal mix[+]	20
Cellulose	6	Dextrin	28
Cod-liver oil	6	Cod-liver oil	6
Mineral mix [Ψ]	4	Mineral mix [Ψ]	4
Vitamin mix [§]	2	Vitamin mix [§]	2
Proximate analysis (% dry matter)			
Crude protein	49.60	Crude protein	49.42
Crude lipid	6.07	Crude lipid	6.16
NFE	30.42	NFE	28.89
Gross energy(kJ g^{-1})[•]	19.34	Gross energy(kJ g^{-1})[•]	19.07
P:E ratio[¶]	25.65	P:E ratio[¶]	25.92

+ Basal mix: 15:4:1 ratio of fish meal, shrimp meal and squid meal

Ψ Mineral mix (g/kg mix): $MgSO_4.7H_2O$, 90.0; $MnSO_4.H_2O$, 1.3; KI, 0.3; $NaH_2PO_4.2H_2O$, 80; $NaHSeO_3$, 0.01; $CoCl_2.6H_2O$, 0.8; $ZnSO_4.7H_2O$, 3.0; NaCl, 25; $CuSO_4.5H_2O$, 0.5; KH_2PO_4. $2H_2O$, 100; $FeSO_4.7H_2O$, 12.5; $CaHPO_4$, 50 (cellulose filler).

§ Vitamin(mg or IU kg^{-1} in diet, Sigma): Vit.A, 5000 IU; cholecalciferol, 3000 IU; tocopherol acetate, 250 IU; menadione, 10; ascorbic acid, 250; thiamine hydrochloride, 25; riboflavin, 15; calcium pantothenate, 50; pyridoxine hydrochloride, 20; folic acid, 6; nicotinic acid, 60; biotin, 0.5; cyanocobalamin, 0.04; choline chloride, 900; inositol, 200.

•Gross energy (KJ g^{-1}) was calculated based on 23.6 kJ g^{-1} for protein, 39.5 kJ g^{-1} for lipid and 17.2 kJ g^{-1} for carbohydrate.

¶ mg protein kJ^{-1} of gross energy.

Preparation of Purified Diet

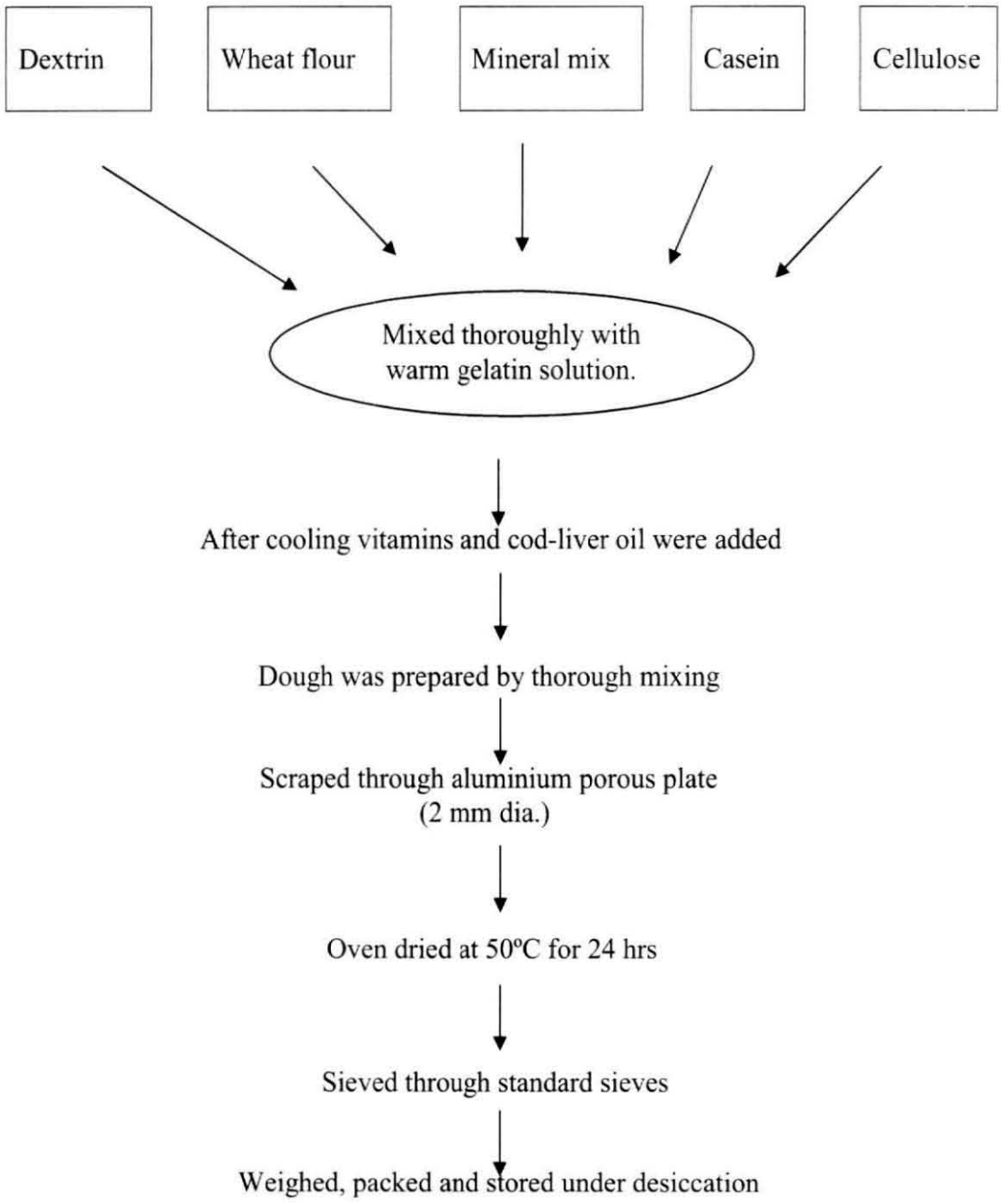


Fig. 6a. Flow chart for the preparation of purified diet

Preparation of Semi-Purified Diet

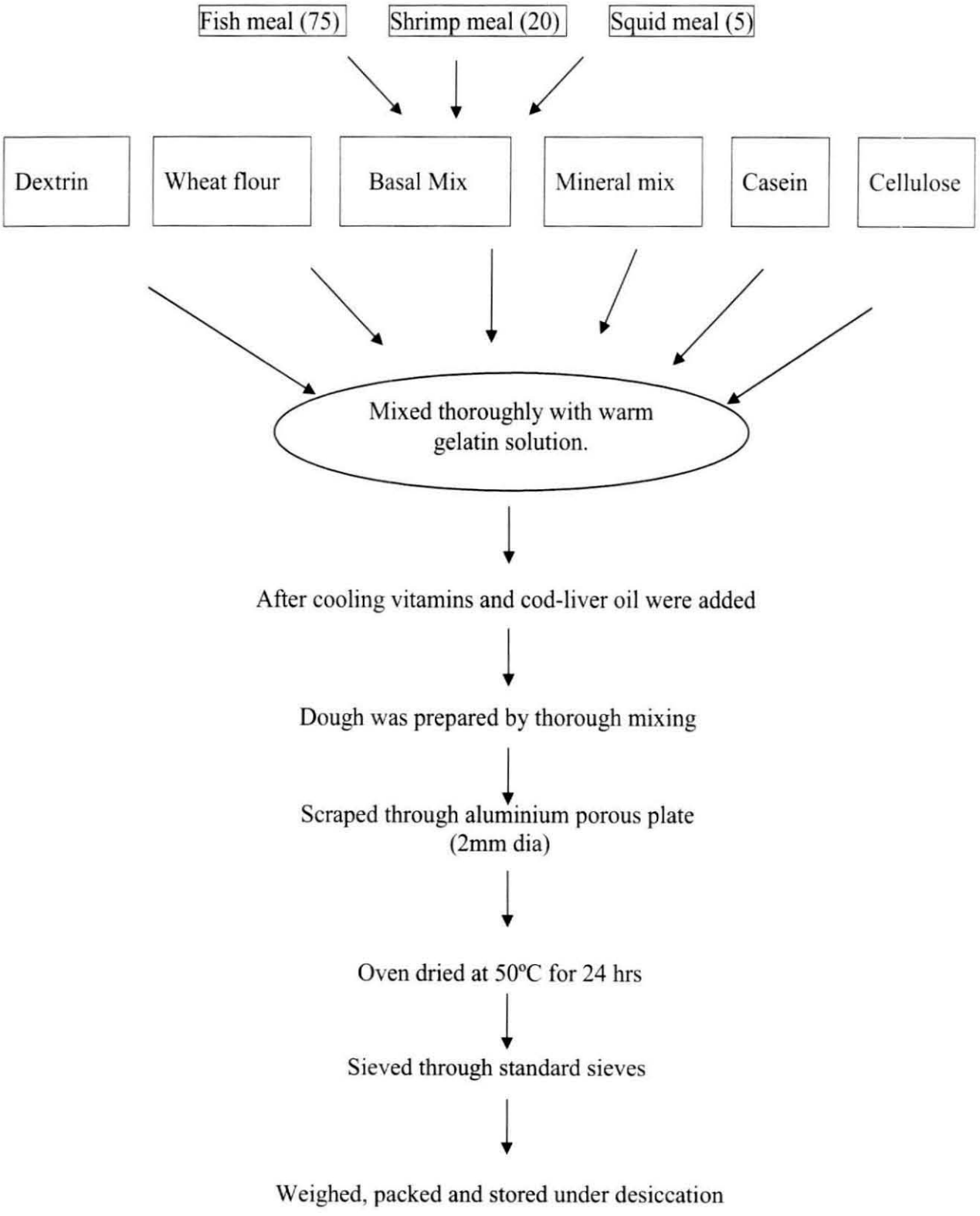


Fig. 6b. Flow chart for the preparation of semi-purified diet

Plate IX. Experimental setup for protein and lipid requirement studies

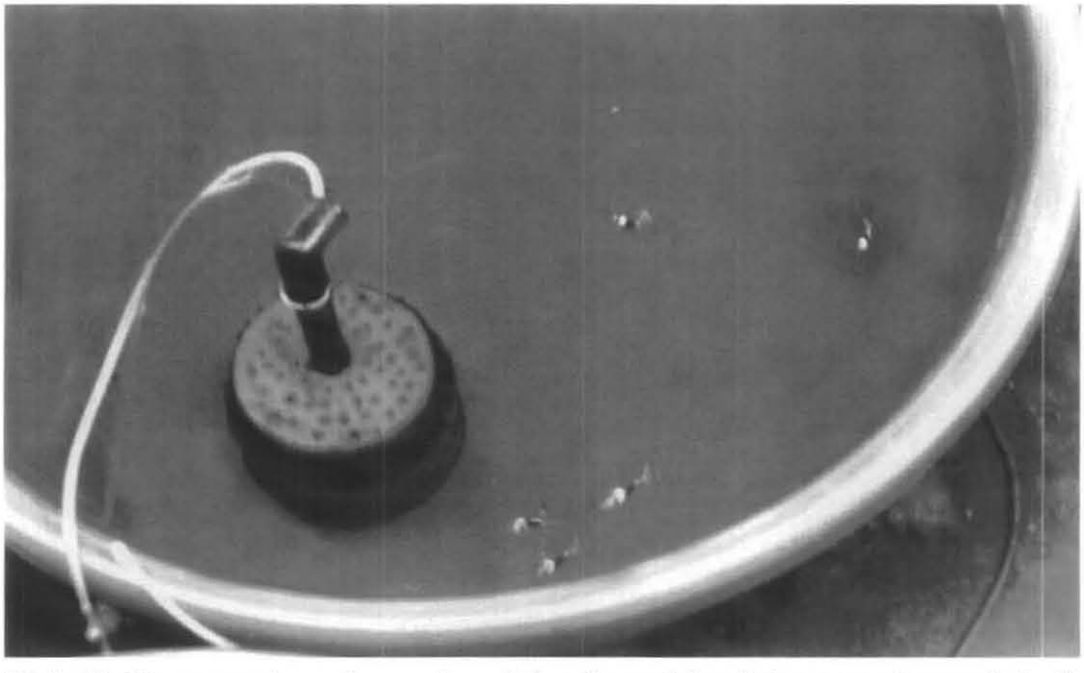


Plate X. Close-up view of experimental unit used for dietary requirement studies

an internal bio-filter set in ½ ℓ plastic bottle (Fig. 5). The containers used for rearing were 70 ℓ circular blue plastic tubs with an internal bio-filter set in ½ ℓ plastic bottle (Fig. 5). Three week old juveniles derived from the same brood were weaned to formulated diets and used for the experiments. Five juveniles of similar length and weight were selected and stocked to minimise differential growth in each treatment. The fishes were acclimatized to the experimental system for one week before the experiment.

Water exchange was carried out daily once in the morning. The exchange was kept low at 10% during the initial 2 weeks of rearing and later increased to 50% till the 7^{th} week and 80% thereafter. The tub was cleaned once a week manually with a scrubber.

3.5.3.2. Diets for protein requirement experiment

Two sets of experiments were conducted to determine the optimum protein requirement of juveniles. The diets used were isoenergetic with the lipid maintained at 6% level. For the first experiment diets were formulated with graded levels of protein from 20% to 50% (D_{20}, D_{30}, D_{40} and D_{50}) maintaining protein increment of 10% (Table VI). Based on the results of the first experiment the second experiment to determine optimum requirement was carried out by formulating six isocaloric diets with protein levels of 33% (D_{33}), 36% (D_{36}), 39% (D_{39}), 42% (D_{42}), 45% (D_{45}) and 48% (D_{48}). The feed ingredients, their inclusion levels and the proximate composition of the diets are given in Table VII. The flow charts of feed preparation are given in Fig. 7.

3.5.3.3. Diets for lipid requirement experiment

Five experimental diets (D_{L3}, D_{L6}, D_{L9}, D_{L12} and D_{L15}) were prepared to achieve graded levels of lipids (Table VIII). The dietary protein level was kept constant (45%) in all the diets. Cod-liver oil was used as the supplementary lipid source. Feed ingredients used and the proximate composition of the diets are given in Table VIII.

Table VI. Ingredient levels, proximate composition and energy levels of the diets used for protein requirement experiment I

	Protein levels (%)			
	D_{20}	D_{30}	D_{40}	D_{50}
Ingredients (g Kg $^{-1}$)				
Basal mix¥	275.0	425.0	555.0	760.0
Wheat flour	150.0	175.0	297.0	50.0
Dextrin	435.0	270.0	30.0	77.5
Cod-liver oil	40.0	30.0	18.0	12.5
Vitamin§	40.0	40.0	40.0	40.0
Mineral mixΨ	20.0	20.0	20.0	20.0
CMC‡	40.0	40.0	40.0	40.0
Proximate composition (% dry matter)				
Crude protein	20.48	30.77	39.90	49.12
Crude lipid	5.98	5.96	5.92	6.06
Crude ash	7.18	10.78	14.07	18.54
N- free extract	52.59	39.03	25.19	12.62
Gross energy*	16.24	16.33	16.09	16.16
P/E ratio¶	12.61	18.84	24.80	30.40

¥ Contains fish meal, shrimp meal and squid meal (15:4:1).
Ψ Mineral mix (g/kg mix): $MgSO_4.7H_2O$, 90.0; $MnSO_4.H_2O$, 1.3; KI, 0.3; $NaH_2PO_4.2H_2O$, 80; $NaHSeO_3$, 0.01; $CoCl_2.6H_2O$, 0.8; $ZnSO_4.7H_2O$, 3.0; NaCl, 25; $CuSO_4.5H_2O$, 0.5; $KH_2PO_4.2H_2O$, 100; $FeSO_4.7H_2O$, 12.5; $CaHPO_4$, 50 (cellulose filler).
§ Vitamin(mg or IU kg^{-1} diet): Vit.A, 5000 IU; cholecalciferol, 3000 IU; tocopherol acetate, 250 IU; menadione, 10; ascorbic acid, 250; thiamine hydrochloride, 25; riboflavin, 15; calcium pantothenate, 50; pyridoxine hydrochloride, 20; folic acid, 6; nicotinic acid, 60; biotin, 0.5; cyanocobalamin, 0.04; choline chloride, 900; inositol, 200.
‡ Carboxymethylcellulose.
*Gross energy was calculated based on 23.6 kJ g^{-1} for protein, 39.5 kJ g^{-1} for lipid and 17.2 kJ g^{-1} for carbohydrate.
¶ mg protein kJ^{-1} of gross energy.

Table VII. Ingredient levels, proximate composition and energy levels of diets used for protein requirement experiment II

	Protein levels (%)					
	D_{33}	D_{36}	D_{39}	D_{42}	D_{45}	D_{48}
Ingredients (g Kg $^{-1}$)						
Basal mix¥	450.0	490.0	530.0	580.0	620.0	670.0
Wheat flour	150.0	150.0	150.0	150.0	150.0	150.0
Dextrin	214.2	178.0	141.0	93.0	56.0	9.0
Cod-liver oil	25.8	22.0	19.0	17.0	14.0	11.0
Vitamin mix§	20.0	20.0	20.0	20.0	20.0	20.0
Mineral mixΨ	40.0	40.0	40.0	40.0	40.0	40.0
CMC‡	50.0	50.0	50.0	50.0	50.0	50.0
Cellulose	50.0	50.0	50.0	50.0	50.0	50.0
Proximate composition (% dry matter)						
Crude protein	33.06	36.54	39.19	41.88	45.17	47.94
Crude lipid	6.04	5.93	5.90	6.03	6.00	6.03
Crude ash	10.85	11.67	12.49	13.51	14.33	15.35
N- free extract	32.75	29.31	25.79	21.23	17.71	13.24
Gross energy*	15.82	16.01	16.02	15.92	16.08	15.97
P/E ratio¶	20.90	22.82	24.46	26.31	28.09	30.02

¥ Contains fishmeal, shrimp meal and squid meal (15:4:1).
Ψ Mineral mix (g/kg mix): $MgSO_4.7H_2O$, 90.0; $MnSO_4.H_2O$, 1.3; KI, 0.3; $NaH_2PO_4.2H_2O$, 80; $NaHSeO_3$, 0.01; $CoCl_2.6H_2O$, 0.8; $ZnSO_4.7H_2O$, 3.0; NaCl, 25; $CuSO_4.5H_2O$, 0.5; KH_2PO_4. $2H_2O$, 100; $FeSO_4.7H_2O$, 12.5; $CaHPO_4$, 50 (cellulose filler).
§ Vitamin(mg or IU kg^{-1} in diet): Vit.A, 5000 IU; cholecalciferol, 3000 IU; tocopherol acetate, 250 IU; menadione, 10; ascorbic acid, 250; thiamine hydrochloride, 25; riboflavin, 15; calcium pantothenate, 50; pyridoxine hydrochloride, 20; folic acid, 6; nicotinic acid, 60; biotin, 0.5; cyanocobalamin, 0.04; choline chloride, 900; inositol, 200.
‡ Carboxymethylcellulose.
*Gross energy (KJ g^{-1}) was calculated based on 23.6 kJ g^{-1} for protein, 39.5 kJ g^{-1} for lipid and 17.2 kJ g^{-1} for carbohydrate.
¶ mg protein kJ^{-1} of gross energy.

Preparation of Diet for Protein and Lipid Requirement Studies

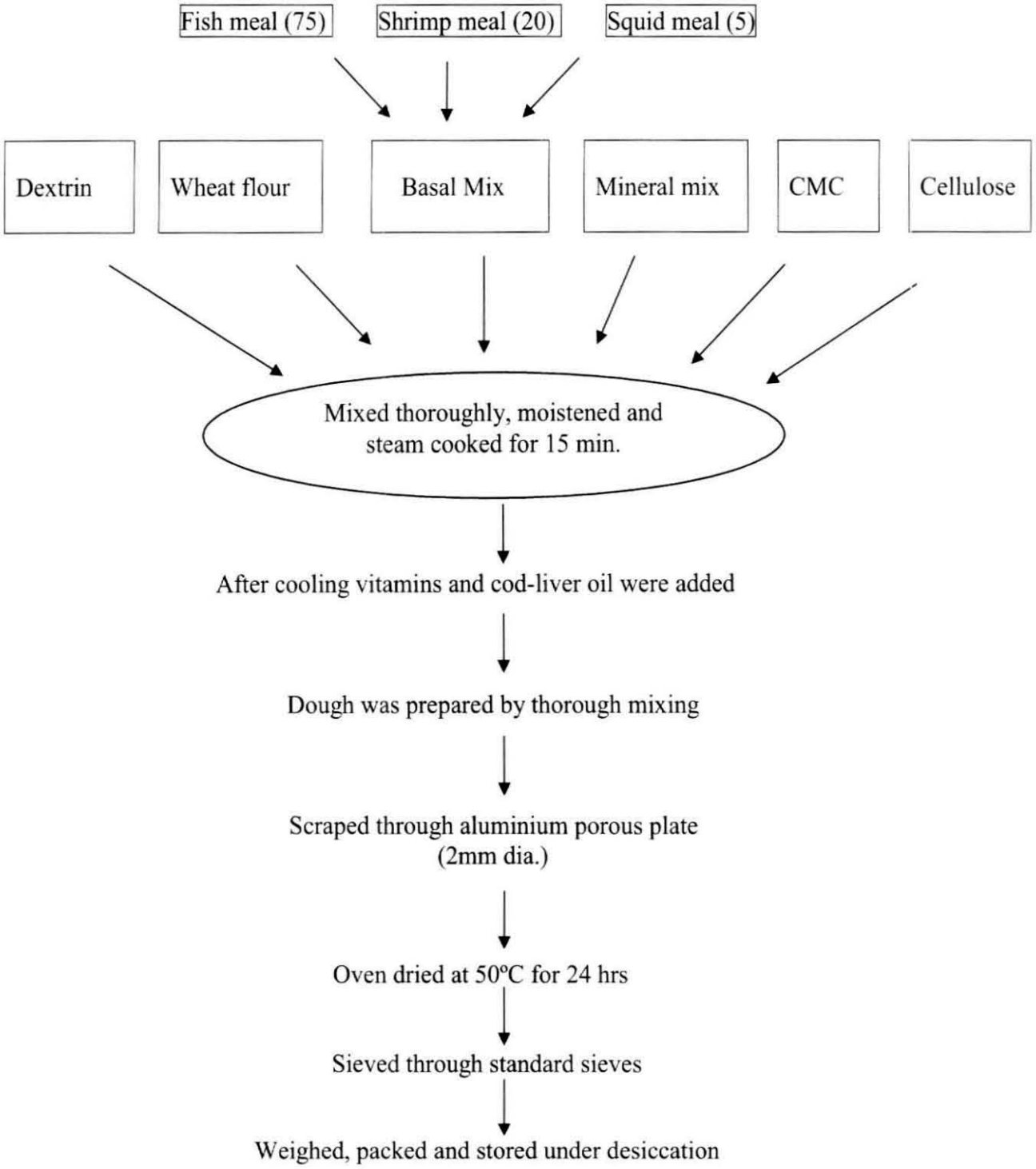


Fig. 7. Flow chart showing the diet preparation for the requirement studies

Table VIII. Ingredient levels, proximate composition and energy levels of the diets used in lipid requirement experiment

	Lipid levels (%)				
	D_{L3}	D_{L6}	D_{L9}	D_{L12}	D_{L15}
Ingredients (g Kg^{-1})					
Basal mix¥	745	745	745	745	745
Starch	5	5	5	5	5
Dextrin	177	146	107	57	26
Cod-liver oil	2	33	63	93	124
Vitamin mix§	20	20	20	20	20
Mineral mixΨ	40	40	40	40	40
CMC‡	10	10	10	20	20
Cellulose	1	1	10	20	20
Proximate composition (% dry matter)					
Crude protein	44.98	45.06	44.93	45.12	45.06
Crude lipid	3.01	6.08	9.05	12.08	15.11
Crude ash	22.40	22.40	22.41	22.47	22.46
N-free extracts	18.89	15.85	12.03	7.09	4.06
Gross energy*	15.05	15.76	16.25	16.64	17.30
P/E ratio¶	29.89	28.59	27.65	27.12	26.05

¥ Contains fish meal, shrimp meal and squid meal (15:4:1).
Ψ Mineral mix (g/kg mix): $MgSO_4.7H_2O$, 90.0; $MnSO_4.H_2O$, 1.3; KI, 0.3; $NaH_2PO_4.2H_2O$, 80; $NaHSeO_3$, 0.01; $CoCl_2.6H_2O$, 0.8; $ZnSO_4.7H_2O$, 3.0; NaCl, 25; $CuSO_4.5H_2O$, 0.5; KH_2PO_4, $2H_2O$, 100; $FeSO_4.7H_2O$, 12.5; $CaHPO_4$, 50 (cellulose filler).
§ Vitamin(mg or IU kg^{-1} in diet): Vit.A, 5000 IU; cholecalciferol, 3000 IU; tocopherol acetate, 250 IU; menadione, 10; ascorbic acid, 250; thiamine hydrochloride, 25; riboflavin, 15; calcium pantothenate, 50; pyridoxine hydrochloride, 20; folic acid, 6; nicotinic acid, 60; biotin, 0.5; cyanocobalamin, 0.04; choline chloride, 900; inositol, 200.
‡ Carboxymethylcellulose.
*Gross energy (KJ g^{-1}) was calculated based on 23.6 kJ g^{-1} for protein, 39.5 kJ g^{-1} for lipid and 17.2 kJ g^{-1} for carbohydrate.
¶ mg protein kJ^{-1} of gross energy.

3.5.4. Hydro-stability of diets

Hydro-stability of diets was estimated by static water method where no pellet or water agitation was provided. The experiment was carried out by accurately weighing 2g of representative feed sample in a bolting silk bag. These bags were hung into a 70 ℓ basin containing seawater using nylon monofilaments from wooden rods. Stability of the diets was estimated after 30 minutes and one hour by rinsing with distilled water and oven drying at 100° C.

3.5.5. Feeding

The fishes were hand fed to apparent satiation *i.e.* till the visual observation of uneaten feed particle. The water filtration was stopped before feeding and resumed after ensuring consumption and removal of excess feed. Feeding was done thrice daily (0900 hrs, 1300 hrs and 1700 hrs) for three weeks with feed granules of 500-1000µm particle size. Thereafter, the feeding was rescheduled and fed twice (0900 and 1600 hrs) daily with granules of size > 2 mm. The excess feed, if any, was carefully siphoned out within an hour into a bolting silk, rinsed with distilled water, and oven dried (50° C).

3.5.6. Data collection

As the fishes were found sensitive to frequent handling measurements of total length and weight were taken before and after the experiment. Fishes were rinsed with freshwater and excess moisture was removed using tissue paper before measuring. The mortalities were recorded on a day to day basis.

3.6. Biochemical Analyses

3.6.1. Proximate composition

Moisture, crude protein and crude fibre levels in feed were determined as per AOAC (1990); crude lipid was estimated by soxhlet extraction with petroleum ether (BP 60-80^0 C); ash content was determined as the residue remaining after incineration of samples at 550^0 C in a muffle furnace for 12 hrs, and the nitrogen free extract (NFE) was computed by difference. Crude protein

estimation was carried out by using the Kjelplus KPS-020 (Pelican, Bio-innovations Pvt. Ltd.) semi-automatic system and the titration using the Titroline 96 (Schott). Flow charts for the proximate analyses are given in Fig. 8a to Fig. 8d.

As the digestible energy values for feed ingredients have not yet been determined for clownfishes, gross energy values were used. The gross energy was calculated using values of 23.6 Kj g^{-1} for protein, 39.5 Kj g^{-1} for lipids and 17.2 Kj g^{-1} for carbohydrates (Blaxter, 1989; Bureau *et al.*, 2002).

3.6.2. Amino acid analysis

Powdered feed/tissue sample 0.1 g with 10 ml of 6 N HCl was digested at 110° C in sealed tubes for 24 hours. The solution was filtered and flash evaporated thrice using distilled water to remove the acid. The acid free sample was then made upto 5 ml with 0.05 N HCl, and filtered in a syringe nylon filter of 0.2 µm. The pre-column derivatisation of amino acids was done with phenylisothiocyanite (PITC) to form phenylthiocarbamyl (PTC) amino acids.

Twenty microlitres (20 µl) of the derivatized sample was injected into HPLC (Waters reversed-phase PICO.TAG amino acid analysis system), fitted with packed column (dimethyloctadecylsilyl bonded amorphous silica). The elution buffer used was sodium acetate trihydrate (pH 6.4) and acetonitrile. The detector (Waters 2487 dual λ absorbance detector) was set at 0.1 AUFS at 254 nm and the column temperature was set at 38°C. Standard (PIERS Amino acid standard H) was run before each sample injection. Samples were injected in triplicates and the output was analyzed using Breeze software.

3.6.2.1. Estimation of tryptophan

Tryptophan was estimated as per the spectrophotometric method (500 nm) of Sastry and Tummuru (1985) after alkali hydrolysis of the sample using 5% sodium hydroxide at 110° C for 24 hrs.

3.6.3. Fatty acid analysis

Lipid extraction was carried out by Folch *et al.* (1957) method. Approx. 20g fresh tissue was homogenized with chloroform methanol mixture (2:1). To analyse feeds, distilled water was added to make the moisture content

Estimation of Dry Matter

Weighed a clean and dry aluminum cup (W)

↓

Weighed approx. 5g sample in the cup (W_1)

↓

Kept in hot air oven at 100°C for 2 hr

↓

Cooled in a desiccator and weighed (W_2)

↓

$$\text{Dry matter (\%)} = \frac{W_2 - W}{W_1 - W} \times 100$$

Estimation of Crude Ash

Weighed clean silica crucible (W)

↓

Weighed approx. 3g sample with crucible (W_1)

↓

Kept in hot air oven at 60-80°C overnight and
Incinerated in a muffle furnace at 550°C

↓

Cooled to room temperature in a desiccator
and weighed (W_2)

↓

$$\text{Crude Ash (\%)} = \frac{W_2 - W}{W_1 - W} \times 100$$

Fig. 8a. Flow charts of dry matter and crude ash estimation

Estimation of Crude Protein

Weighed approx. 0.5g of sample into the digestion tube

↓

Added 1g digestion mixture
(K_2SO_4+$CuSO_4$ in 9:1)

↓

Added 5 ml of concentrated H_2SO_4 and
digested in digester (Kjel-Plus) at 350°C

↓

Cooled to room temperature after solution
became clear and made up to 100 ml

↓

Took 10 ml sample in distillation tube and added 10 ml
40% NaOH in a Distiller (Kjel-Plus)

↓

Distilled for 6 minutes

↓

Released ammonia gas was collected in 10 ml mixed indicator

↓

Resultant green solution was titrated against 0.1N HCl
using an autotitrator (Titroline 96)

↓

$$\text{Crude Protein (\%)} = \frac{\text{Volume of acid (ml)} \times 0.0014 \times 100 \times 100 \times 6.25}{\text{Weight of sample (g)} \times 10}$$

Fig. 8b. Flow chart for crude protein estimation

Estimation of Crude Fat

Weighed 3g sample into a whatman paper thimble and plugged using absorbent cotton (W)

↓

Weighed a round bottom flask (W_1)

↓

Assembled in Soxhlet extraction unit

↓

Added petroleum ether (B.P. 40-60°C) to 1 1/2 times capacity of soxhlet

↓

Started water circulation in the condenser and set hotplates on bench to 50-60°C

↓

Continued extraction for 8 hr (approx. 250 cycles)

↓

Removed the round bottom flask (RBF) from assembly and dried in hot air oven

↓

Cooled RBF in desiccator and weighed to concordance (W_2)

↓

$$\text{Crude Fat (\%)} = \frac{W_2 - W_1}{W} \times 100$$

Fig. 8c. Flow chart for crude fat estimation

Estimation of Crude Fiber

Weighed 2 g fat free sample into 1 liter Berzelius spoutless beaker (W)

↓

Added 200 ml of 1.25% H_2SO_4, covered the mouth with RBF with cold water and digested for 30 minutes

↓

Filtered through 100 µm bolting silk by applying suction and repeatedly washed with warm water

↓

Residue was washed down with 200 ml 1.25% NaOH into the beaker for alkali digestion

↓

Filtered the contents into a Gooch crucible with a final rinse using acetone

↓

Oven dried the Gooch crucible at 70°C overnight (W_1)

↓

Incinerated in Muffle furnace at 550°C, Cooled to room temperature in a desiccator and weighed (W_2)

↓

$$\text{Crude Fibre (\%)} = \frac{W_1 - W_2}{W} \times 100$$

Fig. 8d. Flow chart for crude fiber estimation

approx. 80% and made it into a paste. Added chloroform-methanol mixture (15 times) with 0.01% BHT and mixed thoroughly. The solution was filtered thrice to ensure proper extraction. To the filtrate, distilled water (20% of the total volume of the filtrate) was added and left overnight under refrigeration. Water-soluble residues diffused away from solvent and occupied the top layer in the separating funnel. Solvent containing lipid (bottom layer) was collected by filtering through anhydrous sodium sulphate, evaporated to dryness and later saponified.

Added 5 ml of alcoholic potassium hydroxide (0.5%) into a round bottom flask with sample. Refluxed for five minutes in an atmosphere of nitrogen and 6 ml BF3-MeOH was added and refluxed. In order to recover the dry ester extracted 2-3 times with petroleum ether. Washed the extract with 25 ml distilled water three times (until acid free), dried with sodium sulphate and evaporated the solvent under the stream of nitrogen. The resultant product was transferred into smaller vials and kept in freezer until analysis.

The fatty acid analysis was performed on a Perkin Elmer AutoSystem XL, Gas chromatograph (Perkin Elmer, USA), equipped with a splitless injector and a flame ionization detector (FID). The capillary column was Elite-5 (crossbond 5% diphenyl- 95% dimethyl polsiloxane) with 30 meters, 0.53 mm ID and 0.50 µm film thickness. The column temperature was programmed from 110°C to 160°C at 45°C/min and then to 250°C at 30°C/min and finally to 285°C at 15°C/min. The injector and detector temperatures were kept at 285°C and 290°C respectively. Nitrogen was used as the carrier gas with a pressure of 8 psi. The flow rate of hydrogen and air were maintained at 50psi each. Standard (Supelco, FAME mix C_4-C_{24}) was injected before each sample run. A secondary reference standard of cod-liver oil FAME was used to confirm the peaks. 0.5 – 1 µl of sample was injected in triplicate and the data acquisition was done with TotalChrome 6.X.X software.

3.6.4. Astaxanthin estimation

The extraction of carotenoids was done following the method of Weber and Davoli (2003). 25 g tissue was taken and thoroughly homogenised. The homogenised flesh was covered with 50 g anhydrous Na_2SO_4 and left to dehydrate for at least 30 minutes. It was transferred to a fluted flask containing

200 ml acetone and shaken for 3 hr, followed by filtration and then rotary evaporated to dryness. After complete evaporation it was re-dissolved in 100 ml acetone and filtered and rotary evaporated. 15 ml *n*-hexane was added to this and was transferred to a separating funnel containing 10 ml dimethylsulphoxide (DMSO). The funnel was shaken well to obtain phase separation; with the top phase (*n*-hexane) containing non-polar lipids and the bottom phase (DMSO) of polar carotenoids, including astaxanthin. The bottom phase was collected in a conical flask by placing it above ice followed by the addition of 6 ml distilled water, 3 ml saturated NaCl solution and 10 ml ethanol to increase the polarity of DMSO phase. Mixed well and transferred to a separating funnel, followed by the addition of 20 ml *n*-hexane. The funnel was shaken well to achieve the layer separation and the top (*n*-hexane) phase was collected. Repeated the extraction procedure with another 20 ml *n*-hexane and pooled both the extracts. In order to remove any residual DMSO from the n-hexane phase 40 ml distilled water was added. After shaking and separation, the lower (aqueous) phase was discarded and washed repeatedly twice with fresh distilled water. Collected the *n*-hexane phase, dried over Na_2SO_4 and rotary evaporated. This extract of polar carotenoids was then dissolved in 0.5 ml ethyl acetate and used for astaxanthin estimation.

The astaxanthin was estimated using spectrophotometer (Genesys 10UV, Thermospectronic) at 480 nm, the absorption peak of astaxanthin in visible light. Two cuvettes were filled with 2 ml methanol, and the absorption value was set to zero. To the sample cuvette, few micro-litres (µl) of crude extract was added and mixed thoroughly and reading was taken; added further aliquots to get an absorbance around 0.5. The amount of astaxanthin was estimated by the following equation (Schiedt and Liaaen-Jensen, 1995);

$$X = (A_{480} \times y \times 1000) / (A_{1cm} \times 100)$$

Where x = astaxanthin (mg), A_{480} = absorbance at 480 nm, y = volume of sample (2 ml + crude extract added), and A_{1cm} = specific absorption coefficient of astaxanthin for a 1% solution in a 1 cm cell, in methanol it is 2100 (Fleno *et al.*, 1999). The x value obtained was multiplied with total volume of crude extract for further calculations.

3.7. Formulae used

$$\text{Specific growth rate (SGR)} = \frac{\ln(\text{final weight}) - \ln(\text{initial weight})}{\text{Number of days}} \times 100$$

$$\text{Condition factor (CF)} = (\text{Final weight} / \text{Final length}^3) \times 100$$

$$\text{Average daily gain (ADG)} = (\text{Final weight} - \text{Initial weight}) / \text{No. of days}$$

$$\text{Weight gain (WG \%)} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial mean weight}} \times 100$$

$$\text{Survival (\%)} = (\text{Final fish number} / \text{Initial fish number}) \times 100$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{(Feed intake, g dry weight)}}{\text{(Fish weight gain, g wet weight)}}$$

$$\text{Protein efficiency ratio (PER)} = \frac{\text{(Fish wet weight gain, g)}}{\text{(Protein intake, g)}}$$

3.8. Statistical Analysis of Data

Data were presented as mean ± 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 test (Duncan, 1955). Statistical analyses were performed using the SPSS 7.5 version for WINDOWS and results were treated statistically significant at 5% level. The optimum dietary protein and lipid requirements were estimated by second order polynomial regression.

RESULTS

4. RESULTS

4.1. Water Quality Parameters

Water quality parameters recorded during the different experiments are given in Table IX. All the water quality parameters tested were within the limits recommended for rearing marine fishes. The use of biological filter and proper water exchange effectively reduced the build-up of toxic metabolites and thus helped in maintaining better water quality.

4.2. Broodstock Nutrition

4.2.1. Pair Compatibility

The adult fishes, which were introduced into the broodstock experimental system, were observed for pairing. During the initial two to three weeks of introduction no pairing was observed. In each tank the larger fish exhibited domination over the other and it subsequently became a female and the other a functional male. In most tanks pairs got established within two months of introduction and spawning started two to three months after pairing.

4.2.2. Feeding Behaviour

The fishes were observed to feed actively in the water column, and showed passive feeding response to the feed particles settled at the bottom. The modified feeding strategy employed (disc feeding) resulted in better feed consumption and minimized the feed wastage. Suction or gulping mode of feeding was observed when adult fishes were fed with polychaete worms (90 – 100 mm length and 2 – 3 mm diameter).

Once habituated the fishes immediately approached and consumed the feed as soon as it was dispensed in the water column. A distinct feeding priority was observed among well established pairs with an initial feeding response from the female partner. In such pairs males usually keep away from

Table IX. The mean seawater parameters observed during the experiments (mean ± SD)

Parameter	Temp(^{0}C)	Salinity (‰)	pH	DO (mg/L)	Ammonia (ppm)	Nitrite (ppm)	Nitrate (ppm)
Broodstock experiments							
Broodstock development from juvenile	28.5±1.5	33.2±1.7	8.12±0.4	6.04±0.2	0.009±0.005	0.020±0.008	0.312±0.112
Evaluation of natural diets	27.9±0.9	33.8±1.6	8.07±0.3	6.28±0.3	0.005±0.002	0.008±0.004	0.096±0.056
Evaluation of compounded diets	28.6±1.0	33.5±1.4	8.08±0.2	6.34±0.3	0.006±0.003	0.013±0.003	0.141±0.078
Influence of feed allowance	27.8±1.2	32.8±0.8	8.03±0.2	6.01±0.1	0.008±0.004	0.011±0.004	0.112±0.068
Larval experiments							
Livefeed evaluation	28.7±1.2	32.4±1.1	8.02±0.1	5.72±0.3	0.010±0.004	0.012±0.007	0.396±0.114
Weaning studies	28.1±1.0	31.8±1.4	7.98±0.1	5.96±0.2	0.004±0.002	0.009±0.005	0.238±0.094
Juvenile experiments							
Diet comparison	27.6±1.4	32.1±1.2	8.07±0.1	5.90±0.4	0.004±0.001	0.006±0.003	0.164±0.089
Protein requirement I	28.9±1.0	33.4±1.2	8.11±0.1	6.20±0.2	0.003±0.001	0.004±0.002	0.128±0.056
Protein requirement II	28.4±0.8	32.7±0.9	8.14±0.1	6.00±0.3	0.003±0.000	0.003±0.001	0.096±0.031
Lipid requirement	29.2±1.1	33.8±0.8	8.06±0.0	6.10±0.2	0.002±0.001	0.004±0.001	0.087±0.054

the feed when feeding was done sparingly. However, almost equal feeding was observed among pairs of almost similar size.

4.2.3. Spawning, Fertilization and Parental care

The females spawned after extended courtship behaviours like clearing of nesting site, biting the substratum and parallel swimming by the pairs. Spawning took place usually between 0900 hrs and 1330 hrs with a peak between 1000 hrs and 1200 hrs. During spawning the female attached the eggs on hard substratum with the extended ovipositor by rhythmic movement and slight quivering (Plate XI). The male fertilized the eggs intermittently and the spawning process lasted for 1 to 1½ hours. The eggs were always found to be deposited in proximity to the host anemone.

The pairs exhibited parental care by guarding and fanning the eggs (Plate XII). They removed the unfertilized and infected eggs, if any, from the clutch. The clutch care was mostly done by the male fish.

4.2.4. Broodstock Performance

4.2.4.1. Influence of Natural Diets on Egg and Larval Quality

4.2.4.1.1. Proximate composition

The proximate composition of the tested natural diets is given in Table X. Crude protein content ranged from 49.54 to 70.06% with the lowest and the highest levels in mussel meat and squid meat respectively. Except for the squid diet all other diets had crude protein content in the range 50 to 60%. The crude lipid level varied from 4.71 in polychaete worms to 16.71% in mature mussel.

4.2.4.1.2. Amino acid profile

Amino acid profile (PWM not determined), showed significant ($P<0.05$) difference between the diets (Table XI). The essential amino acid (EAA) profiles of the natural diets also showed significant ($P<0.05$) differences. While cuttlefish had very high levels of arginine, it had very low level of lysine. Leucine and methionine levels were relatively high in squid meat; threonine and

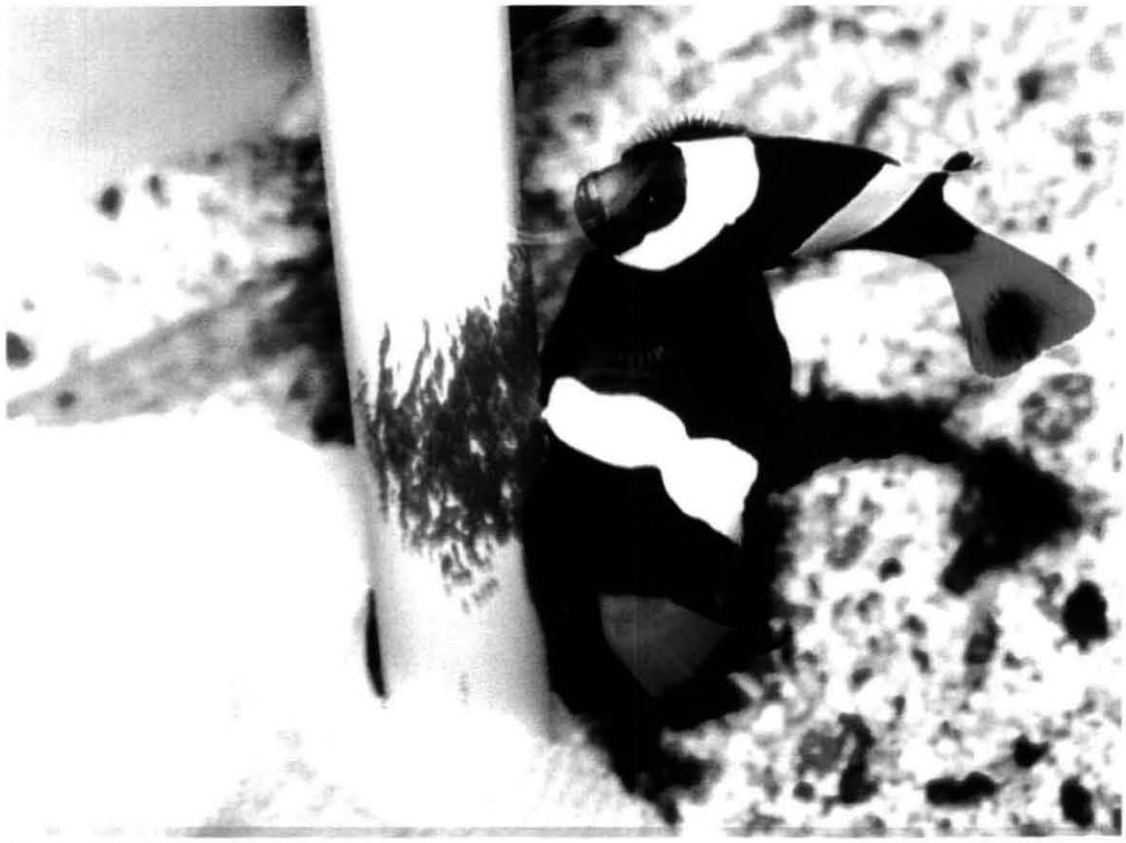


Plate XI. Spawning and fertilization in sebae anemonefish (ovipositor visible)

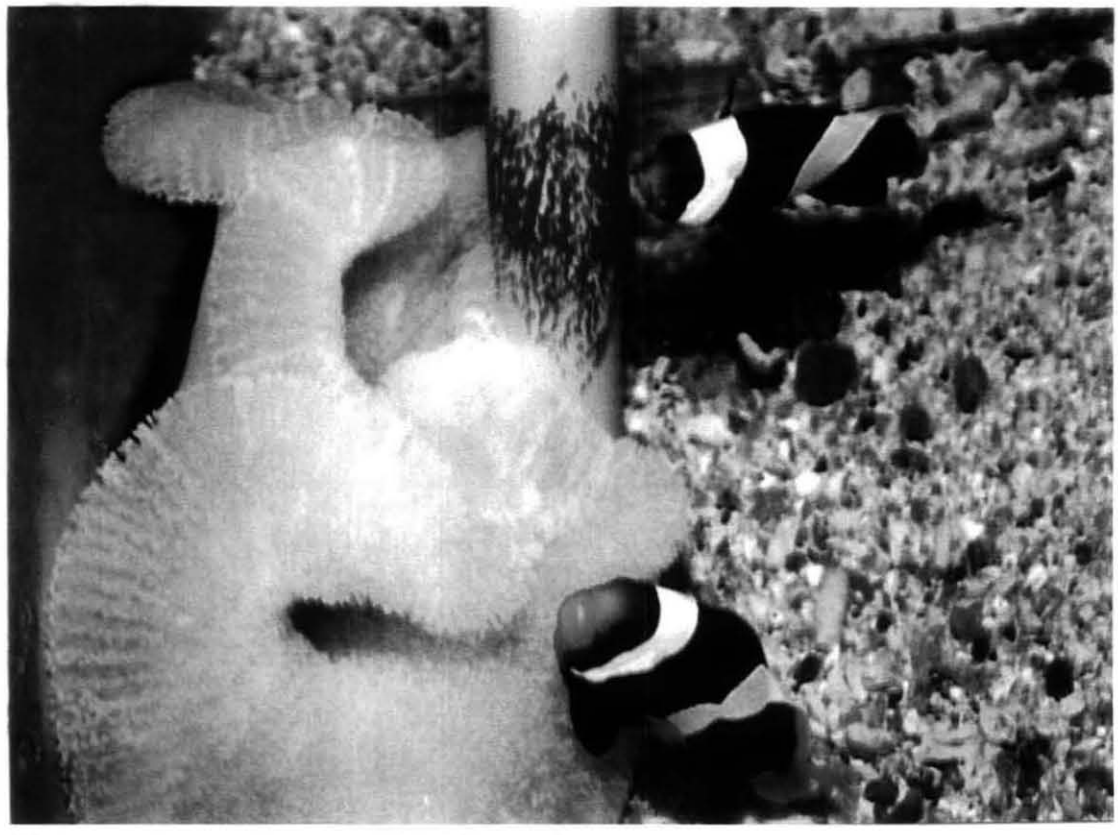


Plate XII. Parental care exhibited by male

Table X. Proximate composition (% dry matter) of natural 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
Polychaete worm	53.56	4.71	*	*
Mussel meat (MSM)	49.54	11.28	23.82	13.32

* not determined

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

Amino acid	DSP	MGD	MSM	SQD	CUT
ARG	5.48^{a}	8.18^{b}	5.29^{a}	4.69^{c}	10.16^{d}
HIS	2.08^{ac}	1.84^{ab}	1.73^{b}	2.30^{cd}	2.62^{d}
ILE	4.14^{a}	4.14^{a}	4.12^{a}	4.65^{b}	4.80^{b}
LEU	8.24^{a}	7.84^{a}	7.56^{a}	9.02^{b}	8.90^{b}
LYS	9.88^{a}	8.67^{a}	15.79^{b}	8.93^{a}	4.48^{a}
MET	2.50^{a}	2.54^{a}	2.55^{a}	3.26^{b}	2.93^{c}
THR	4.23^{a}	5.47^{c}	5.39^{c}	4.72^{b}	4.42^{a}
TRY	1.25^{a}	1.74^{b}	1.67^{b}	1.26^{a}	1.77^{b}
PHE	4.22^{ab}	4.40^{bc}	4.50^{c}	3.94^{a}	3.56^{d}
VAL	4.69^{a}	5.04^{b}	5.23^{b}	4.67^{a}	4.62^{a}
ALA	8.28^{b}	6.24^{a}	5.80^{a}	9.18^{c}	8.13^{b}
ASP	7.11^{a}	9.17^{b}	9.95^{b}	7.86^{a}	7.42^{a}
CYS	0.47^{a}	0.78^{c}	0.64^{bc}	0.71^{bc}	0.60^{b}
GLU	10.43^{a}	9.93^{ab}	9.74^{b}	11.24^{c}	10.45^{ab}
GLY	11.85^{a}	9.98^{c}	7.46^{d}	10.47^{bc}	12.09^{ab}
PRO	5.38^{a}	4.92^{b}	4.23^{c}	5.50^{a}	5.64^{a}
SER	6.48^{a}	5.68^{b}	5.06^{bc}	4.83^{c}	4.70^{c}
TYR	3.30^{a}	3.42^{a}	3.28^{a}	2.75^{b}	2.72^{b}
∑ EAA	46.73	49.88	53.84	47.46	48.25
∑ NEAA	53.27	50.12	46.16	52.54	51.75
EAA/NEAA	0.88	1.00	1.17	0.90	0.93

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
ΣEAA- Total essential amino acids
ΣNEAA- Total non-essential amino acids

tryptophan levels were higher in mature mussel; lysine, phenylalanine and valine were higher in immature mussel meat. Among the essential amino acids lysine content in mussel meat (15.79 g/100g protein) was found to be significantly ($P<0.05$) higher than all other diets. Apart from squid and cuttlefish other diets did not show any significant difference in methionine levels.

The non-essential amino acids profile (NEAA) also showed significant ($P<0.05$) difference between the diets. Glycine, glutamic acid and serine levels were found to be high in the deep-sea prawn (DSP); cysteine and tyrosine levels were high in mature mussel (MGD); aspartic acid content was high in mussel meat (MSM); alanine and glutamic acid levels were high in squid (SQD); and proline level was high in both DSP and SQD.

The total essential amino acids (EAA) content was high in mussel meat (53.84%), which was followed by mature mussel with gonad (49.88%) and the least value was recorded in the deep-sea prawn (46.73%). Consequently, the essential to non-essential amino acid ratios were high in mussel diets (≥ 1) and low in the deep-sea prawn (0.88).

4.2.4.1.3. Fatty acid profile

Fatty acid profile (% total fatty acids) showed considerable difference between the natural diets (Table XII). The total saturated fatty acid levels were significantly ($P<0.05$) high (38.64%) in MSM and low in DSP (23.03%) and SQD (24.51%). Among the saturated fatty acids C16:0 (palmitic acid) level was significantly ($P<0.05$) high in MGD (29.18%) and was low in CUT (17.01%). However, C18:0 (stearic acid) was found to be significantly ($P<0.05$) high (14.95%) in cuttlefish meat.

The total unsaturated fatty acids content also showed significant ($P<0.05$) difference between the diets, with significantly ($P<0.05$) low level in CUT (46.41%). All the other diets had more than 50% unsaturated fatty acids, with DSP containing as high as 62.33%.

The monounsaturated fatty acids content (MUFA) also showed significant ($P<0.05$) difference between the diets with significantly high level in

Table XII. Fatty acid profile (% total fatty acids) of natural diets

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

DSP (31.6%) and low levels in CUT and MSM. Among the MUFA the oleic acid (C18:1 n9) was significantly high in DSP (21.86%); CUT had the least amount (< 1%). However, C20:1 level was very high in CUT (13.71%) as compared to other diets, which had less than 2%.

The polyunsaturated fatty acid (PUFA) levels also showed significant ($P<0.05$) variation between diets with the highest level in MSM (41.12%) and relatively low levels in DSP and CUT, wherein it accounted for less than 30% of the total fatty acids. The essential fatty acid levels (EFA) also showed significant ($P<0.05$) difference among the diets, with significantly ($P<0.05$) higher DHA levels in SQD (28.74%) and CUT (21.39%) than other diets and lower levels in MGD (12.64%) and MSM (12.01%). However, the other important essential fatty acid, EPA level was significantly ($P<0.05$) high in MGD (16.1%) and MSM (21.15%) but did not show significant difference among DSP, CUT and SQD. Arachidonic acid (AA) was significantly ($P<0.05$) higher (7.14%) in DSP than other diets followed by CUT (3.35%).

The total n-3 and *n*-6 fatty acid levels also significantly ($P<0.05$) varied among the diets, with higher levels of n-3 in MSM, whereas the n-6 content was high in DSP. The n-3 highly unsaturated fatty acids content recorded in MSM (34.13%) was about twice that of DSP (17.69%).

The saturated to unsaturated fatty acids ratio was significantly ($P<0.05$) high (0.73) in CUT and low in DSP (0.37) and SQD (0.40). The n-3 to n-6 ratio also showed significant ($P<0.05$) variation among the diets, with MSM giving a ratio of 20.09, which was about ten times higher than that of the DSP (2.04).

The DHA:EPA ratio, an important parameter determining the quality of broodstock diet and its performance, was significantly higher for SQD (7.81), followed by CUT (5.97) and DSP (3.82). Mussel diets (MGD and MSM) gave the least ratios of 0.79 and 0.57 respectively. The EPA:AA ratio was high for SQD (1.53) followed by CUT (1.07) and DSP (0.50).

4.2.4.1.4. Astaxanthin content

Astaxanthin concentration (Table XIII) was found to be high (42.77 μg g^{-1} wet weight) in the deep-sea prawn (DSP). The mature mussel had 23.25 μg g^{-1} of astaxanthin followed by the immature mussel with 17.42 μg g^{-1} wet weight. Cuttlefish gave the least value of 5.13 μg g^{-1} wet weight astaxanthin.

Astaxanthin content in the head portion of the deep-sea prawn (103.48 μg g^{-1} wet weight) was about ten times higher than that of the tail portion (Table XIII). The lower astaxanthin level in mature mussel (female) may be due to the predominance of other carotenoids as the peak absorption in the mussel diets were observed well below 480 nm used for astaxanthin estimation.

4.2.4.1.5. Influence on the egg production

The number of eggs spawned by the fish was significantly ($P<0.05$) influenced by the diets used (Fig. 9). The cuttlefish (CUT) fed group produced the best performance with an average clutch size of 1521 ± 264 eggs followed by those fed the deep sea prawn (DSP, 1300 ± 445), mature mussel gonad (MGD,1150 ± 141), polychaete worm and mussel meat (PWM, 1133 ± 325) and squid meat (SQD, 1025 ± 232). The control group fed the mussel meat (MSM) as diet yielded the least with an average clutch size of 885 ± 55 eggs.

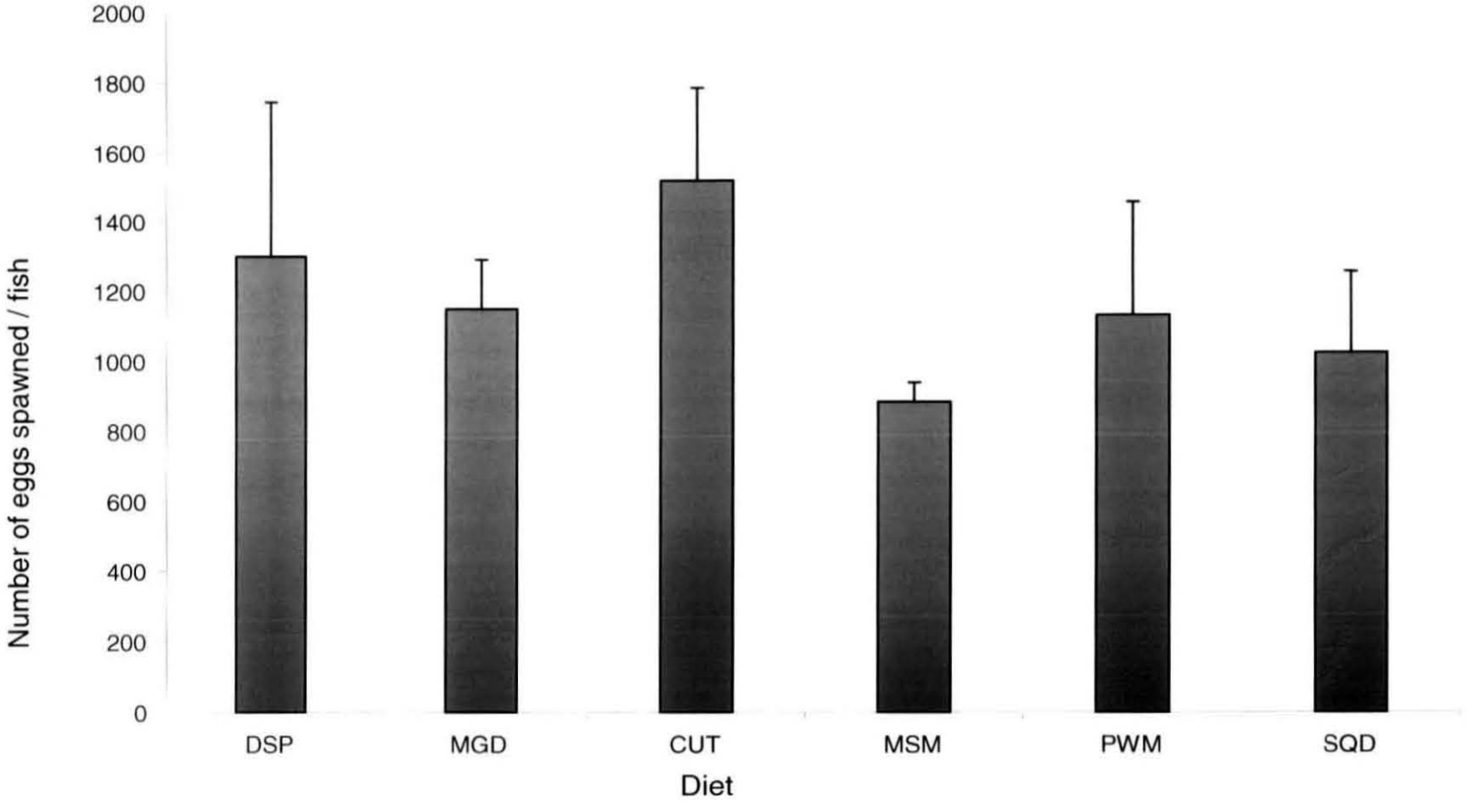
The egg capsule dimensions of the fish from the dietary treatments are presented in Table XIV. The capsule length did not vary significantly between the tested diets, and it ranged from 2.19 (MGD and MSS) to 2.23 mm (SQD). By contrast, the egg capsule width exhibited significant ($P<0.05$) variations among the treatments. The capsule had maximum width (914 μm) when the fishes were fed mature mussel (MGD), closely followed by squid fed fish (910 μm). The capsule showed least width (845 μm) when mussel meat (MSM) was fed.

4.2.4.1.6. Fertilization rate and hatchability

The tested diets did not affect the fertilization rate and hatchability of the eggs in the present study.

Table XIII. Estimated astaxanthin content in tested natural diets

Diet	**Astaxanthin** ($\mu g\ g^{-1}$ wet weight)
Mussel meat (MSM)	17.42
Mature mussel (MGD)	23.25
Cuttlefish meat (CUT)	5.13
Deep-sea Shrimp (whole) (DSP)	42.77
Deep-sea Shrimp (tail)	10.34
Deep-sea Shrimp (head)	103.48



DSP- deep-sea prawn; MGD- mature mussel; CUT- cuttlefish; MSM- mussel meat; PWM- polychaete + mussel; SQD- squid

Fig. 9. The influence of natural diets on the number of eggs spawned by *Amphiprion sebae* (mean ± SD, n=9)

Table XIV. Influence of natural diets on the egg dimensions of *Amphiprion sebae* (mean ± SD, n =108)

Diet	Egg capsule length (mm)	Egg capsule width (µm)
DSP	2.21 ± 0.04	851 ± 37[a]
MGD	2.19 ± 0.05	914 ± 36[c]
CUT	2.21 ± 0.02	855 ± 26[a]
MSM	2.19 ± 0.01	845 ± 20[a]
PWM	2.21 ± 0.01	859 ± 32[ab]
SQD	2.23 ± 0.03	910 ± 10[bc]

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
MSM - Brown mussel meat
PWM - Polychaete worm + Mussel meat (1:1)
SQD - Squid meat

4.2.4.1.7. Larval quality

The total length of newly hatched larvae from the different treatments is presented in Table XV. The differences in mean larval lengths were not statistically significant. Squid meat gave better performance among the diets with a larval total length of 4.31 mm, which was followed by the deep sea prawn meat (4.18 mm). Mussel meat (MSM) produced relatively smaller larvae (3.96 mm) among the tested diets.

The influence of natural diets on the larval survival is depicted in Fig 10. The larval survival did not show any significant variation at initial stage (3 dph), when the broodstock diet was expected to play a major role, or by the termination of the experiment (12 dph). The maximum survival (62.3 ± 6.7%) was obtained with deep-sea prawn which was closely followed by mature mussel (60.3 ± 2.1%) and squid meat (59 ± 5.3%) at the termination of experiment (12 dph). Mussel meat gave comparatively low (44.3 ± 5.7%) survival among the tested diets.

4.2.4.2. Influence of Compounded Diets on Egg and Larval Quality

4.2.4.2.1. Nutrient composition

The crude protein content ranged from 38.33 to 49.62% and crude lipid from 8.56 to 20.09 % in the compounded moist diets CBD_1 to CBD_5 (Table II). Diet CBD_1 was prepared without spirulina and other four diets were with spirulina. The basal mix used in CBD_5 was different from that of other diets in having a ratio of 4:3:3 fishmeal, shrimp meal and squid meal. CBD_1 and CBD_2 had a protein content of about 40% and lipid level of 20%, and CBD_4 and CBD_5 had 50% protein and 10% lipid. CBD_3 had 40% protein and 10% lipid.

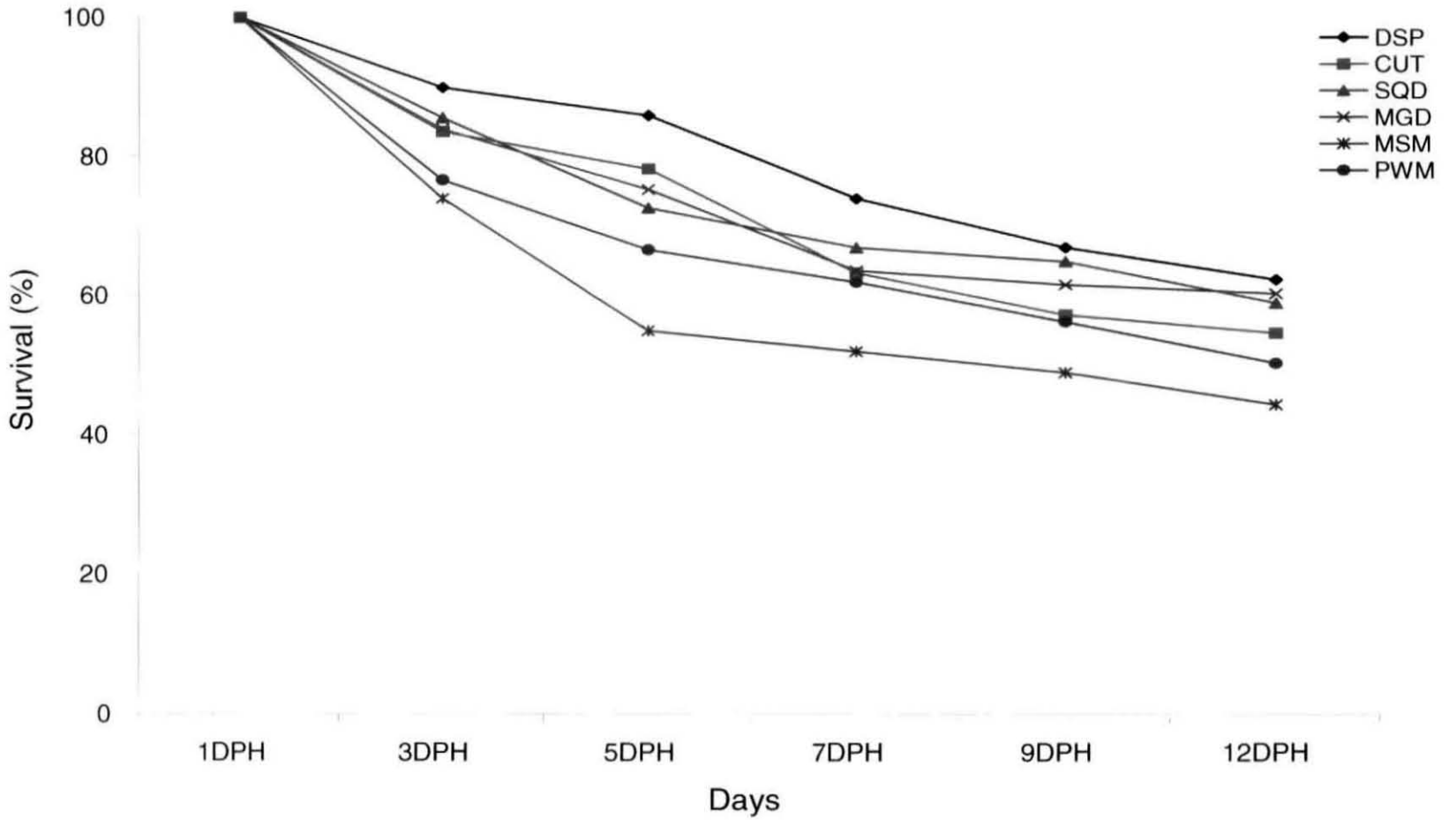
Amino acid profiles (Table XVI) showed significant ($P<0.05$) differences among the diets. The essential amino acid levels, except histidine and phenylalanine were relatively high in diet CBD_4, in which spirulina was a major (45%) ingredient. The dispensable amino acid content was high in diet CBD_5.

Table XV. Influence of natural diets on the total length of newly hatched larvae of o*Amphiprion sebae* (mean ± SD, n =108)

Diet	Larval length (mm)
DSP	4.18 ± 0.15
MGD	4.05 ± 0.19
CUT	4.09 ± 0.12
MSM	3.96 ± 0.10
PWM	4.16 ± 0.07
SQD	4.31 ± 0.09

Statistically insignificant ($P<0.05$)

DSP - Deep sea prawn meat
MGD - Mature brown mussel
CUT - Cuttlefish meat
MSM - Brown mussel meat
PWM - Polychaete worm + Mussel meat (1:1)
SQD - Squid meat



DSP- deep-sea prawn, MGD- mature mussel meat, MSM- mussel meat, SQD- squid, CUT- cuttlefish, PWM - mussel meat + polychaete worms (1:1)

Fig. 10. Influence of natural diets on the survival of *Amphiprion sebae* larvae

Table XVI. Amino acid profiles of moist diets used in the broodstock experiment

	g/100g diet				
	CBD_1	CBD_2	CBD_3	CBD_4	CBD_5
ARG	1.49	1.53	1.56	2.15	2.02
HIS	0.87	0.85	0.82	0.88	1.21
ILE	1.22	1.27	1.30	1.91	1.77
LEU	3.01	3.07	3.22	4.19	3.68
LYS	4.48	4.40	4.37	4.70	4.23
MET	1.03	1.02	1.04	1.14	0.83
THR	2.50	2.48	2.38	2.98	1.71
TRY	0.47	0.47	0.46	0.59	0.47
PHE	1.89	1.89	1.98	2.19	3.13
VAL	1.47	1.55	1.65	2.55	2.28
ALA	3.17)	3.27	3.19	4.90	3.96
ASP	2.55	2.62	2.58	3.87	2.66
CYS	0.20	0.19	0.19	0.14	0.32
GLU	3.75	3.81	4.57	4.96	5.74
GLY	5.44	5.37	5.37	5.88	8.08
PRO	2.39	2.39	2.88	2.74	3.62
SER	1.58	1.65	1.76	2.56	2.03
TYR	1.01	1.04	1.12	1.47	1.30
∑ EAA	18.32	18.38	18.68	23.18	21.23
∑ NEAA	20.01	20.26	21.58	26.44	27.62
EAA/NEAA	0.92	0.91	0.87	0.88	0.77

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
ΣEAA- Total essential amino acids ΣNEAA- Total non-essential amino acids

Fatty acid profiles also showed significant ($P<0.05$) difference between the diets (Table XVII). The levels of saturated, unsaturated, MUFA, PUFA, n-3, n-6 and n-3 HUFA significantly ($P<0.05$) differed between the diets.

Diet CBD_1 had significantly ($P<0.05$) higher percentage (7.96%) of saturated fatty acids viz. C14:0, C16:0 and C18:0. The levels of these fatty acids were found to be low in diet CBD_4. CBD_1 also had a higher (46.87%) content of MUFA and the percentage of C16:1 n7, C18:1 n9, C22:1 and C24:1 n9 was found to be higher than the other diets. The percentage of C18:1 n7 and C20:1 was found to be high in CBD_5. However, MUFA levels were found to be low (29.35%) in CBD_4.

In CBD_4 the PUFA content was dominated by C18:2 n6 and C18:3 n3 and these were significantly higher than the other diets. Diet CBD_5 had significantly higher level (10.14%) of DHA than other diets with relatively low percentage in CBD_4 (4.48%). EPA and AA contents were found to be high in CBD_1 with 1.03% and 8.02% respectively, while diet CBD_4 had the least values with 4.22% EPA and 0.68% AA.

The ratio between saturated and unsaturated fatty acids was similar (0.11) for CBD_1, CBD_2 and CBD_5. n-3/n-6 ratio was found to be high (3.7) for CBD_1 and CBD_5 and it was low (1.59) for CBD_4.

DHA/EPA ratio was significantly ($P<0.05$) high in CBD_5 (2.27) and all other diets had a ratio ranging from 1.06 to 1.09. On the other hand EPA/AA ratio was significantly high in CBD_1 and was found to be low in CBD_5.

4.2.4.2.2. Influence on the egg production

The average number of eggs spawned by the fish fed the moist compounded diets is presented in Fig. 11. The diets significantly ($P<0.05$) influenced the eggs per spawning. Diet CBD_5 gave the maximum egg number (2137 ± 110 eggs) followed by CBD_2 (1683 ± 436) and CBD_3 (1612 ± 517). Diet CBD_1 gave the minimum (1237 ± 295) number of eggs per spawning.

Table XVII. Fatty acid profile (% total fatty acids) of compounded diets used for broodstock diet evaluation

Fatty Acids	CBD_1	CBD_2	CBD_3	CBD_4	CBD_5
14:0	3.31^{a}	3.17^{a}	2.48^{b}	1.85^{c}	2.99^{d}
16:0	1.05^{a}	0.98^{a}	0.91^{a}	0.59^{b}	0.99^{a}
16:1 n7	23.41^{a}	22.12^{ab}	19.78^{c}	14.38^{d}	21.55^{b}
18:0	3.59^{a}	3.40^{c}	3.40^{c}	2.73^{b}	3.33^{c}
18:1 n9	13.26^{b}	12.87^{b}	10.27^{a}	9.38^{a}	13.07^{b}
18:1 n7	1.74^{a}	1.65^{a}	1.34^{b}	0.91^{c}	3.43^{d}
18:2 n6	3.88^{a}	4.37^{a}	4.31^{a}	9.19^{b}	4.00^{a}
18:3 n3	0.87^{a}	1.48^{ab}	1.45^{ab}	6.61^{c}	3.07^{b}
20:1	6.08^{b}	5.65^{a}	5.56^{a}	3.59^{c}	6.87^{d}
20:4 n6	1.03^{a}	0.97^{a}	0.97^{a}	0.68^{b}	0.83^{c}
20:5 n3	8.02^{a}	7.61^{b}	6.13^{c}	4.22^{d}	4.47^{e}
22:1	1.98^{a}	1.90^{a}	1.33^{b}	0.94^{c}	0.66^{d}
22:5 n3	0.73^{a}	0.69^{b}	0.58^{c}	0.39^{d}	0.13^{e}
22:6 n3	8.52^{a}	8.06^{b}	6.66^{c}	4.48^{d}	10.14^{e}
24:1 n9	0.40^{b}	0.39^{c}	0.22^{a}	0.17^{d}	0.22^{a}
$\sum$ Saturated	7.96^{a}	7.55^{b}	6.79^{c}	5.17^{d}	7.32^{e}
$\sum$ Unsaturated	69.91^{a}	67.75^{a}	58.61^{b}	54.92^{c}	68.43^{a}
$\sum$ MUFA	46.87^{a}	44.57^{a}	38.51^{b}	29.35^{b}	45.80^{a}
$\sum$ PUFA	23.05^{a}	23.17^{a}	20.10^{b}	25.57^{c}	22.63^{a}
$\sum$ n-3	18.14^{a}	17.84^{a}	14.82^{b}	15.70^{b}	17.80^{a}
$\sum$ n-6	4.91^{b}	5.33^{b}	5.27^{b}	9.88^{a}	4.83^{b}
$\sum$ n-3 HUFA	17.27^{a}	16.36^{b}	13.37^{c}	9.09^{d}	14.74^{e}
Saturated/ Unsaturated	0.11^{bc}	0.11^{bc}	0.12^{c}	0.09^{a}	0.11^{b}
n3/n6	3.70^{a}	3.34^{b}	2.81^{c}	1.59^{d}	3.69^{a}
DHA/EPA	1.06^{a}	1.06^{a}	1.09^{a}	1.06^{a}	2.27^{b}
EPA/AA	7.75^{a}	7.87^{a}	6.34^{b}	6.17^{b}	5.36^{b}

Values with same superscript in the row are not significantly different from each other ($P<0.05$)
MUFA - monounsaturated fatty acids
PUFA - polyunsaturated fatty acids
DHA - docosahexaenoic acid
EPA - eicosapentaenoic acid
AA - arachidonic acid

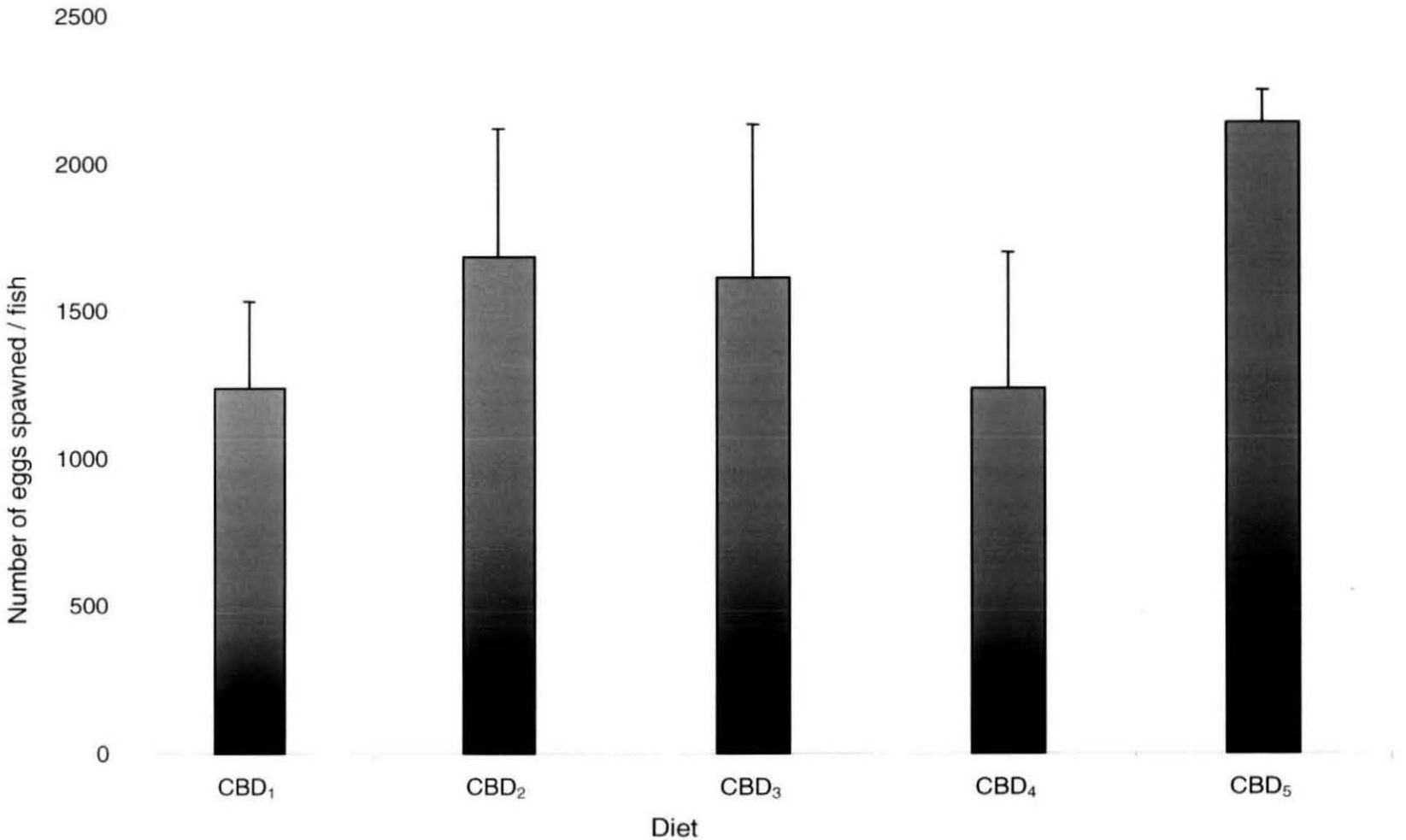


Fig.11. Influence of compounded diets on the number of eggs spawned by *Amphiprion sebae* (mean ± SD, n=9)

The egg dimensions recorded from different treatments are given in Table XVIII. The observed variations in capsule length were not significant. The highest mean egg capsule length (2.27 mm) was recorded in CBD_4 and the least (2.20 mm) in CBD_5 treatment.

The mean width of egg capsule also did not show any significant difference between treatments and it ranged from 857 µm for CBD_1 to 876 µm for CBD_4.

4.2.4.2.3. Fertilization rate and hatchability

The compounded diets did not affect fertilization rate and hatchability of the eggs.

4.2.4.2.4. Larval quality

The mean total length of newly hatched larvae obtained from the dietary treatments is depicted in Table XIX. The length of larvae did not show any significant difference between treatments, and it ranged from 4.20 mm for diet CBD_4 to 4.34 mm for CBD_1.

The larval survival rate at selected time intervals recorded for different treatments is depicted in Fig. 12. Survival rate did not show any significant difference between the dietary treatments on 3 dph or at 12 dph. Though, the feeding schedule followed was same for all the treatments the survival rates differed between the diets. At 12 dph the maximum (60 ± 10%) survival was obtained with CBD_4, which was followed by other spirulina containing diets *viz.* CBD_5 (58.7 ± 10%), CBD_2 (58.3 ± 7%) and CBD_3 (56 ± 14%). The control diet CBD_1 gave the least (35.7 ± 7%) survival among the diets.

4.2.4.3. Effect of Feed Allowance on Egg Production and Size

The level of feeding considerably affected the broodstock performance (Table XX). The restricted feeding ration resulted in substantial reduction in the number of eggs spawned as well as the egg dimension. However, the fertilization and hatchability were not affected by the feed allowance.

Table XVIII. Influence of moist diets on the egg dimensions in *Amphiprion sebae* (mean ± SD, n =108)

Diet	Egg capsule length (mm)	Egg capsule width (µm)
CBD_1	2.22 ± 0.01	857 ± 20
CBD_2	2.21 ± 0.06	857 ± 8
CBD_3	2.23 ± 0.02	860 ± 7
CBD_4	2.27 ± 0.06	876 ± 5
CBD_5	2.20 ± 0.02	861 ± 15

statistically insignificant ($P<0.05$)

Table XIX. Influence of natural diets on the total length of newly hatched larvae of *Amphiprion sebae* (mean ± SD, n =108)

Diet	Larval length (mm)
CBD_1	4.34 ± 0.07
CBD_2	4.21 ± 0.03
CBD_3	4.23 ± 0.07
CBD_4	4.20 ± 0.13
CBD_5	4.22 ± 0.03

statistically insignificant ($P<0.05$)

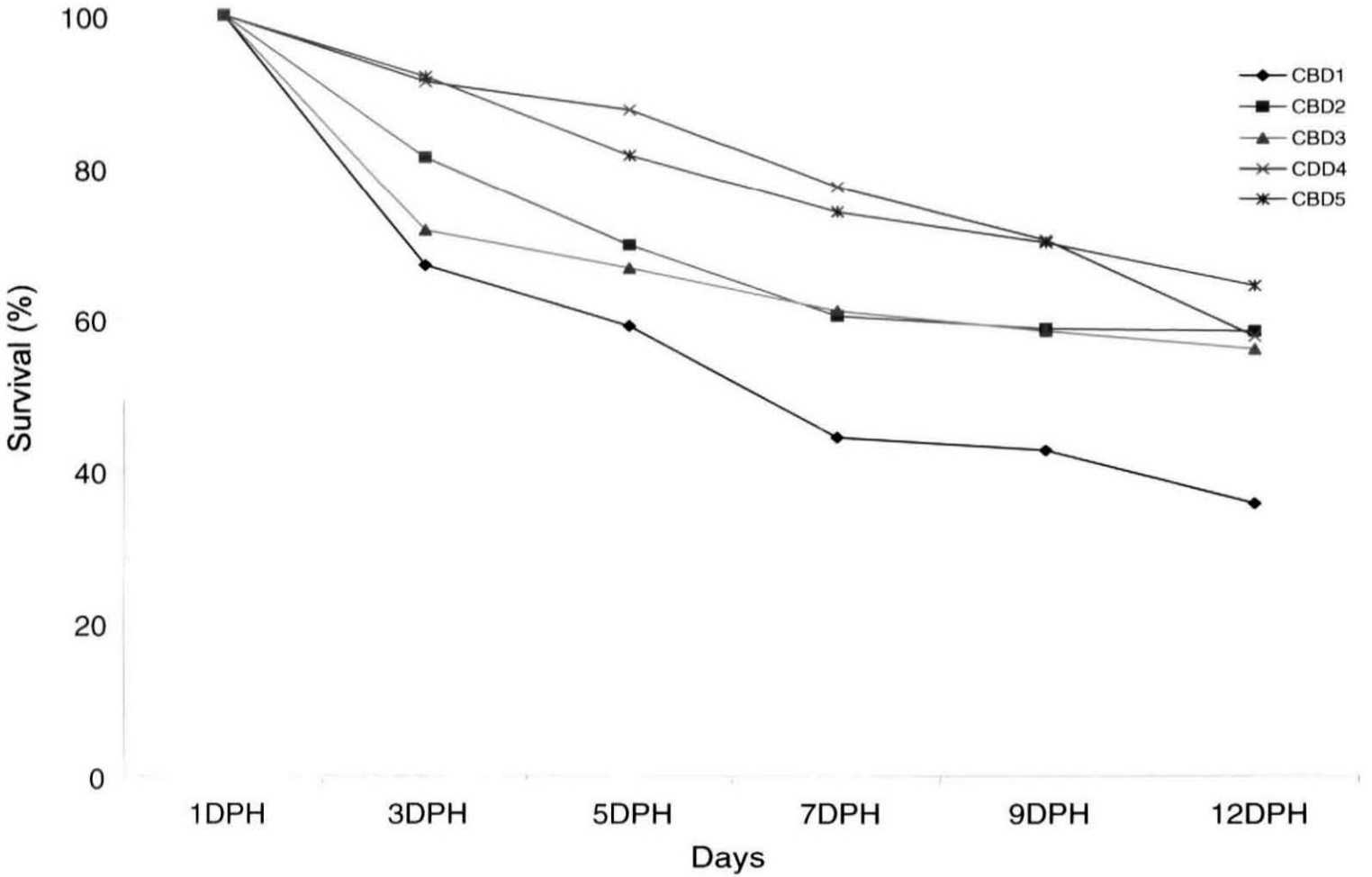


Fig. 12. Influence of compounded diets on the survival of *Amphiprion sebae* larvae

Table XX. Effect of level of feeding on the clutch size and egg dimension (mean ± SD, n = 9)

Parameter	Feeding twice	Feeding once
Clutch size	885 ± 55	416 ± 28
Egg capsule length (mm)	2.19 ± 0.01	2.10 ± 0.12
Egg capsule width (µm)	845 ± 2	811 ± 7
Hatchability (%)	100	100

4.2.4.4. Broodstock Development from Juveniles using Dry Feeds

Successful broodstock development was achieved in captivity using compounded diets and the results are presented in Table XXI. Juveniles of the fish, fed on compounded dry diets attained maturity and started spawning from the 15th month onwards. However, the total length of the two females was comparatively low with 71 and 76 mm at spawning and this was also reflected in the number of eggs spawned which ranged from 200 to 350. However, the egg dimension and hatchability were not affected by the diet used. The viability of larvae was low with 50 - 60% mortality observed on 1dph.

4.2.4.5. General Observations

4.2.4.5.1. Spawn quality

During the experiment, in most instances (> 50%) consumption of the first spawned clutch by the parent fish was observed within two days of spawning. In few cases (<5%) the second batch also was consumed. Often these clutches had higher numbers of unhealthy eggs and translucent appearance. The fecundity increased as the spawning progressed and got stabilised after two to three spawnings in newly formed pairs.

4.2.4.5.2. Egg carotenoids and larval pigmentation

In general, the clutch or egg pigmentation showed resemblance to the external appearance of natural diets (Table XXII). The clutch colouration or pigmentation was influenced by the dietary carotenoids and it varied from pale to bright yellow, pale pinkish to red and pale to deep orange reflecting the colour of the tested natural diets (Plate XIIIa to XIIIe). However, the compounded diets had not much influence on yolk pigmentation, which remained pale yellowish in colour. The clutch which was dark (Plate XIV) on the third day turned silvery on the day of hatching (Plate XV).

A change in diet colouration or carotenoids was found to influence the yolk pigmentation within 48 hours *i.e.* when fed two days prior to spawning. Intensity of yolk colouration was found to be low at first spawning and it became pronounced from the second spawning.

Table XXI. Development of *Amphiprion sebae* broodstock by exclusive use of compounded dry feeds from the early juvenile stage

	Tank 1	Tank 2	Tank 3
Date of spawning	12-7-01	12-7-01	12-7-01
Date of hatching	19-7-01	19-7-01	19-7-01
No. of fishes per tank	2	2	2
Female size (mm)	76	-	71
Spawning started	17-11-02	-	22-11-02
Spawning interval (days)	13-14	-	12-13
Spawnings observed	4	-	10
Average eggs/ spawn	225	-	305
Mortality of female	17/2/03	30/3/02¶	**

** survived ¶ tank broken

Table XXII. Relationship observed between the broodstock diets and egg yolk pigmentation in *Amphiprion sebae*

Diet	Clutch colouration	Diet appearance
DSP	Medium reddish pink	Reddish pink
MGD (female)	Orange to deep orange	Deep orange (boiled)
MSM	Pale to medium yellow	Pale yellowish
SQD	Pale to medium pink	Medium pink (skin)
CUT	Pale to medium pink	Light pink (skin)
PWM	Pale to medium yellow	Brown and pale yellow
Prepared dry diet	Very pale yellow	Brown
Moist spirulina diet	Pale yellow	Light to deep green

DSP - Deep sea prawn meat
MGD - Mature brown mussel
CUT - Cuttlefish meat
MSM - Brown mussel meat
PWM - Polychaete worm + Mussel meat (1:1)
SQD - Squid meat

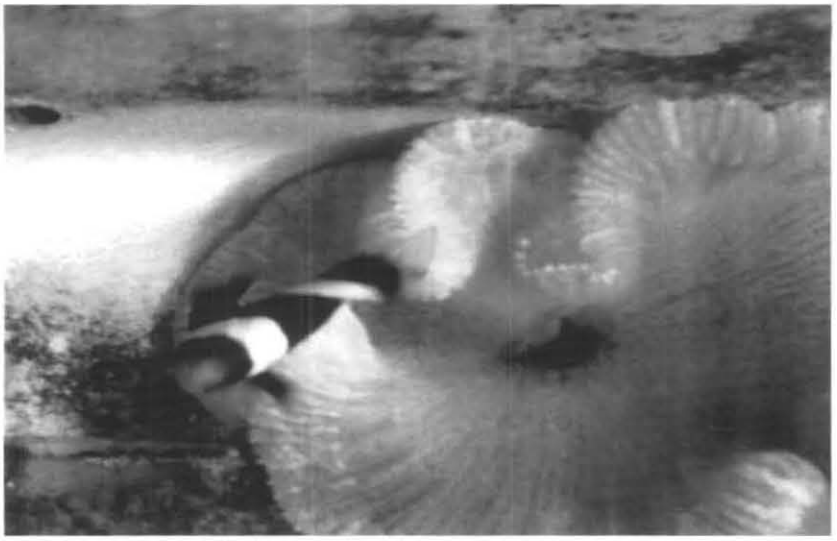


Plate XIIIa. Clutch colouration - Pale yellowish colouration observed when fed mussel meat, polychaete worm and compounded diets

Plate XIIIb. Clutch colouration - Pale pinkish colouration observed when fed squid and cuttlefish meat

Plate XIIIc. Clutch colouration - Reddish pink colouration observed when fed with deep-sea prawn

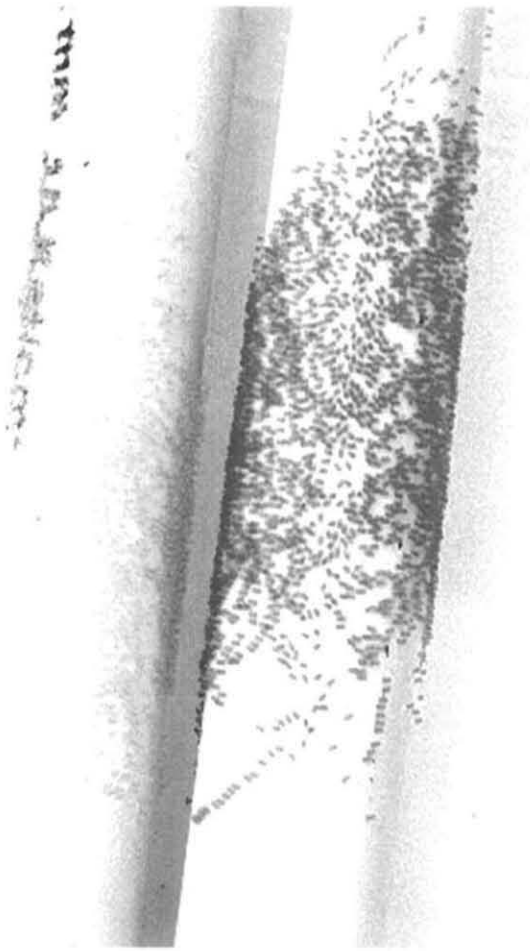


Plate XIIId. Variation in yolk pigmentation with mussel meat (control) and deep-sea prawn fed groups

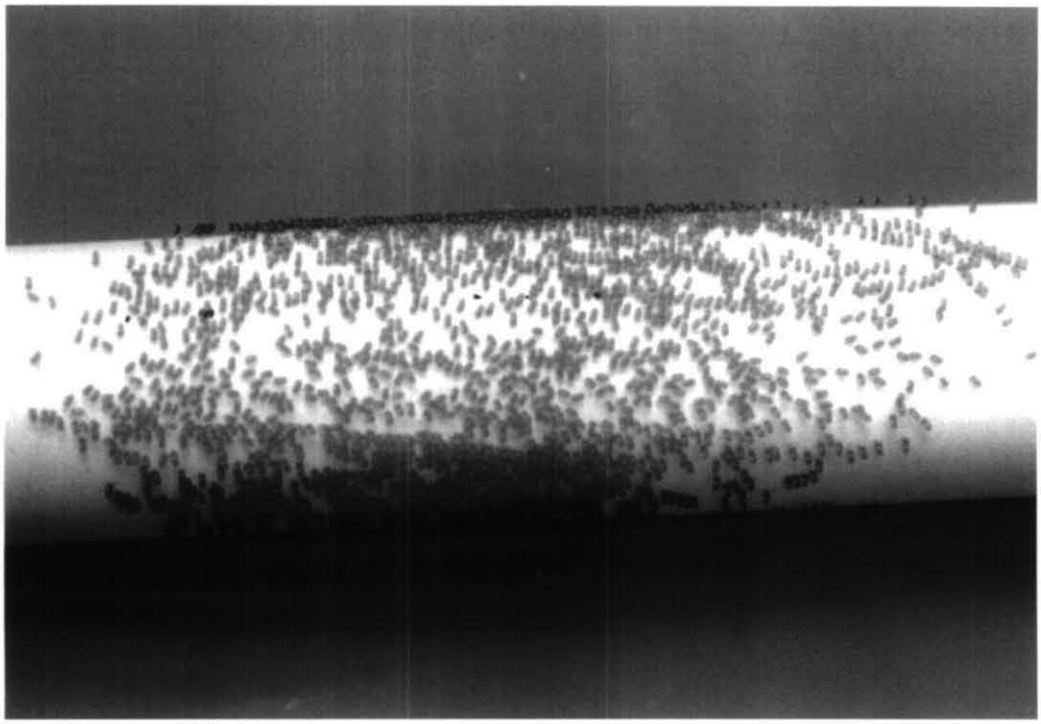


Plate XIIIe. Close-up view of the clutch produced by feeding deep-sea prawn

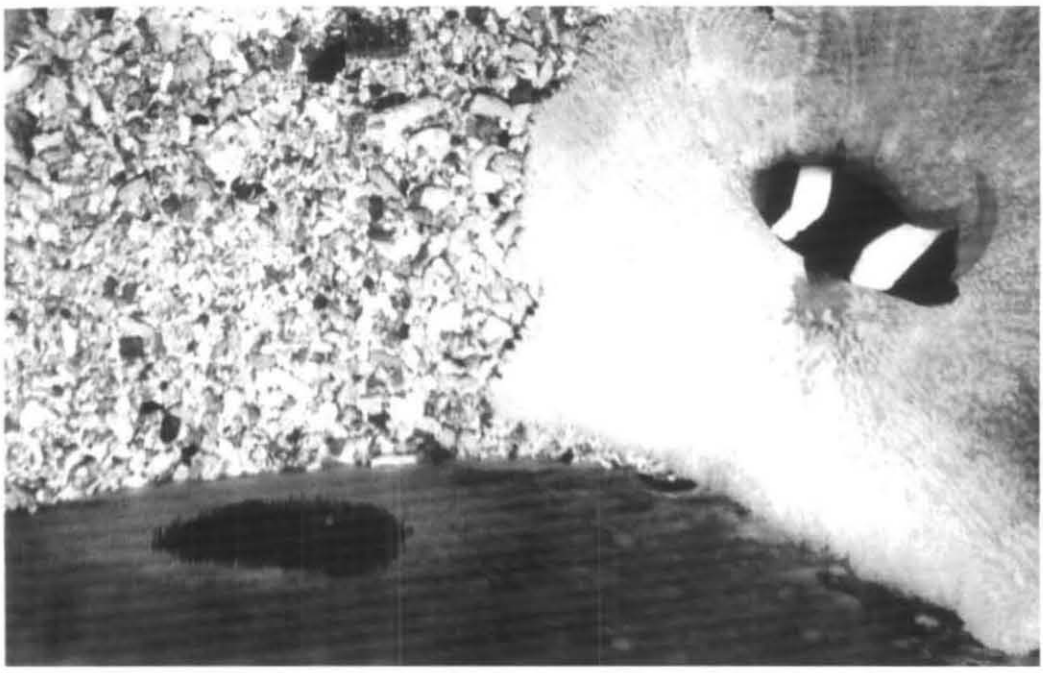


Plate XIV. Dark coloured clutch observed from third day of incubation

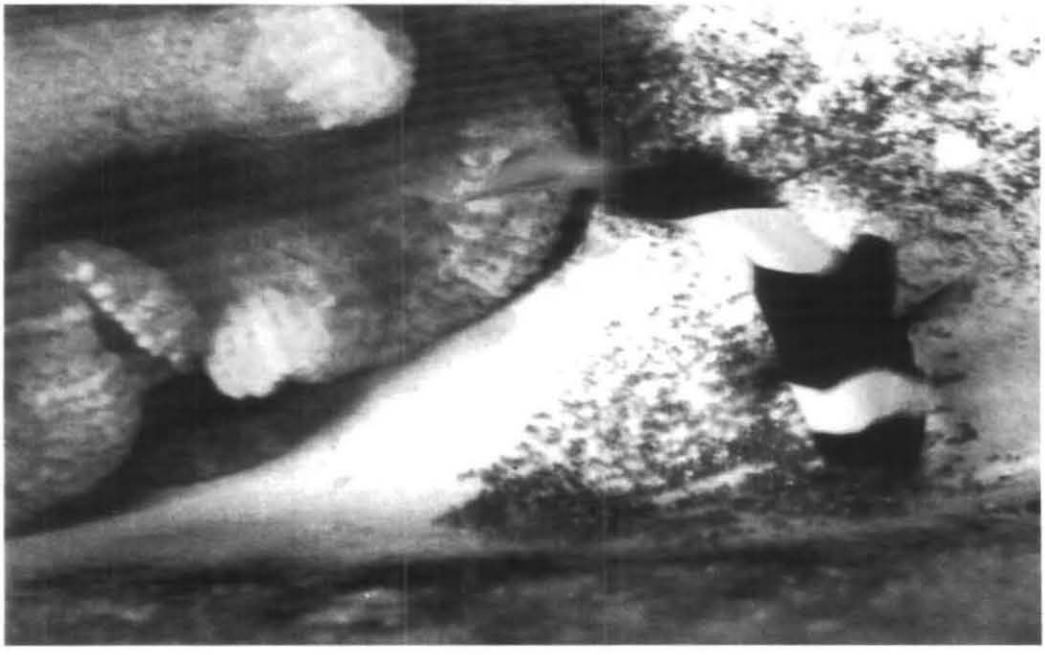


Plate XV. Silvery appearance of clutch on the day of hatching

The larvae obtained from broodstock fed the deep-sea prawn were distinctly different from those on other diets in having greater level of orange/brown pigmentations. The orange/brown pigmentations of the yolk were mobilised to the larvae and were observed as pigment spots on the body. The larvae with normal pigmentation and enhanced pigmentation are given in Plate XVIa to XVId.

The moist diets containing spirulina though increased the clutch size, failed to provide intense pigmentation (remained pale yellowish) even at an inclusion level of 45% (CBD_4).

4.2.4.5.3. Yolk utilization

The yolk utilization pattern during the embryonic development is given in Plate XVIIa & b. The broodstock diets did not have much impact on the intensity of yolk absorption, and in all the experiments similar patterns were observed. The maximum yolk length (YL) was taken as the major criteria. The yolk utilization showed a distinct pattern with the maximum utilization occurring on or after 6^{th} day of incubation. It was also observed that more than 80% of the yolk (in terms of maximum yolk length) was conserved (visual microscopic assessment) until the 6^{th} day of incubation and was later rapidly utilised.

4.3. Larval Nutrition

4.3.1. Gut Anatomy of Larvae

The mouth was opened inside the egg capsule and the normal mouth gape at hatching was 210 – 260 µm. The newly hatched larvae had yolk-sac and oil globule/s as nutrient reserves and pigmented body. The gut was single looped and differentiated into oesophagus, rudimentary stomach, intestine and rectum (Plate XVIII). The teeth were sharp pointed with slight inward curve (Plate XIX).

4.3.2. Evaluation of Livefeed

The performance of larvae under different livefeeds and their combinations is shown in Fig. 13. The survival percentage showed significant

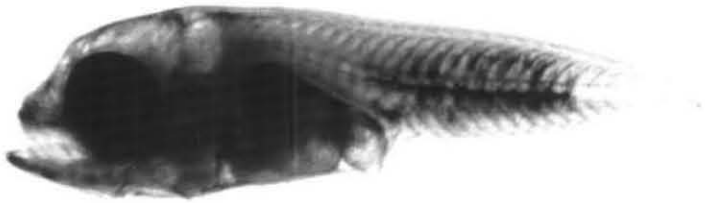


Plate XVIa. Newly hatched larva with normal pigmentation

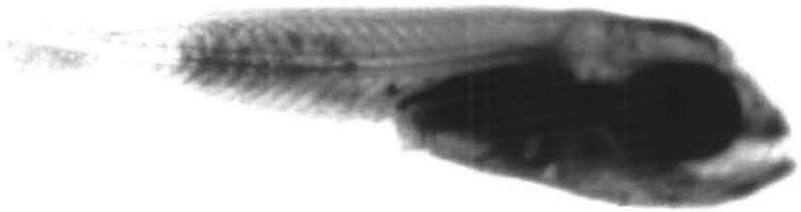


Plate XVIb. Newly hatched larva showing enhanced pigmentation when broods were fed with deep-sea prawn

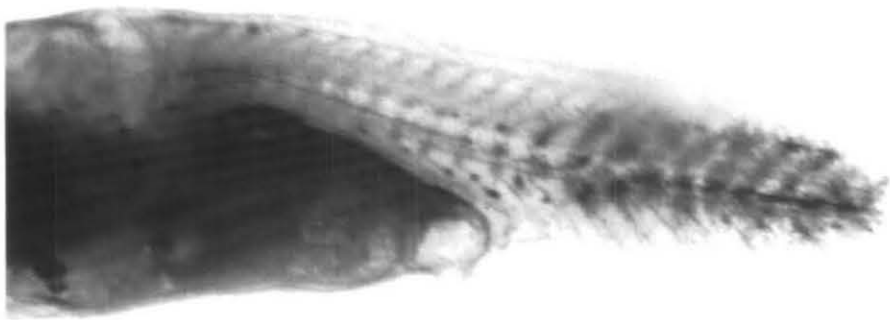


Plate XVIc. Enlarged view of pigmentation pattern (orange spots visible)

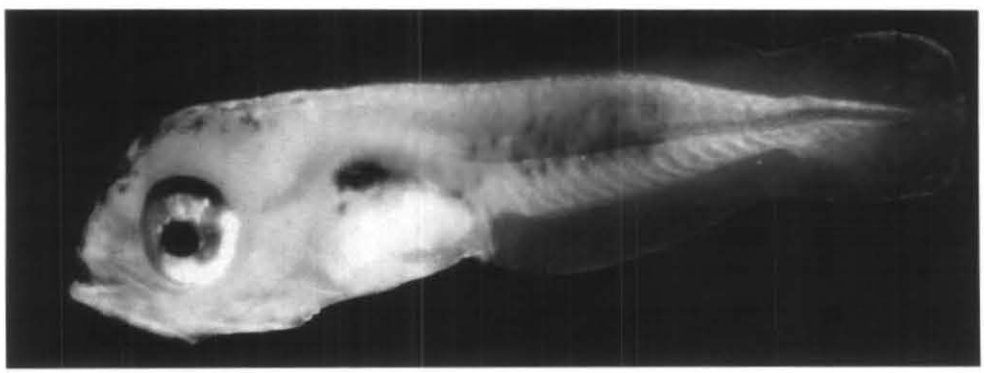


Plate XVId. Opaque view of the larva with enhanced pigmentation

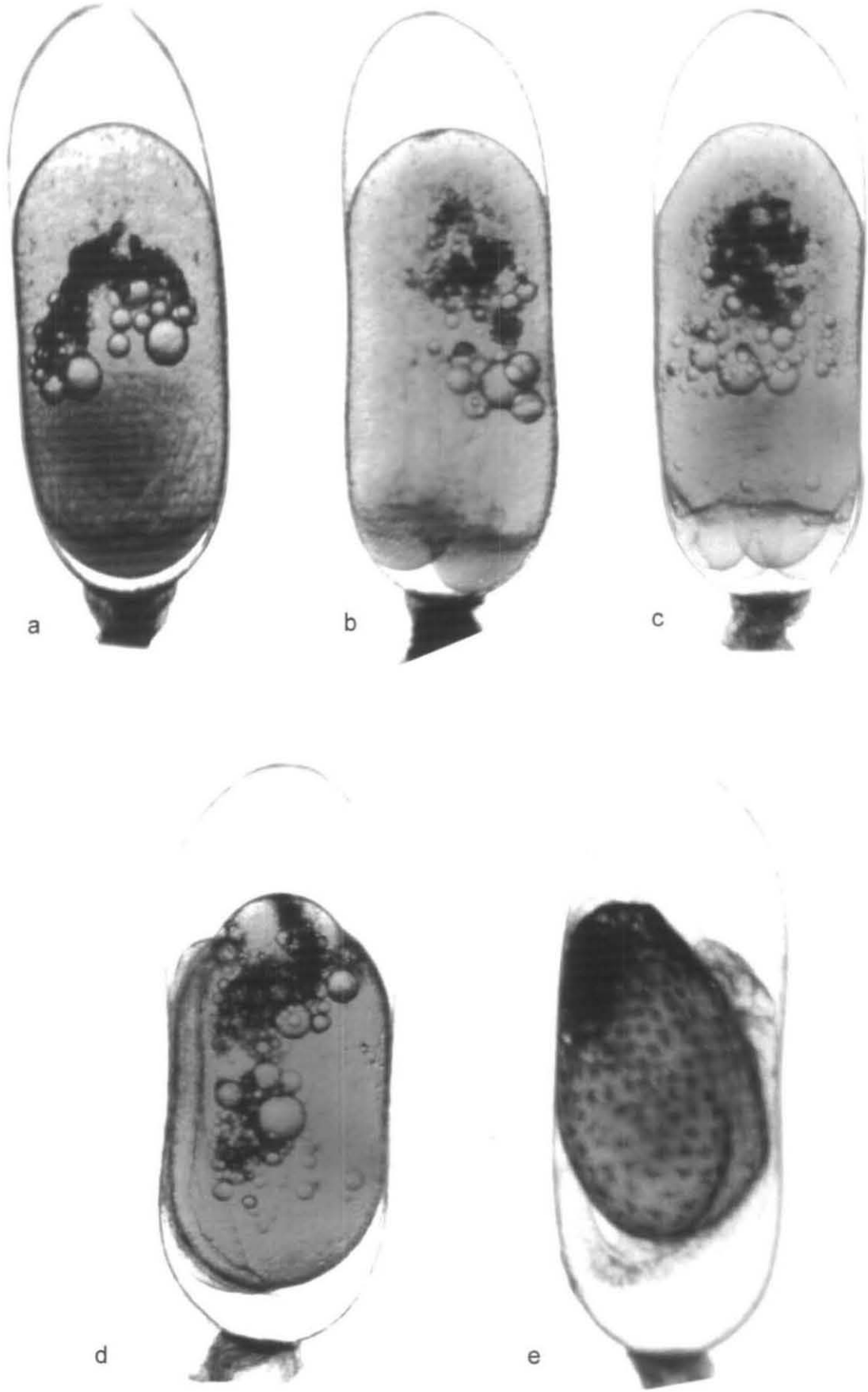


Plate XVIIa. Egg and embryo development and yolk utilization
a) fertilized egg b) first division c) second division d) yolk-plug stage (day 2)
e) neurula (day 3)

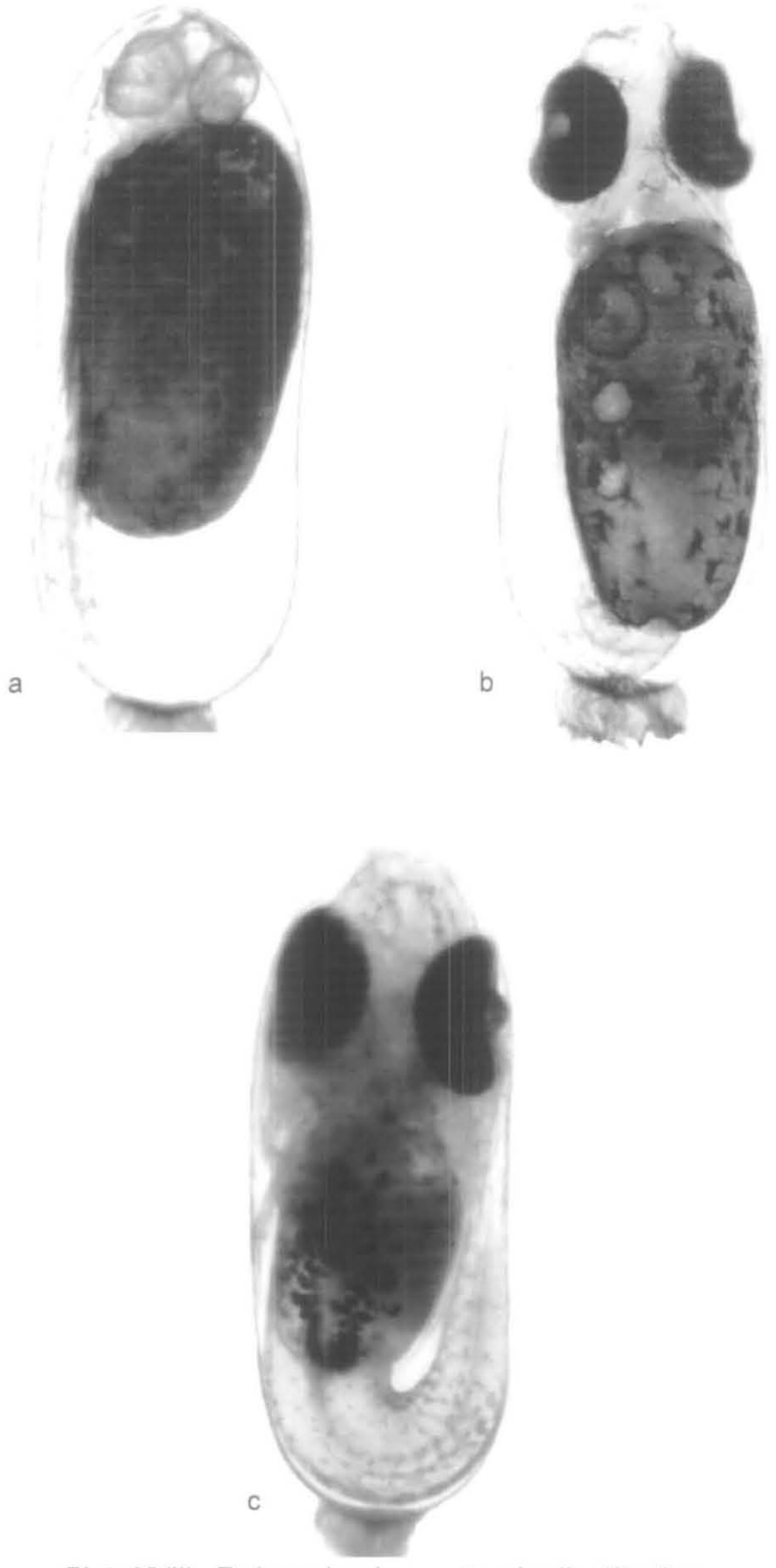


Plate XVIIb. Embryo development and yolk utilization
a) fourth day of incubation b) fifth day of incubation
c) fully developed embryo on the day of hatching

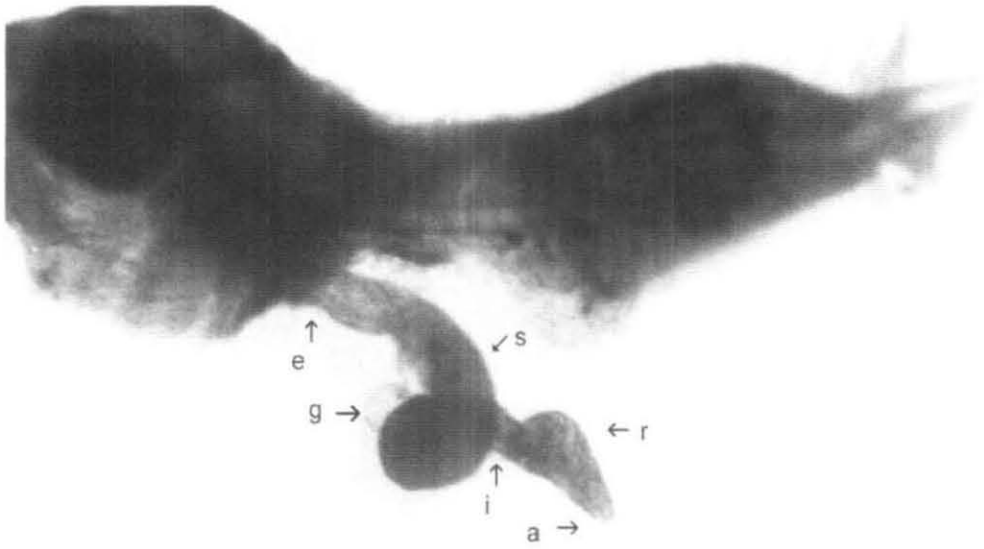


Plate XVII. Dissected gut of first day larva

(e) oesophagus, (g) gall bladder, (s) rudimentary stomach, (i) intestine, (r) rectum, (a) anus

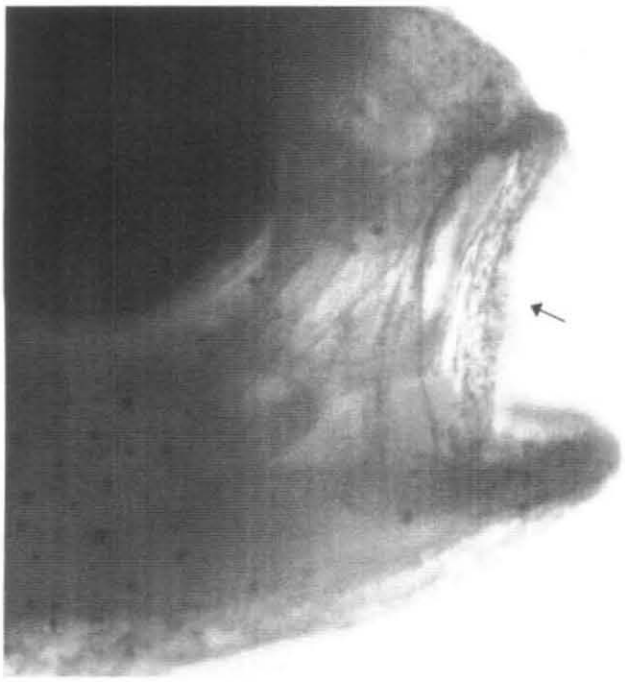
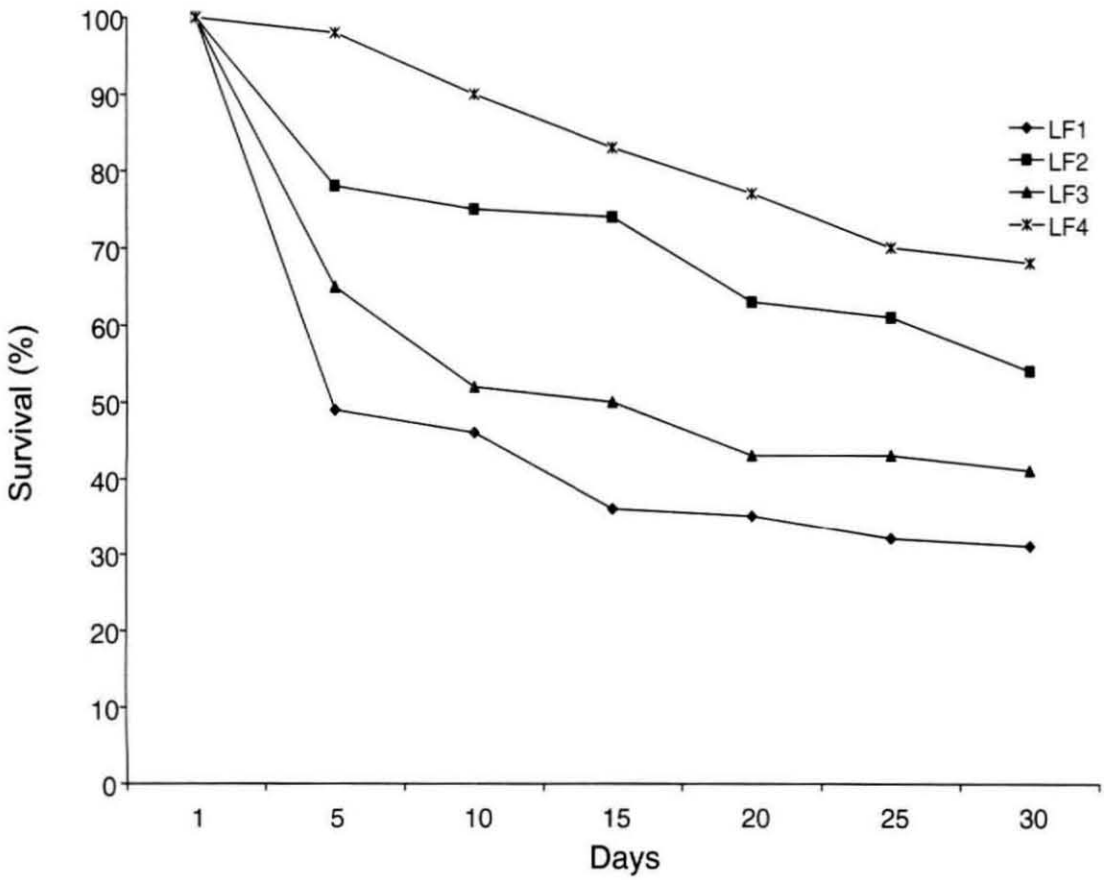


Plate XIX. Opened mouth with sharp inward curved teeth



LF_1 Rotifer alone
LF_2 Rotifer and *Artemia*
LF_3 Rotifer, *Artemia* and *Moina*
LF_4 Green algae with rotifer and *Artemia*

Fig. XIII. Effect of livefeeds on the survival of *Amphiprion sebae* larvae

($P<0.05$) difference between treatments. The larvae reared in green water (LF_4) gave the highest survival rates (68 ± 12.8%) and promoted faster metamorphosis. The onset of metamorphosis (appearance of white head band) in *Artemia* fed groups was observed on 7dph and more than 80% of the larvae metamorphosed by 9dph. The survival rates were low (31 ± 3.5%) in exclusively rotifer fed treatments (LF_1), and the metamorphosis occurred only after two weeks post hatching and took 4 to 5 days more to reach 80% level. In larvae suction formed the predominant feeding habit during early larval stages when rotifers and *Artemia* nauplii were fed.

4.3.3. Weaning

The weaning studies were done using microbound diets with a granule size of 200 - 800µm, containing 45% protein and 12% lipid. Preliminary studies on early weaning and late weaning were conducted. The late weaning was done by introducing wild fishes to a group of juveniles which were already weaned to dry or moist diets.

The results of weaning studies are depicted in Fig. 14. The survival was poor (6 - 11%) when 4dph larvae were weaned to dry diets; the initial mortality being high (70 - 80%) during the first five days of weaning. Better survival rates (> 50%) were obtained when weaning was done after the onset of metamorphosis (14 dph). The initial mortality was also low being 20 - 30% during the first five days of weaning in 14 dph group. The survival rates of post larvae weaned from 21 dph were found to be 75 - 80%. Weaning of month old post larvae (30 dph) showed mortality of less than 5%.

4.4. Nutrition of Juveniles

The diets formulated had a soft texture and had the consistency to float on water surface for longer duration (>5 minutes). The hydrostability also was found to be relatively high (more than 80% retention after one hour). However, since in experiments satiation feeding was employed the feeds were consumed immediately by the fish and this helped to minimise the leaching of nutrients.

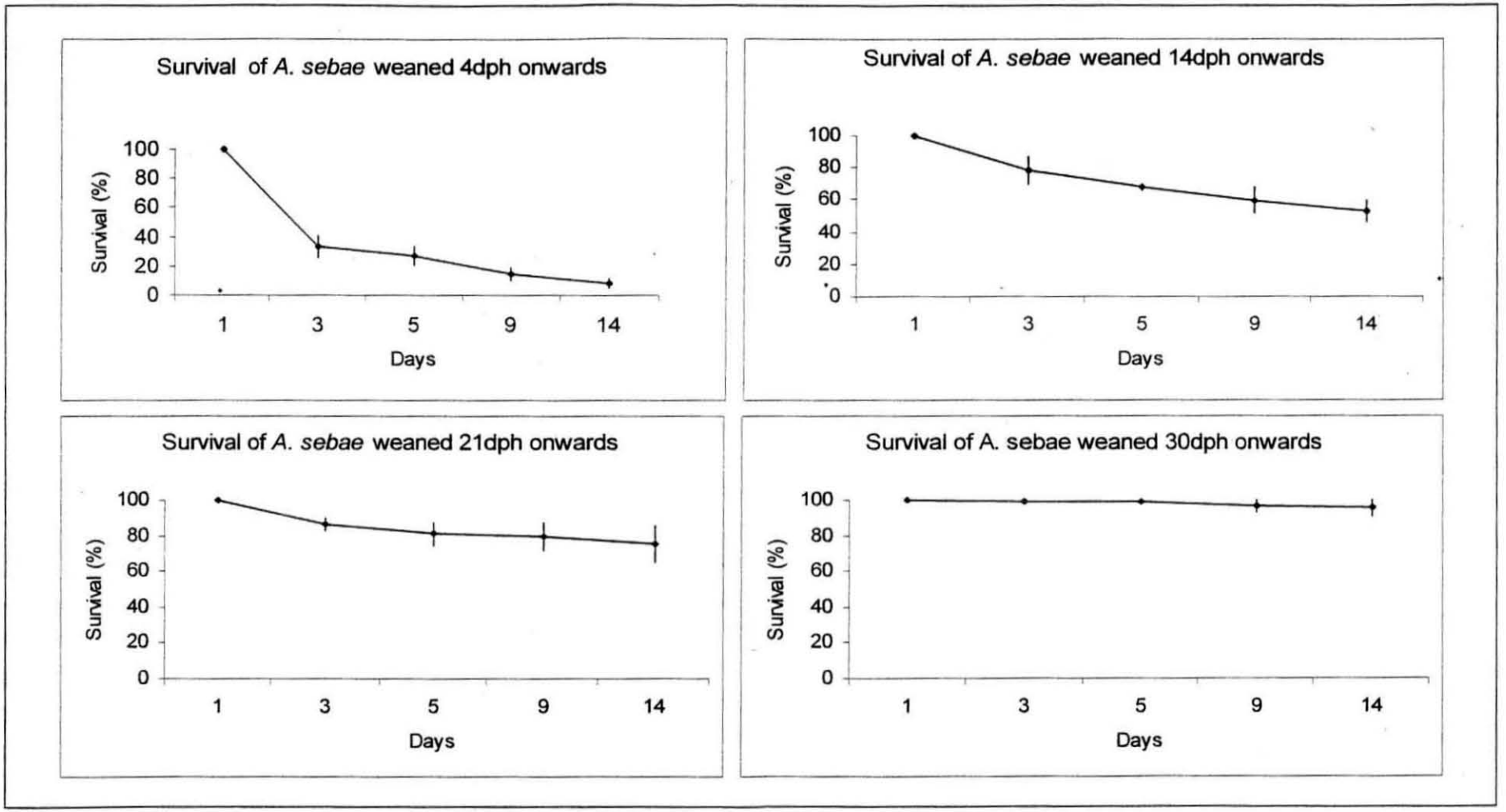


Fig. 14. Effect of weaning age on the survival of *Amphiprion sebae* (mean ± SD, n=3)

4.4.1. Feed Formulation and Acceptability Trial

The basal mix used contained fishmeal, shrimp meal and squid meal in the ratio of 15:4:1, and the amino acid profiles of the mix and that of juvenile sebae anemonefish are presented in Table XXIII.

The mean growth and survival derived from the experiment are given in Table XXIV. The survival and growth of the fish fed on the purified diet were lower than those fed the semi-purified diet. The acceptability was also better for the semi-purified diet. However, the survival and growth did not show any significant difference between the treatments.

4.4.2. Protein Requirement of Juveniles

4.4.2.1. Experiment I

Growth performance of the juveniles of *A. sebae* (mean total length of 11.4 ± 0.2 mm and mean weight of 25.9 ± 3.9 mg) fed diets containing selected protein levels for 9 weeks are presented in Table XXV. Though, the initial length and weight did not differ significantly, size variations were observed among individuals in all the treatments as the experiment progressed, and it continued till the termination of experiment. However, the variations were found to be similar among the replicates of the same dietary treatment.

Survival rates were high (80 - 93.33%) and did not vary significantly among the dietary protein level. The lowest survival rate (80%) was obtained with the diet containing 20% protein (D_{20}).

The weight gain showed significant ($P<0.05$) difference between treatments. The mean weight gain showed an increase with the increase in protein level upto 40% and showed a decrease at 50%. The maximum bodyweight gain was observed with D_{40} (324.2 mg), followed by D_{50}, D_{30} and D_{20} respectively. The length gain also showed significant difference between treatments ($P< 0.05$), with an increasing trend in the tested range, with D_{50} giving the maximum total length gain. The condition factor was significantly ($P<0.05$) higher in D_{40} (1.96) than the other diets.

Table XXIII. Amino acid profiles (g/100g protein) of the basal mix and sebae anemonefish

Amino acid	**Basal mix***	**Sebae anemonefish** (juvenile)
Indispensable amino acids		
Arginine	3.83	4.55
Histidine	2.28	1.51
Isoleucine	3.15	1.87
Leucine	7.72	5.98
Lysine	11.70	10.3
Methionine	2.65	1.62
Threonine	6.57	3.77
Tryptophan	1.22	1.22
Phenylalanine	4.85	2.9
Valine	3.73	2.89
Dispensable amino acids		
Alanine	8.31	10.58
Aspartic acid	6.66	7.82
Cysteine	0.49	0.25
Glutamic acid	9.27	8.79
Glycine	14.21	20.89
Proline	5.89	8.09
Serine	4.04	4.91
Tyrosine	2.54	2.17

*Basal mix – 15:4:1 ratio of fish meal, shrimp meal and squid meal

Table XXIV. Growth and survival of *Amphiprion sebae* fed on purified and semi-purified diets (mean ± SD)

	PFD_{50}	**$SPFD_{50}$**
Initial weight (mg)	115.1 ± 16.6	90.7 ± 23.1
Initial length (mm)	18.0 ± 1.2	17.7 ± 1.4
Weight gain (mg)	204.2 ± 38.7	262.1 ± 42.1
Length gain (mm)	9.4 ± 1.5	12.1 ± 1.1
SGR	2.27 ± 0.22	3.02 ± 0.17
CF	1.64 ± 0.09	1.66 ± 0.14
ADG	4.54 ± 0.86	5.82 ± 0.94
WG (%)	177.4 ± 77.2	288.9 ± 157.3
Survival (%)	68.75 ± 8.8	81.25 ± 8.8

Table XXV. Growth and survival of juvenile Amphiprion sebae fed graded levels of protein in experiment I (mean ± SD, n=9)

	Diets			
	D_{20}	D_{30}	D_{40}	D_{50}
Initial weight (mg)	26.24 ± 1.9	22.7 ± 1.6	24.3 ± 3.5	30.5 ± 3.5
Initial length (mm)	11.4 ± 0.2	11.2 ± 0.2	11.3 ± 0.1	11.6 ± 0.2
Weight gain (mg)	167.4 ± 17.10^{a}	217.4 ± 22.94^{ab}	324.2 ± 54.84^{c}	287.5 ± 46.45^{bc}
Length gain (mm)	11.0 ± 1.11^{a}	13.1 ± 1.17^{ab}	14.8 ± 1.25^{b}	15.3 ± 1.89^{b}
SGR	3.17 ± 0.04^{a}	3.74 ± 0.25^{b}	4.23 ± 0.14^{c}	3.72 ± 0.39^{b}
CF	1.72 ± 0.12^{a}	1.67 ± 0.08^{a}	1.96 ± 0.04^{b}	1.63 ± 0.11^{a}
ADG	2.66 ± 0.27^{a}	3.45 ± 0.36^{ab}	5.12 ± 0.87^{c}	4.56 ± 0.74^{bc}
WG (%)	636.9 ± 18.39^{a}	968.2 ± 170.86^{a}	1336.0 ± 132.46^{b}	962.4 ± 262.77^{a}
Survival (%)	80	93.33±1.55	86.67±11.55	86.67±11.55

Means having same superscript in the row are not significantly different from each other ($P<0.05$)

Specific growth rate (SGR; % day^{-1}) ranged from 3.17 to 4.23, with a maximum at 40% protein level (D_{40}), and the minimum at 20% protein. There was no significant ($P<0.05$) difference in SGR between diets D_{30} and D_{40}. The average daily gain (ADG) in weight also differed significantly with D_{40} (5.12 mg d^{-1}) giving the maximum gain followed by D_{50}. The percentage weight gain was also significantly ($P<0.05$) higher with the 40% diet. There was no significant difference between other diets in the tested range.

The ANOVA results also suggested the best performance of juveniles at 40% dietary protein. Therefore, a second experiment with closer range of protein levels (33 to 48%, with 3% increment) was conducted to optimise the protein requirement.

4.4.2.2. Experiment II

Early juveniles (14.7 ± 0.5 mm and 62.58 ± 5.94 mg) were used for this protein optimisation study, which lasted for 9 weeks. The mean growth response and feed performance data derived from the experiment are given in Table XXVI.

Survival rates were high (86.7 to 100%) and did not vary significantly with the dietary protein level. The mortalities recorded were due to the increased aggression during the final phase of the experiment.

Weight gain showed significant ($P<0.05$) difference between the dietary treatments. The weight gain showed ten fold increase over the initial weight when fed 45% dietary protein (Table XXVI). The body weight gain increased upto 45% dietary level and later declined at 48% protein level. The diet D_{33} containing 33% protein elicited less than one third of the growth with D_{45}. There was no significant difference in weight gain between D_{42} and D_{48}. The length gain also showed significant ($P<0.05$) difference among the treatments. The highest gain in total length was for D_{42} closely followed by D_{45}. The condition factor was significantly ($P<0.05$) higher in D_{36} (1.92) than other tested levels. The D_{36} was followed by D_{39}, D_{45} and D_{48} but these were not significantly different from each other.

Table XXVI. Growth performance and feed utilization of juvenile *Amphiprion sebae* fed protein optimizing diets (mean ± SD, n=3)

	Diets					
	D_{33}	D_{36}	D_{39}	D_{42}	D_{45}	D_{48}
Initial weight (mg)	51.64 ± 5.4	60.5 ± 2.5	65.96 ± 3.1	63.82 ± 5.0	68.21 ± 5.9	65.34 ± 3.2
Initial length(mm)	13.8 ± 0.4	14.6 ± 0.4	15.07 ± 0.5	14.4 ± 0.2	15.4 ± 0.1	14.7 ± 0.1
Weight gain (mg)	188.53 ± 37.67^{a}	266.21 ± 9.89^{a}	387.38 ± 72.1^{b}	562.84 ± 22.89^{c}	697.28 ± 90.92^{d}	486.36 ± 37.44^{c}
Length gain (mm)	11.3 ± 0.12^{a}	11.1 ± 0.95^{a}	14.6 ± 0.94^{b}	21.1 ± 1.55^{c}	20.4 ± 0.96^{c}	17.8 ± 1.73^{d}
SGR	2.44 ± 0.17^{a}	2.68 ± 0.01^{a}	3.06 ± 0.23^{b}	3.63 ± 0.17^{cd}	3.84 ± 0.32^{d}	3.39 ± 0.18^{bc}
CF	1.52 ± 0.19^{ab}	1.92 ± 0.18^{c}	1.78 ± 0.21^{bc}	1.40 ± 0.13^{a}	1.67 ± 0.12^{abc}	1.61 ± 0.15^{ab}
ADG	2.99 ± 0.60^{a}	4.23 ± 0.16^{a}	6.15 ± 1.15^{b}	8.93 ± 0.36^{c}	11.07 ± 1.44^{d}	7.72 ± 0.59^{c}
WG (%)	365.1 ± 46.77^{a}	440.0 ± 2.99^{ab}	587.3 ± 96.07^{bc}	881.9 ± 106.76^{de}	1022.2 ± 221.03^{e}	744.4 ± 91.64^{cd}
Survival (%)	93.33±11.55	100	86.67±11.55	93.33±11.5	86.67±11.5	86.67±23.09
FCR	2.41 ± 0.06^{a}	2.04 ± 0.09^{b}	1.67 ± 0.03^{c}	1.40 ± 0.01^{d}	1.60 ± 0.02^{e}	2.19 ± 0.10^{c}
PER	1.26 ± 0.01^{a}	1.34 ± 0.07^{b}	1.53 ± 0.11^{c}	1.71 ± 0.02^{d}	1.38 ± 0.05^{c}	0.95 ± 0.08^{e}

Means having same superscript in the row are not significantly different from each other ($P<0.05$)

The SGR ranged between 2.44 and 3.84 and showed significant (P<0.05) difference among the tested levels. The SGR increased with increasing dietary protein level up to 45% protein and further it decreased. The average daily gain (ADG) in weight differed significantly (P<0.05) among the diets with D_{45} giving the highest (11.07 mg d^{-1}) and D_{33} the lowest gains. The ADG of juveniles fed D_{45} was almost three times higher than D_{33}. The 45 % dietary protein also supported significantly (P<0.05) higher weight gain percentage.

The feed utilization parameters like FCR and PER also differed significantly (P<0.05) between diets. The higher and lower dietary protein levels resulted in higher FCR. The most efficient FCR was achieved with D_{42} (1.40). The low protein diet (D_{33}) resulted in higher FCR (2.41), thus lower efficiency. The PER was high (1.71) for the 42% protein diet followed by 36, 45, 33, 39 and 48%, and the least PER (0.95) was observed with the highest protein inclusion.

ANOVA results suggest the best performance of juveniles at 45% dietary protein. The second order polynomial regression analysis of weight gain data, $y = -2.5451x^2 + 235.25x - 4861.1$; $r = 0.90$, showed the optimum protein requirement for maximum weight gain at 46.2% (Fig. 15). The equation for second order polynomial regression of SGR is $y = -0.0091x^2 + 0.8162x - 14.767$; $r = 0.90$, and gave the optimum protein level at 44.9% (Fig. 16).

The amino acid profiles of the diets are given in Table XXVII. In D_{45} which gave best response the essential amino acids constituted 46.11% of the total amino acids and the ratio between essential to non-essential was 0.86:1. The ratio remained almost similar in all the diets, as the only variable in the protein source was that of the basal mix.

4.4.3. Lipid Requirement of Juveniles

Juveniles of *A. sebae* used for the lipid requirement study had mean length of 12.4 ± 0.2 mm and mean weight of 37.1 ± 0.7 mg. The growth performance of fish fed the experimental diets containing various lipid levels for 9 weeks are presented in Table XXVIII.

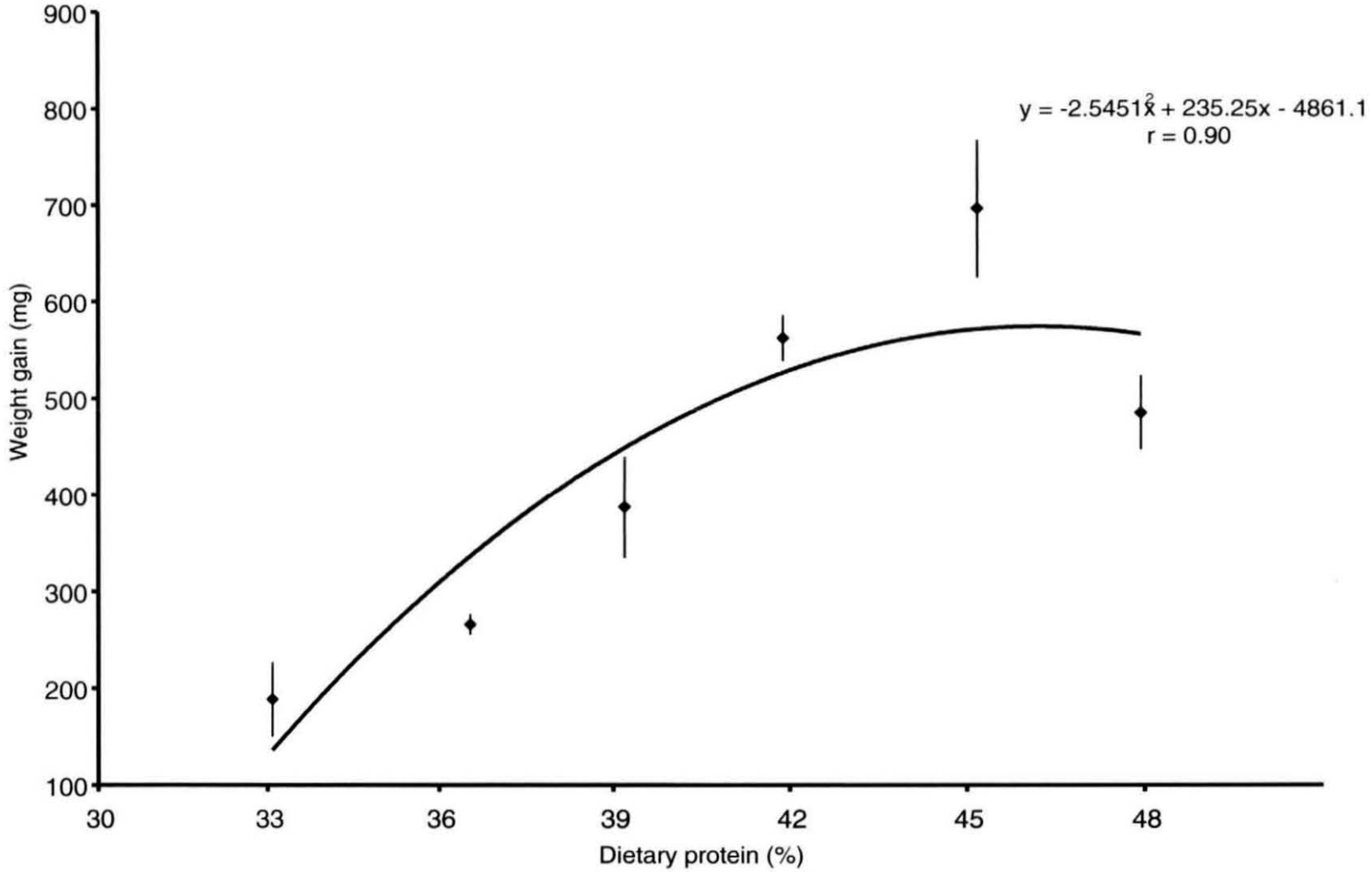


Fig. 15. Optimum dietary protein requirement for the juvenile *Amphiprion sebae*

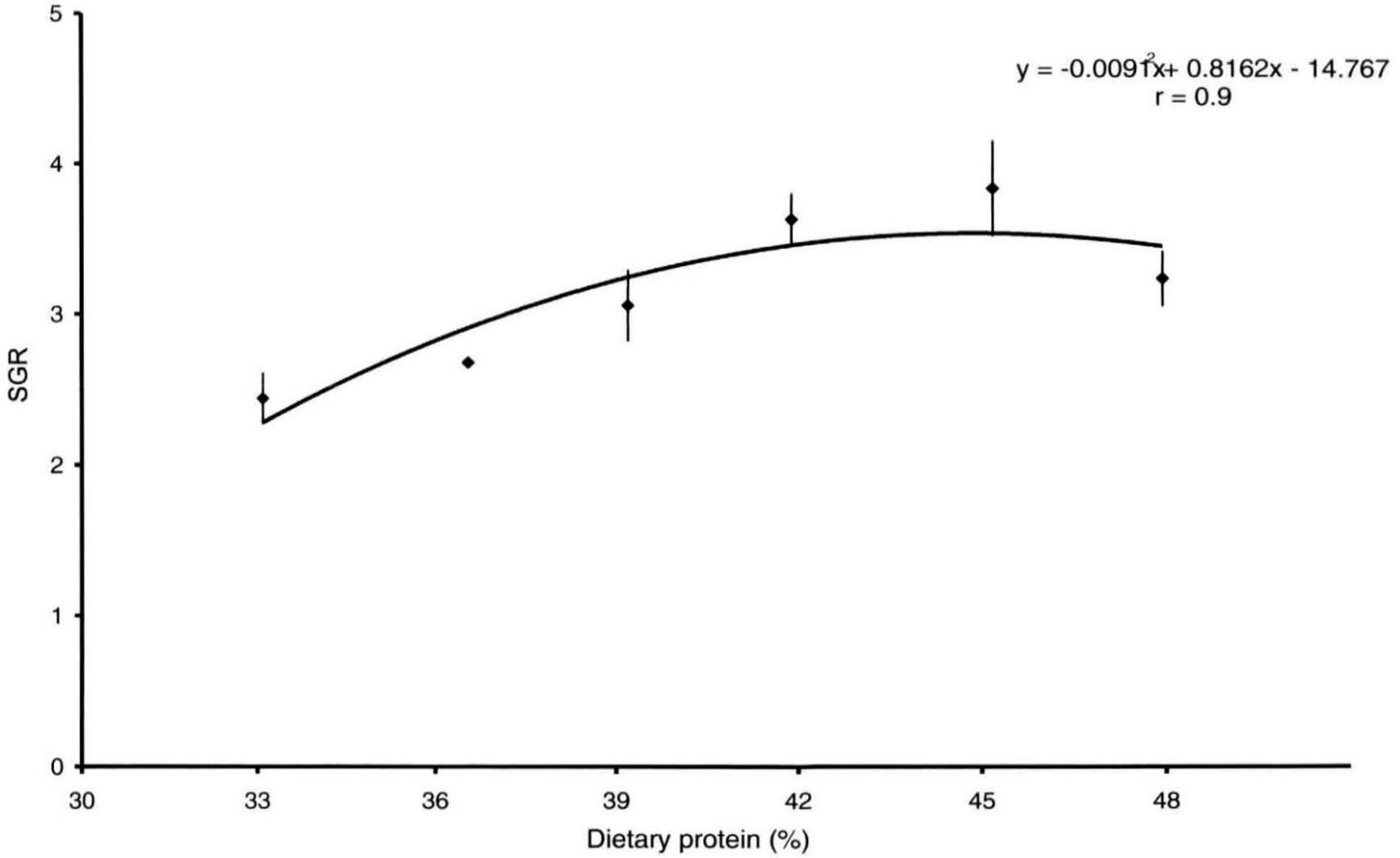


Fig. 16. Effect of dietary protein level on the specific growth rate of juvenile *Amphiprion sebae*

Table XXVII. Amino acid profiles of the diets used for protein requirement (experiment II) of *Amphiprion sebae*

Diet / Amino acid	(g/100g diet)					
	D_{33}	D_{36}	D_{39}	D_{42}	D_{45}	D_{48}
ARG	1.24	1.37	1.47	1.58	1.70	1.81
HIS	0.65	0.72	0.78	0.84	0.92	0.98
ILE	1.02	1.13	1.21	1.30	1.40	1.49
LEU	2.63	2.91	3.11	3.32	3.58	3.80
LYS	3.51	3.91	4.22	4.54	4.92	5.25
MET	0.86	0.95	1.02	1.09	1.18	1.25
THR	1.83	2.04	2.22	2.40	2.61	2.79
TRY	0.35	0.39	0.42	0.45	0.49	0.53
PHE	1.65	1.83	1.96	2.09	2.25	2.39
VAL	1.32	1.45	1.55	1.66	1.78	1.89
ALA	2.42	2.70	2.91	3.14	3.41	3.64
ASP	1.97	2.20	2.37	2.56	2.77	2.96
CYS	0.16	0.17	0.19	0.20	0.22	0.23
GLU	4.13	4.49	4.75	4.99	5.32	5.58
GLY	4.33	4.82	5.19	5.58	6.04	6.44
PRO	2.64	2.87	3.03	3.19	3.40	3.56
SER	1.44	1.58	1.69	1.80	1.93	2.05
TYR	0.93	1.02	1.09	1.16	1.24	1.32
ΣEAA	15.05	16.70	17.96	19.26	20.83	22.16
ΣNEAA	18.01	19.84	21.23	22.62	24.34	25.78
ΣEAA/ΣNEAA	0.84	0.84	0.85	0.85	0.86	0.86

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
ΣEAA- Total essential amino acids ΣNEAA- Total non-essential amino acids

Table XXVIII. Growth response and feed utilization of juvenile *Amphiprion sebae* fed graded levels of dietary lipid (mean ± SD, n=3)

	Diets				
	D_{L3}	D_{L6}	D_{L9}	D_{L12}	D_{L15}
Initial weight (mg)	36.22 ± 10.5	37.64 ± 12.0	36.76 ± 7.7	36.96 ± 7.0	37.04 ± 6.2
Initial length (mm)	12.2 ± 0.8	12.6 ± 0.5	12.2 ± 0.8	12.4 ± 0.8	12.2 ± 0.4
Weight gain (mg)	214.6 ± 14.7^{a}	309.4 ± 15.5^{b}	322.9 ± 23.4^{b}	335.6 ± 26.4^{b}	309.6 ± 21.6^{b}
Length gain (mm)	13.0 ± 0.28^{a}	14.5 ± 0.44^{c}	14.4 ± 0.21^{bc}	14.3 ± 0.42^{bc}	13.6 ± 0.00^{ab}
SGR	3.07 ± 0.10^{a}	3.52 ± 0.08^{b}	3.62 ± 0.09^{b}	3.67 ± 0.11^{b}	3.55 ± 0.10^{b}
CF	1.57 ± 0.14^{a}	1.75 ± 0.01^{ab}	1.91 ± 0.06^{bc}	1.96 ± 0.05^{bc}	2.02 ± 0.13^{c}
ADG	3.41 ± 0.23^{a}	4.91 ± 0.24^{b}	5.13 ± 0.37^{b}	5.33 ± 0.42^{b}	4.91 ± 0.35^{b}
WG (%)	592.5 ± 40.4^{a}	821.9 ± 41.2^{b}	878.4 ± 63.6^{b}	908.1 ± 71.3^{b}	835.8 ± 58.4^{b}
Survival (%)	80.0 ± 20.0	100	100	100	86.67 ± 23.1
FCR	1.37 ± 0.01^{a}	1.44 ± 0.01^{b}	1.26 ± 0.02^{c}	0.90 ± 0.02^{d}	1.56 ± 0.01^{e}
PER	1.63 ± 0.00^{a}	1.54 ± 0.01^{b}	1.73 ± 0.02^{c}	2.51 ± 0.02^{d}	1.41 ± 0.00^{e}

Means having same superscript in the row are not significantly different from each other ($P<0.05$)

Survival rates were high and did not vary significantly with the dietary lipid level. The weight gain showed significant ($P<0.05$) differences between dietary treatments, and increased upto 12% dietary lipid level and later showed a decline at 15% (D_{L15}). The mean weight gain ranged from 214.6mg (D_{L3}) to 335.6mg (D_{L12}). Apart from diet D_{L3} containing 3% lipid there were no significant differences in the growth between other diets. The second order polynomial regression on weight gain, $y = -1.8899x^2 + 41.428x + 112.65$; $r = 0.98$, revealed the optimum dietary lipid requirement for maximum weight gain at 10.96% (Fig. 17).

The total length gain varied significantly ($P<0.05$) among the treatments, and the maximum gain was attained with 6% dietary lipid level closely followed by 9% and 12% levels. There was no significant difference between the highest (D_{L15}) and the lowest (D_{L3}) lipid diets tested in terms of length gain, both the diets resulted in least gain. The ADG levels were also significantly lower at the 3% lipid level and the observed differences between other treatments were not significant. The condition factor showed significant ($P<0.05$) differences between the tested lipid levels, with an increasing trend from 1.57 for diet D_{L3} to 2.02 for diet D_{L15}.

The SGR was found to vary significantly ($P<0.05$) with the dietary lipid concentration; and the highest SGR of 3.67 was obtained with D_{L12} followed by D_{L9}, D_{L15}, D_{L6} and D_{L3}. The application of second order polynomial regression on the SGR data showed the optimum dietary lipid concentration at 11.05% (Fig. 18).

The FCR also varied with diets ($P<0.05$) and the best conversion was achieved by using diet D_{L12}. The FCR of less than 1 is generally considered as highly efficient. The PER values varied significantly ($P<0.05$) among the treatments with a maximum (2.51) for diet D_{L12} and the minimum (1.41) for diet D_{L15}.

The fatty acid profiles of the diets are given in Table XXIX. The percentage of fatty acids in the diets showed an increase in DHA, EPA, C22:5 n-3, C24:1, C18:1 n7, C18:1 n9, C16:1 n7 and C14:0 with increase in the dietary

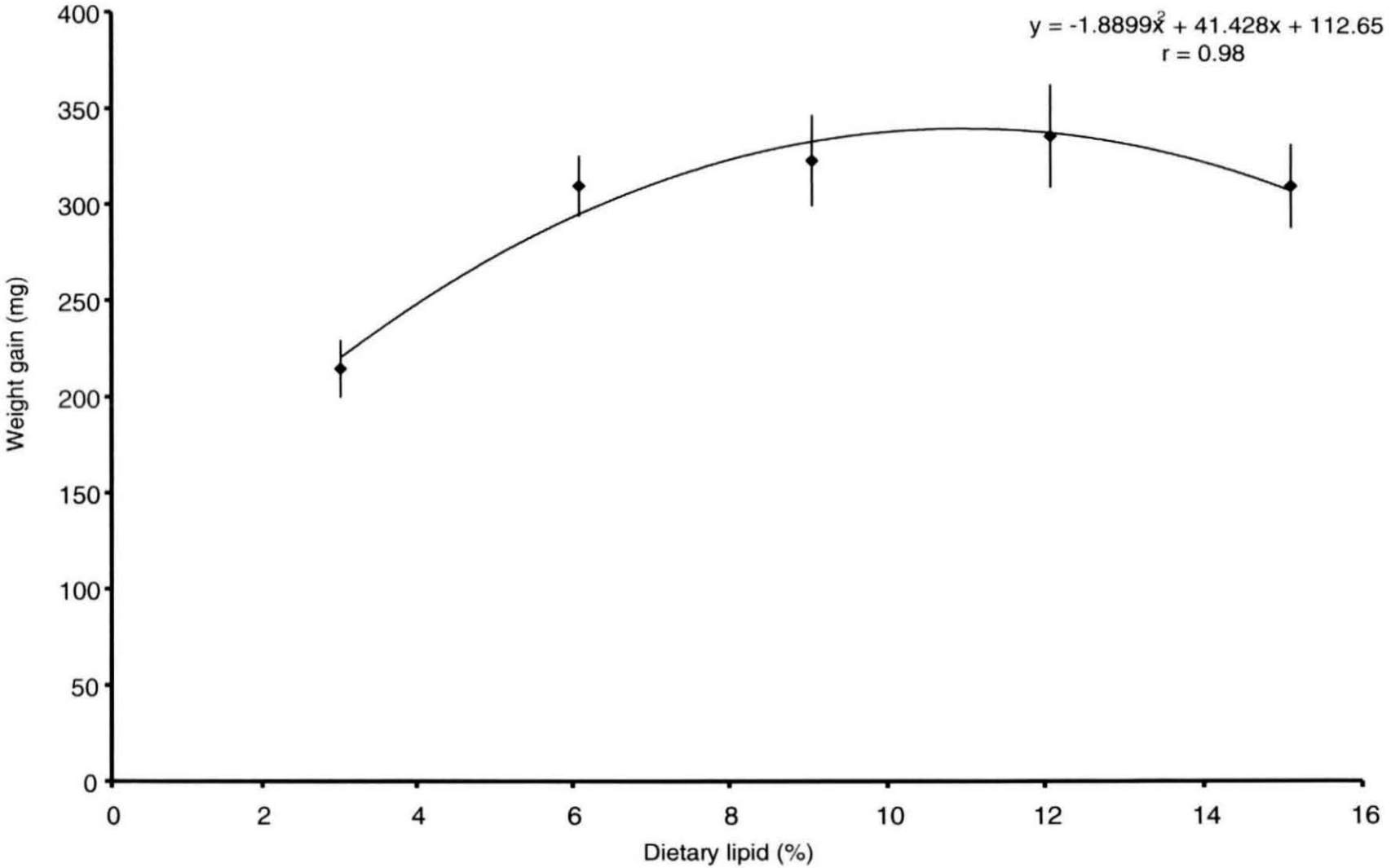


Fig. 17. Optimum lipid requirement for the juvenile *Amphiprion sebae*

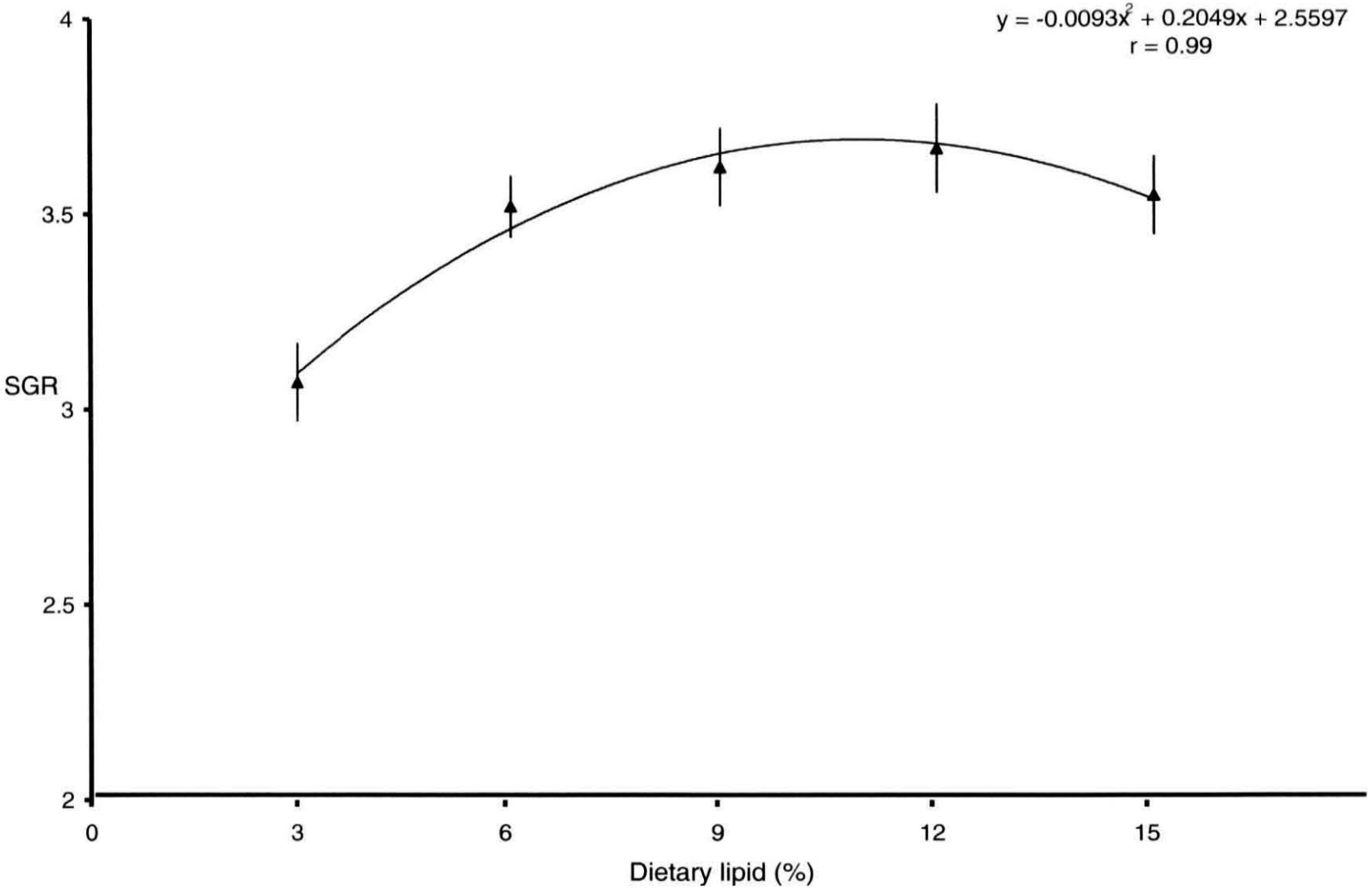


Fig. 18. Effect of dietary lipid on the specific growth rate of juvenile *Amphiprion sebae*

Table XXIX. Fatty acid profile of the diets used for lipid requirement in juvenile *Amphiprion sebae*

Fatty Acids (%)	D_{L3}	D_{L6}	D_{L9}	D_{L12}	D_{L15}
C14:0	3.02	3.16	3.28	3.39	3.50
C16:0	1.24	1.22	1.20	1.18	1.16
C16:1 n7	26.26	26.09	25.94	25.80	25.67
C18:0	4.75	4.56	4.39	4.23	4.07
C18:1 n9	12.35	12.84	13.28	13.68	14.07
C18:1 n7	1.69	1.74	1.78	1.82	1.86
C18:2 n6	5.00	4.82	4.66	4.51	4.37
C18:4 n3	1.08	1.05	1.02	0.99	0.97
C20:1	7.86	7.58	7.32	7.09	6.86
C20:4 n6	1.37	1.31	1.26	1.22	1.17
C20:5 n3	7.66	7.91	8.14	8.34	8.55
C22:1	1.49	1.65	1.78	1.91	2.04
C22:5 n3	0.75	0.76	0.77	0.78	0.79
C22:6 n3	8.48	8.67	8.84	8.99	9.14
C24:1 n9	0.20	0.26	0.30	0.35	0.39
∑ **Saturated**	9.00	8.93	8.86	8.80	8.74
∑ **Unsaturated**	74.19	74.67	75.10	75.49	75.87
∑ **MUFA**	49.86	50.15	50.41	50.65	50.89
∑ **PUFA**	24.33	24.52	24.69	24.84	24.99
∑ **n-3**	17.97	18.39	18.76	19.11	19.44
∑ **n-6**	6.36	6.13	5.92	5.73	5.54
∑ **n-3 HUFA**	16.89	17.34	17.74	18.12	18.48
Saturated/ Unsaturated	0.12	0.12	0.12	0.12	0.12
n3/n6	2.82	3.00	3.17	3.34	3.51
DHA/EPA	1.11	1.10	1.09	1.08	1.07
EPA/AA	5.60	6.03	6.45	6.86	7.29

Values are means of triplicate analysis
MUFA mono unsaturated fatty acids
PUFA poly unsaturated fatty acids
DHA docosahexaenoic acid
EPA eicosapentaenoic acid
AA arachidonic acid

lipid content. On the other hand the levels of AA, C18:3 n3, C18: 2 n6, C20:1, C16:0 and C18:0 showed a decrease with increasing dietary lipid supplementation in the diet.

There was not much difference among the diets in the total saturated fatty acids, total unsaturated fatty acids, total MUFA and total PUFA levels. However, n-3 and n-6 fatty acids showed variation between the diets. The saturated to unsaturated fatty acid ratio remained almost same in the diets. The DHA/EPA ratio ranged from 1.07 to 1.11 and EPA/AA ratio varied from 5.60 to 7.29.

DISCUSSION

5. DISCUSSION

5.1. Broodstock Nutrition

Brood fishes normally showed a preference for feeds in the water column and they seldom preferred settled feed particles. The new feeding strategy employed using the disc surface of the anemone (disc feeding) is successful in improving the feed consumption and the reproductive performance of sebae clownfish. This suggests the efficacy of the new feeding strategy for improving broodstock performance as well as in minimising feed wastage and thereby in maintaining good water quality.

The gut content analysis of adult fishes (n-7) collected from Rameswaram (June-July) showed relatively high percentage (>80%) of worms. The occurrence of worms in such high proportion may be either due to their availability at that particular time or due to selective feeding by the fish. The occurrence of worms in the gut content was also reported in clownfishes of Eniwetok (Allen, 1972). The feeding territories of clownfishes are limited to a few meters around their host anemones. Therefore, the food preferences, if any, in clownfishes are rather restricted to food availability within the territorial limits.

Adult fishes were observed to have the ability to feed by gulping or suction mode. This is evident from the occurrence of intact worms (2.5 - 3.5 mm diameter and length greater than that of the fish) in the gut contents. This mode of feeding is further confirmed in the laboratory trials by feeding the fish with polychaete worms. The gut to body length ratio of adults is found to be 1.2:1 in sebae anemonefish; however, the gut contents are highly skewed towards a carnivorous feeding habit.

Pairing in clownfishes is relatively easy due to their protandrous hermaphroditism and social hierarchies. In general, the dominant individual within a group functions as the female and the next dominant in the hierarchy becomes a functional male. However, in the broodstock development study two juveniles are found to be adequate to establish a breeding pair as reported for the same species (Sreeraj, 2002), and for other clownfishes (Shapiro, 1984).

The first spawning usually results in smaller clutch with brightly pigmented eggs, but often have relatively higher percentage of unfertilised eggs, which are consumed by the parent fish. The fecundity increases with the spawning and gets stabilised after the first 2 to 3 spawnings. Clownfishes may have the ability to distinguish bad quality eggs and remove them from the clutch by eating. Allen (1972) frequently observed the presence of clownfish eggs from the gut of Eniwetok clownfishes. Whether such consumption of eggs has any nutritional significance needs to be studied.

The major problem in hatchery production of marine fishes is the unpredictable reproductive performance of the broodstock. This unpredictability depends on various biotic and abiotic factors and among these factors nutrition of brood fishes plays a major role. The diets given to the broodstock are known to influence the fecundity, embryo development and larval quality (Bromage, 1998; Furuita, 2000; Izquierdo *et al.*, 2001).

The impact of diets on reproductive performance of various marine food fish species has been well documented (Luquet and Watanabe, 1986; Bromage and Roberts, 1995; Bromage, 1998). In many fishes reproduction involves a dramatic decrease in their food intake and a substantial transfer of nutrients from various body stores in to the developing oocytes (Aksnes *et al.*, 1986; Nassour and Leger, 1989). However, in continuous spawners with short vitellogenic period, which continue to feed during spawning, the diet considerably influences the egg quality (Watanabe *et al.*, 1985; Tandler *et al.*, 1995; Izquierdo *et al.*, 2000). Parameters frequently used to assess dietary influence of broodstock performance are the fecundity, egg dimension, fertilization, embryonic development, hatchability and larval quality.

In sebae clownfish dietary influence on reproductive performance is found to be mainly associated with clutch size, egg quality and larval survival. The number of eggs spawned (clutch size) is significantly ($P<0.05$) influenced by the dietary treatments. Of the five compounded diets tested three (CBD_5, CBD_2 and CBD_3) produced higher average clutch size (>1600 eggs) than that of cuttlefish meat (CUT), which gave the best results (1521 eggs) among the tested

natural diets (fig. 9). This shows that the compounded diets are superior to the single exclusive natural diets in promoting egg production.

The clutch size obtained in the present investigation is the highest ever reported for clownfishes under captive conditions. The clutch sizes reported for different species of clownfishes under captive conditions are given below:

Species	Average clutch	Largest clutch	Reference
Amphiprion akallopisos	301	392	Hoff, 1996
Amphiprion clarkii	668	981	Hoff, 1996
Amphiprion ephippium	583	869	Hoff, 1996
Amphiprion frenatus	440	551	Hoff, 1996
Amphiprion melanopus	249	359	Hoff, 1996
Amphiprion ocellaris	236	313	Hoff, 1996
Amphiprion percula	331	649	Hoff, 1996
Amphiprion polymnus	526	1217	Hoff, 1996
Amphiprion rubrocinctus	428	817	Hoff, 1996
Amphiprion sebae	569 (n-236)	1450	Sreeraj, 2002
Amphiprion sebae	961 (n-121)	2400	Present study

In the present study, variations observed in the egg and larval sizes were insignificant and the yolk utilization followed a similar pattern irrespective of the diets. The egg dimensions of the sebae anemonefish did not vary much between diets, except in the case of squid meat fed groups where the capsule width was significantly higher than other natural diets, suggesting the limited role of the diet in determining the egg size in sebae anemonefish.

The present study revealed the distinct influence of dietary carotenoids on eggs within a shorter duration (48 hrs). The change in yolk pigmentation was evident when females were fed carotenoid rich diets like the deep-sea prawn (42.77 µg astaxanthin/g wet wt.). When the fishes fed mussel meat (control diet) were switched over to the deep-sea prawn diet the egg yolk pigmentation changed from yellowish to reddish even when fed two days prior to spawning. This faster mobilisation of carotenoids indicates that by taking advantage of the protracted spawning as well as the short vitellogenic period it

should be possible to enhance the reproductive performance in clownfishes by dietary manipulations as reported in seabreams, where a duration of fifteen days was found to influence the egg quality (Watanabe *et al.*, 1984e; 1985; Tandler *et al.*, 1995). However, in the case of batch spawners like the salmonids, where vitellogenesis extends to several months (Fremont *et al.*, 1984), diets must be given continuously for more duration to assess its impact on broodstock performance.

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, 1997; Rodriguez *et al.*, 1998). Unlike other fishes the fertilization rate and hatchability of the sebae anemonefish were found unaffected by the broodstock diets used. The extensive parental care exhibited by the clownfishes makes these parameters insignificant as determinants of egg quality. Whether these parameters are affected by feeding diets with inferior nutrient profiles needs to be studied. Hatchability was found affected only when the eggs were artificially incubated without the parents.

Parent fishes invariably consumed the unfertilized and unhealthy eggs (turn opaque within a few hours), which help maintain the health of the clutch. The parent fish also vigorously fanned the egg mass using pectoral and caudal fins, which enabled water circulation around the densely packed clutch and aided in the removal of metabolic wastes. Male did most of the egg care, and on the day of hatching it vigorously fanned the eggs, which eased the process of hatching. The hatchability is affected when the brooders are distracted or disturbed just before or during the hatching process as their vigorous fanning is necessary for the larvae to break open the thick walled egg capsule. Complete hatching occurs within a day, though rarely the hatching was found extended to two successive days.

The differences in egg production observed among anemonefishes fed the natural diets can be attributed to the variations in their nutritional profile. The energy requirements of sebae anemonefishes are high during spawning, because of their continuous spawning nature, continuous growth even after initiation of spawning, and extensive parental care. All these energy demanding

processes need to be catered through the diet in order to maintain high spawning performance.

Among the nutrients dietary protein is known to significantly influence the process of reproduction in fishes (Smith *et al.*, 1979; Watanabe *et al*, 1984a; Cerda *et al.*, 1994). Watanabe *et al.* (1984b) suggested 45% dietary protein as optimum for the broodstock of red seabream. In *D. labrax* reduction in dietary protein from 51% to 34% significantly affected the broodstock performance (Cerda *et al.*, 1994). Decrease in reproductive performance was also reported in red seabream (Watanabe *et al.*, 1984b). Protein content in the natural diets used in the present study ranged from 50 to 70% (Table X). Within this range the cuttlefish meat (60% protein and 11% lipid) gave the best performance in terms of clutch size followed by the deep-sea prawn (56% protein and 10% lipid), and the least was obtained with mussel meat (50% protein and 11% lipid). Squid meat though had the highest protein (70%) content had relatively low levels of dietary lipids (5%) and thereby caused a decrease in egg production perhaps due to the lower availability of dietary fatty acids essential for reproductive processes or due to nutrient imbalances.

If dietary protein is considered as an individual variable influencing the fecundity in sebae anemonefish, higher egg production can be achieved by 55 to 60% protein in the diet. The dietary lipid levels that provided better performance falls in the range of 9 to11% and it is found that higher dietary lipid levels do not affect the clutch size. These inferences suggest that protein and its constituent amino acids are the major nutrients influencing the clutch size, provided that lipid and fatty acids levels are adequate. So, it is suggested that marine natural diets with 55 to 60% protein and 9 to 12% lipid are suited for sebae anemonefish broodstock in maximising egg production.

Fresh natural diets are traditionally used to feed broodstock and are proved to be the most effective way of meeting the nutritional needs of fish and ensuring good quality eggs (Bruce *et al.*, 1999). Dietary protein source is also known to influence the reproductive performance in fishes (Watanabe *et al.*, 1984a; Harel *et al.*, 1992; Tandler *et al.*, 1995). However, in the present study the compounded diets yielded better performance than the natural diets,

especially in terms of egg production. In the compounded moist diets protein ranged from about 38 to 50% (Table I). Among the tested diets, including natural diets, the diet CBD_5 (48.9% protein and 9.2% lipid) gave the highest average number of eggs per spawning. Though, CBD_4 (45% spirulina) had comparable protein (49.6%), lipid (8.6%) and energy levels as that of CBD_5, it resulted in poor performance. This clearly indicates the importance of balanced nutrients profile like fatty acids and amino acids in the diet. The performance of the diet CBD_4 was lower than that of CBD_3 (40.3% protein and 9.8% lipid), suggesting the need for animal matter of marine origin in the broodstock diets of *A. sebae*. The higher performance provided by CBD_5 is mostly due to its superior and balanced nutrient profile contributed by the basal mix (4:3:3 ratios of fishmeal, shrimp meal and squid meal) and supplementation of lower level of spirulina (20%) and 5% cod-liver oil.

The results of broodstock nutrition studies, both with natural and compounded diets, clearly indicate the need for higher dietary protein level (50%) together with moderate level of dietary lipids (10%) for promoting higher egg production in *A. sebae*. It is also evident that diets with high (20%) lipid content do not have much influence on egg production, once the essential fatty acids needs are met. Therefore, it can be concluded that dietary proteins and amino acids have a major role in determining the fecundity in sebae anemonefish, once the essential fatty acids needs are satisfied.

The higher level of egg production and egg quality observed in sebae anemonefish with the cuttlefish diet is in conformity to the observations in other marine fishes (Watanabe *et al.*, 1985, 1991; Harel *et al.*, 1992; Tandler *et al.*, 1995; Fernandez-Palacios *et al.*, 1997; Vassallo-Agius *et al.*, 2001b). The superiority of cuttlefish diet in enhancing the egg production and improving the viability of eggs in red seabream is attributed to the fat insoluble portion (Watanabe *et al.*, 1991).

The wide variations in fecundity of sebae anemonefish observed with the tested diets may be due to the differences in vitellogenin synthesis and its uptake. It may be affected by the availability (dietary) and synthesis of amino acids and fatty acids. The role of amino acids and fatty acids in the vitellogenin

synthesis is described in fishes (Sargent, 1995); wherein the hydrolysis of triacylglycerols by lipoprotein lipase generates free fatty acids (FFA) and these are transported via serum to the liver and the free amino acids (FAA) enter the liver following breakdown of muscle protein by proteases to generate FAA. The association of newly synthesized lipids with egg specific apoprotein in the liver enables the formation of vitellogenin and is exported to the eggs via the serum, which is then proteolytically cleaved into smaller yolk proteins (Specker and Sullivan, 1994). However, in the case of protracted spawners with short vitellogenic period like sebae anemonefish it is likely that the dietary proteins may be a major source of amino acids required for vitellogenesis. Moreover, unlike fishes like salmon which cease feeding upon attainment of maturity and mobilise body reserves for the gonadal development and vitellogenesis, the sebae anemonefish continues to feed and grow even after initiation of spawning. Thus there is need for increased supply of amino acids and fatty acids during the process of vitellogenesis. Therefore, it can be speculated that the rate of vitellogenesis as well as egg production in fishes with protracted spawning (2-3 spawnings per month continues for more than a year) may be markedly influenced by the broodstock diet. The process of vitellogenesis needs more in depth studies in protracted spawners like sebae clownfish to establish the role of dietary nutrients and factors controlling it.

The newly spawned marine fish eggs have a total amino acid content of 40-60% in their dry mass (Fyhn, 1989; Ronnestad and Fyhn, 1993; Thorsen *et al.*, 1993). Information on amino acid composition of marine fish with demersal eggs are scarce, and it is known that in these eggs the free amino acid pool is marginal with 2 to 5% when compared to 50-60% in pelagic eggs (Thorsen *et al.*, 1993). In the present study though amino acids profile of eggs was not determined, its importance in the egg production is quiet evident. In cuttlefish meat, which gave the best performance among natural diets, the essential to non-essential amino acid ratio was found to be 0.93:1(Table XI). The higher percentage of essential amino acid (53.84%) in the mussel meat with an EAA/NEAA ratio of 1.17:1 resulted in least egg production among the diets. Similarly the mature mussel with 1:1 ratio also resulted in comparatively low egg production. However, the deep-sea prawn with a ratio of 0.86:1 produced better

result, suggesting the possible role of non-essential amino acids in egg production.

The poor performance obtained with mussel meat might have been due to the significantly higher level of lysine (15.8%), and the amino acid imbalances caused due to such higher level of one essential amino acid. The cuttlefish which gave superior egg production, however, had the least content of lysine (4.5%). These results suggest that lysine may have an important role in the process of egg production. A similar trend was also observed with compounded moist diets, where the diet CBD_5 which gave superior performance had the lowest level (8.65%) of dietary lysine when compared to diet CBD_1 which had 11.65% lysine gave the least performance (Table XVI).

Among the compounded moist diets, CBD_4 had the best essential amino acid profile (Table XVI), owing to the higher content (45%) of spirulina. But the best performance was recorded with diet CBD_5 which had higher content of dispensable amino acids like cysteine, glutamic acid, glycine and proline apart from essential amino acids like histidine and phenylalanine, with the essential to non-essential ratio of 0.77:1. The highest EAA/NEAA ratio (0.92) obtained with CBD_1 gave the least spawning performance among compounded diets. These results suggest that though, fishes can synthesise dispensable amino acids adequately for normal protein synthesis, dietary supplementation may help improve the egg production in continuous spawners like the clownfishes where its requirement may be high during the process of vitellogenesis as a source of energy and as egg constituents. The analysis of juvenile sebae anemonefish, 6-7 cm (Table XXIII) also showed the preponderance (63.5%) of NEAA, suggesting them to be a major protein component in this species. This may also be a reason for the better performance obtained with diets having high NEAA composition.

The vitellogenin is known as the main yolk protein precursor in teleosts and its amino acid composition is characterised by high content of alanine, glutamic acid and leucine and a lower content of serine (Fernandez-Palacios *et al.*, 1997). The vitelline envelope proteins of gilthead seabream were found to have high content of proline and glutamic acid and a relatively low

content of cysteine (Hyllner *et al.*, 1995). Further studies are needed to confirm the essentiality and role of dispensable amino acids in brood fishes.

The importance of essential amino acids (EAA) in the broodstock diet has been established by Harel *et al.* (1995) in an *in vitro* binding assay study in gilthead seabream. They found that the dietary composition of EAA significantly affects the plasma vitellogenin (Vg) level and its binding capacity to oocyte membrane, and suggested that dietary EAA may affect egg quality through the control of Vg synthesis and its uptake, without any apparent effect on egg EAA composition and dietary essential fatty acids (EFA) on the other hand affect egg quality, mainly through changing the egg EFA composition without any apparent effect on Vg synthesis. However, Silversand *et al.* (1995) reported striking similarities between the fatty acid composition of eggs and vitellogenin in cod, suggesting that vitellogenin plays a fundamental role in the process of lipid accumulation. The exact role of fatty acids in the vitellogenesis is yet to be clarified, though it is known that dietary lipids influence the fatty acid composition of eggs in many species. Thus, the variations observed in the number of eggs spawned by the anemonefish in the present study can be attributed to the EAA and NEAA profiles of the diet and their influence on the vitellogenin synthesis and uptake.

Lipids are one of the major constituents in broodstock diet and have a direct influence on the egg and larval quality (Watanabe, 1985). They form an important membrane constituent in egg and larvae and act as an energy source. They are also known to play an important role in eicosanoid formation (Henderson and Sargent, 1985; Bell and Dick, 1990; Sargent *et al.*, 1994). Low lipid level in broodstock diet is known to influence the egg and larval quality (Watanabe *et al.*, 1984a; Verakunpiriya *et al.*, 1996; Vassalo-Agius *et al.*, 1998). Duray *et al.* (1994) observed improved fecundity and hatchability in rabbit fishes with an increase in dietary lipid.

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, b; Harel *et al.*, 1994; Watanabe and Kiron, 1995) and the egg fatty acid profiles in fishes (Mourente and Odriozola,

1990). EFA of total egg lipids from most fish are richer in n-3 PUFA than parental body oils (Sargent et al., 1989), indicating the essentiality of dietary n-3 PUFA for broodstock.

The EFA requirement is known to be high during spawning, as large quantities of fatty acids are needed for egg production (Sargent, 1995; Zohar *et al.*, 1995; Navas *et al.*, 1997). Harel *et al.* (1994) showed that in gilthead seabream the EFA composition of eggs quickly responded to the dietary change with higher lipid turnover in organs (ovary, liver and digestive tract tissue) associated with reproduction. In the European seabass, administration of a high EFA diet during vitellogenesis produced eggs of similar quality to those of groups fed high EFA diet throughout the year (Navas *et al.*, 1997). On the other hand in fishes like coho salmon, with long period of vitellogenesis, eggs reflect the dietary fatty acid profile only after two months of feeding (Hardy *et al.*, 1990). However, continuous spawners like sebae anemonefish need a consistent dietary supply of EFA to maintain the egg and larval quality. The dietary EFA composition was found to be reflected in fish eggs (Lasker and Theilacker, 1962).

The oil globules, rich in triglycerides, are shown to be an energy source for developing eggs and larvae (Watanabe, 1985; Mourente and Odriozola, 1990). The amount of lipids in eggs generally correlates with the incubation period (Blaxter, 1969; Kaitaranta and Ackman, 1981). The sebae clownfish have multiple oil globules and they incorporate high amount of lipids in their eggs to satisfy the energy requirements during incubation, which normally extends from 6 to 8 days, and it also acts as an energy source for the early larvae. As clownfishes spawn continuously, the mobilization of body EFA reserves may not be adequate enough to meet the demand and hence dietary supply is needed to maintain high fecundity and spawn quality. Nassour and Leger (1989) found that about 50% of total lipids deposited in the oocytes of rainbow trout are of dietary origin. The dietary EFA level is known to have greater influence on egg production than other parameters (Watanabe *et al.*, 1984a; Fernandez-Palacios *et al.*, 1995; Furuita *et al.*, 2000).

The n-3 HUFA play an important role in the maintenance of membrane fluidity and correct functions of bound membrane enzymes (Bell *et al.*,

1986) and n-3 HUFA are the major constituent of egg fatty acids and account for 30-40% in many marine fishes (Harel and Place, 1998). The n-3 HUFA level between 1.5 and 2% improved the spawning quality in marine fishes (Watanabe *et al.*, 1985; Fernandez-Palacios *et al.*, 1995; Furuita *et al.*, 2000). Deficiency of n-3 HUFA in the broodstock diet critically affects the fecundity, hatchability and viability (Mourente and Odriozola, 1990; Fernandez-Palacios *et al.*, 1995; Rodriguez *et al.*, 1998; Almansa *et al.*, 1999). 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; Lavens *et al.*, 1999; 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). Fernandez-Palacios *et al.* (1995) found that excess n-3 HUFA in the broodstock diet causes decreased fecundity and yolk-sac hypertrophy in newly hatched *S. aurata* larvae and they recommended a level of 16 g n-3 HUFA per kg diet for better egg and larval quality. The results from the present study show that the upper level of n-3 HUFA needed in sebae anemonefish broodstock diet to produce high quality eggs is less than 30% of the total fatty acids and it ranged from 17 to 25% in natural diets and 14 to 18% in moist diets. Thus, it is concluded that a diet with 15 to 20 % of n-3 HUFA in the total fatty acid fraction may give better reproductive performance in *A. sebae*. Besides, a high level of PUFA (>35%) and n-3 HUFA (>30%) appears to have a detrimental effect in sebae anemonefish resulting in significantly low egg production.

DHA is typically reported to be high in marine fish eggs (Watanabe, 1993; Watanabe and Kiron, 1995), and it accumulates faster than EPA in the lipids of fish eggs. DHA serves as a metabolic energy reserve during the development of eggs and it also occurs in the neural cell membranes and forms an integral part of brain and in eye formation (Mourente *et al.*, 1991; Bell *et al.*, 1995). Being an essential fatty acid it needs to be supplied continuously through the diet, especially in the case of continuous spawners like the sebae anemonefish. This clearly points towards the importance of DHA in the broodstock diets.

Delbare *et al.* (1995) observed that the fatty acid profile of larvae from bad quality eggs (BQE; <50% survival on first day) of tomato clownfish, *A. ephippium* showed higher EPA:DHA ratio (7.3:1). Dominguez *et al.* (2001) reported changes in fatty acids during incubation and found a decrease in concentration of monounsaturated fatty acids and an increase in PUFA, especially the DHA, indicating its role in egg and larval quality.

In sebae anemonefish the egg production was found apparently unaffected by the DHA/EPA ratio. The maximum production was obtained using a compounded moist diet CBD_5 with a ratio of 2.3:1, while all other diets had ratios in the range 1.06-1.09:1. Among the natural diets cuttlefish with a higher ratio of about 6:1 gave better 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. These results suggest that the influence of DHA:EPA ratio on egg production is marginal, except at very high and very low levels.

Arachidonic acid is known to be involved in eicosanoid formation (Henderson and Sargent, 1985; Bell and Dick, 1990; Sargent *et al.*, 1994), and thereby influences reproductive process. The arachidonic acid received comparatively low research attention compared to that of other EFAs. In Atlantic salmon a 2% dietary level improved egg production and larval quality (Bromage *et al.*, 2001). AA and EPA are known to influence fertilization rate in gilthead seabream (Fernandez-Palacios *et al.*, 1995).

The lower EPA/AA ratio together with higher DHA/EPA ratio among the compounded moist diets resulted in superior egg production. However, it did not follow any specific pattern.

Fernandez-Palacios *et al.* (1995) reported that higher level of n-3 HUFA (3.15% of the diet) decreased the fecundity and induced yolk sac hypertrophy in newly hatched out larvae of gilthead seabream resulting in poor survival.

According to Fernandez-Palacios *et al.* (1995) the n-3 HUFA of eggs are not the sole criterion determining egg quality, as both lower and higher

levels of n-3 HUFA in eggs are associated with low egg quality and larval survival. The higher egg lipid content caused poor egg viability in turbot, sole and seabass (Devauchelle *et al.*, 1982). Analysing the natural maturation diets employed by breeders of freshwater aquarium fishes Tamaru *et al.* (1997) reported that broodstock of many freshwater aquarium fishes do not seem to have an absolute requirement for long chain PUFA. Instead they reported higher levels of 18:2n-6 and 20:4n-6 in the diets.

Marine fish oils are rich in long chain HUFA and are known to improve the egg quality in fishes (Bell *et al.*, 1997; Navas *et al.*, 1998; Bruce *et al.*, 1999). In a study on the larvae of coral reef damselfish, *Acanthochromis polyacanthus* the use of cod-liver oil as fatty acid source in the diet gave higher survival and growth (Southgate and Kavanagh, 1999). The use of cod-liver oil, which is a rich source of n-3 HUFA, might have influenced the egg production in sebae anemonefish as it formed the major lipid source in the compounded moist diets.

Carotenoids are reported to influence the reproductive performance in marine fishes (Watanabe and Miki, 1993; Watanabe and Kiron, 1995; Verakunpiriya *et al.*, 1997 a, b; Vassallo-Agius *et al.*, 2001a). In many fish species pigment accumulation occurs during maturation and is directly influenced by broodstock diets (Hubbs and Strawn, 1957; Craik, 1985; Miki *et al.*, 1984; Verakunpiriya *et al.*, 1996). Sebae anemonefish is found to actively mobilize the dietary carotenoids to eggs and such active transfer of carotenoids from the broodstock diets to eggs within a short span (48 hrs) indicates their importance in eggs; though the functions of carotenoids in eggs are not yet clearly established. Carotenoids are very effective antioxidants against the peroxidation of long-chain fatty acids typical of fish eggs (Watanabe and Kiron, 1995).

In the present study, the yolk pigmentation in sebae anemonefish generally resembled the external appearance of the natural diet. Pickova *et al.* (1999) also showed that the carotenoid composition of feed given to females would be reflected in the yolk content and composition of their eggs.

Dietary astaxanthin content is known to influence the egg quality in fishes. In red seabream improved reproductive performance and egg quality are achieved by supplementing diets with synthetic astaxanthin (Watanabe and Miki, 1993; Watanabe and Kiron, 1995). The dietary inclusion levels recommended for better reproductive performance are 20 mg /kg for red sea bream (Watanabe and Miki, 1993), and 30 mg/kg diet for yellowtail (Verakunpiriya *et al.*, 1997b). However, an inclusion of 20% krill meal in the diet for yellowtail caused inferior reproductive performance (Verakunpiriya *et al.*, 1997b). In the present study on sebae clownfish, dietary astaxanthin level (Table XIII) was about 45 mg/kg (wet weight) in the deep-sea prawn; however, it did not affect the egg production, though higher numbers of egg were obtained with a dietary level 5 mg/kg (wet weight). The least number of eggs was obtained with mussel meat which had a astaxanthin content of about 17 mg/kg. These results clearly indicate that dietary astaxanthin does not have any significant influence on the egg production in sebae clownfish. However, astaxanthin has a positive influence on the egg and larval quality, as the survival of larvae from broodstock fed the deep-sea prawn and mature mussel meat were found to be higher than those obtained with other diets.

Egg colour observations made during present study indicate that like many other clownfish species viz. *A. ocellaris*, A. *percula*, *A. clarkii*, *A. akallopisos*, *A. polymnus* and *A. sandaracinos* (Hoff, 1996), *A. sebae* produced predominantly orange coloured nests in nature. It is also evident that the yolk pigmentation in *A. sebae* reflects the diet colours, *i.e.*, different intensities of yellow, orange and red. The bright reddish pink colouration in the eggs of fish fed the deep-sea prawn based diet is due to the presence of high levels of astaxanthin. Similarly, the differences observed in the intensity and pattern of yolk colouration *viz.* pale pink colouration given by cuttlefish and squid; and the yellow to orange colouration induced by the mussel meat clearly indicate differences in the incorporation of quantity and quality of carotenoids. Spirulina though is a good source of zeaxanthin (Miki *et al.*, 1986), its supplementation is ineffective in imparting yolk pigmentation in sebae anemonefish. In *Oreochromis niloticus*, feeding raw spirulina alone imparted bright orange colour to the egg as compared to the whitish appearance of those fed with compounded diet (Lu and

Takeuchi, 2004). Zeaxanthin was the main carotenoid converted from dietary astaxanthin and mobilized into yellowtail eggs (Verakunpiriya *et al.*, 1996). But, carotenoids were not detected in the fertilized eggs when spirulina was fed to striped jack (Vassallo-Agius *et al.*, 1999).

The clutch colouration in sebae anemonefish is found to fade under captivity after initial few spawnings, if not fed with adequate carotenoid rich diet. Incorporation of spirulina at levels as high as 45% (CBD_4) in the diet failed to provide brighter yolk pigmentation suggesting that dietary β-carotene is not adequately utilized by the sebae clownfish.

Hoff (1996) reported that addition of astaxanthin into the gelatin based diet results in bright coloured nests within two weeks. He also compared the hatching percentage and found that those fed with pigment supplementation showed an increased hatching percentage of 84.04% as compared to 68.75% without supplementation. However, in the present study the hatchability was unaffected by the dietary treatments.

It was often observed that the yolk pigmentation pales out when a particular diet is stopped. With a change in diet the new dietary carotenoid profile gets its complete expression in the eggs usually from the second spawning. So, in order to sustain particular yolk pigmentation continuous dietary supply is necessary. This also points at the lower carotenoid retention/storage abilities in the ovary, and other organs or tissues. These characteristics along with the influence of carotenoids on the pigmentation of larvae make clownfish an ideal species for carotenoid nutrition studies.

Vitamins, especially vitamin E, C and A, are important in fish reproduction and in achieving egg and larval quality (Watanabe, 1985; Watanabe *et al.*, 1991b; Fernandez-Palacios *et al.*, 1996; Dabrowski and Ciereszko, 2001; Ikeda, 1985; Ishibashi *et al.*, 1994; Santiago and Gonzal, 2000; Furuita *et al.*, 2003). In the present study, the levels of vitamin E and C used in the diets of broodstock were higher than that used for juveniles being 350 IU and 300 mg/kg diet respectively. These levels were found effective in supporting superior reproductive performance when compounded moist diets were used.

Considering all the above aspects it can be speculated that egg production in continuous spawners like sebae anemonefish is controlled largely by the broodstock diet. Among the nutrients amino acids (both essential and non-essential) with adequate level of EFA in the diet is found to facilitate superior egg production. However, the DHA/EPA ratio and astaxanthin level do not influence the egg production, instead they may influence the egg and larval quality.

The pattern observed in the yolk utilization during the embryonic development did not vary among the treatments. The maximum yolk utilization occurs during the late embryo development, followed by the initial egg cell divisions. Both yolk and oil globules provide the energy needs of developing embryo. The endocytosis followed by intracellular digestion in the yolk syncytial layer (YSL) is the major mechanism of yolk utilization in teleosts (Heming and Buddington 1988). All the nutrients from the yolk pass through the YSL to reach the embryo.

Sebae clownfish egg has multiple oil globules with varying dimensions. There are numerous oil-globules scattered in the yolk with few larger ones (1 to 3 with a diameter>150 µm). The smaller globules are utilized during the course of embryo development and 1 or 2 oil globules formed due to the fusion of smaller globules remain on the day of hatching. Thus, the newly hatched larvae generally have 1 or 2 oil globules. The fusion of oil globules was also observed during the embryo development in *Lateolabrax japonicus* (Makino *et al.*, 1999).

In the present investigation, incubation period was found to be a major factor determining the availability of yolk nutrients to larvae. The relative advantages of shorter incubation period and early hatching are evident from the yolk utilization pattern. Shorter incubation helps retain considerable amount of yolk reserves which contributes to the larval survival and viability. On the other hand, delayed hatching causes substantial reduction in yolk reserves and produce weaker larvae resulting in low survival rates.

The diets fed to brood fish are known to influence the larval quality in many fishes (Duray *et al.*, 1994; Fernandez-Palacios *et al.*, 1995; Tandler *et*

al., 1995; Aby-ayad *et al.*, 1997). Though the 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 endogenous nutrient reserves are utilized within a short span of two days in *A. sebae* (Sreeraj, 2002) when compared to three days for other clownfishes like, *A. percula* (Gordon and Hecht, 2002) and *A. melanopus* (Green and McCormick, 2001). The faster utilization of yolk observed in *A. sebae* may be due to the higher metabolism and growth rate in this tropical species. In larvae with yolk and oil globules, the latter always seems to be resorbed much slower than the former (Houde *et al.*, 1976; Bagarinao, 1986).

The survivability of *A. sebae* larvae was high in almost all the treatments at 3 dph, when the influence of broodstock diet is expected to be high. However, those fed the mussel meat and the compounded diet CBD_1, resulted in low survival (< 70%) on 3 dph, perhaps due to the excess n-3 HUFA levels in these diets, which is known to have negative effect on larval survival (Furuita *et al.*, 2000).

The pigmentation of larvae is influenced by the dietary pigments, the mature mussel as well as deep-sea prawn rendered orange pigmentation to the larvae, which were seen as pigment spots on the body. Hoff (1996) attributed the hatching success and early larval survival in clownfishes to the egg pigmentation. The higher survival rates of larvae from the broodstock fed on the deep-sea prawn and mature mussel as diets may be due to the higher astaxanthin content which is known to influence immunity in fishes (Thompson *et al.*, 1994; Paripatananont *et al.*, 1999). The improved survival may also be due to the vitamin A activity of the carotenoids, as they form precursor for vitamin A, which in turn can increase the visual capabilities of the larvae and thereby resulting in increased prey strike success. Supplementation of astaxanthin in the diet increased the vitamin A level in rainbow trout ovary (Guillou *et al.*, 1989). The mobilization of carotenoids to the ovaries and then to larvae was also reported (Torrissen and Chrishtiansen, 1995; Choubert *et al.*, 1998). Large scale mortality was observed when Atlantic salmon was fed astaxanthin deficient diets

(Christiansen *et al.*, 1995). Besides, the higher rate of mortalities in newly hatched larvae during their transfer to larval rearing system may be due to the insufficiency or imbalance in EFA.

Apart from producing higher fecundity, diet CBD_5 gave better larval survival, possibly due to the higher DHA content and better DHA/EPA ratio of the diet. Selective retention of DHA during the process of embryogenesis is often observed in fishes (Izquierdo, 1996)

5.1.1. Influence of level of feeding on spawning

Under restricted feeding or one time *ad libitum* feeding, *A. sebae* exhibited considerable reduction in their reproductive output and the number of eggs spawned was almost half to that of the control treatment fed twice *ad libitum*. Similar observations were also reported in other fishes like rainbow trout (Scott, 1962), goldfish (Sasayama and Takahashi, 1972), European seabass (Cerda *et al.*, 1994a) and in Atlantic salmon (Berglund, 1995). The comparatively lower egg dimensions observed in the present study with feed restriction are in conformity with the reports in European seabass (Cerda *et al.*, 1994a).

5.1.2. Broodstock development from juveniles

Though many species of clownfishes have been bred successfully in captivity, even by many home aquarists; the feeding of the brood fish is mostly carried out with a combination of natural and commercial diets. In the present study, for the first time successful broodstock development and spawning was achieved by feeding dry formulated diets to early juveniles (from 21 dph) from 15^{th} month (Table XXI). Sreeraj (2002) reported spawning of *A. sebae* broodstock developed from about 3 month old juveniles (24-36 mm) in 12^{th} month by feeding fresh natural diets. The duration for first spawning in both these studies is comparable as the duration in the present study is calculated from the day of hatching.

However, the major difficulties encountered were the slow growth and smaller size of fish at maturity when fed exclusively on dry diets from the early juveniles. The number of eggs per clutch also was a paltry two to three

hundreds which is quite low for this species. The exclusive use of dry diets for broodstock development though sufficient to initiate spawning did not improve the reproductive performance, though hatchability was not affected. Decrease in egg production and viability was also observed with seabass fed pelleted diets (Cerda *et al.*, 1994). However, the results of the experiment to develop broodstock from the early stages by feeding exclusively on dry diets clearly indicate the possibility of developing off the shelf diets for the broodstock development in sebae anemonefish and related species with further intensification of research and refinement of diets. Broodstock development from hatchery reared juveniles can be an ecofriendly alternative by reducing wild collection for the purpose of juvenile production.

5.2. Larval Nutrition

The successful rearing of clownfish owes greatly to the emergence of comparatively well developed larvae, which are capable of exogenous feeding immediately after hatching, and the relatively easy larval rearing protocols. These features enabled aquarium hobbyists in rearing larvae of many clownfishes successfully though larval feeding remains a problem largely due to the inadequate knowledge of specific nutritional needs and diet preferences of the larvae.

The nutritional requirements of larvae are difficult to determine using classical procedures. Consequently, compounded feeds for the larvae are generally formulated empirically (Abi-Ayad and Kestemont, 1994). Poor feed acceptability, low survival and growth rates are usually observed with artificial diets. If dietary nutrients available for metabolic activities are insufficient the body reserves get metabolised and prolonged feeding with nutritionally incomplete diets may cause irrecoverable damage to early larval stages.

The alimentary system of newly hatched larvae of sebae anemonefish had a single looped gut, oesophagus, stomach, intestine, rectum, kidney, liver, *etc.* on the day of hatching, which is similar to the reports for other clownfishes (Green and McCormick, 2001; Gordon and Hecht, 2002). The mouth opening is sufficient to accept rotifers as first feed, and the larvae readily feed on

Artemia nauplii by 4 dph. Calcification of teeth has been reported after 4 dph in *A. melanopus* (Green and McCormick, 2001), which helps the larvae in grabbing the prey.

The livefeed provided to the larvae and nutrient availability critically affects the survival of larvae after the exhaustion of endogenous nourishment. Though in the present study livefeeds were not enriched, about 50% of the larvae successfully metamorphosed in most treatments.

Rotifers are generally used as first feed for marine fish larvae and continued till the initiation of *Artemia* feeding. *A. sebae* larvae readily accept rotifers as confirmed by their presence in the gut. During the early larval period, sebae anemonefish larvae were found to capture the prey predominantly by suction and this mode of feeding has been well documented with *A. perideraion* by Coughlin (1994).

When *A. sebae* larvae are fed rotifers as exclusive livefeed, survival and metamorphoses rates were affected. Large scale mortality (upto 50%) was observed within the first five days of rearing, by then yolk reserves were utilized. Besides, the metamorphosis took almost two weeks to reach 50% level resulting in smaller larvae. This comparatively poor performance might have been due to the inadequacy of rotifers as livefeed for advanced larval stages, as the larvae need to spend more energy in foraging to satisfy their requirement on these small preys. Thus, insufficient availability of dietary nutrients and energy might have been responsible for poor growth and delayed metamorphosis of larvae fed rotifers as exclusive diet.

Artemia nauplii forms the preferred livefeed for the larvae after the rotifer feeding stage and can be fed from fourth day post hatching to sebae clownfish larvae. As the cysts are commercially available it's the most preferred livefeed in most commercial hatchery operations. Acceptability of *Artemia* from 4 dph as observed in the present study is in conformity to that reported for *Amphiprion melanopus* (Green and McCormick, 2001). In a preliminary experiment, when excess nauplii were fed to *A. sebae* larvae voracious feeding was observed, with a larva (6 dph) consuming more than 75 nauplii within a short

span of 10-12 minutes. This often resulted in poor digestibility followed by death. The mortality due to this "gluttony" is characterised by the presence of partially digested nauplii packed gut and the emergence of orange trail of faeces. Ignatius *et al.* (2001) reported poor digestibility of *Artemia* nauplii for the 7 dph larvae of *A. sebae*.

In the present experiment using *Artemia* nauplii as exclusive feed after first three days of rotifer feeding (LF_3) resulted in poor larval survival (Fig. 13). This might have been due to the abrupt shifting to larger size prey like *Artemia* and the discontinuation of rotifer feeding. Though most of the larvae are capable of accepting and assimilating *Artemia* nauplii from 4 dph, many are incapable of accepting *Artemia* at this early stage leading to eventual death due to starvation.

The lower survival obtained also suggests the inadequacy of any single feed as exclusive livefeed (e.g. rotifer). This may be due to the nutrient insufficiency of *Artemia* nauplii as an exclusive livefeed immediately after three days of rotifer feeding, when endogenous reserves also serve as source of energy. However, feeding with *Moina* a freshwater cladoceran and *Artemia* after 20 dph did not influence survival, though *Moina* is known to be a poor source of EFA (Tamaru *et al.*, 1997). Exclusive feeding with *Moina* after metamorphosis during preliminary attempts often resulted in large scale mortality due to shock syndrome or fainting especially when they were handled or disturbed. Even two month old juveniles were found to have acute shock syndrome when they were fed continuously with *Moina* as major livefeed. A similar observation on mortality was also reported in larvae of coral reef damselfish (Southgate and Kavanagh, 1999), and suggested to be caused mainly due to the deficiency of HUFA in the diet.

After metamorphosis (appearance of two white bands and epibenthic habit) the rate of mortality is found to decline in all the treatments. The higher mortality during metamorphosis may be due to the increased energy and nutrient demands during this complex physiological process.

The treatment LF_4 in which a mixed schedule of rotifers and *Artemia* were fed to the larvae with microalgae in the rearing medium performed better in terms of survival than other treatments. The use of microalgae in the rearing medium during the early feeding stages is widely practised for marine fish larvae (Naess *et al.*, 1990; Reitan *et al.*, 1993; Oie *et al.*, 1997) and is believed to improve the nutritional conditions of the larvae, either directly (Moffatt, 1981) or through improving the nutritional value of the rotifers (Lubzens, 1987), it also have several other advantages like, light attenuation or shading, provide contrast to feeding, may increase appetite and have growth promoting effects (Naess *et al.*, 1990; Reitan *et al.*, 1993; Oie *et al.*, 1997). Higher survival and growth were also reported in other marine fish species when larvae were reared on microalgal environment (Naas *et al.*, 1992; Reitan, 1994). It has also been proposed that bacteria controlling functions of microalgae as more important than their nutritional effects (Stottrup *et al.*, 1995). The larvae of *A. sebae* reared under these conditions also exhibited almost uniform growth and synchronous metamorphosis.

These results suggest that rotifer feeding for first four days followed by a combination of rotifer with *Artemia* nauplii in the green water medium can sustain higher larval survival in sebae anemonefish. As marine finfish are incapable of synthesizing polyunsaturated fatty acids it is important to improve the nutritional content of livefeeds through enrichment to achieve better larval survival (Leger *et al.*, 1986; Sorgeloos *et al.*, 2001). The use of enriched livefeeds and a co-feeding with micro-diets after a weeks rearing can benefit large scale production of this species.

5.2.1. Weaning

Weaning of larvae to dry diets is a laborious and time consuming process in marine fish hatcheries. The advantages of early weaning of larvae are assurance of nutrient quality, reduction in labour needs and operational costs *etc.* Micro-particulated diets have been used with varying levels of success in weaning marine fish larvae (Teshima *et al.*, 1982; Walford *et al.*, 1991; Lopez-Alvarado *et al.*, 1994). Even a partial replacement of livefeed or earlier weaning to dry diets can result in considerable cost saving in hatchery production (Jones

et al., 1993; Lavens *et al.*, 1995). Critical factors that influence efficiency of dry diets to larvae are particle size, feed acceptability and nutrient availability.

The digestive systems in most larvae are under developed on hatching and are expected to be fully functional after metamorphosis (Kolkovski *et al.*, 1993; Kolkovski, 2001). The first feeding larvae generally lack functional stomach, acid secretion, and peptic enzyme activity until metamorphosis (Munilla-Moran and Stark, 1989; Miwa *et al.*, 1992; Bisbal and Bengston, 1995), and this makes the weaning process difficult.

The exogenous enzymes from livefeeds supposedly influence the larval digestion process (Lauff and Hofer, 1984; Munilla-Moran *et al.*, 1990), and in activating endogenous enzymes of larvae (Kolkovski *et al.*, 1993). In *A. percula* extracellular digestion and absorption across the lumen occurs on 9 dph and by that time larvae could utilize prepared diets (Gordon and Hecht, 2002).

The results of weaning experiments on sebae anemonefish larvae to dry diets suggest that weaning after three weeks of rearing, when they fully metamorphose and become epibenthic, is better for achieving good survival. The survival rate after this period was considerably higher and it is assumed that the larvae are well adapted to assimilate nutrients from the dry diets. In the present study, the ideal age of weaning to dry diets, without affecting growth was found to be 30 dph (Fig. 14). Gordon *et al.* (1998) reported comparatively better survival for *A. percula* when the larvae were weaned 7 dph onwards; however they found that 15-20 dph as optimal time to wean towards formulated dry diets without affecting growth as they found that digestive secretions by the gastric glands were adequate for efficient digestion and assimilation of formulated diets by then. Juvenile seahorse, *Hippocampus abdominalis* of 1-2 months age was successfully weaned to frozen and artificial foods, but was unsuccessful in the case of newborns (Woods, 2003).

The weaning experiments on 14 dph larvae showed promising results with more than 50% survival. After 3 weeks of hatching (21 dph) the larval survival was found to be above 75%. The lower survival rate of larvae weaned from 4 dph may be attributed to the inefficient utilization of the diet, either due to

unsuitable particle size or texture of the diet (Dabrowski, 1984; Le Ruyet *et al.*, 1993), or their inability to digest and assimilate at required levels as a result of inadequately developed digestive system (Lauff and Hofer, 1984; Smith, 1989).

5.3. Juvenile Nutrition

5.3.1. Protein Requirement

As there is only scanty information available on the nutritional requirements of marine ornamental fishes including clownfishes, the present work gives a basic understanding of the nutrition of these fishes in captivity. Juvenile production is the most important factor determining the commercial viability and profitability of clownfish hatcheries and is reported as the major cause identified for the failure of pioneering rearing projects like the Instant Ocean Hatcheries (Hoff, 1996).

Protein being the most expensive component in fish diet needs to be optimised for any viable aquaculture venture. Studies on the protein requirements are particularly important for new species of commercial aquaculture importance. Though, hatchery production technologies for clownfishes have been developed, their nutritional requirements are still unknown.

One important observation made during the course of this experiment is the substantial size variation among the fish within each replicate. Though, the initial individual size was almost uniform there were distinct growth variations at the termination of the experiment within treatment replicates, which may be due to the social hierarchies. Though higher growth rates were observed in fishes fed high protein diets, there were greater growth variations and an increased aggression among individuals within the replicates. A similar pattern was observed in all the replicates, with 2 or 3 individuals growing faster than the others. Growth variations were also reported in juvenile *Amphiprion percula* when reared in captivity (Gordon *et al.*, 1998). Ochi (1986) observed considerable difference in the growth of 0-year old juvenile anemonefishes in natural habitat. The social hierarchies in anemonefishes have been well documented (Moyer, 1976; Moyer and Nakazono, 1978; Hattori and Yanagisawa, 1991), and are

mainly induced by the aggressive dominance of larger fishes (Fricke and Fricke, 1977). The magnitude of aggression depended upon the relative social ranking of the individuals present in the anemone. The slower growth rates observed in some individuals within a treatment may be due to the excessive energy used for evasion of attacks and the consequent lower foraging time (Allen, 1972; Fautin and Allen, 1992).

Variation in individual growth is a common phenomenon in many cultured fish stocks (Huntingford *et al.*, 1990; Stefansson *et al.*, 2000; Smith and Fuiman, 2003). Interactions are known to influence the growth in many fish species leading to the formation of feeding hierarchies, thereby decreasing the growth of the low ranking individuals (Koebele, 1985). Among the social interactions, size-related dominance is considered to be one important factor that determines the aggressive behaviour, feeding and growth performance (Abbott and Dill, 1989). The differences in feed intake between individuals explain much of the variation in growth found within groups of fish (McCarthy *et al.*, 1992, 1993). However, difference in protein metabolism also influences the efficiency with which individuals use food (Carter *et al.*, 1993; MaCarthy *et al.*, 1994). Consequently, there may be subtle differences in whole-body or organ specific protein synthesis related to social rank which reflects the stress imposed by a particular rank (Carter and Houlihan, 2001).

Stocking density is also found to be an important determinant factor in rearing *A. sebae*. The stocking density in the present study was decreased from eight individuals per 70 ℓ to five individuals to reduce the aggression. In the commercial rearing facilities aggression is minimised by keeping very low or very high stocking densities (Hoff, 1996), where the individuals may (low stocking) or may not (high stocking) have enough space for separate territories resulting in decreased territorial aggression.

The growth performances of fishes are generally poor when fed with purified diets compared to practical diets (Tacon and Cowey, 1985). In the present study also, growth performance was found to be low with the purified diet. This situation is further exacerbated when early juveniles were used.

Because of the high level of growth variability semi-purified diets were used to define protein and lipid requirements.

The results of the present study on early juveniles showed better growth performance at about 45% dietary protein, which is similar to the protein requirement reported for freshwater ornamental fishes like discus (Chong *et al.*, 2000) and swordtail (Kruger *et al.*, 2001). Johnston *et al.* (2003) obtained comparatively lower growth than the present study when fed crushed flake food containing 46% protein and 5% crude fat to *A. percula* and probably due to the lower dietary lipid. In an earlier attempt to wean *A. percula* Gordon *et al.* (1998) used a formulated diet with 43.8% protein and 8.4% lipid. The dietary protein requirement observed for juvenile *A. sebae* is significantly higher than that of other aquarium fishes such as dwarf gourami (Shim *et al.*, 1989), goldfish (Lochmann and Philipps, 1994), and tinfoil barb (Elangovan and Shim, 1997).

The optimum protein level observed in the present study is comparable to those observed for other marine fishes like Japanese eel (Nose and Arai, 1972), rainbow trout (Zeotoun *et al.*, 1973), Asian seabass (Sakaras *et al.*, 1989), European seabass (Perez *et al.*, 1997), and Japanese flounder (Lee *et al.*, 2002). The dietary protein level above the optimum led to growth depression in sebae anemonefish, as reported in many other fishes (Dabrowski, 1977; Teng *et al.*, 1978; Jauncey, 1982; Siddiqui *et al.*, 1988; Vergara *et al.*, 1996; Lee *et al.*, 2002). Jauncey (1982) attributed the poor growth in high protein diet to the decreased availability of dietary energy for growth due to the increased energy requirement for deamination and excretion of excess amino acids absorbed. However, occurrence of growth plateau after a linear increase was also observed with dietary protein in fishes (El-sayed and Teshima, 1992; Lee *et al.*, 1993; Kang *et al.*, 1998). The decreased weight gain above the optimum was also suggested to be due to the reduction in available dietary energy for growth because of the non-availability of non-protein energy necessary to deaminate and excrete the excess amino acids absorbed (Lim *et al.*, 1979; Jauncey, 1982; Vergara *et al.*, 1996). Prather and Lovell (1973) indicated the possible toxic effects of high protein diets with low non-protein energy to catfishes.

In the sebae anemonefish growth exhibited significant variation between treatments and the weight gain improved with increasing protein levels upto 45% and later it decreased (Table XXVI). Similar trends were also observed with many other species (Jauncey, 1982; Cho *et al.*, 1985; Vergara *et al.*, 1996; Bai *et al.*, 1999). The polynomial regression analysis showed the optimum requirement at 46.2% with weight gain and 44.9% using SGR (Fig. 15-16).

The specific growth rate showed an increasing trend upto 45% dietary protein, beyond which it declined (Table XXVI). The lower SGR value for diet D_{48} indicates inefficient utilization of protein above the optimum level. The feed conversion was found to be better in diets with 42 and 45% dietary protein indicating better assimilation and conversion of the ingested nutrients. The lower (33%) and the highest (48%) protein diets yielded comparatively poor response. The highest protein diet (D_{48}) though, did not affect consumption caused growth depression leading to higher FCR value.

Diets used for the protein optimisation experiments were formulated to have the P/E ratio between 20 and 30 mg/kJ^{-1} (Table VII). Diet D_{45} with a ratio of 28.09 mg/kJ^{-1} resulted in the best response in terms of growth and protein sparing, diet D_{48} with a P/E ratio of 30.02 mg/KJ^{-1} led to decreased growth and protein efficiency. The diet D_{33} with a P/E ratio of 20.90 mg/kJ^{-1} resulted in low growth rate, possibly due to the increased protein utilization as energy source for metabolic activities. Though this species is considered an omnivore, its carbohydrate utilization seems to be low, the diet with maximum carbohydrate inclusion (32.75% in D_{33}) producing the least growth.

The PER decreased with the increase in dietary protein level, indicating inefficient utilization of higher levels of dietary protein. The results are in agreement with the general pattern observed in most other fishes (Ogino and Saito, 1970; Dabrowski, 1977; Siddiqui *et al.*, 1988; Lee *et al.*, 2002).

5.3. 2. Lipid Requirement

Adequate levels of non-protein energy source, such as lipid and carbohydrate, in the diet can minimise the use of protein as source of energy

(Cho and Kaushik, 1990). The protein sparing effect of lipids is also reported in many fishes (Tacon and Cowey, 1985; DeSilva *et al.*, 1991; Kim and Kaushik, 1992; Vergara *et al.*, 1996; Weatherup *et al.*, 1997; Hillestad *et al.*, 1998; Chan *et al.*, 2002; Lee *et al.*, 2002). Dietary lipid also influences the appetite and palatability of diets besides supplying the essential fatty acids. Lipid optimisation is essential in fish diets as it is known to affect growth either in lower or higher levels (Metailler *et al.*, 1981; Nematipour *et al.*, 1992). In the present study with juvenile clownfish increased growth rate is observed upto 12% dietary lipid when fed with isoproteic (45%) diets. The polynomial regression analysis showed the optimum lipid level at 11% in the diet (Fig.17-18).

Weight gain was found to be influenced significantly by the lipid level, though growth was affected only when the lipid content of the diet was very low (3%). This shows that when dietary protein and energy requirements are met the dietary lipid influences the growth only at extremely low levels. The study also indicates that inclusion of higher lipid levels does not significantly improve the growth in high protein and high calorie diets. Ineffectiveness of excess dietary lipid was also reported for other species (Kikuchi *et al.*, 2000; Lee *et al*, 2000; DeSilva, 2002). Kiron (1989) observed that when protein was kept optimum dietary lipid did not influence the survival in the mullet *Liza parsia*.

Protein to energy ratio in the diets must be balanced for better utilization of non-protein nutrients as energy sources for maintenance and activity allowing the maximum use of protein for growth (Lovell, 1989). In the present study treatment D_{L12} with 12% lipid gave the best FCR (0.90) and PER (2.51) than the lower and higher lipid diets (Table XXVIII), suggesting superior protein sparing action and protein utilization for growth. All the diets used in the present study were isoproteic (45% CP) and yielded better FCR (<1.60). The improved protein utilization may be due to the effective use of the non-protein energy source (lipid) to meet the energy needs and the consequent lower level of protein catabolism.

P/E ratio of 27.12 mg/kJ^{-1} for the 12% lipid diet was found to be the best among the tested diets and the lower (26.05 mg/kJ^{-1} in D_{L15}) and higher

(29.89 mg/kJ^{-1} in D_{L3}) ratios resulted in comparatively poor performance. The low performance of 3% lipid diet may also be due to the inadequacy of dietary lipids and fatty acids as energy source or as structural components and higher reliance on dietary protein for growth. The FCR and PER recorded for 12% lipid and 45% protein (0.90) diet was found to be superior suggesting this to be ideal for juvenile rearing.

From the protein and lipid requirement studies it is concluded that P/E ratio between 27 and 28 mg protein kJ^{-1} of gross energy in the diets is optimum for better growth performance in juvenile sebae anemonefish.

5.3.3. Influence of carotenoids

Fishes like other animals are unable to synthesize carotenoids *de novo* (Goodwin, 1984). The clownfish juveniles were often found to exhibit faded hues if not fed with proper carotenoid rich diets in captivity. Until recently this was a major problem in the captive maintenance of most aquarium species, especially the marine ornamentals. Incorporation of β-carotene in the diets did not improve the faded appearance of *A. sebae* juveniles. This probably indicates lack of proper biochemical pathways for the bioconversion of β-carotene to other carotenoids in clownfishes. The ineffectiveness in imparting yolk pigmentation was also observed when broodstock were fed with diets having high spirulina content. According to Tanaka (1978), ingested pigments may be deposited in fish tissue directly without modification or after biotransformation, depending on the tissue and species specificities in carotenoid assimilation. Astaxanthin and canthaxanthin are generally the end products of carotenoid metabolism in marine and freshwater fishes. Depending on their ability to transform carotenoid pigments Simpson and Kamata (1989) classified fishes into three catagories *viz.* those that transform β-carotene to astaxanthin, those that deposit carotenoids without transformation and those that can biosynthesise astaxanthin from sources other than β-carotene. Hoff (1996) suggested that clownfishes belonged to the third category. The present observations also confirm this status as dietary β-carotene was not adequately utilized by juveniles and adults of sebae anemonefish.

The information generated from the study will facilitate the development of high performance diet for broodstock and weaning diet for larvae and juveniles. The study also points out the prospects of utilizing sebae anemonefish as a reference model for marine fish broodstock nutrition studies. The advantages of broodstock research on clownfishes are their smaller size, easy domestication under controlled condition, early attainment of maturity, hermaphroditism, incessant spawning, extensive incubation period and the emergence of developed larvae.

SUMMARY

SUMMARY

The anemonefish, *Amphiprion sebae* Bleeker 1853, is one of the popular species in marine aquarium hobby. In the present study, a series of laboratory experiments were carried out on the broodstock, larvae and juvenile stages of this fish with the focus to developing suitable feeds for each of these stages. Studies on broodstock nutrition were focussed on evaluating the efficacy of selected natural diets and formulated moist diets on the egg and larval quality. An experiment was also conducted to determine the effect of dietary allowance on broodstock performance. The larval nutrition studies were focussed on assessing performance of selected livefeeds and their combinations and to determine the optimum weaning age. The acceptability of formulated feeds, and optimum dietary protein and lipid levels were determined for juveniles.

Broodstock nutrition experiments were done in triplicate for each treatment and the duration of the study extended to three consecutive spawnings. The efficacy of diets on broodstock performance was tested using six natural and five formulated moist diets. The six natural diets used for this study were brown mussel meat, mature brown mussel, cuttlefish meat, squid meat, deep-sea prawn and a mixture of polychaete worm and mussel meat (1:1). The salient results and observations are as follows:

- The highest feeding activity was observed in the water column followed by that on the disc surface of host anemone. The feed particles settled at the bottom were seldom preferred.

- The 'anemone disc feeding' strategy substantially improved the feed consumption.

- The suction or gulping type of feeding was observed when fed with polychaete worms and streaks of cuttlefish or squid.

- The mean number of eggs spawned significantly varied with the quality of natural diets used ($P<0.05$). Among the fresh natural diets tested cuttlefish (59.5% protein and 10.7% lipid) gave the best performance with an

average clutch of 1521 ± 264 eggs (mean ± SD, n=9), followed by the deep-sea prawn (53.23% protein and 8.98% lipid) with 1300 ± 445 eggs.

- The egg capsule length ranged from 2.19 mm for the fishes fed mussel diets (MSM and MGD) to 2.33 mm for those fed squid meat, and the observed variations in egg capsule length between treatments were not significant.

- The egg capsule width, on the other hand, exhibited significant ($P<0.05$) variations between treatments. The capsule showed maximum width (914 µm) when mature mussel with gonad was fed and minimum width (845 µm) when fed with mussel meat.

- The variations in mean larval lengths among treatments were not significant, and it ranged from 3.96 mm for the mussel meat to 4.31 mm for the squid meat.

- The amino acid and fatty acid profile of the natural diets showed significant ($P<0.05$) difference between them. The higher levels of essential amino acids, especially lysine, in the mussel meat (MSM) significantly reduced the egg production. Cuttlefish meat showed a significantly lower level of lysine and a higher level of arginine.

- The essential to non-essential amino acid ratio of <1 improved the egg production and that >1 was found to decrease egg production in sebae clownfish. Cuttlefish diet that gave higher egg production had a ratio of 0.93 and deep-sea prawn 0.88. Thus, balance in essential amino acids together with an essential to non-essential ratio of <1 significantly influenced egg production.

- High levels of PUFA (>35%) and *n*-3 HUFA (>30%) significantly reduced egg production in sebae clownfish. Imbalances in the levels of DHA and EPA critically affected the efficacy of squid meat and mussel diets.

- Cuttlefish that gave the highest mean number of eggs had a DHA/EPA ratio around 6.0 and had almost equal proportion of EPA and AA.

- The clutch colouration showed variation with the diets from pale to bright yellow (mussel meat and its combination with polychaete worms), pale pinkish (cuttlefish and squid) to reddish pink (deep-sea prawn) and pale to deep orange (mature mussel with gonads).

- The diets with higher astaxanthin levels *viz.* deep-sea prawn (42.77 µg/g wet weight) and mature mussel (female) with gonads (23.25 µg/g wet weight) gave brightly pigmented larvae. Feeding brood fishes with deep-sea prawn was found to influence the pigmentation of spawned eggs within 48 hrs of feeding.

- The newly hatched larvae obtained from deep-sea prawn and mature mussel fed treatments had higher orange-brown pigmentations in addition to the black pigmentation, which was distinctly different from other dietary treatments.

With a view to developing a compounded diet for broodstock five moist diets were prepared and their efficacy on reproductive performance was studied. The salient results and observations are as follows:

- The mean number of eggs per spawning showed significant ($P<0.05$) difference between treatments. The diet CBD_5 with a basal mix containing fishmeal, shrimp meal and squid meal in the ratio 4:3:3 and spirulina (20%) with about 50% protein and 10% lipid produced the best result (2137 ± 110 eggs). Diet CBD_1 with about 40% protein and 20% lipid without spirulina supplementation gave the lowest number of eggs per spawning (1237 ± 110 eggs).

- The dietary level of 50 - 60% protein and 10% lipid with balanced amino acid and fatty acid combination was found to facilitate better reproductive performance in sebae anemonefish. However the diets did not have any significant influence on egg capsule length (2.20 to 2.27 mm), egg capsule

width (857 µm to 876 µm) and the length of newly hatched larvae (4.20 mm to 4.34 mm).

- Diet CBD_4 with comparatively higher essential amino acid content (23.18 g/100g diet) with an essential to non-essential amino acid ratio of 0.88:1 gave lower number of eggs. However, diet CBD_5 with an essential amino acid content of 21.23 g/100g diet and a low EAA:NEAA ratio of 0.77 produced superior result among all the tested diets. Dietary lysine content in the compounded diets showed an inverse relationship with egg production with increased lysine content resulting in decreased egg production.

In order to study the effect of feed allowance on broodstock performance an experiment was conducted by feeding the fish *ad libitum* either once or twice daily. The salient results of the study are:

- The daily feed allowance was found to significantly ($P<0.05$) influence the number of eggs spawned and the egg dimensions. The mean number of eggs spawned and the egg dimensions were found to be significantly low (416 ± 28 eggs) for the once *ad libitum* feeding as compared to twice *ad libitum* (885 ± 55 eggs). However, the embryo development and hatchability were not affected by the dietary regime.

An experiment was conducted using dry diets on 3 weeks old larvae until their maturation and spawning. The fishes matured and spawned successfully when fed the dry diets and the first spawning was observed from 15^{th} month after hatching. However, the mean number of eggs per spawning was substantially lower than the control diets.

In the newly hatched larvae the mouth was open inside the egg capsule itself and the normal mouth gape (without stretching) at hatching was 210-260 µm. This enabled larvae to consume smaller predators like rotifers as first feed immediately after hatching. The larvae had yolk sac and oil globule/s as nutrient reserve. The gut was single looped and differentiated into oesophagus, rudimentary stomach, intestine and rectum.

The efficacy of selected live feeds for larval rearing was evaluated for a month. The salient results and observations are as follows:

- The maximum survival (68%) and faster onset of metamorphosis (80% by 9 dph) were obtained when larvae were reared in green water (*Chlorella* sp.) and fed with a combination of rotifers and *Artemia* nauplii.

- When larvae were fed rotifers alone the survival was 31% and the duration of metamorphosis also got extended to more than two weeks to reach 80% level.

Trials were carried out for two weeks to find out the optimum age for weaning hatchery reared larvae to formulated diets.

- When 4 dph larvae were weaned the survival was poor (6 - 11%) after two weeks and the mortality reached 70 - 80% during the first five days of feeding.

- The survival rate (75 - 80%) substantially increased when 21 dph post larvae were weaned to microdiets.

- The best results were obtained when the post larvae were weaned after one month (30 dph), and the mortality was less than 5%.

The optimum protein requirement of the juveniles was determined by two successive experiments of nine weeks duration. In the first experiment four isocaloric semi-purified diets formulated to contain protein levels of 20%, 30%, 40% and 50% were used. Based on the results of the first experiment, in the second experiment the protein levels used were 33%, 36%, 39%, 42%, 45% and 48%. The salient results of these experiments are given below:

- In the first experiment survival rates were high (80 - 93.3%) and did not vary significantly with the dietary protein level. But the weight gain and SGR showed significant ($P<0.05$) differences between treatments. The analysis of the response data suggested the best performance at 40% dietary protein.

- In the second experiment the weight gain, length gain, SGR, FCR and PER showed significant ($P<0.05$) differences between treatments. The second order polynomial regression analysis of weight gain at the selected protein levels showed the optimum requirement as 46.2% and the regression analysis of SGR showed the optimum level at 44.9% dietary protein. Thus it is concluded that dietary level of 45% protein is adequate in formulated diets of juveniles of the sebae anemonefish.

Lipid requirement of juveniles were studied with five isocaloric and isoproteic (45%) semi-purified diets, containing lipid levels of 3%, 6%, 9%, 12% and 15%, for nine weeks duration. Cod-liver oil was used as the supplementary lipid source. The salient results are listed below.

- The survival rates obtained were high (80 - 100%) and did not show significant variation among the lipid levels.

- The weight gain, length gain, SGR, FCR and PER showed significant ($P<0.05$) differences between treatments.

- The second order polynomial regression of the weight gain and SGR data showed the optimum dietary lipid concentration at 10.96% and 11.05%. The results suggest that about 11% lipid is required for best response in juvenile sebae anemonefish.

From the protein and lipid requirement experiments it is concluded that P/E ratio between 27 and 28 mg protein kJ^{-1} of gross energy in the diets is optimum for better growth performance in juvenile sebae anemonefish.

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Appendix - I

EXPERIMENTAL SCHEDULE

Broodstock Nutrition Studies

Broodstock development from juveniles	-	July 2001 – February 2003
Natural diet evaluation	-	June 2002 – January 2003
Feed allowance on egg production	-	July 2002 – September 2002
Compounded diet evaluation	-	March 2003 – October 2003

Larval Nutrition Studies

Weaning studies	-	August 2001 – January 2002
Livefeed evaluation studies	-	March 2002 – July 2002

Juvenile Nutrition Studies

Purified Vs semi-purified diet evaluation	-	January 2001 – March 2001
Protein requirement experiment I	-	March 2001 – June 2001
Protein requirement experiment II	-	August 2001 – November 2001
Lipid requirement experiment	-	March 2002 – June 2002

Appendix II

ANOVA table for the protein requirement experiment II

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
ADWG	Between Groups	135.440	5	27.088	38.006	.000
	Within Groups	8.553	12	.713		
	Total	143.992	17			
CF	Between Groups	.541	5	.108	3.931	.024
	Within Groups	.330	12	2.751E-02		
	Total	.871	17			
FCR	Between Groups	.745	5	.149	279.27	.000
	Within Groups	.000	12	1.60E-03		
	Total	.745	17			
LG	Between Groups	291.493	5	58.299	42.902	.000
	Within Groups	16.307	12	1.359		
	Total	307.800	17			
PER	Between Groups	.315	5	1.017E-03		
	Within Groups	.000	12	0.189	57.733	0.000
	Total	.315	17			
SGR	Between Groups	4.523	5	.905	22.315	.000
	Within Groups	.486	12	4.054E-02		
	Total	5.010	17			
WG	Between Groups	537593.6	5	107518.7	38.158	.000
	Within Groups	33812.471	12	2817.706		
	Total	571406.0	17			
WGPERCEN	Between Groups	1019491	5	203898.2	15.278	.000
	Within Groups	160152.1	12	13346.009		
	Total	1179643	17			

Appendix III

ANOVA table for lipid requirement studies on juveniles

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
LTGAIN	Between Groups	4.956	4	1.239	23.827	.000
	Within Groups	.520	10	5.200E-02		
	Total	5.476	14			
WTGAIN	Between Groups	27760.394	4	6940.098	32.077	.000
	Within Groups	2163.583	10	216.358		
	Total	29923.977	14			
SGR	Between Groups	.692	4	.173	35.834	.000
	Within Groups	4.827E-02	10	4.827E-03		
	Total	.740	14			
CF	Between Groups	.394	4	9.859E-02	22.964	.000
	Within Groups	4.293E-02	10	4.293E-03		
	Total	.437	14			
ADG	Between Groups	6.991	4	1.748	31.960	.000
	Within Groups	.547	10	5.469E-02		
	Total	7.538	14			
WGPERC	Between Groups	187136.2	4	46784.043	29.478	.000
	Within Groups	15870.566	10	1587.057		
	Total	203006.7	14			
FCR	Between Groups	.767	4	.192	1250.543	.000
	Within Groups	1.533E-03	10	1.533E-04		
	Total	.769	14			
PER	Between Groups	2.242	4	.560	4424.026	.000
	Within Groups	1.267E-03	10	1.267E-04		
	Total	2.243	14			