CULTURE AND NUTRITIONAL ENRICHMENT OF THE ROTIFER *BRACHIONUS ROTUNDIFORMIS* (TSCHUGUNOFF) FOR THE REARING OF MARINE FIN FISH AND SHRIMP LARVAE

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BY

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CENTRAL MARINE FISHERIES RESEARCH INSTITUTE,

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Dedicated

to

My parents

and

Kochu Mamman

DECLARATION

I hereby declare that the thesis entitled "Culture and Nutritional enrichment of the rotifer *Brachionus rotundiformis* (Tschugunoff) for the rearing of marine fin fish and shrimp larvae" is an authentic record of research work carried out by me under the guidance and supervision of Dr. C.P. Gopinathan, Principal Scientist, Central Marine Fisheries Research Institute, in partial fulfillment of the requirements for the Ph.D. degree in Marine Biology under the Faculty of Marine Sciences of the Cochin University of Science and Technology and no part there of has been previously formed the basis for the award of any other degree in any University.

Date: 5-08-2004.

(S.D. Gopakumar)

CERTIFICATE

This is to certify that this thesis entitled "Culture and Nutritional enrichment of the rotifer *Brachionus rotundiformis* (Tschugunoff) for the rearing of marine fin fish and shrimp larvae" is an authentic record of research work carried out by S.D. Gopakumar (Reg. No. 1952) under my guidance and supervision in Central Marine Fisheries Research Institute; in partial fulfillment of the requirements for the Ph.D. degree in Marine Biology under the Faculty of Marine Sciences of the Cochin University of Science and Technology and no part of this has previously formed the basis for the award of any other degree in any University.

Date 5-8-2004

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PREFACE

The flourishing culture of marine finfish in various parts of the world can be partly attributed to the successful mass cultivation of the rotifers *Brachionus plicatilis* and *B. rotundiformis*. Marine fish require live feed at early life stages for survival and proper development, in contrast to most edible fish species that are cultured in fresh and brackish water and salmonids cultured in seawater. Most of the mass cultured rotifers are known to lack adequate amounts of essential nutrients and must be enriched before being offered to the fish larvae. Among the nutrients, lipids are found, unequivocally to have the greatest influence on growth and survival of marine fish larvae. Since rotifers lack the essential fatty acids (EFA), required by marine fish larvae, EFA enrichment is vital for their use as ideal live feed during early stages of the larvae.

The present study is an attempt to standardize the environmental condition like pH, salinity and photoperiod, and also the feed for the maximum production of rotifers. Considering the deficiency of essential fatty acids in rotifers, enrichment experiments were carried out and fatty acids profile were analysed. Attempts were made to improve the production of clown fish (*Amphiprion sebae*) juveniles using enriched rotifers.

Attempts were also made to rear various larval stages of *Penaeus monodon* with enriched rotifers as a substitute for *Artemia* nauplii.

The thesis is organized in **five Chapters** with a **General Introduction** to the topic and review of literature with special reference to fatty acid nutrition in **Chapter I**.

In **Chapter II** the culture of rotifer *B. rotundiformis* using different micro algae and yeast, are presented with the effect of environmental parameters like pH, salinity and photoperiod on mass production of rotifers.

Chapter III deals with the fatty acid enrichment and biochemical analysis of enriched rotifers.

In **Chapter IV** the larval rearing of clown fish *Amphiprion sebae* with enriched rotifers is presented.

In **Chapter V** the larval rearing of *Penaeus monodon* with enriched rotifers is given.

In **Chapter II** to **V**, details have been presented under sections such as separate introduction, material and methods, results and discussion.

The **Summary** of important findings of the study follows the five chapters and literature cited in the thesis are listed in the **Reference** section after the Summary.

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List of Abbreviations

AA	- Arachidonic Acid
ANOVA	- Analysis of variance
AOAC	- Association of Official Analytical Chemist
BF_3MeOH	- Boron trifluoride methanol
CIFT	- Central Institute of Fisheries Technology
CL*Cl*R	- Clown fish larvae reared with *Chlorella marina* enriched rotifers
CL*Ig*R	- Clown fish larvae reared with *Isochrysis galbana* enriched rotifers
CL*Na*R	- Clown fish larvae reared with *Nannochloropsis salina* enriched rotifers
CLOER	- Clown fish larvae reared with shark liver oil enriched rotifers
*Cl*R	- *Chlorella marina* enriched rotifer
CLYR	- Clown fish larvae reared with *Saccharomyces cerevisiae* enriched rotifers
CMFRI	- Central Marine Fisheries Research Institute
DHA	- Docosahexaenoic acid
DMRT	- Duncan's multiple range test
DO	- Dissolved Oxygen
EFA	- Essential fatty acids
EPA	- Eicosapentaenoic acid
FAME	- Fatty acid methyl esters
FEMD	- Fishery Environment and Management Division
FID	- Flame ionization detector
FRP	- Fibreglass Reinforced Plastic
HCl	- Hydrochloric Acid
HUFA	- Highly unsaturated fatty acids

*Ig*R	- *Isochrysis galbana* enriched rotifer
'K'	- Instantaneous growth rate
KOH	- Potassium Hydroxide
LA	- Linoleic acid
LNA	- Linolenic acid
L-type	-Large type
MUFA	- Mono unsaturated fatty acid
NaOH	- Sodium hydroxide
*Na*R	- *Nannochloropsis salina* enriched rotifer
NSM	- Non saponifiable matter
OER	- Oil emulsion enriched rotifer
PE	- Petroleum ether
pH	- Potential hydrogen
Ppt	- Part per thousand
PUFA	- Poly unsaturated fatty acids
PVC	- Poly Vinyl Chloride
SEM	- Standard error mean
SM	- Small medium
S-type	- Small type
YR	- *Saccharomyces cerevisiae* enriched rotifer

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Chapter 1
General Introduction

1. General Introduction

It is an established fact that feed is a vital essential component and major constraint in the progress of aquaculture practices. This is especially true for the larval rearing of many cultivable species of finfish and shellfish. Proper understanding of amino acid profile and fatty acid requirements of animals has enabled us to produce nutritionally well balanced diet to the rearing larvae in a hatchery system. Live feed organisms of both phytoplankton and zooplankton are successfully used as food for rearing the larvae of finfish and shellfish. Live feeds are an important food source for many species of fish, and can provide an inexpensive alternative to other commercial feeds. Most fish and prawn larvae depend on zooplankton at some stage of their life span and some even feed exclusively on zooplankton during entire life. Zooplankton have been used to rear fish and larvae (De Pauw *et al.*, 1984; Watanabe *et al.*, 1983), especially for species which do not accept artificial feeds (Bryant and Matty, 1980). Among various zooplankters, the most important groups are rotifers, copepods, *Artemia* and some cladocerans.

Rotifers are a group of aquatic microscopic invertebrates comprising about 2000 species of unsegmented, bilaterally symmetrical, pseudocoelomates. They are commonly referred to as 'Wheel animal cules' as their disc like anterior end (corona) bears resemblance to a pair of revolving wheels due to the synchronized beating of their coronal cilia. Many

species coming under the family *Brachionidae* are widely used in larviculture. *Brachionus plicatilis* was first identified as pest in the pond culture of eels in the fifties. Japanese researchers soon realized that this rotifer could be used as suitable live feed organisms for the early larval stages of marine fish. Ito (1960) introduced the culture of the euryhaline rotifer *Brachionus plicatilis*, as a source of live feed for fish larvae. Since then rotifers have become the most extensively used food organisms for a large variety of finfish and crustacean larvae and are now indispensable in raising marine fish larvae (Lubzens, 1987). They are the first food of the initial larval stages of many fish species grown in commercial marine hatcheries (James *et al.*, 1983).

Two morphotypes S (small) and L (large) are distinguished based on morphological and physiological differences (Fu *et al.*, 1991 a, b; Rumengan *et al.*, 1991; Fu *et al.*, 1993). These strains could be selectively employed for fish larvae depending on the mouth size of the larvae. The length of lorica of L type is about 130-340 μm (238.9 μm on average) and that of S type is 100 to 210 μ m (160.3 μm on average). Moreover, the lorica of the S type shows pointed spines, while of the L-type has obtuse-angled spine.

Studies on morphology, karyotype/genetics including allozyme constitution and reproductive behaviour of S and L type of *B. plicatilis* showed that these types are best treated as different species. A re-examination of existing available names revealed *B. plicatilis* (O. F. Muller,

1786) and *B. rotundiformis* (Tschugunoff, 1921) as correct names for the L and S type respectively (Segers, 1995; Gomez and Serra, 1995; Hagiwara *et al.*, 1995; Munuswamy *et al.*, 1996). Serra *et al.* (1998) studied the ecological genetics of *Brachionus* spp population in Torreblanca marsh. Allozyme and morphometric analysis showed that *Brachionus* group *plicatilis* (formerly, *Brachionus plicatilis* and currently split into *B. plicatilis* and *B. rotundiformis* was composed of three groups of genotypes with no evidence of gene flow between them (*B. plicatilis*, *B. rotundiformis* SM and *B. rotundiformis* SS). Accordingly *B. plicatilis* is a euryhaline, low temperature group, *B. rotundiformis* SM is adapted to high temperature and low salinity condition. Mating shows that most copulation occurred within a group. *B. plicatilis* has a mating recognition system different from those of either *B. rotundiformis* SM or SS, whereas the two *B. rotundiformis* groups had partially differentiated mating preferences made to classify the genotypes into three clonal groups (called SS, SM and L). The isozyme pattern of S and L types is different and allowed clear separation in two distinctive groups (Fu *et al.*, 1991 b) chromosome number is also different (Rumengan *et al.*, 1991). *B. plicatilis* and *B. rotundiformis* are unable to produce cyst after cross-mating (Fu *et al.*, 1993) and they have different isozyme patterns (Fu *et al.*, 1991 b), morphology (Fu *et al.*, 1991 a), chromosomal number (Rumengan *et al.*, 1991) and binding of a mating pheromone to male receptors (Rico and Snell 1995). Differences in mating behavior are expected between these species.

Brachionus plicatilis can withstand wide salinity range from 1 to 97 ppt; optimal reproduction can only take place at salinities below 35 ppt (Lubzens, 1987). Rotifer size was also inversely proportional to increasing salinities in the culture system. The fatty acid composition varies especially the total *n*-3 HUFA content of the S and L type rotifers in relation to different salinity regimes. 15-20 ppt salinity will be more conducive to produce nutritionally enriched S type rotifers. Lipid synthesis was more conducive at 30 ppt salinity to the L type rotifers. The *n*-3 HUFA in rotifers, which are indispensable to the growth and survival of marine fish larvae (Watanabe *et al.*, 1983; James *et al.*, 1983), shows that adequate quantities of these EFA are present in both strains of rotifers at the above mentioned salinities, when using *Nannochloropsis* in the culture system. Rotifer filtration rates vary with salinity and are reduced at high salinities. High rotifer productivity was obtained at low salinity regimes (up to 5 ppt salinity), a salinity of 20 ppt would be the most conducive for the production of rotifers, since 100% recovery of that population was observed on transfer from 20 to 30 ppt salinity. L type rotifers, in contrast, are more sensitive to changing salinities in the culture system, since 100% recovery was observed only at and above 25 ppt salinity. Thus, although lower salinity regimes yield higher rotifer biomass, the rotifers in marine fish hatcheries should be produced at 20 ppt salinity for the S type and 30 ppt for the L type.

Rotifers are often fed to larval fish cultivated at temperatures and salinities different from the rotifer cultures (Blaxter, 1988; Lubzens *et al.*, 1989). This change in conditions can affect mobility and availability of rotifers (Gatesoupe and Luquet, 1981; Lubzens, 1987; Oie and Olsen, 1993). Rotifers should be cultured at lower temperature and similar salinities to the fish larval rearing tanks or acclimated for at least 6 hours to larval rearing conditions before transfer (Fielder *et al.*, 2000).

Techniques for rotifer cultures are classified into three types, batch culture, semi-continuous culture and intensive culture using chemostats. Batch culture method, the entire culture in a tank is harvested at once, and part used as the innoculum for the next culture. Batch cultures are commonly used in live food aquaculture to produce rotifers as food for fish larvae. This technique creates highly variable conditions both in abundance as well as in biochemical content of the rotifers.

Semi continuous culture method is also known as "thinning culture", in which rotifer density is kept constant by periodic harvesting. First, micro algae culture is introduced into a tank (similar to the batch culture method) and additional feeding commences the following day. After the rotifer attains the prescribed density, part of the culture is harvested and replaced with dense or diluted micro algal culture. In many cases, yeast is provided to supplement the micro algae. Contrary to the batch culture method, this long-

term culture is maintained at a low density for a period of 7-14 days without water quality treatment. With the use of biofilters, the period can extend to two or three months. The size of the culture tank is larger than that used in batch culture method.

Intensive rotifer culture using chemostat, is the production of nutritionally enriched live rotifers at the lowest possible cost for feeding the larval stages of finfishes and shellfishes. In this system there are two stages; stage I and stage II. The stage I is continuous algal culture (using filtered and diluted sea water enriched with nutrients at 30 ppt salinity) (James *et al.*, 1988). The stage II rotifer culture system consisted of three 100 litre capacity and two 1 m^3 capacity chemostats provided each with 50 litre and 500 litre capacity mixing reactors. The desired food level in the rotifer chemostats were synchronized by metering pumps from the mixing reactors. The wash out from the chemostat was collected using 100 litre and 1 m^3 capacity rotifer concentration tanks to facilitate daily harvest. The rotifer chemostats were kept in a temperature-controlled room provided with aeration and temperature controllers to maintain the temperature at 25°C. Fatty acid composition of rotifers produced under this type of system is significantly higher especially when *Nannochloropsis* is used as feed. The 1 m3 capacity rotifer chemostats could be adapted as suitable units for large-scale production of rotifers in aquaculture.

For stable production of rotifers, the turbidostat is a very attractive system. It combines both early warning monitoring and effective regulation of algae and rotifer densities in the two stages of the system. In the turbidostat of Boraas and Bennet (1988), algal densities in the rotifer stage are held constant, as regulated by turbidity measurements. The modified turbidostats (Walz *et al.*, 1997) provides turbidity sensors to regulate algal concentration in both stages. The turbidostat is a very new tool for the production of rotifers. This is a very good tool for experiments because it functions optimally at maximum growth rates where as the chemostat is better for lower or middle range growth rates.

Recent development of a high-density mass culture system for rotifer *B. rotundiformis* (Yoshimura *et al.*, 1997) resolved the problems like low dissolved oxygen, foaming separation and ammonia toxicity by using a filtering equipment for removing particulate debris in the culture medium. Quantitative determination of rotifers by a centrifugation, and measuring their packed volume (PV, ml/lit). PV of rotifers is easier and more accurate than direct count of the density.

Fu *et al*, (1997) developed an automatic continuous culture system. The system consists of filtration unit, a culture unit and a harvest unit. In this system filtered water and food are continuously supplied into a rotifer culture tank at a pre-determined rate, and the same amount of culture water is

transferred into a harvest tank to obtain rotifers at a significant biomass. The average production was about 2.1 billion rotifers/day from a 1 m^3 S type continuous culture in which high rotifer densities ranging from 3000 to 6000 ind/ml was maintained.

Rotifers are filter feeders and can be fed a variety of food types, including algae, yeast, bacteria or inert foods such as microcapsules and detritus. The type of feed used for culturing rotifers can have a significant effect on the cost of operations and on the nutritional value of rotifers. (Carnic *et al.*,1993). Micro-algae are the principal component of most cultured rotifer diet. Many species of algae are employed according to availability under local conditions. The most commonly used species are *Nannochloropsis salina*, *Tetraselmis gracilis, Isochrysis galbana, Chlorella marina* and *Dunaliella salina.* Species high in *n*-3 HUFA's such as *Nannochloropsis* sp. are regarded as very good feed. The main draw back in using phytoplankton is the huge amount of labour, time and facilities that must be developed for producing the large quantities needed to feed rotifer. Alternately marine yeast (*Candida* sp.*),* Baker's yeast (*Saccharomyces cerevisiae*), and cake yeast (*Rhodotorula* sp) has also been successfully used for rearing rotifers. But yeast has no nutritional value and they lack the much-needed HUFA's (Walford and Lam, 1992). The use of bacteria as feed for *B. plicatilis* is also investigated, which revealed that addition of vitamin B_{12} producing bacteria could greatly enhance the growth of cultured. *B. plicatilis*

(Yu *et al.*, 1989). Relatively high reproductive rates were found in two strains of rotifers fed with frozen *Nannochloropsis* biomass (Lubzens *et al.*, 1995).

Different types of yeast is also given as feed for rotifers such as the baker's yeast, ω yeast or marine yeast (*Candida* spp) and caked yeast *Rhodotorula* sp. Baker's yeast (*Saccharomyces cerevisiae*) are fed to rotifers and found that rotifers could grow well as an when fed with micro-algae during the first week; but declined during the second week of culture, probably as a result of nutritional deficiency (Hirata, 1979). Hirayama and Funamoto (1983) tested the dietary value of baker's yeast, and the supplementary effect of Vitamin B_{12} on rotifers, which grow under bacteria free conditions. Rotifers fed with the yeast alone could not grow, and their eggs were not viable. However, when supplemented with vitamin B_{12}, the rotifers grew well and their eggs hatched successfully.

The marine yeast *Candida* sp. was cultured in 600 litre capacity fermenters using a synthetic culture medium containing 0.15% ω feed oil (fish oil) (Al-Hinty and James, 1983; James *et al.*, 1983). The culture *n*-3 density of rotifers using marine yeast is higher than baker's yeast fed rotifers. The *n*-3 (HUFA's) is higher in marine yeast fed rotifers than other yeast. In fish hatcheries ω yeast (yeast supplemented with fish oil) is used to improve the nutritional quality of rotifers for feeding fish larvae rather than promoting population growth in rotifers (Imada *et al.*, 1979; Fukusho *et al.*, 1989 a,b).

Hirayama and Funamoto (1983) observed that omega feed oil (fish oil) also supplements the nutritional deficiency of baker's yeast and improves the population growth of the rotifer.

Bacteria growing in the culture water of rotifers play an important role in forming a complicated ecosystem. Some strains of bacteria serve as a food source and were found to be eaten by the rotifers (Hino, 1993). Yasuda and Taga (1980) examined 300 strains of bacteria and isolated two strains (P 1 and P 7) of *Pseudomonas*, which increased rotifer population. Sakamoto and Hirayama (1983) reported that the photosynthetic bacteria *Thiocapsa roseopersicina* contributed to the population growth of rotifers when provided alone and even more effectively when provided with algae or yeast. B_{12} producing bacteria due to its nutritive values is the best strain for rotifer growth during mass culture. Rotifers can be enriched with DHA from different bacterial strains (Lewis *et al.*, 1998). Bacterial strains *Shewanella gelidimarina* and *Colwellia psychroerythrus* are rich in either EPA or DHA. EPA incorporation to a level equivalent to 1.8% dry weight after exposing rotifers to 10^9 cell/ml of the marine bacterium for 12 hours (Nichols *et al.*, 1996) reported a maximum EPA enrichment equivalent to 1.4% dry weight for rotifers followings 24 hours exposure to 10^8 cells/ml of *Shewanella gelidimarina*.

The nutritional quality of the food offered to cultured marine organisms is crucial during the first few weeks of larval life. (Ben-Amotz *et al.*, 1987). Watanabe *et al*, (1983) suggested that lipids in general and specifically *n*-3 highly unsaturated fatty acids (HUFA) have an essential role in the larval diet. Although rotifers have been found to synthesize some *n*-3 HUFA by *denovo* synthesis (Lubzens *et al.*, 1985), the amount accumulated is small and insufficient to meet the possible demand of the finfish larvae. Fatty acids must be provided to the rotifers via their food, which in most cases is supplied by unicellular algae or through enrichment of commercial or home made oil emulsion. Enrichment techniques currently in use include (1) the French technique; microencapsulated oils containing high concentration of *n*-3 HUFA's (Sakamoto *et al.*, 1982; Ozkizilcik and Chu 1994), Japanese technique; bakers yeast and yeast in emulsion, Belgium technique; emulsified marine oils rich in *n*-3 HUFA's, (Watanabe *et al.*, 1980; Leger *et al.*, 1987; Kissil and Koven, 1990; Sorgeloos and Leger, 1992; Ozkizilcik and Chu, 1994); and the British technique; live micro algae, (Watanabe *et al.*, 1980, 1982; Millamena *et al.*, 1988; Whyte and Nagata 1990; Ozkizileik and Chu, 1994).

The nutritional value of rotifers fed with different diets are influenced by many factors which include the cell density of micro algae; the temperature and pH of the micro algal culture, different systems of producing micro algae and also with different medium used for algal culture. Rezeq and

James *et al.* (1987) observed that the micro algal cell density in rotifer culture not only influence the rotifer population but also the presence of HUFA. At food density of 37.5 x 10^6 cells/ml of *Chlorella* there is no inhibition in the biosynthesis of these essential fatty acids. There is a decline of 18.3 *n*-3 and 20.5 *n*-3 acids at 50 x 10^6 *Chlorella* cells/ml. James *et al*, (1989) stated that the total *n*-3 HUFA in *Chlorella* shows an increasing trend with the decrease in temperature and the total *n*-3 HUFA in *Nannochloropsis* shows an increasing trend with increasing temperature of up to 25 °C, the *n*-3 HUFA in *Chlorella* constituted mostly of linolenic acid (18:3 *n*-6) whereas *Nannochloropsis* contained eicosapentaenoic acid (EPA) as a major constituent of *n*-3 HUFA. The percentage of *n*-3 HUFA decreased significantly on the increase in the temperature. The decrease in the *n*-3 fatty acid percentage seems to be associated with the increase in the *n*-6 fatty acid percentage. Thus, the higher temperature may activate the *n*-6 pathway more than the *n*-3 pathway for fatty acid synthesis.

Variation in the composition of the culture medium can cause changes in the biochemical content of micro algae especially in those of protein, carbohydrate and lipid. (Sakamoto *et al.*, 1998). This in turn reflects the nutritive value of rotifer and ultimately to the growth and survival of the larvae fed by them. Mainly two enrichment media that are extensively used for the growth of most of the algae are the 'Walne and the Guillard's F/2 medium. Agricultural fertilizers like urea are also used for the production of micro

algae in out door culture system, but the nutritive value of such algae is inferior to that of the enrichment medium.

Schizochytrium sp, a DHA rich heterotrophic golden algae is effective in enriching *Artemia* nauplii and rotifers (Barclay and Zeller, 1996). The effectiveness of enrichment achieved with this strain of micro algae is due to several factors 1) the high content of *n*-3 HUFA in the spray dried cells, 2) the small size of the cells which readily facilitated ingestion by rotifers, 3) the excellent suspension characteristics exhibited by the spray dried cells in seawater, which kept them available for ingestion. Spray dried *Schizochrytrium* with its unique *n*-3 and *n*-6 HUFA profile may also be a candidate for replacing much of the live algae used in the culture of penaeid shrimp larvae.

Park *et al*, (1999) studied the growth and fatty acid composition of rotifers cultured in high density by the various enrichments and culture methods. The rotifers are fed with condensed freshwater *Chlorella* was enriched with ω yeast, Algamac, super selco and marine *Chlorella*. The density of rotifer and dissolved oxygen levels in the groups of rotifers enriched by super selco, ω yeast or Algamac were drastically decreased. The *n*-3 HUFA contents of rotifers enriched by super selco were higher than those of rotifers enriched by either ω yeast or Algamac in both methods. The supplementation of condensed marine *Chlorella* for 24 hours by the semi

continuous culture was effective for the improvement of the nutritional value of rotifers and it could provide stable growth condition for rotifer culture in high density. Enrichment of rotifers to improve its nutritive value can be carried out with other products like selco, super selco, home made oil emulsion and by feeding different forms of bacteria.

Rotifers grown on the culture selco replacement diet are an excellent HUFA composition. The use of culture selco allows direct enrichment of the rotifers without the need of a cumbersome bioencapsulation treatment, complementary diets such as protein selco and DHA culture selco have been developed in order to incorporate higher levels of protein and DHA. .

One of the cheapest ways to enrich rotifers is by using oil emulsions. Home made emulsions can be prepared with egg lecithin and fish oils (Watanabe *et al.*, 1982). The first emulsion was made from (*n*-3) HUFA rich fish oils (i.e. cuttlefish oil, pollack liver oil, cod liver oil, menhaden oil, etc.) and emulsified with egg yolk and seawater (Watanabe *et al.*, 1982, 1983). Recently, more purified oils containing specifically high levels of the EFA 20:5 *n*-3 has been used. Since the stability and storage possibility of these products is relatively low they are usually made on the spot and used immediately.

The dietary requirements of marine fish larvae has evolved from consideration of optimal dietary levels of *n*-3 HUFA to consideration of optimal dietary ratio of the two principal HUFA's, 22-6*n*-3, 20:5*n*-3 and 20:4*n*-6. Ideal marine fish larval diet is one containing circa 10% of the dry weight as *n*-3 HUFA rich marine phospholipids with less than 5% triacylglycerols, as exemplified by the lipid compositions of marine fish egg yolk, marine fish larvae themselves add their natural zooplankton prey. Such diet provide 22:6*n*-3, 20:5*n*-3 and 20:4*n*-6 in the desired levels and ratio and simultaneously satisfy known requirements for phospholipids, inositol and choline (Sargent *et al.*, 1999). For most of the fish and crustacean species the estimated phospholipid requirement of larvae are in the range of 1-3% phosphatidylcholine + Phosphatidylinositol of diet dry weight (Couthean *et al.*, 1997). The requirement for *n*-3 HUFA is about 0.5% for both larval and juvenile red sea bream 2.0% for juvenile yellow tail 0.8% for turbot 2.0% for flounder, 1.8% for juvenile striped jack 1.0% for seabass and gilt head sea bream (Watanabe *et al.*, 1989).

Marine fish can neither biosynthesize 22:6 *n*-3 *de novo* nor from shorter chain precursors such as 18:3*n*-3. Therefore 22-6*n*-3 and 20:5*n*-3 are essential dietary constituent for marine fish (Sargent *et al.*, 1999). The DHA: EPA ratio and their individual content in absolute terms are important for fish larval nutrition. The specific roles of DHA and EPA during larval development are different (Watanabe, 1993). The specific role of DHA in the development

of neural tissue as brain and retina has been well documented (Mourente *et al.*, 1991 and Bell *et al.*, 1995). Thus, the high content DHA in the developing larvae is obvious since the head of the larva constitutes significant part of the body mass. Rodriguez *et al.*, 1994 reported that higher dietary content of DHA than EPA during the rotifer stage improved the growth and survival of the larvae of gilt head sea bream. Yoshimatsu *et al.* (1995) larvae of *Mugil cephalus* *n*-3 HUFA like EPA: DHA as their essential fatty acids. Larvae receiving enriched rotifers with linoleic acid and linolenic acid showed typical symptoms of EFA deficiency syndromes, and exhibited poor growth and high mortality. The larvae reared in the tanks with *Nannochloropsis* supplement showed better growth than those of non-or fewer supplement growth.

Marine fish larvae are usually small at hatching (Theilacker and Dorsey, 1980; Kissil, 1984/85) and except for a few species, their size ranges between 2 – 7 mm. Rotifers offered to them must meet their nutritional requirements for optimization of growth and survival. These include (1) the size (2) the distribution and concentration of rotifers in the larval tanks (3) the total amount available (4) digestibility and absorption and (5) nutritional quality.

The live marine ornamental trade is a rapidly growing industry that relies almost exclusively on the collection of animals from coral reef ecosystems (Chapman *et al.*, 1997). The long term sustainability of the

marine ornamental industry is being threatened by environmental pressures that are severely degrading the health of coral reef ecosystems. The commercial culture of marine ornamental fin fish is very much in its infancy, but advances can be made more rapidly using insights from years of research and development with marine food fish species. The tropical marine ornamental fishes (Pomacentridae) are important in the trade for ornamental fish (Wilkerson, 1998) and are popular subject of research (Fautin, 1991). Over the last 20 years, mariculture centres and scientific laboratories have started rearing these fishes in large quantities (McLarney 1985, 1986; Miyagawa, 1989; Hoff, 1996; Young 1996; Job *et al.*, 1997).

Among the tropical marine ornamental fishes the clown fishes are the most popular due to their generally small and hardy nature, attractive colours, and high adaptability to life in captivity and the interesting display of behavior due to their association with sea anemones. Important clown fish species are *Amphiprion chrysogaster*, *A. clarkii*, *A. frenatus*, *A. melanopus*, *A. ocellaris*, *A. percula*, *A. sebae*, and *A. perideraion*. A technology for the hatchery production of the clown fish, *A. seba* was developed for the first time in India (Gopakumar *et al.,* 1999). The two key bottlenecks that currently limit expansion of the marine ornamental industry are the control of captive maturation and spawning and the identification of appropriate first-feed items for marine ornamental fish larvae.

The present study focused mainly on the nutritional quality of rotifers, which is being fed with different feeds. The main objective of the study includes

1. To study the relative efficacy of different feeds (micro algae, yeast.) and environmental parameters like salinity, pH and photoperiod for the mass culture of rotifers,
2. To study the nutritional profile (fatty acids) of rotifers which is being enriched with different diets.
3. To study the growth rate and survival of *Penaeus monodon* larvae and *Amphiprion sebae* larvae (number of days taken for pigmentation and metamorphosis), which is being fed with, enriched rotifers.

Chapter 2

2. Culture of the rotifer *Brachionus rotundiformis* (Tschugunoff) using different feeds

2.1. Introduction

The rotifer *Brachionus rotundiformis* is indispensable for aquaculture since it is the first food of the initial larval stages of many finfish and crustaceans. In recent years, many attempts have been made to improve rotifer culture conditions and the nutritional quality of the rotifers produced for aquaculture. Rotifers were found as an adequate food source for the following reasons: their shape, size and colour; their relatively slow motility; their chemical content that can be manipulated to meet the nutritional requirements of the fish larvae, and the ease with which they can be cultured at high densities to provide the large numbers required for raising larvae in captive systems. Moreover, rotifers can actually serve as a bio-capsule or vehicle, for transferring therapeutic agents to the fish larvae. (Lubzens *et al.*, 2001). Recent experiments show that rotifers can be used for transferring probiotic bacteria to fish larvae (Markridis *et al.*, 1999, 2000; Rombaut *et al.*, 1999 a, b).

The present trend in mass production of rotifers for aquaculture is the use of high quality and high-density biomass input as a means to increase maximum production. Conventional rotifer culture methods like batch culture method, semi-continuous culture methods, now practiced only

for research purposes and commercial units are adopting the new intensive culture techniques using turbidostats and chemostats for mass propagation of rotifers. In these improved techniques all the physico-chemical parameters are maintained at optimum condition with a steady supply of microalgae. The nutritional quality of cultured rotifers for rearing larval fish depends on the transfer of dietary compounds from phytoplankton or yeast to the rotifers. Temperature, salinity and pH have variable effects on the productivity of different strains of rotifers. (Miracle and Serra, 1989). *B. rotundiformis* is most productive at high temperatures (> 30°C) while *B. plicatilis* is most productive at lower temperatures (<25°C) (Fukasho, 1983). Many fresh water and marine algae are given as feed for rotifers in both live and in condensed suspension. Among this *Chlorella vulgaris,* a fresh water alga is an excellent food for rotifer if supplemented with Vitamin B_{12}. Condensed suspension of *C. vulgaris* was used for the food of rotifer *B. plicatilis* and *B. rotundiformis* in place of *Nannochloropsisis oculata* (Maruyama *et al.*, 1997). Another fresh water alga *Selenastrum capricornatum* which has three times greater concentration of unsaturated fatly acids than saturated fatty acids is a potential feed for rotifers (King *et al.*, 2002).

Algal diets can be fed to the rotifer singly or in combination of two or more. In an experiment fed with *Isochrysis galbana* (diet A) singly and *I. galbana* and *Nannochloropsis gaditana* (diet B) to rotifers reveals that there is an increase in neutral lipid content fed upon diet B, compared to diet A

which increased the phospholipid content. Diet B-fed rotifers had the highest content in 20:4*n*-6 and 20:5*n*-3, whereas rotifers fed diet A have highest 22:6*n*-3 content. *Isochrysis galbana* was found to contain substantial amount of DHA and a low EPA content (Fernandez-Reiriz *et al.*, 1989) whereas *N. gaditana* contained substantial amounts of EPA and 20:4*n*-6 (Sukenik *et al.*, 1993).

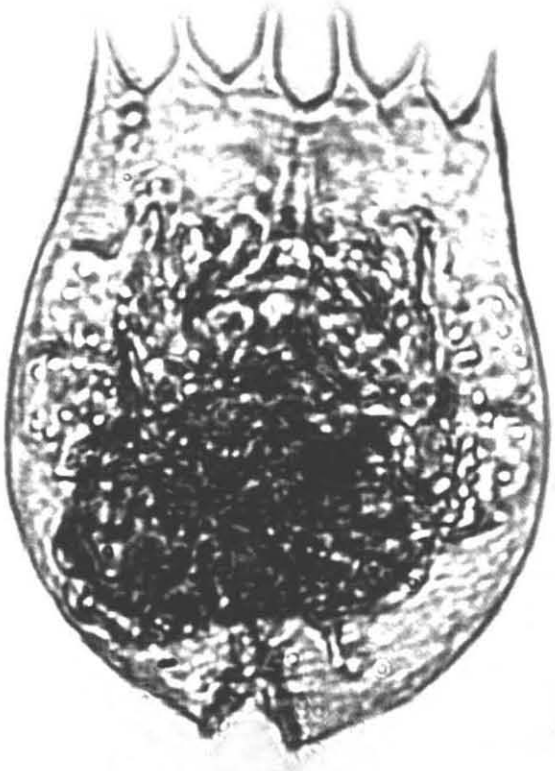
2. 2. Material and methods

The experiment was conducted in the Fisheries harbour laboratory of CMFRI, Thoppumpadi at Kochi. Stock cultures of rotifers and different microalgae were collected from Crustacean Division and FEM Division of CMFRI, Cochin respectively. The rotifer, *B. rotundiformis*, with lorica length of (100-120 μ) was employed for the experiment (Plate I) was brought from Mandapam. The stock culture of rotifers in 250ml flask is transferred into three 20 litre capacity translucent white buckets with ¼th filled with filtered seawater of salinity 35 ppt and is well aerated. The bucket is covered with lid in order to check the contamination of ciliates. The supply tubes of blower is connected with ciliate filter, which is made of activated charcoal and coral sand, both of the ends are covered tightly with 20 μm mesh. Microalgae like *Nannochloropsis salina, Chlorella marina* and *Isochrysis galbana* are inoculated separately into a 5 litre sterilised flask filled with filtered seawater. Walne's medium is enriched into the flask and it is kept in the indoor A/c room, which is maintained at a temperature between 28 - 23°C and provided with white fluorescent lights. Every day the flasks are

Plate – I

Brachionus rotundiformis (Tschugunoff)

Plate I



shaken properly for aeration. The rotifers in the bucket were fed with a mixture of algae, which include *Nannochloropsis, Isochrysis* and *Chlorella* on every morning and evening. After three days, sample from each bucket was taken and the rotifer population was counted using a Coulter counter under the microscope. One ml of the sample was taken and live rotifers were killed using a drop of diluted formalin. Five replicates were conducted and counted under the microscope for more reliability and finally average of samples were taken as the final value. When sufficient rotifer population was reached the mass culture of microalgae *Nannochloropsis salina*, *Isochrysis galbana, Chlorella marina* was started in the indoor system (Plate II b, c and d). Baker's yeast in the form of frozen block, also was procured from market and kept in the freezer. For the culture of microalgae white translucent buckets of 20 litres capacity and rectangular perspex tanks of 40 litres capacity were used. While buckets are maintained inside the A/c room and perspex tank were kept in non-A/c room. Ultra-violet filtered water was used inside the A/c room for both mass culture and stock culture. In the perspex tanks, seawater treated with chlorine was used after passing through 10 μ filter bags. Proper aeration and sufficient light using white fluorescent tubes for 24 hrs were provided. Seawater of salinity 35 ppt was used to culture *Nannochloropsis salina* and *Chlorella marina*. *Isochrysis galbana* is cultured using seawater of 25 ppt salinity. Enrichment medium used for the mass culture and stock culture was the modified Walne's medium (Walne, 1974).

Plate – II

Micro algae and Yeast used for enriching rotifers

a) *Saccharomyces cerevisiae*

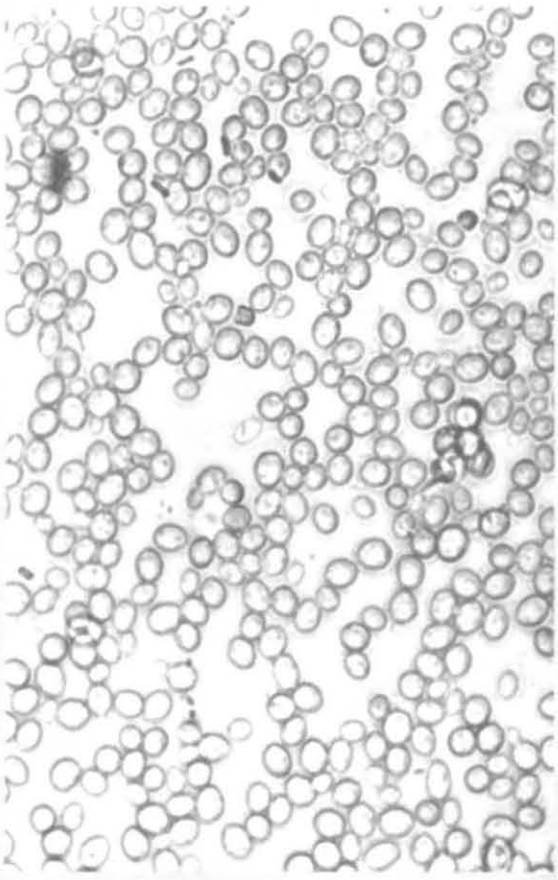
b) *Chlorella marina*

c) *Isochrysis galbana*

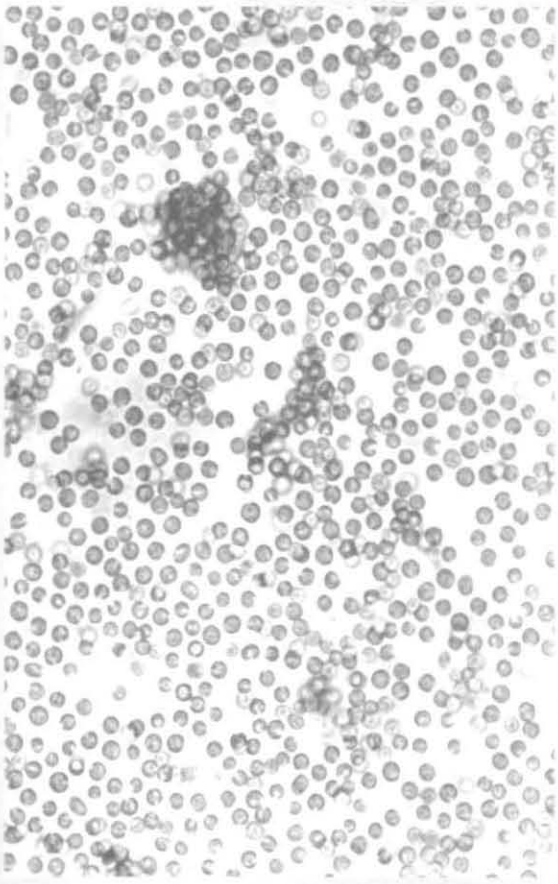
d) *Nannochloropsis salina*

Plate II

a



b



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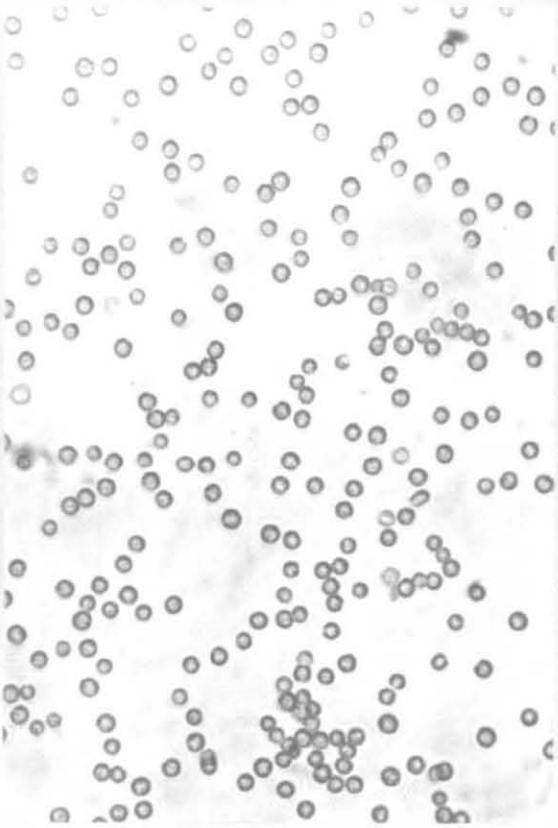
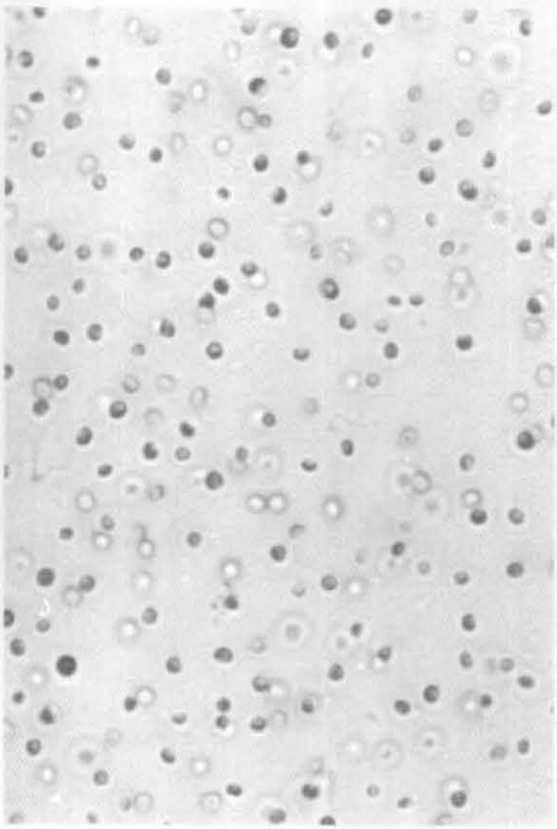


d



Mass culture of rotifers

Two litres of algal stock culture were added to the 20 litre capacity buckets for each algal species in separate buckets and 3 litres of algal stock culture to the 40 litre capacity rectangular perspex tanks enriched with nutrients (Plate III a, b and c). After 24 hrs, it was noticed that 40 litre tanks bloom first, but the 20 litre buckets maintained inside the A/c room had taken 36-48 hrs for blooming. Among 3 species of micro-algae, *Nannochloropsis* and *Chlorella* bloom within 24 hrs but *Isochrysis* blooms only after 24 hrs. When the algae were about to bloom, the experimental set up for the rotifer culture was done. Rotifer culture experiments were carried out in 50 litre capacity perspex tubs with white base with a lid and well aerated. Each treatment was carried out in triplicates (Plate IV a, b, c and d). So 12 tubs were arranged in platform very near to the window, so that most of the time natural light was supplied to the culture animals. Tube-lights were also arranged parallel to the tanks and light was provided for 24 hrs. The tub was filled one-fourth with filtered and chlorinated seawater. Rotifer from the mass culture was collected using a micropipette and 25 ± 5 numbers of rotifer with eggs (8 - 10 nos) were introduced in each tub. Initial concentration of rotifers per ml was maintained at 60-70 nos per ml in all the experiments. The rotifer tubs are named as *Na* R (NaR_1, NaR_2, NaR_3) i.e. *Nannochloropsis* fed rotifers, *Ig* R (IgR_1, IgR_2, IgR_3) as *Isochrysis* fed rotifers, *Cl* R (ClR_1, ClR_2, ClR_3) as *Chlorella* fed rotifer, Y R (YR_1, YR_2, YR_3) as yeast fed rotifer. These tubs are allocated as NaR_1 followed by IgR_2,

Plate – III

a) **Stock culture of micro algae.**

b) **Indoor mass culture of micro algae.**

c) **Indoor mass culture of micro algae.**

Plate – IV

a) Experimental set up for Semicontinuous culture of rotifers

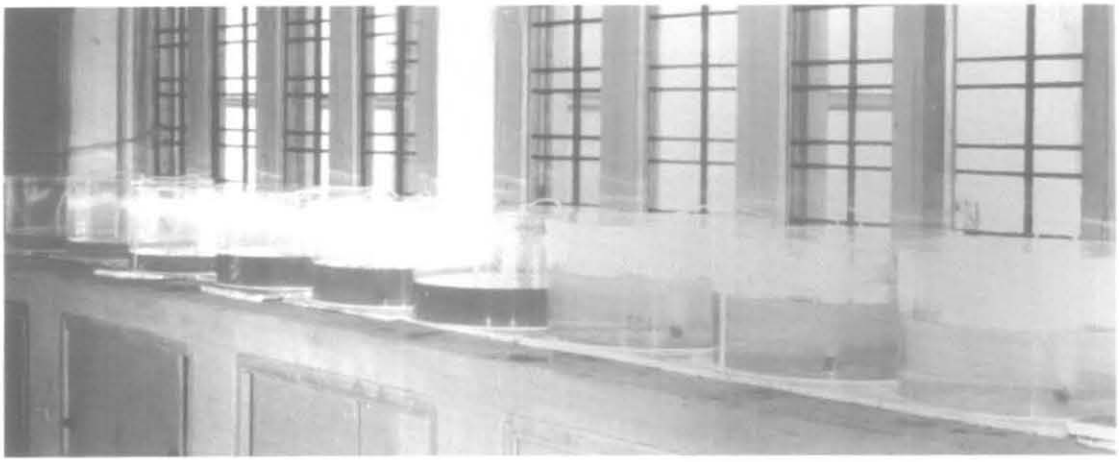
b) Culture of rotifers. Day 1

c) Culture of rotifers. Day 2

d) Culture of rotifers. Day 4

Plate IV

a



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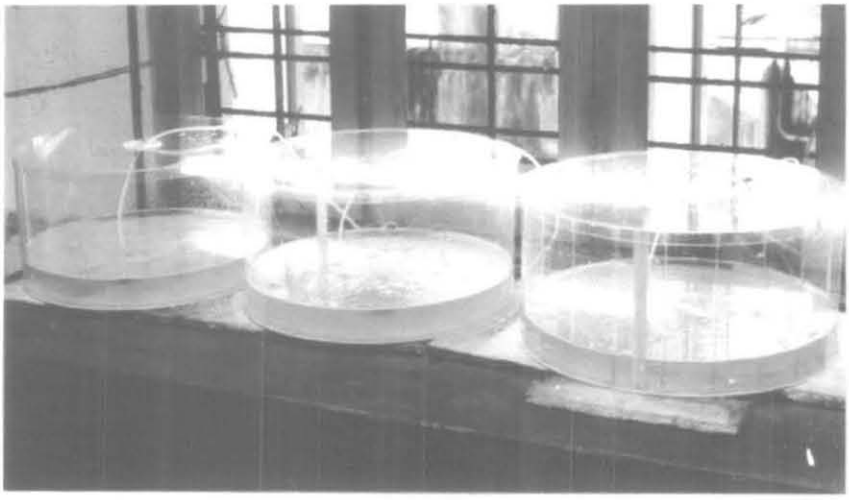

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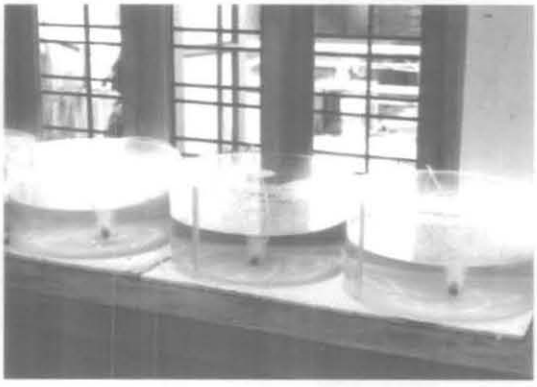


C/R_2 so that minimum error due to climatic factors will not affect the individual treatments. All the tubs where rotifers were introduced were fed with particular microalgae maintained at optimum level i.e., *Nannochloropsis* (4 - 5 x 10^6 cells/ml), *Isochrysis* (5 - 6 x 10^6 cells/ml), *Chlorella* (4 - 5 x 10^6 cell/ml) by adding the algae twice daily. As rotifers fed with *Isochrysis* had a prolonged lag phase, algae were not given on the second day. On day 6 during late evening hours counting of rotifers in the individual tubs were done. Samples from each tub was taken initially in a 100 ml glass beaker from that I ml sub sample were taken using a graduated dropper and was fixed in a Coulters counting chamber. One drop of diluted formalin was added to the chamber and waited for five minutes so that all the rotifers become dead, which made the counting easy. The samples were counted under a microscope. From each tubs a minimum of five sub samples were counted and the average was taken as the final value. In the case of yeast fed rotifers the feeding protocol was quite different from that of microalgae. On day 1 of the experiment, 1 gm yeast cells/million rotifers are maintained in YR treatments (Plate II a). This was done by mixing the weighed quantities of yeast in 1 litre water and supplied to rotifer tubs twice a day. All these experiments were done simultaneously and repeated ten times in different environmental conditions. Among ten experiments conducted for studying, the relative efficacy of different feeds for rotifer culture, Exp 1 to 3, the rotifer culture was maintained at three different pH levels (6.8 – 8.2) keeping other parameters at optimum level. In experiment 4 to 6 rotifer culture was maintained at three different salinities

(20 ± 2, 25 ± 2and 30 ± 2) with other parameters at optimum. Experiment 7 to 9, light intensity at varying photoperiod (12 l: 12 d, 18 l: 6 d and 15 l: 9 d) keeping other parameters at optimum. In experiment 10, all the environmental parameters were kept at optimum level. On day 6 evening, after the counting, rotifers were collected using a 100 μm mesh. The collected sample was allowed to drain the water completely and the sample transferred to a separate container and was stored in freezer at –82°C. Ten experiments were conducted with optimum environmental factors and feed concentrations to find out population growth rate, doubling time and instantaneous growth rate. The DO level was maintained between 3.8 to 4.6 mg/litre in all tubs. The temperature of all the experiments were maintained between 29-32°C. Population growth rates was calculated from initial and final aliquot samples from each experiment. Doubling times were calculated by dividing $\log_e 2$ by the instantaneous growth rate (K) of pooled data of ten experiments. $K=[(\ln N_t - \ln N_0) /t]+D$, Where N_0=Initial number of rotifers, N_t=number of rotifers after t days, D= dilution rate (m^3d^{-1}). The whole experiment was statistically designed with triplicates.

2. 2. 1. Statistical analysis

a) The transformed values of rotifer production were subjected to one factor analysis of variance (ANOVA) with feed at four levels as factor. Similarly transformed values of rotifer production were subjected to separate one-way analyses of variance (ANOVA) with pH and salinity levels as the

factors in them. Among the ten experiments, experiment 1 and experiment 2 were analyzed separately and the rest of the experiments (3 to 10) were analysed after combining their respective data. In all the above cases the means of the various levels of factors were grouped by Duncan's multiple range test at a level of significance equally 0.5 ($P<0.05$)(Duncan, 1955).

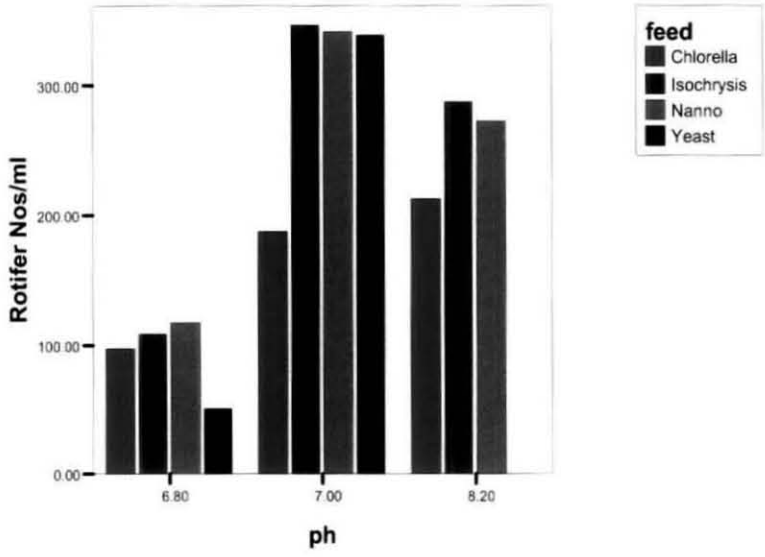
b) Instantaneous growth rate (K), doubling time and per day growth of rotifers were pooled for the ten experiments and were subjected to one way analyses of variance (ANOVA) with feed as the source of variations. The means of four feeds were compared using DMRT.

c) The growth rate of rotifers for each feed was subjected to linear, quadratic and cubic regression over time in days, with an aim to find the best regression equation based on the R^2 values.

2. 3. Results

The best feed for rotifer in terms of growth and optimum physico-chemical parameters. (Salinity, pH and light intensity) were studied during this series of experiments (10 experiments; Table 2. 14). In the first three sets of experiments where three different pH levels were studied, (Exp1 - pH 6.8; Exp 2 - pH 8.2; Exp 3 - pH 7) maximum rotifer growth rate was produced with a pH of 7 (Mean 346.6 ind.ml^{-1}) followed by a pH of 8.2 (Mean-287.6 ind.ml^{-1}). Rotifer production in the experiment with pH-6.2 is very low compared with other two (mean 116.6 ind.ml^{-1}) (Fig 2.1).

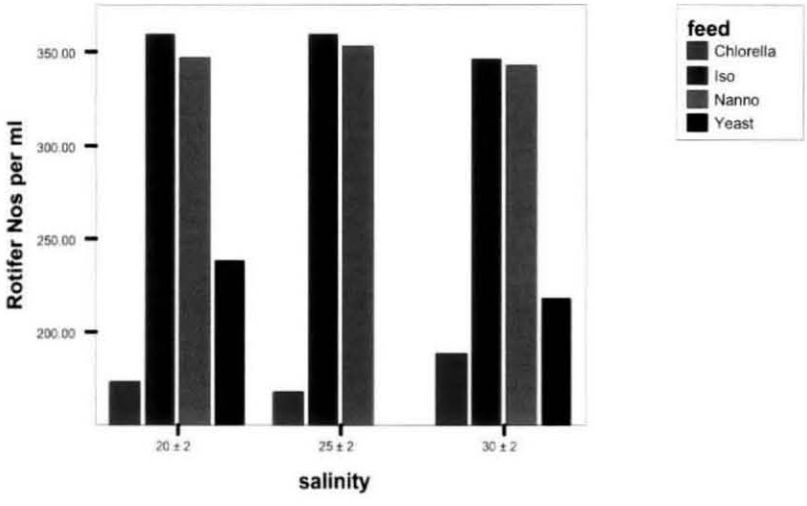
Fig. 2. 1. Rotifer production at different pH



The experiments on relative efficacy of feeds (*Na*R, *Cl*R, *Ig*R and YR) for rotifer growth had proved *Nannochloropsis* and *Isochrysis* to be the best feeds. But the growth rate of *Ig*R was very slow during first three days and afterwards there was a quick spurt in the growth rate and population density. In the case of *Na*R the very next day onwards growth rate and population density had increased. Regarding YR a steady growth rate was observed but the rate of increase was not that much impressive and if no water exchange was given the culture will be fully collapsed. *Cl*R, the growth rate was not that much impressive. The culture density decreases from day 5 onwards. The mean population density of rotifers was also comparatively lower compared with other treatments.

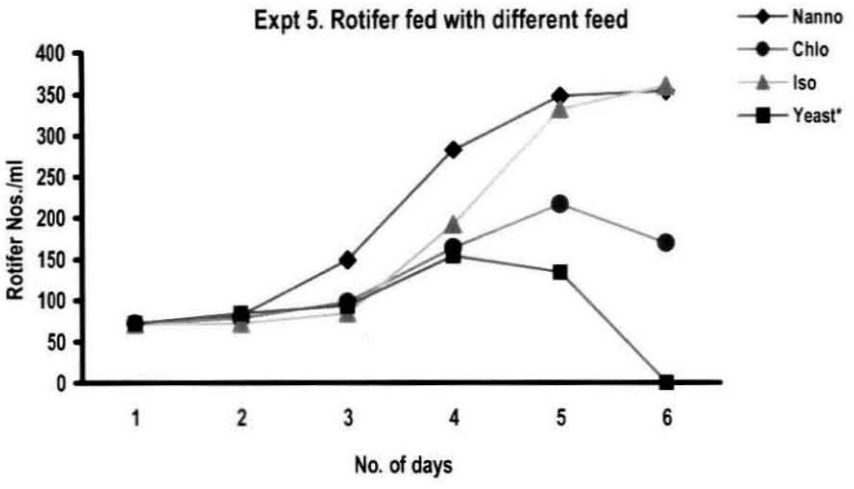
During the next set of experiments (Exp 4, 5, 6) different salinity regimes for the optimum growth of rotifers were studied (Exp 4, 20 ± 2ppt, Exp 5, 25 ± 2 ppt, Exp 6, 30 ± 2ppt). A maximum culture density of rotifers was produced with a salinity of 20 ± 2 (mean 360 ind.ml^{-1}). There was no significant difference in the rotifer density with a salinity of 25 ± 2 (mean 359 ind.ml^{-1}). But rotifers reared in salinity 30 ± 2 the mean is 346.6 ind.ml^{-1} was less compared with other two levels (Fig 2.2).

Fig. 2. 2. Rotifer production in different salinity



Rotifer production was maximum in *lg*R (360 ind ml^{-1}) followed by *Na*R (353 ind ml^{-1}) in all the three experiments. But there is no significant difference within these two treatments. Rotifer production was much lower for other two treatment viz. *Cl*R (189 ind ml^{-1}) and YR (238 ind ml^{-1}). The Exp 5, water was not exchanged in the yeast fed rotifer tanks, which led to the total collapse of the culture on day 6. The culture system was severely attacked by ciliates (Fig. 2.3).

Fig. 2.3. Rotifer culture crashed due to ciliate attack

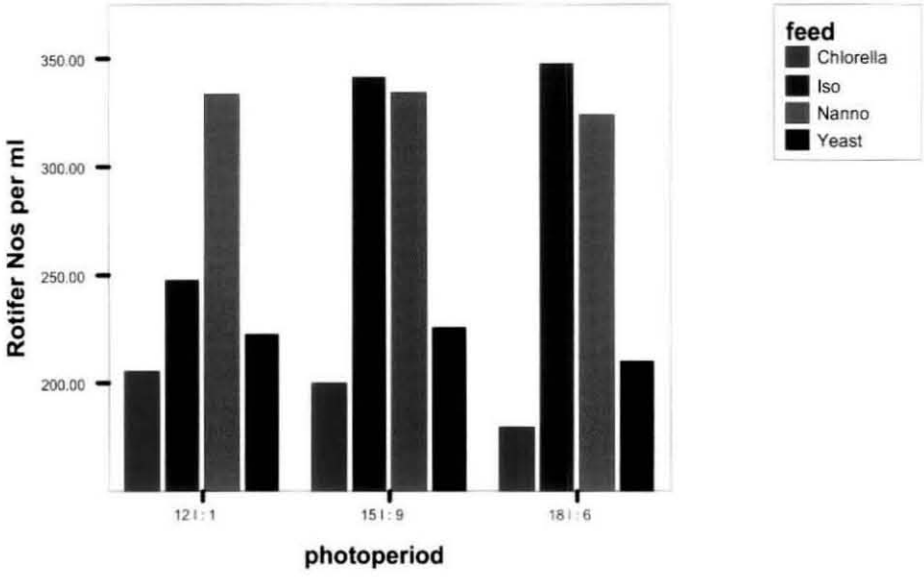


* No water exchange during culture

Rotifer production in relation to different photoperiods was tried in the next set of experiments (Exp 7, 8, 9). Exp 8 with a photoperiod of 12 l:12d and Exp 9 with a photoperiod of 15l: 9d and Exp 7 with a photoperiod of 18 l:6 d was tried. An increased rotifer production was observed with 18 l:6 d as photoperiod (mean 347 ind.ml^{-1} followed by 15 l:9 d as photoperiod (341 ind.ml^{-1}) (Fig. 2. 4).

Rotifer production was maximum in *lg*R (348 ind ml^{-1}) treatment during Exp 7 and Exp 9. During Exp 8 rotifer production was maximum in *Na*R (334 ind ml^{-1}) treatment. The same trend followed in the case of *Cl*R (206 ind ml^{-1}) and YR (226 ind ml^{-1}) treatment as that of previous experiments. In all the three experiments YR treatments showed better growth rate than *Cl*R treatments.

Fig. 2. 4. Rotifer production at different photoperiod



The optimum environmental parameters obtained from the earlier experiments were combined in the Exp 10 with salinity 20 ± 2 ppt, pH 7.1 and photoperiod 181:6d. A maximum rotifer production of (353.3 ind.ml^{-1}) was produced when *Isochysis galbana* was given as feed at a cell density of (5-6x10^{6}cell/ml) followed by (mean 320.3 ind.ml^{-1}) *Nannochloropsis salina* fed rotifer maintained at a cell density of (4-5x10^{6}cell/ml) was given as. In the case of *chlorella* fed rotifers the mean rotifer production was (201.6 ind.ml^{-1}) and yeast fed rotifer the mean production was (232 ind.ml^{-1}) at a cell density of 4-5 x10 ^{6} cells/ml and 1gm yeast cells/million rotifers (Fig. 2. 5).

Fig. 2. 5. Rotifer production at optimum condition

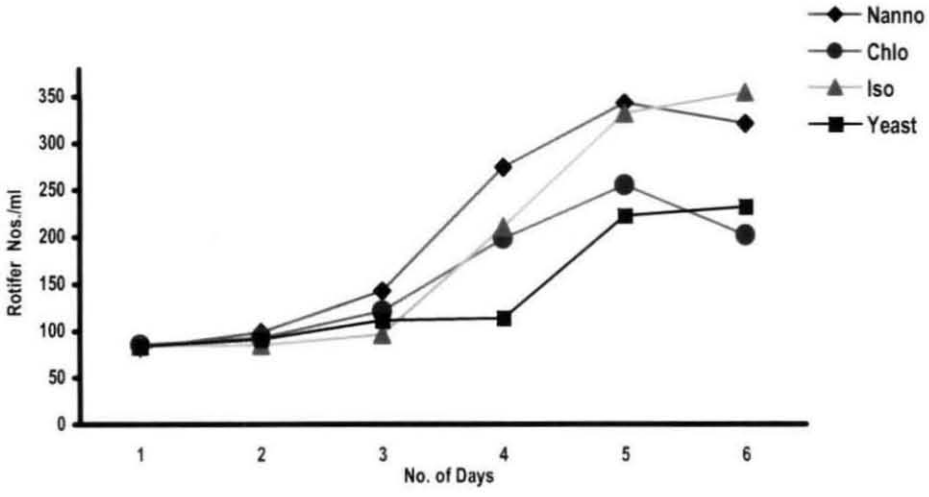
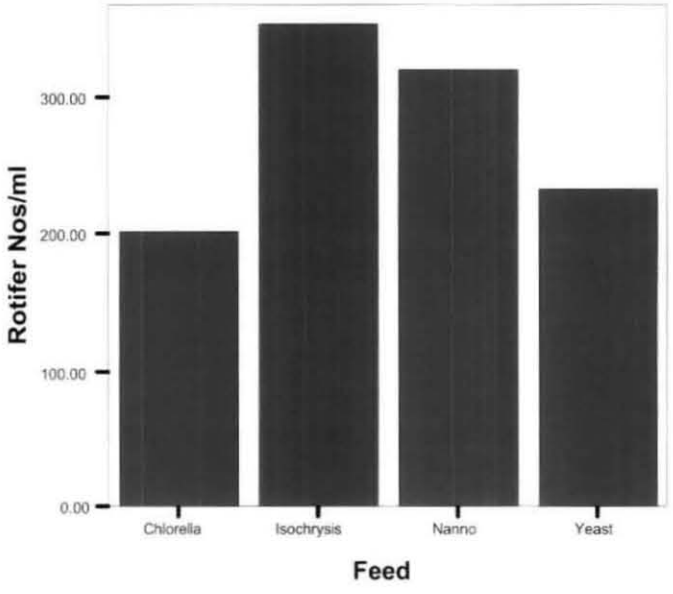


Fig. 2. 6. Rotifer production with different feeds



The rotifer production was maximum when *Nannochloropsis salina* and *Isochrysis galbana* were fed to rotifers. The calculated instantaneous growth rate (K) (pooled data of ten Experiments in Table) for rotifers fed

with different feeds showed that rotifers fed with *Nannochloropsis salina* was higher (0.30893 ± 0.004) followed by *Isochrysis galbana* fed rotifers (0.3042.5 ± 0.004). The instantaneous growth rate for bakers yeast and *chlorella* fed rotifers were (0.04616 ± 0.0595) and (0.21599 ± 0.004) respectively.

The per day growth of rotifers fed with different feeds were calculated using the pooled data of ten experiments. A maximum production of 40.18 ± 1.979 rotifers $ml^{-1}d^{-1}$ was recorded when *Isochrysis* was fed to rotifers. Rotifer growth rate was equally good when *Nannochloropsis salina* was fed to them (39.95 ± 1.687 rotifers $ml^{-1}d^{-1}$). The per day growth calculated for *chlorella* and bakers yeast fed rotifers were 18.23 ± 0.548 rotifers $ml^{-1}d^{-1}$ and 16.416 ± 2.595 $ml^{-1}d^{-1}$ respectively.

The population doubling time had shortened when *Nannochloropsis salina* was fed to rotifers (2.254 ± 0.0289 days). For rotifers fed with *Isochrysis galbana* the doubling time did not vary much from that of the previous treatment (2.2966 ± 0.0407 days). The population doubling time for *Chlorella marina* and bakers yeast fed rotifers were (3.250 ± 0.0662 days) and (2.525 ± 0.3626 days) respectively. The population doubling time decreased from 3.250 ± 0.0662 day to 2.254 ± 0.0289 days when *Nannochloropsis salina* was fed to rotifers (Plate V a, b, c and d).

Plate – V

a) **Mass culture of *Brachionus rotundiformis***

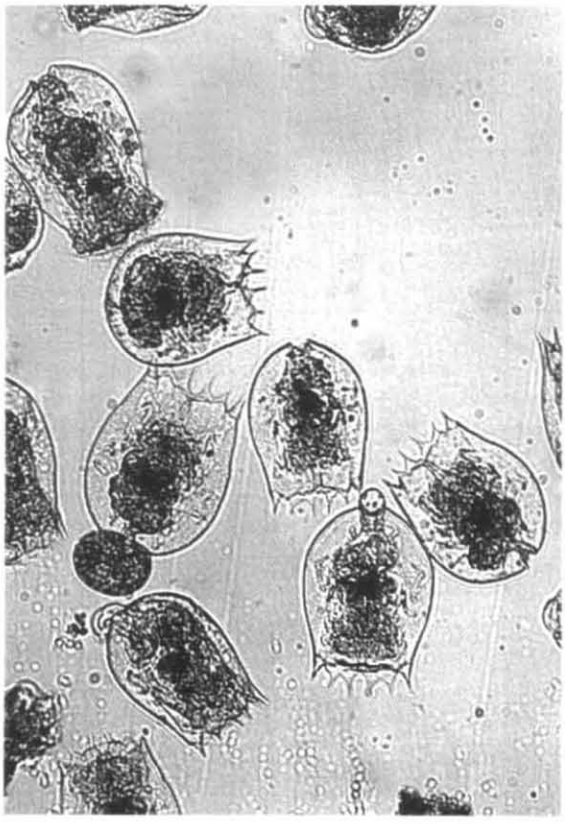
b) ***B. rotundiformis* with single egg**

c) ***B. rotundiformis* with empty egg capsule**

d) ***B. rotundiformis* with two eggs one hatched**

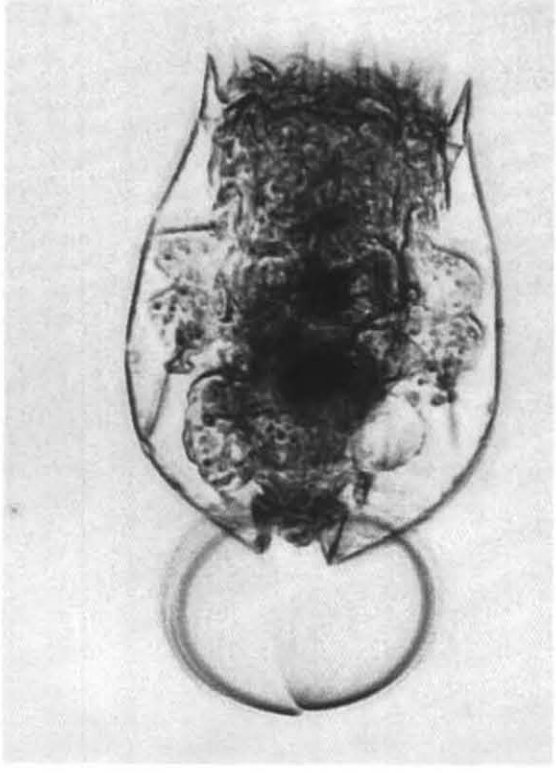
Plate V

a

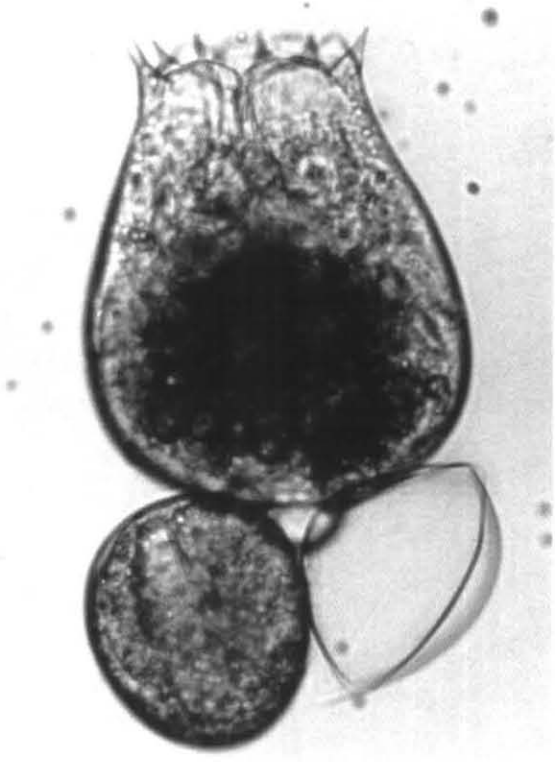


b

c



d



2. 3. 1. Statistical analysis

Average number of rotifers over culture period, fed with different feed such as *Nannochloropsis salina*, *Chlorella marina*, *Isochrysis galbana* and *Saccharomyces cerevisiae* was computed from the observations recorded with three replication (Fig. 2. 7 – 2. 10).

The growth rate was regressed on the culture period in days, to find out the best curve fit. The analysis revealed that the R^2 estimate of the quadratic relationship was the best among the tested methods viz: linear, quadratic and cubic. So the equation selected was quadratic and is as follows Growth rate = $b_0 + b_1 \cdot day + b_2 \cdot day^2$. Growth rate was the average number of rotifers per ml/day corresponds to the culture period, and b_0, b_1 and b_2 are the regression coefficients. The R^2 values were high enough to be treated as significant in three out of four feeds. The estimated regression coefficients and R^2 for different feeds are given in the table 2.1.

Table 2. 1. Quadratic curve estimates for rotifer culture with different feeds

Feed	b_0	b_1	b_2	R^2
1	-282.060	190.114	-23.914	0.87820
2	-226.447	148.128	-23.914	0.82072
3	-299.547	181.559	-21.026	0.63948
4	-88.207	59.655	-7.262	0.27925

Fig. 2. 7. Growth rate (per ml/day) of rotifers fed with *N. salina*

Growth rate = -282.060+190.114*day - 23.914 * day^2

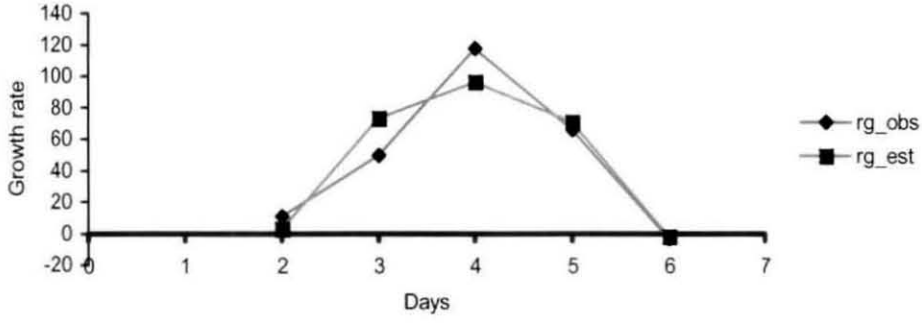


Fig. 2. 8. Growth rate (per ml/day) of rotifers fed with *C. marina*

Growth rate = -226.447+148.128*day - 19.121*day^2 .821

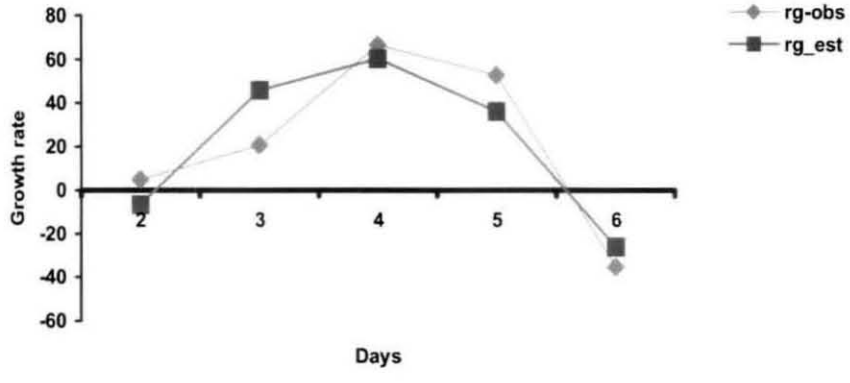


Fig. 2. 9. Growth rate (per ml/day) of rotifers fed with *I. galbana*

Growth rate = -299.547+181.559*day -21.026*day^2

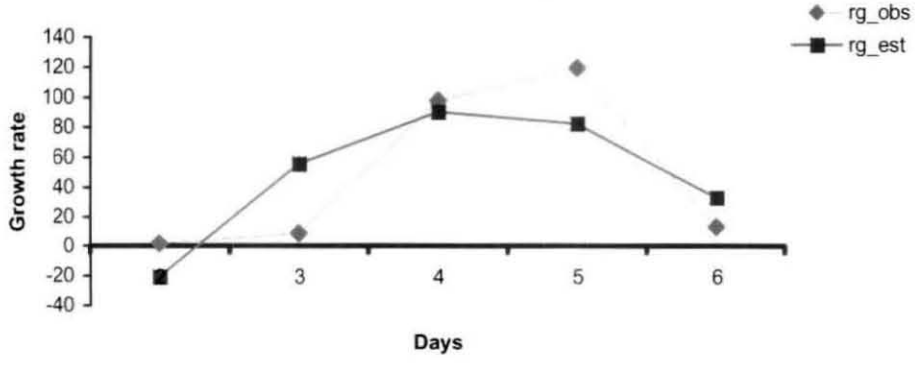
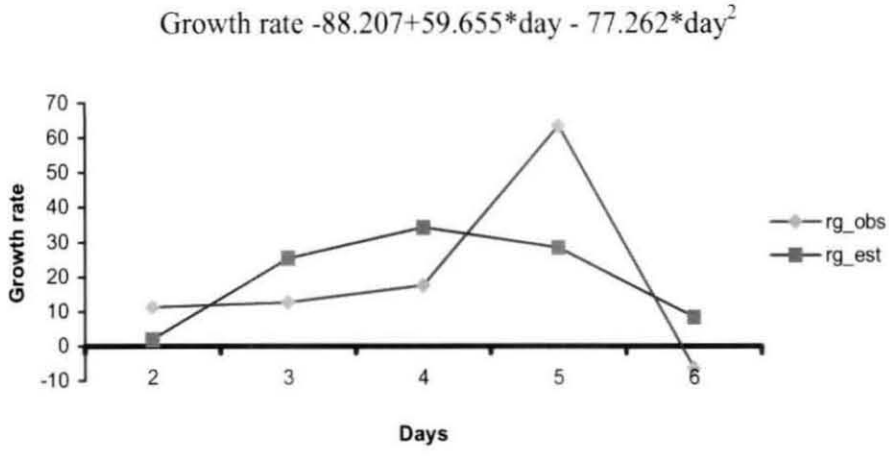


Fig. 2. 10. Growth rate (per ml/day) of rotifers fed with *S. cerevisiae*



Experiment I

The one-way analysis of variance (ANOVA) performed on the transformed rotifer nos/ml data (transformation was done by taking the natural log of the original numbers of rotifer/ml added with one) and the ANOVA table given below. It can be observed that the feed have a very significant contribution to the total variation in the data ($P<0.01$). The post Hoc Duncanís multiple range test (DMRT) revealed the grouping of the four levels of feeds into two sub sets which are significantly ($p<0.01$) different from each other. (Means of the transformed values are given in the Table 2. 2 and 2. 3).

Table 2. 2. ANOVA for rotifer culture with different feeds

Source of variation	Sum of squares	df	Mean square	F	Significance
Feed	1.28	3	0.428	39.370	0.000
Error	0.087	8	0.011		

Table 2. 3. DMRT grouping for rotifer culture with different feeds

Sl. No.	Name of the feed fed to rotifers	*Mean of transformed values of rotifer nos/ml
1	*Nannochloropsis salina*	4.767^{a}
2	*Chlorella marina*	4.581^{a}
3	*Isochrysis galbana*	4.687^{a}
4	*Saccharomyces cerevisiae*	3.938^{b}

*The means with similar superscripts do not differ significantly.

It can be seen in the DMRT grouping that the mean corresponding to yeast fed rotifers is low and is significantly different from each of the other three feeds.

Experiment 2

In this experiment the feed had a very significant contribution to the total variation in the data ($P < 0.01$) (Table 2. 4). Three homogenous groups were elucidated by DMRT analysis in which *Nannochloropsis* fed rotifers and *chlorella* fed rotifers formed one group and other group formed by *Nannochloropsis* and *Isochrysis* fed rotifers. The yeast fed rotifers differed significantly from other three feeds, thus formed the third group (Table 2. 5).

Table 2. 4. ANOVA for rotifer culture with different feeds

Source of variation	Sum of squares	df	Mean square	F	Significance
Feed	69.119	3	23.040	1042.388	0.000
Error	0.177	8	0.022		

Table 2. 5. DMRT grouping for rotifer culture with different feeds

Sl. No.	Name of the feed fed to rotifers	Transformed *mean value of rotifer nos/ml
1	*Nannochloropsis salina*	5.598^{ab}
2	*Chlorella marina*	5.346^{b}
3	*Isochrysis galbana*	5.663^{a}
4	*Saccharomyces cerevisiae*	0^{c}

* Means with similar superscripts do not differ significantly

Experiment 3 to 10

It was observed that the feed had a very significant contribution to the total variations in the data ($P<0.01$) (Table 2. 6). Two homogenous groups have been elucidated by DMRT analysis in which *Nannochloropsis* fed rotifers and *Isochrysis* fed rotifers formed one group and *Chlorella* fed rotifers and yeast feed rotifers formed the other group (Table 2. 7).

Table 2. 6. ANOVA for rotifer culture with different feeds

Source of variation	Sum of squares	df	Mean square	F	Significance
Feed	19.311	3	6.437	7.60	0.000
Error	77.856	92	0.846		

Table 2. 7. DMRT grouping for rotifer culture with different feeds

Sl. No.	Name of the feed fed to rotifers	*Mean of transformed values of rotifer nos /ml
1	*Nannochloropsis salina*	5.824^{a}
2	*Chlorella marina*	5.241^{b}
3	*Isochrysis galbana*	5.824^{a}
4	*Saccharomyces cerevisiae*	4.748^{b}

* The means with similar superscripts do not differ significantly

ANOVA for instantaneous growth rate (K), doubling time and per day growth of rotifers with different feeds.

The instantaneous growth rate (K), which was calculated by the formulae detailed in material and method, was subjected to one-way analysis of variance (ANOVA) (Table 2. 8) with feed at four levels as the group variable. The ANOVA table is given below, and it can be seen that feed component was highly significant ($P < 0.01$). The DMRT performed on the 'K' values showed the existence of three homogenous groups among the four levels of feeds. The groups were *Nannochloropsis* fed rotifers and *Isochrysis* fed rotifers, *Chlorella* fed rotifers, yeast fed rotifers. The means are given in the Table 2. 9 a.

Table 2. 8. ANOVA for instantaneous growth rate (K), doubling time and Per day growth of rotifers fed with different feeds

Source of variation		Sum of Squares	df	Mean Square	F	Significance
K	Feed	1.357	3	.452	16.708	.000
	Error	3.141	116	.0271		
Doubling time	Feed	19.143	3	6.381	6.148	.001
	Error	120.395	116	1.038		
Per day growth	Feed	15565.823	3	5188.608	51.396	.000
	Error	11710.603	116	100.953		

The values for doubling time and per day growth was subjected to one way analysis of variance (ANOVA) with feeds at four levels as the group valuable. The ANOVA table is given below and it can be seen that feed component was highly significant for both cases ($P< 0.01$). The DMRT performed on the doubling time and per day growth values showed the existence of two homogenous sub set group among the four levels of feeds

(Table 2. 9 b). The groupings are for doubling time (1) *Nannochloropsis* fed rotifers, *Isochrysis* fed rotifer and *Saccharomyces* fed rotifers (2) *Chlorella* fed rotifers, with mean values shows that *Isochrysis* and *Nannochloropsis* fed rotifers are superior. The DMRT grouping for per day growth was as follows. (1) *Nannochloropsis* and *Isochrysis* fed rotifers (2) *Chlorella* and *Saccharomyces* fed rotifers. The mean values showed that *Nannochloropsis* and *Isochrysis* feed were superior for rotifers in terms of per day growth (Table 2. 9 c).

Table 2. 9 a. DMRT grouping for instantaneous growth rate (K) of rotifers

Sl. No.	Name of feed fed to rotifers	*Mean values of rotifers
1	*Nannochloropsis salina*	0.308^{a}
2	*Chlorella marina*	0.215^{b}
3	*Isochrysis galbana*	0.304^{a}
4	*Saccharomyces cerevisiae*	0.046^{c}

* The means with similar superscripts do not differ significantly

Table 2. 9 b. DMRT grouping for doubling time of rotifers

Sl. No.	Name of feed fed to rotifers	*Mean values of rotifers
1	*Nannochloropsis salina*	2.255^{a}
2	*Chlorella marina*	3.250^{b}
3	*Isochrysis galbana*	2.296^{a}
4	*Saccharomyces cerevisiae*	2.525^{a}

* The means with similar superscripts do not differ significantly

Table 2. 9 c. DMRT grouping for per day growth of rotifers

Sl. No.	Name of feed fed to rotifers	*Mean values of rotifers
1	*Nannochloropsis salina*	39.950^{a}
2	*Chlorella marina*	18.233^{b}
3	*Isochrysis galbana*	40.183^{a}
4	*Saccharomyces cerevisiae*	16.416^{b}

* The means with similar superscripts do not differ significantly

The one-way ANOVA performed on rotifer growth with different pH levels (3 levels) as the factor and presented in the Table no 2. 10. The ANOVA indicates that the varying pH levels had significant ($P < 0.01$) impact on the rotifer population. DMRT performed as a post Hoc measure indicated the existence of two homogenous subsets, which is given in the Table 2. 12.

The one-way analysis of variance (ANOVA) was performed on with different salinity levels (3 levels) as the factor and ANOVA in Table 2. 11.

The ANOVA indicated that the varying salinity levels had significant ($P < 0.05$) impact on the rotifer multiplication. The DMRT performed as a post Hoc measure indicated the existence of two homogenous subsets, which is given in the following Table 2. 13.

Table 2. 10. ANOVA for pH levels for Rotifer culture

Source of variation	Sum of Squares	df	Mean Square	F	Significance
Ph level	121020.1	2	60510.044	7.887	.001
Error	283885.3	37	7672.576		

Table 2. 11. ANOVA for Salinity levels for Rotifer culture

Source of variation	Sum of Squares	df	Mean Square	F	Significance
Salinity level	64388.979	2	32194.490	3.498	.041
Error	340516.4	37	9203.146		

Table 2. 12. DMRT grouping for rotifers in different pH levels

Sl. No.	pH level	*Mean of rotifer nos/ml
1	6.80	92.995^{b}
2	7.00	268.63^{a}
3	8.20	192.995^{ab}

* The means with similar superscripts do not differ significantly

Table 2. 13. DMRT grouping for rotifers in different salinity levels

Sl. No.	Salinity levels for Rotifer culture	*Mean of rotifer nos/ml
1	20	275.718^{a}
2	25	206.704^{ab}
3	30	183.661^{b}

*The means with similar superscripts do not differ significantly

Table 2. 14. Environmental parameters and rotifer production with different feeds

Exp.	Feed	Rotifer Nos./ml (Mean)	Salinity (ppt)	pH	Photoperiod (Hrs.)
1	*Nannochloropsis salina*	116.66	20 ± 2	6.8	18 l : 6 d
	Chlorella marina	96.66	20 ± 2	6.8	18 l : 6 d
	Isochrysis galbana	107.66	20 ± 2	6.8	18 l : 6 d
	Baker's Yeast	51.00	20 ± 2	6.8	18 l : 6 d
2	*Nannochloropsis salina*	272.66	20 ± 2	8.2	18 l : 6 d
	Chlorella marina	211.66	20 ± 2	8.2	18 l : 6 d
	Isochrysis galbana	287.66	20 ± 2	8.2	18 l : 6 d
	Baker's Yeast	0	20 ± 2	8.2	18 l : 6 d
3	*Nannochloropsis salina*	341.33	20 ± 2	7.0	18 l : 6 d
	Chlorella marina	187.66	20 ± 2	7.0	18 l : 6 d
	Isochrysis galbana	346.66	20 ± 2	7.0	18 l : 6 d
	Baker's Yeast	338.66	20 ± 2	7.0	18 l : 6 d
4	*Nannochloropsis salina*	347.00	20 ± 2	7.0	18 l : 6 d
	Chlorella marina	173.66	20 ± 2	7.0	18 l : 6 d
	Isochrysis galbana	359.66	20 ± 2	7.0	18 l : 6 d
	Baker's Yeast	238.66	20 ± 2	7.0	18 l : 6 d
5	*Nannochloropsis salina*	353.66	25 ± 2	7.0	18 l : 6 d
	Chlorella marina	168.66	25 ± 2	7.0	18 l : 6 d
	Isochrysis galbana	359.33	25 ± 2	7.0	18 l : 6 d
	Baker's Yeast	0	25 ± 2	7.0	18 l : 6 d
6	*Nannochloropsis salina*	343.33	30 ± 2	7.0	18 l : 6 d
	Chlorella marina	188.66	30 ± 2	7.0	18 l : 6 d
	Isochrysis galbana	346.66	30 ± 2	7.0	18 l : 6 d
	Baker's Yeast	218.66	30 ± 2	7.0	18 l : 6 d
7	*Nannochloropsis salina*	324.33	20 ± 2	7.0	18 l : 6 d
	Chlorella marina	180.33	20 ± 2	7.0	18 l : 6 d
	Isochrysis galbana	347.66	20 ± 2	7.0	18 l : 6 d
	Baker's Yeast	210.66	20 ± 2	7.0	18 l : 6 d
8	*Nannochloropsis salina*	334.00	20 ± 2	7.0	12 l : 12 d
	Chlorella marina	206.00	20 ± 2	7.0	12 l : 12 d
	Isochrysis galbana	248.33	20 ± 2	7.0	12 l : 12 d
	Baker's Yeast	222.66	20 ± 2	7.0	12 l : 12 d
9	*Nannochloropsis salina*	334.66	20 ± 2	7.0	15 l : 9 d
	Chlorella marina	200.33	20 ± 2	7.0	15 l : 9 d
	Isochrysis galbana	341.66	20 ± 2	7.0	15 l : 9 d
	Baker's Yeast	226.00	20 ± 2	7.0	15 l : 9 d
10	*Nannochloropsis salina*	320.33	20 ± 2	7.0	18 l : 6 d
	Chlorella marina	201.66	20 ± 2	7.0	18 l : 6 d
	Isochrysis galbana	353.33	20 ± 2	7.0	18 l : 6 d
	Baker's Yeast	232.00	20 ± 2	7.0	18 l : 6 d

2. 4. Discussion

The type of feeds, its concentration and environmental factors like pH, salinity and photoperiod have vital impact in rotifer growth and multiplication. In the present investigation a highly significant ($P<0.001$) increase in rotifer population density and per day growth when *Isochrysis galbana* and *Nannochloropsis salina* were fed to the rotifers. The algal foods can yield substantially different reproductive rates (Hirayama *et al.*, 1979; Snell *et al.*, 1983; James and Rezeq, 1988). James and Rezeq (1989) on their observation show that increased rotifer productivity could be achieved while *Nannochloropsis* strain as compared to *chlorella* strain and also showed increased rotifer productivity with higher cell densities of micro algae. This is in accordance with the observation of James and Rezeq (1988). Reproductive rates of rotifers were higher when fed live or frozen *Nannochloropsis* than when fed Baker's yeast (Tamaru *et al.*, 1991; Hamada *et al.*, 1993; Lubzens *et al.*, 1995). When *Nannochloropsis* is fed to rotifer cultures the culture density starts increasing from the next day onwards and the doubling time was shortened to 2.254±0.0289 days. The instantaneous growth (K) for rotifers fed *Nannochloropsis salina* and *Isochrysis galbana* was higher (0.308±0.004) and (0.304±0.004) when compared with yeast and *Chlorella marina* enriched rotifers in the present study. The growth rate ranged from 0.193 and 0.325 when *Chlorella* density of 12.5 million/ ml and 50 million cells / ml was fed to rotifers (Razeq and James 1987). Per day growth of maximum 40.18±1.97 and 39.95± 1.68

was recorded when *I galbana* and *N salina* was fed to rotifers in the present study. A maximum production of 36.33±1.5 rotifers /ml /day was recorded and a population doubling time of 3.59±0.09 days observed when *Chlorella* sp. was fed to rotifers (Razeq and James 1987). It was also observed that the rotifer culture density reached its peak on day 5 when *Nannochloropsis* is fed to rotifers. In all other groups (different diets) it has taken six days to produce maximum culture density. *Isochrysis* fed rotifers are also equally good as that of *Nannochloropsis* fed rotifer group in term of per day growth and culture density, but there is a lag in the culture density of rotifer during the first two days of culture, it is also observed that *Isochrysis* cell density in the tubs remains unchanged during the first two days indicating there is some negligence in the feeding by rotifers. It is believed that *Isochrysis galbana* which is a biflagellate which moves very quickly in the medium that the rotifers may not filter it. Park *et al.*, 1999 evaluated the growth and nutritional quality of rotifer, *Brachionus rotundiformis* fed by different diets (fresh water *Chlorella*, Marine *Chlorella*, presently called as *Nannochloropsis* spp., and Omega yeast) for the high density cultivation which indicated that marine *Chlorella* could be appropriate diet for high density cultivation of rotifers.

In the present investigation the use of baker's yeast as feed for rotifers is also evaluated. It is observed that the per day growth and doubling time was considerably lower than that of other feeds. But baker's yeast fed rotifers is superior to *Chlorella* fed rotifers in culture density. A

total collapse of rotifer culture was observed when there was no water exchange from the rearing containers (Exp 2 and 5). The main cause of collapse is due to ciliate attack. Contamination of rotifer mass cultures with the ciliate *Euplotes sp.* reduces population growth of *B. rotundiformis* Tschugunoff (Hagiwara, *et al.*, 1995). Hirayama (1987) stated that mass cultures of rotifers were subjected to a sudden unexpected decrease or suppressed growth of the population, especially when fed with baker's yeast. Apart from the nutritional deficiencies in baker's yeast, another problem, which may cause the sudden decrease of rotifer population or suppressed growth, is the accumulation of faeces and uneaten food with consequent pollution of the culture medium (Hirata, 1979; Hirayama, 1987). Hirata (1979), Yufera and Pascual (1980) reported that rotifers cultured with baker's yeast alone could not be maintained for long periods and had lower reproductive rates than those cultured on algae. Problems encountered with the use of yeast include more frequent rotifer culture crashes and poor survival in target species that have high HUFA requirement (Fukusho, 1989 a; Hirayama and Funamoto, 1983). Hirayama and Funamoto (1983) found that vitamin B_{12} was essential for rotifer growth and baker's yeast was deficient in this vitamin. However, when yeast is supplemented with vitamin B_{12} the rotifers grew well and their eggs hatched successfully. The dietary value of baker's yeast supplemented with vitamin B_{12} was still much lower than that of *chlorella.*

Apart from the above mentioned feeds for rotifer culture there are many other feeds, which are now in use. There may be in dried form, or algal pastes certain bacteria, marine yeast or ω yeast and algal species like *Dunaliella* spp, *Phaedactylum tricornutum*, *Tetraselmis* spp., *Asteromonas gracilis*, *Chlorella vulgaris*. The rotifer *B. plicatilis* can be effectively fed on the halo tolerant algae *Asteromonas gracilis* (Hotos and Arvanidon1995), which is one of the biggest algae in terms of cellular volume. Rotifer does not exhibit any trend of avoidance for relatively large sized algal cells (*Asteromonas*: 16-20 μ m, *Dunaliella*: 10 μ m, *Tetraselmis*: 8-12 μ m) when supplied in combination with small microalgae like *Chlorella* and *Nannochloropsis*. A blue green alga (Cyanobacterium) *Synechocystis* sp. that can be cultured at temperature between 25°C and 35°C in simple economical medicines for mass culture was found to be useful feed for the mass culture of rotifers, giving better growth of the rotifers than other micro algae. (Sakamoto *et al.,* 1998). The dietary value of rotifers cultured with *Synechocystis* is lower in *n*-3 HUFA. The level of *n*-3 HUFA of such rotifers was enhanced by secondary culture with *Nannochloropsis* for 24 hrs and these rotifers are nutritionally rich (*n*-3 HUFA) when compared with rotifers, which are fed only with *Nannochloropsis*. While selecting feed for culturing rotifers one should keep in mind that the particular feed not only hikes the population density of the mass culture but also improves rotifers nutritional quality especially the fatty acid profile since the developing fin fish and shell fish larvae require fatty acids (*n*-3 and *n*-6) for their normal growth and the development of neural tissues. James *et al.* (1987) suggest that the

nutritional quality of marine yeast is better than that of baker's yeast. Gatesoupe and Robin (1981) who obtained 35 - 55 rotifers $ml^{-1}d^{-1}$ while using commercial single cell proteins such as *Spirulina, Chlorella* and methanol grown yeast, for feeding rotifer. Maruyama *et al.*, (1997) states that condensed suspension of *Chlorella vulgaris* was used for the food of the rotifer *B. plicatilis* and *B. rotundiformis* in place of *Nannochloropsis* sp. The cell compounds of *C. vulgaris* such as protein content, amino acid minerals and vitamins are generally similar to those of *N. oculata.*

Regarding the physico chemical parameters like salinity, pH and photoperiod, which were also included during the present study, revealed that *B.rotundiformis* reared in less salinity 20 to 25 ppt salinity, produces more rotifer nos/ml compared with higher salinities. Rotifer growth and productivity were inversely related to increasing salinity in the culture system. Rotifer filtration rates vary with salinity and are reduced at high salinities. 15 - 20 ppt salinity will be more conducive to produce nutritionally enriched S. type rotifers. Growth rate responses to salinity and temperature of *B. rotundiformis* are better adapted to low salinity – high temperature (Serra *et al.*, 1998). Fielder *et al.*, (2000) stated that the effect of salinity on the availability of rotifers in the water column was greater than the effect of temperature.

Acidic media are best for rotifer culture pH with 7 gives maximum rotifer density (Exp III). The pH adjustment was done by the addition of HCl

(35%) and NaOH (10) and also with buffer capsules. By keeping the pH at 7 the concentration of undissociated ammonia can be controlled below lethal. Yoshimura *et al.*, (1995) states that rotifer culture medium with pH has the undissociated ammonia less than 10 ppm and the rotifer population in this media is remarkably higher. The algal productivity (*Chlorella*) and the *n*-3 PUFA are considerably higher when the pH was lowered from 8.5 to 6.5 (James *et al.*, 1988). The total *n*-3 PUFA content was significantly elevated at pH 6.5 compared with other pH regime. Further more, the content increased with decreasing pH of up to 6.5. The total lipid content also showed an increase with decreasing pH from 8.5 to 6.0.

Reproductive rates are affected by pH of the culture and a pH ranging from 7 to 8 is considered adequate for culturing rotifers (Furukawa and Hidaka, 1973; Lubzens, 1987; Fulks and Main, 1991).

Regarding the photoperiodicity for rotifer culture an 18 h light to 6 hrs darkness had given maximum rotifer production. White fluorescent lights are provided during night hours, which also stimulated the multiplication of algal diets in the rotifer culture tubs.

It is being concluded that rotifers can be fed with *Nannochloropsis salina* for high population density in a shorter time. It is also suggested that *Isochrysis galbana* is equally good, the best culture condition for the growth

of rotifer *B. rotunidiformis* strain having the temperature >30°C, pH 6.8-7.2 salinity 20-25 ppt and photoperiod 18l: 5d.

3. Fatty acid enrichment and biochemical analysis of enriched rotifers

3. 1. Introduction

Lipids play an important role in fish eggs and larvae as sources of metabolic energy and as essential materials for the formation of cells and tissue membranes. About 34-43% of the lipids in rotifers are phospholipids and 20-55% are triacylglycerols, diacylglycerols, sterols, sterol esters and free fatty acids (Teshima *et al.*, 1987; Frolov *et al.*, 1991; Nagata & Whyte, 1992; Fernandez-Reiriz *et al.*, 1993; Rainuzzo *et al.*, 1997). Rotifers are regarded as 'living food capsules' for transferring nutrients to fish larvae (Lubzens et al,.1989). These nutrients in the rotifers include highly unsaturated fatty acids (mainly 20:5*n*-3 and 22:6*n*-3) essential for survival of marine fish larvae. By using a high quality feed, that is a mixture of algae and other enrichments, the essential fatty acids and other dietary components could be transferred from algae via the rotifers to the marine fish larvae (Watanabe *et al.*, 1983; Segner *et al.*, 1984). Two types of products were used for enrichment, the commercial ones (Selco) and the one produced in the laboratory with different vegetable and animal oils (i.e., soya bean, sun flower, peanut, squid, cod-liver (Narciso *et al.*, 1999); shark liver (present study); sardine, tuna orbital (Navarro *et al.*, 1995, Bruce *et al.*, 1999); and *Boops boops* (Thrush *et al.*, 1993). More recently, bacteria isolated from Antarctica and containing high levels of EPA were suggested as a potential alternative enrichment food for rotifers (Nichols *et al.*, 1996).

The lipid content of rotifers is usually lower than that of their food sources, indicating that lipids are utilized by the rotifers. Rotifers utilize relatively more DHA in highly reproducing cultures (Oie and Olsen, 1997) and lipid utilization is temperature dependent (Olsen *et al.*, 1993). They accumulate about 3-5 times more total lipids when they are kept at 10^0 C than at 25^0 C (Lubzens *et al.*, 1995). These results suggest that higher enrichment levels could be obtained if this procedure is performed at relatively low temperatures (depending on the rotifer strain), where reproductive rates and utilization rates are slowed down.

Enriched rotifers have been shown to be able to maintain their improved nutritional value for several hours at 10°C during their application as live-feed for marine fish larvae (Rainuzzo *et al.*, 1989) at this temperature. Olsen *et al.* (1993) demonstrated that daily losses of total lipids and (*n*-3) fatty acids are about 4 and 10% per day respectively.

3. 2. Material and Methods

3. 2. 1. Enrichment of rotifers with oil emulsion, micro algae and yeast

The enrichment strategy was to incorporate globule generated from a blend of 10 ml egg yolk (as emulsifying agent), 5 ml shark liver oil and 100 ml seawater into rotifers. The mixture was blended using a hand blender for 15-20 minutes at a speed of 3000 rpm (Plate VI a) to

Plate – VI

a) **Set up for oil enrichment**

b) **Oil globules for enriching rotifers**

c) **Rotifer enriched with oil globules**

produce fine oil globules of size range 8-14 μm (Plate VI b). This mixture was then passed through 20 μm mesh to separate bigger particles. The oil emulsion was collected in small plastic containers and kept in the freezer for daily use. The oil emulsion taken out of the freezer for daily enrichment was hand blended prior to drawing of aliquots for even distribution of oil globules. Rotifers, which were fed only with yeast, were used for oil enrichment purpose (Plate VI c and VII b). Enrichment of rotifers was carried out by taking 1.5ml of this mixture per litre of enrichment medium at a maximum density of 300-350 rotifers/ml (starved rotifers) for a period of 12 hrs (Plate VII a, and c and Plate VIII a and b). After bio-encapsulation the rotifers were properly washed using filtered seawater and were collected in small vials for fatty acid analysis. Bio encapsulation of rotifers using oil emulsion was done with triplicates. Bio encapsulated rotifers were stored in the freezer at –82°C.

The enrichment strategy to incorporate micro algae and yeast is as follows. Starved rotifers were introduced at 450-500 nos/ml in five different plastic buckets containing *N. salina* (15-20x10^6 cells/ml); *I. galbana* (10-15x10^6 cells/ml); *C. marina* (15-16x10^6 cells/ml), and *Saccharomyces cerevisiae* (1gm/million rotifers). All the enrichment containers were well aerated and kept for 12 hrs (Plate V11 a and c; Plate VIII a and b). These enriched rotifers were collected, washed properly and stored in vials under -82°C. Total lipids were extracted from

Plate – VII

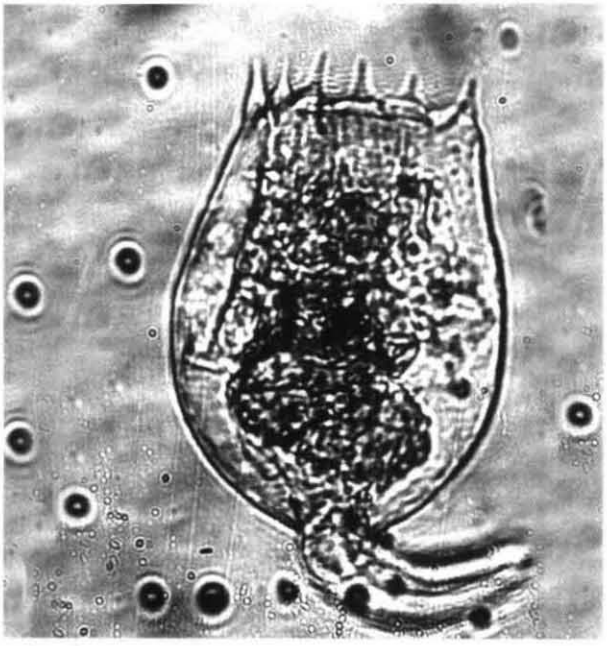
a) **Enrichment of rotifers with *Chlorella marina.***

b) **Enrichment of rotifers with Oil emulsion.**

c) **Enrichment of rotifers with *Nannochloropsis salina.***

Plate VII

a



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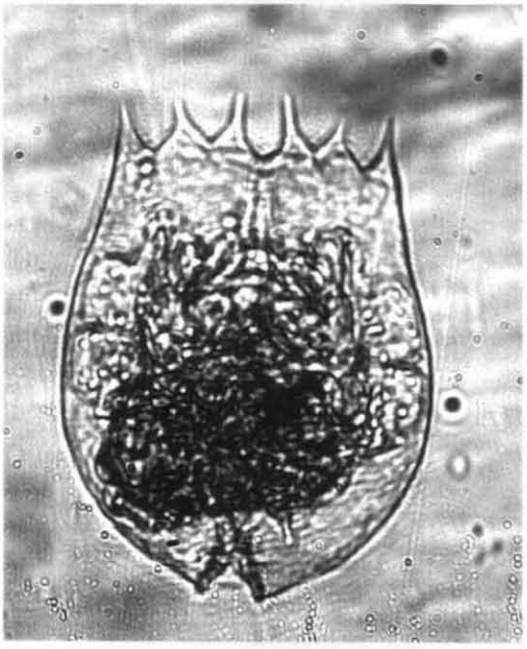

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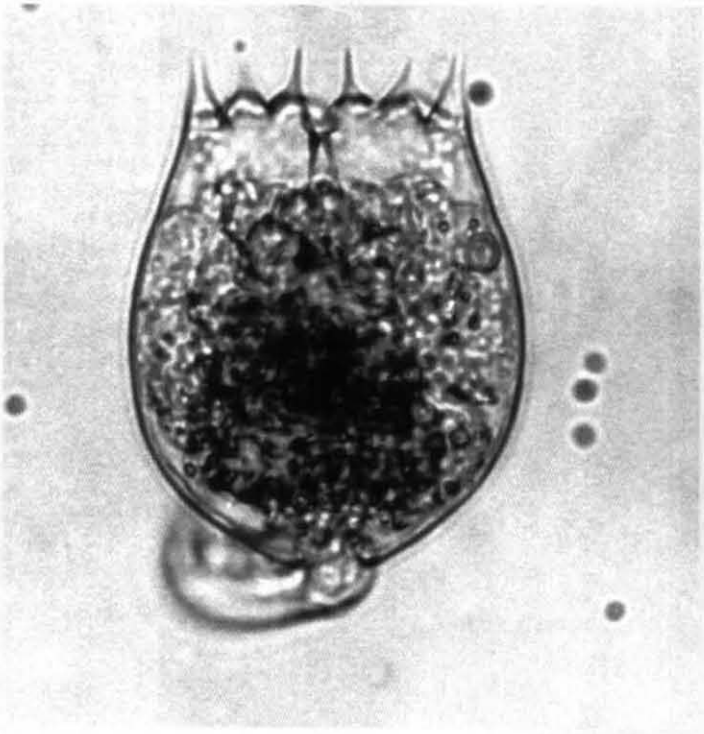


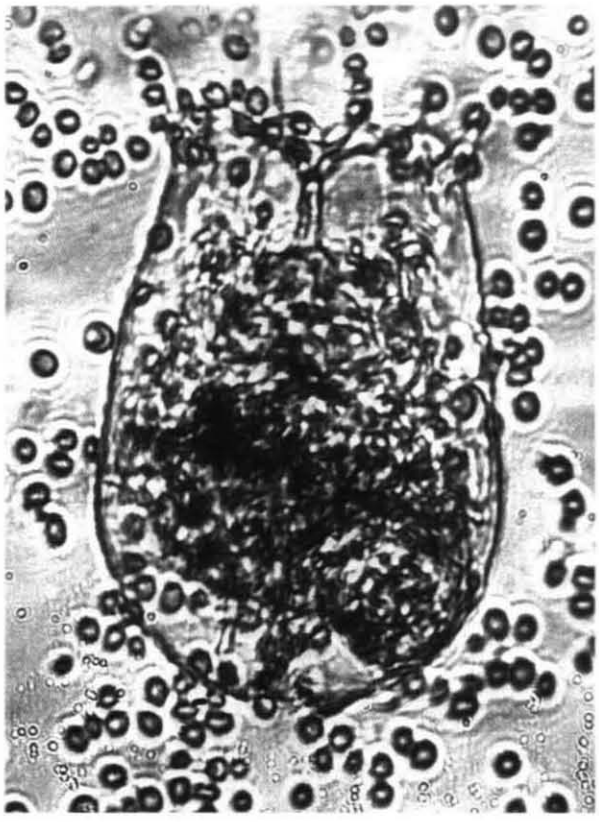
Plate – VIII

a) Enrichment of rotifers with yeast.

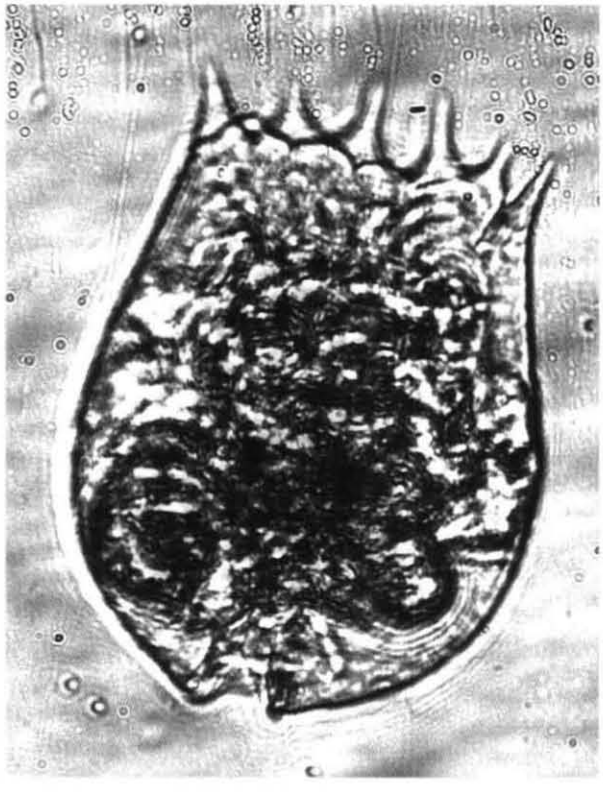
b) **Enriched rotifer with *Isochryisis galbana.***

Plate VIII

a



b



the enriched rotifers using Bligh and Dyer method (1959) at CMFRI laboratory and the fatty acid analysis using Gas Chromatography (Chrompak CP- 9001, Gas Chromatograph) was done in CIFT.

3. 2. 2. Fatty acid analysis of Enriched/ Bio encapsulated rotifers

a. Sample collection

Microalgae enriched rotifers, yeast enriched rotifers and bio encapsulated rotifers (oil emulsion) were used for fatty acid analysis (AOAC 2000).

b. Sample protection

Precautions were taken to ensure that there is no degradation of lipids during storage, extraction and saponification. All the solvents were flushed with N_2 before being placed in freezers.

c. Sample preparation

Two samples were analysed from each experiment. Total lipids were extracted from the rotifers by the method of Bligh and Dyer (1959). After saponification, saponifiable materials were recovered and fatty acids were converted to fatty acid methyl esters (FAME) (Metcalfe *et al.*, 1966).

d. Lipid extraction

Moist filter paper was used to remove excess moisture. About (1-3 gm) sample weighed and homogenized using a porcelain mortar with a solvent mixture consisting of 15 volumes of Chloroform-Methanol (2:1 V/V). The mixture was filtered through a Buchner funnel, transferred to

a measuring cylinder and noted the total volume. To this about 0.2 of its volume (V/V) of water was added to facilitate phase separation and the whole extract was transferred to a separating funnel shaken thoroughly and allowed to separate overnight at low temperature in nitrogen atmosphere. From the resultant biphasic solution, bottom chloroform layer containing the purified lipid was collected and dried with anhydrous sodium sulphate till the solution became clear and filtered. The total volume of the chloroform extract was noted and small quantity was taken in a pre-weighed vial and the lipid content was estimated by gravimetry. The chloroform extract was evaporated in a vacuum flash evaporator and lipid was stored in a small volume of chloroform in a deep freezer till further analysis.

e. Saponification

About 0.5 gm of lipid sample was accurately weighed and evaporated to dryness in a round bottom flask. To this 30 ml methanol and 1.5 ml 150% (15 gm in 100 ml) KOH were added and refluxed for 2 hrs. When the contents of the flask were still warm extracted with 30 ml petroleum ether (60°- 80°), in a separating funnel to remove the non-saponifiable matter (NSM). On separation, the bottom aqueous layer contained fatty acid and top petroleum ether layer contained NSM. Both the layers were collected separately and the aqueous layer was extracted twice more to remove the NSM completely.

The combined aqueous layer was acidified with concentrated HCl (about 1-2 ml HCl was added drop by drop till the solution became acidic and tested for pH and extracted again with 30ml petroleum ether to recover the fatty acids. The ether extract was washed with water, dried over anhydrous sodium sulphate and filtered.

f. Esterification

The fatty acid portion was taken in a round bottom flask, solvent was evaporated and to this 15 ml BF_3MeOH was added. The contents of the flask were refluxed for 6 minutes, cooled and 6 ml of saturated sodium chloride solution was added. From this methyl esters were separated by extraction (3 times) with 25 ml petroleum ether (PE). The combined ether extract was washed with water, dried over anhydrous sodium sulphate, filtered and evaporated in a rotary evaporator. The residual Fatty Acid Methyl Esters (FAME) were re-dissolved in chloroform (2-3 ml), collected in small Teflon copper vials, flushed with N_2 and stored in deep freezer until further analysis.

g. Gas Chromatograph analysis

Separation and quantification of fatty acid methyl esters were carried out on Gas Chromatograph varian equipped with FID and Heliflex AT- 225 capillary column (30m x 0.53 mm i.d. 0.5 μ m). The column was programmed after an initial hold of 1 min from 120^0C to 200^0C at a rate of 16^0C per minute, from 200^0C to 220^0C at a rate of 2^0c per minute and maintained 220^0C for10 min. The injector and detector

were maintained at 250^{0}Cand 280^{0}C respectively . Nitrogen was used as carrier gas at a pressure of 6 psi. The FAME were identified according to their retention times and quantified by external standard methods. 1 µl of the sample was injected into the gas chromatograph for analysis.

3. 2. 3. Statistical analysis

One-way analysis of variance (ANOVA) was used to detect differences ($P< 0.05$) in the fatty acid composition of rotifers enriched with different diets, which are the sources of variation (unsaturated fatty acids, *n*-3 fatty acids, HUFA's, PUFA's and ratios like *n*-3/*n*-6, DHA/EPA, EPA/AA were tried). The means of five diets were compared using Duncan's multiple range test (DMRT), by which homogenous grouping of the different diets were identified.

3. 3. RESULTS

3. 3. 1. Enrichment of rotifers with oil emulsion, micro algae and yeast

Rotifers after 12 hrs enrichment were collected and the samples were observed under microscope and confirmed that rotifer gut were full of respective enrichment diets.

3. 3. 2. Fatty acid analysis of Enriched/ Bio encapsulated rotifers

Bio-encapsulated rotifers (shark oil emulsion) were fed, to the larvae of *Amphiprion sebae* and *Penaeus monodon* respectively. Rotifer

samples were analysed in duplicates and both saturated and unsaturated fatty acid percentage in total area were detected (Table 3. 1). Recognising the importance of unsaturated fatty acids only MUFA's, PUFA's and HUFA's were taken into consideration. (palmitoleic acid) 16:1-*n*7 content was high in *Nannochloropsis salina* (*Na*R) and *Isochrysis galbana* (*Ig*R), fed rotifers (17.19 ± 0.99, 16.005 ± 0.805) followed by yeast fed rotifers (14.51 ± 0.21) (YR). Palmitoleic acid content was very low in oil enriched rotifers (OER) (4.09 ± 0.02). It was absent in *Chlorella* fed rotifers (*Cl*R).

The highest concentration of oleic acid (18:1*n*9) was recorded in yeast fed rotifers (28.96 ± 0.08) followed by *Isochrysis* fed rotifers (12.7 ± 0.55). Oleic acid was also present in good quantity in *Nannochloropsis* fed rotifers (9.02 ± 0.28) and oil enriched rotifers (7.26 ± 0.025). *Chlorella* fed rotifers contained only trace amounts of oleic acid (0.26 ± 0.02).

The total MUFA content was high in yeast enriched rotifers (43.47 ± 0.13) followed by *Isochrysis galbana* enriched rotifers (28.705 ± 0.25) and *Nannochloropsis* enriched rotifers (26.21 ± 0.71). In oil enriched rotifers the total MUFA content was (11.84 ± 0.005). In *Chlorella* enriched rotifers the total MUFA was significantly low (0.26 ± 0.002).

Table 3. 1. Fatty acid composition (area percentage) of total lipids of rotifers nutritionally enriched with different enrichment diets

Fatty acids	*N.salina*	*C.marina*	*I.galbana*	Shark oil emulsion	*S.cerevisiae*
8 : 0	0.495 ± 0.025	0.69 ± 0.06	0.085 ± 0.015	0	0.05 ± 0.015
10 : 0	0	0	0	0	0
12 : 0	0.31 ± 0.03	0.455 ± 0.025	0.36 ± 0.06	0	0.29 ± 0.015
14 : 0	6.45 ± 0.35	4.615 ± 0.495	6.25 ± 0.36	0.32 ± 0.035	4.54 ± 0.16
16 : 0	36.61 ± 1.19	28.925 ± 0.575	32.185 ± 0.625	8.12 ± 0.28	16.16 ± 0.43
16 : 1*n*7	17.19 ± 0.99	0	16.005 ± 0.805	4.09 ± 0.02	14.51 ± 0.21
18 : 0	0.56 ± 0.04	6.32 ± 0.31	7.335 ± 0.385	6.69 ± 0.07	4.16 ± 0.085
18 : 1*n*9	9.02 ± 0.28	0.26 ± 0.02	12.7 ± 0.55	7.26 ± 0.025	28.96 ± 0.08
18 : 2*n*-6	7.13 ± 0.37	29 ± 1.3	6 ± 0.2	8.62 ± 0.295	8.17 ± 0.12
18 : 3*n*-3	0	17.55 ± 2.35	0	21.03 ± 0.22	1.46 ± 0.185
20 : 0	0	0	0	0.32 ± 0.035	7.62 ± 0.13
20 : 1	0	0	0	0.49±0.04	0
20 : 4*n*-6	0.405 ± 0.025	0	0.12 ± 0.02	2.09 ± 0.055	0
20 : 5*n*-3	16.685 ± 0.385	0	13.925 ± 0.725	29.12 ± 0.17	0
22 : 6*n*-3	0.79 ± 0.03	0	0.38 ± 0.03	4.08 ± 0.27	0
Unidentified	4.08 ± 0.26	8.535 ± 0.145	2.245 ± 0.155	7.71 ± 0.055	12.11 ± 0.125
∑ saturated	44.425 ± 0.795	41.005 ± 0.195	46.215 ± 1.295	15.45 ± 0.42	32.82 ± 0.665
∑ unsaturated	51.22 ± 0.78	46.81 ± 3.67	49.13 ± 1.17	76.78 ± 0.465	53.1 ± 0.065
∑ HUFA	17.475 ± 0.415	0	14.305 ± 0.695	33.2 ± 0.1	0
∑ *n*-3 fatty acids	17.475 ± 0.415	17.55 ± 2.35	14.305 ± 0.695	54.23 ± 0.12	1.46 ± 0.185
∑ *n*-6 fatty acids	7.535 ± 0.345	29 ± 1.3	6.12 ± 0.22	10.71 ± 0.35	8.17 ± 0.12
∑ MUFA	26.21 ± 0.71	0.26 ± 0.02	28.705 ± 0.255	11.84 ± 0.005	43.47 ± 0.13
∑ PUFA	7.535 ± 0.345	46.55 ± 3.65	6.12 ± 0.22	31.74 ± 0.57	9.63 ± 0.065
n-3/*n*-6	2.326576 ± 0.161	0.602751 ± 0.054	2.33636 ± 0.030	5.06349 ± 0.165	0.178703 ± 0.026
DHA/EPA	0.047348 ± 0.07792	0	0.027289 ± 0.041	0.14011 ± 1.588	0
EPA/AA	41.19753 ± 15.4	0	116.0417 ± 36.25	13.933 ± 3.090909	0

(Values are means for two replicate samples ± S.E.M) DHA: EPA: AA ratios: *Nannochloropsis* fed rotifers - 0.4:8.4:0.2, *Chlorella* fed rotifers - 0, *Isochrysis* fed rotifers - 0.2:7.0:0.05, Shark liver oil fed rotifers - 1:7:0.5,*Saccharomyses* fed rotifers- 0.

The major PUFA's present in rotifers enriched with different microalgae, yeast and oil emulsion were 18:2*n*-6 (Linoleic acid) 18:3*n*-3 (Linolenic acid) and 20:4*n*-6. (Arachidonic acid. AA). 18:2*n*-6 was present in maximum quantity in *Chlorella* enriched rotifers (29 ± 1.3). In *Nannochloropsis* enriched rotifers it was significantly low (7.13 ± 0.37), and was comparatively lower in *Isochrysis* enriched rotifers (6 ± 0.2). In oil enriched rotifers percentage was slightly high (8.62 ± 0.295) followed by yeast-enriched rotifers (8.17 ± 0.12). Linolenic acid (18:3*n*-3) was high in oil-enriched rotifers (21.03 ± 0.22) followed by *Chlorella* enriched rotifers (17.55 ± 2.35). Trace quantity was detected in yeast enriched rotifers (1.46 ± 0.185) and was not present in detectable levels in *Nannochloropsis* and *Isochrysis* enriched rotifers.

Arachidonic acid (AA) was relatively high in oil-enriched rotifers (2.09 ± 0.055) followed by *Nannochloropsis* enriched rotifers (0.405 ± 0.025). The mean percent in *Isochrysis* enriched rotifers was (0.12 ± 0.02). Arachidonic acid was totally absent in *Chlorella* and yeast enriched rotifers.

The total PUFA fraction was highest in *Chlorella* enriched rotifers (46.55 ± 3.65) followed by oil enriched rotifers (31.74 ± 0.57). In the yeast enriched rotifers the mean percent was (9.63 ± 0.065). In *Nannochloropsis* enriched rotifers the mean percent was (7.535 ± 0.345) and in *Isochrysis* enriched rotifers the mean percent was (6.12 ± 0.22).

The major HUFA's present in the analysed rotifers were 20:5*n*-3 (eicosapentaenoic Acid, EPA) and 22:6*n*-3 or (docosahexaenoic acid DHA). EPA was very high in oil enriched rotifers (29.12 ± 0.17) followed by *Nannochloropsis* enriched rotifers (16.685 ± 0.385), and *Isochrysis* enriched rotifers (13.925 ± 0.725). EPA was totally absent in *Chlorella* and yeast enriched rotifers. The mean percent of DHA in oil-enriched rotifers was 4.08 ± 0.27 followed by *Nannochloropsis* enriched rotifers (0.79 ± 0.003) and *Isochrysis* enriched rotifers (0.38 ± 0.03).

The total HUFA fraction was highest in oil enriched rotifers (33.2 ± 0.1) followed by *Nannochloropsis* enriched rotifers (17.475 ± 0.415), and *Isochrysis* enriched rotifers (14.305 ± 0.0695). HUFA's were not detected in *Chlorella* and yeast fed rotifers.

The total saturated fatty acid fraction in each sample was calculated and was found to be high in *Isochrysis* enriched rotifers (46.215 ± 1.295), followed by *Nannochloropsis* enriched rotifers (44.425 ± 0.795) and *Chlorella* enriched rotifers (41.005 ± 0.195). The mean percent in yeast and oil enriched rotifers were 32.82 ± 0.665 and 15.45 ± 0.424 respectively.

The total unsaturated fatty acid fraction in the enriched rotifers was calculated and is as follows follows. Oil enriched rotifers 76.78 ±

0.465; *Nannochloropsis* enriched rotifers 51.22 ± 0.078; yeast enriched rotifers 53.1 ± 0.065; *Isochrysis* enriched rotifers 49.13 ± 1.17; and *Chlorella* enriched rotifers 46.81 ± 3.67.

The total *n*-3 fatty acid fraction present in each sample was calculated. In oil enriched rotifers the n-3 fatty acid content was high (54.23 ± 0.12) followed by *Chlorella* enriched rotifers (17.55 ± 2.35) and *Nannochloropsis* enriched rotifers (17.475 ± 0.415), and *Isochrysis* enriched rotifers (14.305 ± 0.695) and low in yeast fed rotifers (1.46 ± 0.185).

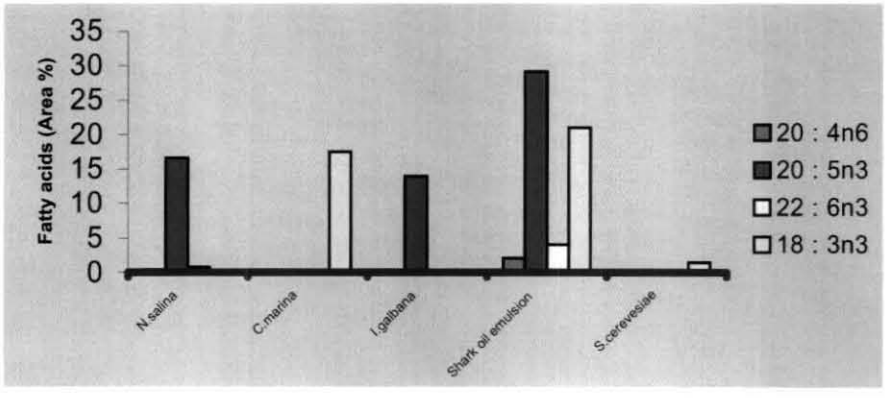
The total *n*-6 fatty acids fraction was high in *Chlorella* enriched rotifers (29 ± 1.3) followed by oil-enriched rotifers (10.71 ± 0.35), yeast enriched rotifers (8.17 ± 0.12), and in *Nannochloropsis* and *Isochrysis* enriched rotifers it was 7.535 ± 0.345 and 6.12 ± 0.255 respectively.

The *n*-3/*n*-6 ratio was high in oil-enriched rotifers (5.0634 ± 0.16505) followed by *Isochrysis* enriched rotifers (2.3363 ± 0.02957) and *Nannochloropsis* enriched rotifers (2.32657 ± 0.161602). The n-3/n-6 ratio was very poor in *Chlorella* enriched rotifers (0.60275 ± 0.05401) and in yeast enriched rotifers (0.178703 ± 0.026033). The DHA/EPA ratio was comparatively high in oil enriched rotifers (0.14011 ± 1.588) followed by *Nannochloropsis* fed rotifers (0.047348 ± 0.07792) and *Isochrysis* fed rotifers (0.027289 ± 0.041).

The EPA/AA ratio was very high in *Isochrysis* enriched rotifers (116.0417 ± 36.25) followed by *Nannochloropsis* enriched rotifers (41.19753 ± 15.4) and oil enriched rotifers (13.933 ± 3.09).

The post ñenrichment DHA: EPA: AA ratios rotifers for were also studied. Shark liver oil enriched rotifers was found to be superior to other diets with the ratio of 1:7:0.5.The ratios for other diets were as follows: *Nannochloropsis* enriched rotifers the 0.4:8.4:0.2; and *Isochrysis* enriched rotifers, 0.2:7.0:0.05. Since the level of DHA, EPA and AA were not in detectable levels, no ratio can be developed for *Chlorella* and yeast enriched rotifers. Variations of essential fatty acids of rotifers enriched with selected diets are given in the Figure 3. 1.

Fig. 3. 1. Variation of essential fatty acids of rotifers enriched with selected diets



3. 3. 3. Statistical analysis

ANOVA (Table 3. 2.) shows the fatty acid profile of rotifers enriched with various micro algae, yeast and shark liver oil emulsion. The enrichments effects are highly significant ($P < 0.01$). The mean values of total unsaturated fatty acids, PUFA's, HUFA's, *n*-3 fatty acids and ratios like *n*-3/*n*-6, DHA/EPA, EPA/AA were significantly higher ($P < 0.05$), in the oil emulsion enriched rotifers compared with other diets. Exceptionally high PUFA content was recorded in rotifers enriched with *Chlorella marina* (46.55) and the ratio of EPA/AA in rotifers enriched with *Isochrysis galbana* (118.321). The high PUFA content in *Chlorella marina* enriched rotifers was mainly due to the contribution of 18:3*n*-3 to the total PUFA content. The DMRT performed on the total unsaturated fatty acid levels showed the existence of two homogenous groups among the five levels of diets. The groupings are (1) Diet 4 i.e., shark liver oil emulsion enriched rotifers alone forming one group and (2) Diets 1, 2, 3 and 5 forming another group with different mean values i.e., *N. salina, C. marina, I. galbana* and *S. cerevisiae* respectively (Table 3. 3).

Table 3. 2. ANOVA for selected fatty acids of rotifers enriched with different diets

Source of variation		Sum of squares	df	Mean Square	F	Significance
Unsaturated fatty acids	Feed	1,147.112	4	286.778	45.762	0.000
	Error	31.333	5	6.267		
HUFA	Feed	1,543.635	4	385.909	1,450.239	0.000
	Error	1.331	5	0.266		
***n*-3**	Feed	3,080.076	4	770.019	309.176	0.000
	Error	12.453	5	2.491		
PUFA	Feed	2,567.332	4	641.833	116.114	0.000
	Error	27.638	5	5.528		
***n*-3/*n*-6**	Feed	31.442	4	7.860	339.814	0.000
	Error	0.116	5	0.023		
DHA/EPA	Feed	0.031	4	0.008	164.305	0.000
	Error	0.000	5	0.000		
EPA/AA	Feed	19,726.12	4	4,931.529	64.974	0.000
	Error	379.50	5	75.900		

Table 3. 3. DMRT grouping for total unsaturated fatty acid fraction of rotifers enriched with various diets

Sl.No	Name of the diet fed to rotifers	*Mean values of unsaturated fatty acids
1	*Nannochlorpsis salina*	51.22^{b}
2	*Chlorella marina*	46.81^{b}
3	*Isochrysis galbana*	49.13^{b}
4	*Shark liver oil emulsion*	76.31^{a}
5	*Saccharomyces cerevisiae*	53.04^{b}

*Means within the row not bearing the same letter superscripts are significantly different

The DMRT performed on the total HUFA's showed the existence of four homogenous groups among the five levels of diets. The groupings are (1) Diet 4 i.e., shark liver oil emulsion enriched rotifers (2) Diet 1 i.e., *N. salina* enriched rotifers, (3) Diet 3 i.e., *I. galbana* enriched

rotifers and (4) Diet 2 and 5 i.e., *C. marina* and *S. cerevisiae* respectively (Table 3. 4).

Table 3. 4. DMRT grouping for total HUFA fraction of rotifers enriched with various diets

Sl. No.	Name of the diet fed to rotifers	*Mean values of highly unsaturated fatty acids
1	*Nannochlorpsis salina*	17.48^{b}
2	*Chlorella marina*	0.00^{d}
3	*Isochrysis galbana*	14.30^{c}
4	*Shark liver oil emulsion*	33.30^{a}
5	*Saccharomyces cerevisiae*	0.00^{d}

*Means within the row not bearing the same letter superscripts are significantly different

Table 3. 5. DMRT grouping for total PUFA fraction of rotifers enriched with various diets

Sl. No	Name of the diet fed to rotifers	*Mean values of poly unsaturated fatty acids
1	*Nannochlorpsis salina*	7.54^{c}
2	*Chlorella marina*	46.55^{a}
3	*Isochrysis galbana*	6.12^{c}
4	*Shark liver oil emulsion*	31.17^{b}
5	*Saccharomyces cerevisiae*	9.70^{c}

*Means within the row not bearing the same letter superscripts are significantly different

The DMRT performed on the total PUFA's showed the existence of three homogenous groups among five levels of diets. The groupings are (1) Diet 2 i.e., *C. marina* enriched rotifer, (2) Diet 4 i.e., shark liver oil

emulsion enriched rotifer and (3) Diet 5, 1 and 3 i.e., *S. cerevisiae, N. salina* and *I. galbana* enriched rotifer respectively (Table 3. 5).

The DMRT performed on the total *n*-3 fatty acids showed the existence of 3 homogenous groups among the five levels of enrichment. The groupings are: (1) Diet 4 i.e., Shark liver oil emulsion enriched rotifers (2) Diet 1, 2 and 3 i.e., *N. salina, C. marina* and *I. galbana* respectively and (3) Diet 5 i.e., *S. cerevisiae* enriched rotifer (Table 3. 6).

Table 3. 6. DMRT grouping for total *n*-3 fatty acids of rotifers enriched with various diets

Sl. No.	Name of the diet fed to rotifers	*Mean values of *n*-3 fatty acids
1	*Nannochlorpsis salina*	17.46^{b}
2	*Chlorella marina*	17.55^{b}
3	*Isochrysis galbana*	14.31^{b}
4	*Shark liver oil emulsion*	54.11^{a}
5	*Saccharomyces cerevisiae*	1.65^{c}

*Means within the row not bearing the same letter superscripts are significantly different

The DMRT performed on the *n*-3/*n*-6 ratio showed the existence if four groupings among the five levels of diets. The groupings are: (1) Diet 4 i.e., shark liver oil emulsion enriched rotifers, (2) Diet 1 and 3 i.e., *N. salina* and *I. galbana* enriched rotifers, (3) Diet 2 i.e., *C. marina* enriched rotifers and (4) Diet 5 i.e., *S. cerevisiae* enriched rotifers (Table 3. 7).

Table 3. 7. DMRT grouping for *n*-3/*n*-6 fatty acid ratio of rotifers enriched with various diets

Sl.No.	Name of the diet fed to rotifers	*Mean values of *n*-3/*n*-6 fatty acids ratio
1	*Nannochlorpsis salina*	2.33^{b}
2	*Chlorella marina*	0.60^{c}
3	*Isochrysis galbana*	2.34^{b}
4	*Shark liver oil emulsion*	5.23^{a}
5	*Saccharomyces cerevisiae*	0.20^{d}

*Means within the row not bearing the same letter superscripts are significantly different

The DMRT performed on the DHA/EPA ratio showed the existence of four homogenous groups among the five levels of diets. The groupings are: (1) Diet 4 i.e., shark liver oil emulsion enriched with rotifers, (2) Diet 1 i.e., *N. salina* enriched rotifers, Diet 3 i.e., *I. galbana* enriched rotifers and (4) Diet 2 and 5 i.e., *C. marina* and *S. cerevisiae* enriched rotifers respectively (Table 3. 8).

Table 3. 8. DMRT grouping for DHA/EPA fatty acid ratio of rotifers enriched with various diets

Sl.No.	Name of the diet fed to rotifers	*Mean values of DHA/EPA ratio
1	*Nannochlorpsis salina*	0.05^{b}
2	*Chlorella marina*	0.00^{d}
3	*Isochrysis galbana*	0.03^{c}
4	*Shark liver oil emulsion*	0.15^{a}
5	*Saccharomyces cerevisiae*	0.00^{d}

*Means within the row not bearing the same letter superscripts are significantly different

The DMRT performed on the EPA/AA ratio showed the existence of three homogenous groups among the five levels of diets. The groupings are: (1) Diet 3 i.e., *I. galbana* enriched rotifers (2) Diet 1 i.e., *N. salina* enriched rotifers and (3) Diets 4, 5 and 2 i.e., Shark liver oil emulsion, *S. cerevisiae* and *C. marina* enriched rotifers respectively (Table 3. 9).

Table. 3. 9. DMRT grouping for EPA/AA fatty acid ratio of rotifers enriched with various diets

Sl. No.	Name of the diet fed to rotifers	*Mean values of EPA/AA ratio
1	*Nannochlorpsis salina*	41.30^{b}
2	*Chlorella marina*	0.00^{c}
3	*Isochrysis galbana*	118.32^{a}
4	*Shark liver oil emulsion*	14.23^{c}
5	*Saccharomyces cerevisiae*	0.00^{c}

*Means within the row not bearing the same letter superscripts are significantly different.

3. 4. Discussion

Rotifer's produced from the mass culture for the larval rearing of fin fishes and shell fishes lack adequate amounts of nutrients especially fatty acids and must be enriched with various marine oils and micro algae. The fatty acid composition of rotifers largely reflects their food, indicating that the ingested lipids are hydrolysed in the gut, reabsorbed, metabolised and incorporated into body phospholipids. The fatty acids are stable and do not change considerably after 1 or 2 days of starvation

(Lubzens *et al*, 1985). De *novo* synthesis of PUFA by rotifers was suggested by Lubzens *et al.* (1985) but the rate of synthesis is very low. In order to supply large amounts of PUFA to marine fish larvae; rotifers must be fed a PUFA rich food.

The nutritional quality of rotifers was improved by feeding them on algae or, on emulsified Pollock or cuttle fish oil prior to their transfer into fish tanks (Watanabe *et al.*, 1983). Several commercial oils of plants and animal origin were tested in order to improve the HUFA content and the DHA: EPA ratio. The relationship between the *n*-3 and *n*-6 fatty acid series, and more recently, the DHA/ EPA ratio, EPA/AA ratio, and DHA: EPA: AA ratio seems to be indicators of best survival and growth of marine fish larvae (Sargent *et al.*, 1997). Plant oils like linseed oil, peanut oil, sunflower oil, soyabean oil and the animal oils derived from tuna orbital, squid, sardine, cod, shark etc Selco, Super Selco and Topal emulsions (Artemate systems, Belgium), are used for enrichment. Oil emulsions of plant origin give very poor results in relation to either HUFA content or DHA: EPA ratio and EPA: AA ratio. All the oil emulsions from animal origin resulted in HUFA incorporation (Narciso *et al.*, 1999). Shark liver oil which was used as one of the enrichment diets in the present study is a very good source of HUFA's (32.2%) and the total unsaturated fatty acids percentage is high (76.78%). Shark liver oil is very cheap next to sardine oil and is available through out the world with an uninterrupted supply. Compared to sardine oil and squid liver oil the

Table 3. 10. Fatty acid composition of certain sharks, oil sardine and squid collected from various literature

Source					
Fatty acid	*Shark (*Carcharhinus wheeleri*) liver	*Shark (*Carcharhinus amboinensis*) liver	*Shark (*Scoliodon sorrakowah*) liver	Oilsardine (*Sardinella longiceps*)	**Squid (*Loligo duvaucelli*)
Saturated C12:0					
C14:0	4.2	3.7	3.0	0.4	0.1
C15:0	1.7	1.1	1.3	0.1	0.1
C16:0	47.0	12.7	21.8	2.9	5.4
C17:0	5.4	3.0	1.4	0.7	0.1
C18:0			5.5	15.2	11.9
C19:0	4.2	3.5		0.9	1.8
Total	62.5	24.0	33.1	27.5	31.1
Monounsaturated					
C16:1 *n*7	1.8	25.5	5.0	4.1	2.4
C18:1 *n*9	23.1	22.0	12.9	11.2	11.9
C20:1	0.5	0.3	0.2	0.8	0.0
C22:1	0.4	1.2		0.0	0.0
Total	25.9	49.0	18.1	16.5	17.5
Polyunsaturated					
C18:2 *n*-6		1.0	2.4	2.0	3.8
C18:3 *n*-3	1.9	1.0	0.2	0.6	0.5
C18:4 *n*-3			0.2	0.6	2.3
C20:2	0.4	1.0	0.7	0.5	1.0
C20:4 *n*-6	2.5	0.8	5.2	0.0	0.0
C20:5 *n*-3	2.1	1.2	4.5	5.4	8.3
C22:3 *n*-3			1.8	10.2	10.7
C22:4 *n*-3				2.4	0.0
C22:5 *n*-3	0.5	2.7		0.0	3.3
C22:6 *n*-3	2.5	16.6	26.5	24.7	21.3
Total	9.7	24.2	41.5	56.1	51.8
Unidentified	1.5	2.9	7.3		

*CIFT 1997; ** Ackman 1999.

total HUFA's and unsaturated fatty acids percentage is high. Table 3.10 shows that in shark liver oil DHA is high (26.5%) than EPA and the AA percentage is present in higher levels than in any other marine fish oils like sardine oil, squid liver oil, cod liver oil, menhaden, anchovy etc.

However with one known exception is the oil from the eye socket of the tuna (Sawada *et al.*,1993). It is observed that shark liver oil is the only marine fish oil with good levels of AA, which is very important as an eicosanoid precursor. Virtually every tissue in the body produces eicosanoids and they have a wide range of physiological actions.

As the shark liver oil met the requirement, of HUFA or DHA/EPA ratio and EAA/AA ratio as far as fatty acid composition is (Table3.9) concerned, it was selected for this study.

Regarding the enrichment period early workers had tried (9, 12, 24, 33 and 48 hours). The HUFA content and DHA: EPA ratio increased with enrichment periods up to 33 hours (Narciso *et al.*, 1999). In the present study 12 hours duration was done so that the rotifer mortalities after enrichment was minimum. Moderately starved rotifers exhibited a two-phased increase in *n*-3 fatty acids when they were fed a diet rich in these fatty acids. The first 20-30 minute of enrichment, the increase in *n*-3 fatty acids was primarily due to increased gut content. The subsequent slow increase was due to an incorporation of *n*-3 fatty acids into rotifer tissues. Saturation was achieved before 24 hrs of exposure and the saturation level was independent of the initial content of *n*-3 fatty acids in the rotifers. The *n*-3 fatty acids were assimilated with high efficiency from the feed and were not metabolised faster than other groups of fatty acids. Enriched rotifers retained their nutritive value for a

sufficient period after enrichment to serve well as live feed for marine fish larvae (Olsen *et al.*, 1989). The increase in *n*-3 HUFA levels of enriched rotifers fed on capelin oil or by low temperature crystallization separation for rotifer enrichment reached a maximum between 6 and 12 h. of enrichment and did not change significantly thereafter (Kissil and Koven, 1990).

In the present study rotifer fed with *Nannochloropsis* gave a very good fatty acid profile especially HUFA, and *n*-3 fatty acids, the *n*-3/*n*-6, DHA/EPA and EPA/AA ratios. The total PUFA fraction in the enriched rotifers revealed that *C marina* enriched rotifers contain maximum percentage of PUFA fraction, infact the major contribution is from the linoleic and linolenic fatty acids which the marine fish lack the enzyme Δ – 5 desaturase to convert into 18:3*n*-3 to 20:5*n*-3 or 18:2*n*-6 to 20:4*n*-6 (Sargent et al., 1997). *C marina* enriched rotifers lacks 20:4*n*-6, which has an essential function in producing eicosanoids. Arachidonic acid is essential for certain marine fin fishes (Ostrowski and Divakaran, 1990; Castell *et al.*, 1994). In turbot, dietary deficiencies in AA have resulted in high mortality and obvious pathology (Bell *et al.*, 1985a), while Castell *et al.* (1994) reported a positive effect of AA on survival from levels ranging from 0.5- 1.0%. Shark oil enriched rotifers and *N salina* enriched rotifers contained the required levels of AA.

The richest sources of 22:6*n*-3 among marine protists are the dinoflagellates and the phototrophic prymnesiophyceans, which include *Phaeocystis poucheti*, *Isochrysis galbana* and *Pavlova lutheri* (Sargent *et al.*, 1997). The latter two organisms have long been used as *n*-3 PUFA sources in mariculture; specifically they are sources of 22:6*n*-3 and not 20:5*n*-3 although *I. galbana* contain very significant amounts of 22:5*n*-6. Some members of the Prasinophyceae, which are much smaller organisms than either the dinoflagellates or haptopyceans, are also good sources of 22:6*n*-3 (Dunstan *et al.*, 1992).

Traditionally the commonest organism used in mariculture as a source of 20:5*n*-3 is the so-called marine *Chlorella*; strictly a Eustigmatophyte *Nannochloropsis occulata*. In the present study *Nannochloropsis salina* instead of *N. occulata* was used to enrich the rotifers, which satisfied all the fatty acid requirements of the larvae. Fatty acid profile of *N. salina* is superior to other algae used in the present study. Diatoms are invariably rich in 20:5*n*-3 though they are deficient in 22:6*n*-3. One marine protist rich in 20:4*n*-6 is the phototrophic red alga *Porphyridium cruentum* (Wood, 1974). Fernandez-Reiriz and Labarta (1996) reported a high DHA/EPA ratio in rotifers enriched with *Isochrysis galbana*. *Nannochloropsis gadiatana* contains substantial amounts of EPA and 20:4*n*-6 (Sukenik *et al.*, 1993). The gross chemical composition and fatty acid composition of marine unicellular algae, yeast and rotifers were studied by different authors

(Ben-Amotz *et al.*, 1987, Whyte and Nagata, 1990, Renaud *et al.*, 1999, Fernandez and Labarta, 1996; Kaladharan *et al.*, 1999). The fatty acid composition of the algae was species-specific with the highest ratio of Polyethylene to saturate and monoethylenic acid in *Isochrysis galbana* and *Phaeodactylum tricornatum*, and the highest content of *n*-3 HUFA in *Nannochloropsis salina* and *P. tricornutum*.

The fatty acid composition of the total lipids of rotifers fed on the algae or yeast is as follows. The major fatty acids present in rotifers fed on yeast were 16:0, 16:1*n*-7, 18:0, 18:1 and 18:3*n*-3; in rotifers fed on *N. salina* 16:0, 16:1*n*-7. 18:0, 18:1, 18:2*n*-6 and 20:5*n*-3; and in rotifers fed on *P. tricornutum* 14:0, 16:0, 16:1*n*-7, 18:1 and 20:4*n*-3. Long PUFA of 20:4*n*-3 and 20:5*n*-3 were detected in rotifers fed on *P. tricornatum*. The major fatty acids in *Thalassiosira pseudonana* and rotifers were 14:0, 16:0, 16:1*n*-7, 18:4*n*-3, 20:5*n*-3 and 22:6*n*-3 and in *Tetraselmis suecica* and corresponding rotifers contained 16:0,16:4*n*-3, 18:1*n*-9, 18:1*n*-7, 18:2*n*-6and 18:3*n*-6 acids. Among these algal diets for rotifers, *N. salina, T. pseudonana, P. tricornatum* and *I. galbana* are the preferred species in terms of fatty acids profiles.

The nutritive values of rotifers fed with *Chlorella marina* and *S. cereviciae* are inferior when compared with other feeds especially in the HUFA levels. DHA, EPA and AA are absent in these two feeds. Biochemical constituents of six species of micro algae studied and

indicated that *Chlorella* have very low lipid levels when compared with other species (Kaladharan *et al.*, 1999).

Regarding the DHA: EPA: AA ratio in the present study which is very important for marine finfish larvae, oil enriched rotifers gave a promising ratio with 1:7:0.5 followed by *Nannochloropsis* fed rotifers which is 0.4:8.4:0.2. Except in oil enriched rotifers the DHA level is generally very low but the EPA levels are very high. In marine finfish larvae the conversion of EPA to DHA is possible which can meet the requirement of DHA. The results suggest a strong possibility of retro conversion of DHA to EPA in rotifers, since the high content of DHA in shark liver oil was not reflected after enrichment of rotifers. *Artemia* nauplii and rotifers are capable of readily retro converting 22:6*n*-3 to 20:5*n*-3 through the process of β- oxidation a well known process in mammals (Braclay and Zeller, 1996). It was suggested that brine shrimp nauplii have marked propensity to retro convert DHA to EPA (Navarro *et al.*, 1999). The present study revealed that commercial oils with homemade emulsions are the best for rotifer enrichment. While selecting oils, tuna orbital oil, squid liver oil, shark liver oil, cod liver oil and sardine oil are the preferable ones for marine finfish and shrimp larvae. Certain micro algae like *Nannochloropsis* spp., *Isochrysis* spp. are also good in terms of fatty acids profile but the maintenance is highly laborious.

Chapter 4

4. Larval rearing of *Amphiprion sebae* Bleeker with enriched rotifers

4. 1. Introduction

The marine ornamental fish trade is rapidly expanding and tropical marine aquarium fishes are in great demand in the international markets. About 20 species of Pomacentrids which are important in the global ornamental fish trade have been reared in captivity the world over (Arvedlund *et al.*, 2000) and in India there are three reports of successful larval rearing of anemone fishes (Gopakumar *et al.*, 1999; Ignatius *et al.*, 2001; Madhu and Rema Madhu, 2001). The anemone fishes are symbionts on sea anemone and the nature of symbiosis is well documented in early reports (Mariscal, 1970 a, 1970 b, 1972; Allen, 1972; Fautin, 1986). Most of the anemone fishes are colorful, hardy and easy to feed. Among the anemone fishes, *Amphiprion percula*, the most colourful clown fish is the most popular marine fish species in aquarium trade. It is also considered as a reference fish for scientific studies on nutrition and egg and larval quality (Delbare *et al.,* 1995).

Clown fish larvae are fed usually with the rotifer *B. rotundiformis* from the same day of hatching even though complete yolk exhaustion occurrs after 2 days. Pomacentrid larvae are very sensitive to light and in the presence of bright light reflection, they exhibit 'head butting' syndrome and consequent mass mortality (Job *et al.*, 1997). Green water technique widely

employed in the rearing of marine finfish larvae has also been successfully used for pomacentrid larvae. (Danilowez and Brown, 1992; Job *et al.*, 1997; Arvedlund *et al.*, 2000). The Green water reduces light penetration in the larval rearing tank. The addition of various microalgae to the water during early first feeding of marine fish larvae frequently has resulted in improved growth and survival during the larval stage (Howell, 1979; Scott and Middleton, 1979; Jones *et al* 1981; Bromley and Howell, 1993; Vasquez-Yeomans *et al.*, 1990; Naas *et al.*, 1992). Improvement of the rearing practice for first feeding larvae also includes the addition of microalgae together with the enriched rotifers to the rearing tanks. This has been shown to improve growth and survival during early larval stages (Naas *et al.*, 1992; Reitan, 1994).

The introduction of rotifers marked the first regular successes in the mass larval rearing of several marine species of economic value such as red sea bream (*Pagrus major*) in Japan (Fujita,1979), grey mullet (Nash *et al.*, 1974), sole (Howell, 1973; Girin, 1974; Fuchs, 1978, 1982; Dendrinos and Thorpe, 1987), gilthead sea bream (Person and Verilland, 1980, 1981; Tandler and Helps, 1985), sea bass (Barnabe, 1974; Girin, 1975), turbot (Kuhlmann *et al.*, 1981; Olsen and Minck, 1983; Witt *et al.*, 1984), flounder (Fukusho *et al.*, 1985) and Milkfish (Liao *et al.*, 1979; Juario and Storch, 1984). Most of the marine fish are raised on basically the same methods, where by *B. plicatilis* is provided as the first food during the first day of

exogenous feeding. In this chapter, the impact of feeding enriched rotifers to larvae of *Amphiprion sebae* are reported.

4. 2. Material and Methods

4. 2. 1. Rotifer enrichment

The experiment was conducted at the Vizhinjam Research Centre of CMFRI, Trivandrum. The "semi- continuous" system was used for culturing the rotifer *Brachionus rotundiformis*. The diets used for lipid enrichment are different micro-algae, yeast and shark liver oil emulsion. Micro algae species *Nannochloropsis salina, Isochrysis galbana* and *Chlorella marina* were cultured in mass scale for enriching rotifers. Fresh bakers yeast was used as another source of feed for rotifers at 1 g yeast per million rotifers. Oil emulsion was prepared with shark liver oil and the composition of the oil emulsion and its preparation is same as explained in Chapter 2, i.e., *Na* R, rotifer fed with *Nannochloropsis*; *Cl* R rotifer fed with *Chlorella*; *Ig* R rotifer fed with *Isochrysis*; YR rotifer fed with yeast and OER rotifer fed with oil emulsion. The fatty acid profile of the enriched rotifers is given in chapter 3. Required quantity of rotifers were sieved through 100 micron mesh from the mass culture tubs to the enrichment vessels which contained a good concentration of enrichment medium. 20 litre translucent tubs with good aeration were used. Average temperature in the enriching containers was 26 $\pm$ 1.5° C. The enrichment duration was 10-12 hrs. The rotifers used for

testing larval survival growth, metamorphosis and pigmentations were nutritionally enhanced with the following five different treatments (Table 4. 1).

Table 4. 1. Enrichment of rotifers with different diets for 12 hrs

Enrichment medium	Concentration
Nannochloropsis salina	15-20x10^6 cells/ml
Isochrysis galbana	10-15x10^6 cells/ml
Chlorella marina	15-16x10^6 cells/ml
Saccharomyces cerevisiae	1gm yeast per million
Oil emulsion	*Shark liver oil- 5 ml egg yolk15ml in 1.5 litres of sea water.

*1.5 ml in 1 litre of seawater for enriching rotifers.

These nutritionally enriched rotifers were washed again, counted and then introduced in to larval rearing tanks on the same day after hatching of larvae.

4. 2. 2. Larval rearing of *Amphiprion sebae*

The brood stock of *A. sebae* was developed by collecting the fish along with the anemone belonging to the genus *Stoichactis* from Tuticorin/Mandapam area. They were kept in rectangular FRP tanks, (2.2 m x 1.2 m x 1m) along with sea anemones. The tanks were installed with biological filters. In each tank 6-8 numbers of fishes were introduced. They were fed with boiled mussel meat, fresh squid meat, formulated feeds twice daily. The spawning activity commenced with cleaning of the nesting site by both male and female. This behavior starts on the previous day or the same

day of spawning. Just prior to spawning the fishes exhibited parallel swimming usually with belly touching and biting on the substratum. The spawning occurred between 09.00 hrs and 13.00 hrs and lasted for 1 to 1.5 hours by this time females start attaching eggs to the substratum, followed by fertilization by the male. The eggs were attached to small earthen pots, granite slabs, walls of the PVC tank and to the PVC pipes of the biological filter of the tank (Plate IX a). The eggs were yellow or orange in color initially and turned to light grey on third day and the silvery color of eyespot was visible from 6th day (Plate IX b). Both male and female took part in aerating the eggs. They remove dead eggs from the egg mass (Plate IX c). The eggs usually hatched in the late evening hours of the 6th and 7th day after hatching. The average number of eggs laid in one spawning ranges from 550-1200 nos.

The larval rearing experimental units consisted of 200 litre capacity rectangular glass tanks/glass compartments situated in the indoor hatchery (Plate X d). The exterior of the glass tanks were covered with brown plastic coated sheets to reduce light reflection. Aged seawater from the brood-stock tank was filtered using a filter bag and filled 75% of the total volume of the tank. Air passing through an air stone maintained water circulation and aeration. The temperature in the tank was maintained at 27.5-31.0 °C, pH, 8.0-8.2, salinity 29.5-35.0 ppt and ammonia < 0.01 ppm. There were five treatments arranged in triplicates CL*Na*R (T-1), CL*lg*R(T-2), CL*Cl*R(T-3), CLYR (T-4), CLOER(T-5). In (T-1), the phytoplankton *Nannochloropsis*, in (T-

Plate – IX

a) Eggs of *Amphiprion sebae* Day 1

b) Eggs of *A. sebae* Day 6

c) Fanning of eggs by mother fishes

Plate – X

a) **Pigmented larvae of *A. sebae* Day 5**

b) **Metamorphosed larvae of *A. sebae* with single white band. Day 15.**

c) ***A. sebae* larvae Day 1**

d) **Larval rearing of *A. sebae* in glass tanks covered with brown coloured paper.**

2), *Isochrysis*, in (T-3) *Chlorella* and in (T-4) and (T-5), yeast were used to green up the tanks until the bottom of the tank could no longer be seen "Green water technique". These methods of reducing light stopped the "head butting syndrome" of the fish and improved the water quality. This also increased the contrast for feeding and acted as food for the rotifers. The enriched rotifers from each tube were filtered, washed and introduced into the larval rearing tanks prior to the introduction of larvae. The rotifers were maintained at 4-6 nos/ml in the larval rearing tanks.

The hatching of clown fish eggs occurred on late evening hours, when the mother fishes fans the eggs continuously and vigorously. All the lights in the hatchery were switched off and in a period of 10-20 minutes all the eggs hatched and the larvae moved towards the corners of the tank in groups (Plate X c). The healthy larvae were collected using a torch light in a 1.5 litre plastic mug. The wet weight of the larvae was measured to the nearest 0.1 mg. Sixty numbers of larvae were individually counted and introduced slowly into each of the tanks. White fluorescent lights were provided up to 23.00 hrs daily. The larvae were pelagic in the early stages of life cycle. A maximum of 10% mortality was observed on the 1st day due to the handling stress. The larvae started feeding rotifers on the same day of hatching even though complete yolk utilization occurred after 2 days. Every morning tanks were monitored by torchlight and dead larvae and debris settled in the bottom were siphoned off. Water exchange was done up to 20 % in the first three treatment tanks (green water algae) and up to 30 % in the fourth and

fifth treatment tanks (green water yeast) from the third day of larval rearing onwards. Enriched rotifers were given on early morning and early evening hours so as to keep the rotifer population at 4-6 nos/ml. The experiment was continued upto 15 days and the final wet weight were taken. During the experimental period, growth in terms of weight (mg) survival and number of days taken for pigmentation and metamorphosis were studied in different treatments. The larval rearing experiments were repeated three times with larvae of three brood stocks (each treatment having triplicates).

4. 2. 3. Statistical analysis

(a) The final weight of clown fish larvae after fifteen days was analysed using two factor analysis of variance (ANOVA) model involving feed and experiment and their interaction as factors and the means of the major factors was grouped using Duncan's multiple range test (DMRT).

(b) The transformed survival rates of clown fish larvae were analysed using two-factor interaction model with feed at five levels and experiments at three levels along with their interaction as the factor. The means of five levels of feeds were compared using Duncan's multiple range test (DMRT).

(c) Analysis of variance (ANOVA) was applied on the metamorphosis and pigmentation data (in hours) with feed and experiment being the factors of a two-factor interaction model. The homogenous means

among the five levels of feed were grouped separately for metamorphosis and pigmentation using Duncan's multiple range test (DMRT).

4. 3. Results

4. 3. 1. Rotifer enrichment

Rotifers enriched with different diets for 12 hrs were collected using a 100 μ sieve, washed with filtered seawater, and observed under microscope to know whether the gut is filled with various enrichment diets. Enriched rotifers were immediately transferred to larval rearing tanks.

4. 3. 2. Larval rearing of *Amphiprion sebae*

Experiment I

The initial average weight (wet weight) of the larvae day -1 ranged between 0.79 ± 0.2 mg. The growth gained by the larvae after 15 days varies in different treatments (Fig. 4. 1). Larvae fed with rotifers enriched with oil emulsion (CLOER, T-5) gained maximum growth 65.38 ± 1 mg on day 15; followed by larvae fed with rotifers enriched with *Nannochloropsis salina* 43.807 ± 1 mg (CL*N*aR; T-1). Larvae fed with *Chlorella marina* enriched rotifers gained an average weight of 24.2 ± .04 mg, (CL*C*lR; T-3). Larvae fed with *Isochrysis galbana* enriched rotifers gained an average weight of 31.01 ± 2 gm, (CL*Ig*R; T-2). The growth gained by larvae fed rotifers enriched with yeast was very low 12.07 ± 1 mg, (CLYR; T-4) when compared with other treatments (Table 4. 2). The number of days taken for the first pigmentation

of larvae, i.e., from translucent colour to dull black colour, was also observed (Plate X a). In T-1 (CL*Na*R) and T-2 (CL*lg*R) larvae took only 4 days for first pigmentation as against 5 days in T-5 (CLOER), 6 days in T-3 (CL*Cl*R) and 9 days (216 hrs) in T-4 (CLYR) (Table 4. 3 and Fig. 4. 3). After this pigmentation some physiological changes were observed in the larvae, which feed more voraciously. When food was visually located, the larvae formed a 'S' shape and propelled themselves forward to capture the food. The larvae also moved to the water column and spend most of the time in the water column.

In this experiment the number of days taken by the clownfish larvae for initial signs of metamorphosis (Table 4. 3 and Fig 4. 3) varied: T-5; CLOER took only 7 days (191.3 hrs), followed by T-1 CL*Na*R; 8 days (211.3 hrs), T-2 CL*lg*R; 10 days (240 hrs), T-3 CL*Cl*R; 12 days (309.6hrs), and T-4 CLYR, nearly 15 days (357.3 hrs) for first metamorphosis (Plate X b). Prior and during metamorphosis, day 7 to 10, the larvae drop to the bottom and cluster in groups just off the bottom. Swimming pattern changed from a jerky, darting motion to a smooth paddle – like motion. Clustering was more evident towards the evening. During day light there was no cluster formation and the larvae swim up in the water column or at midstream in counter clockwise circles. Healthy larvae in good quality water conditions usually metamorphose over a three-day period. Delayed metamorphosis was a serious problem often resulting in weak and defective individuals with semi-

opaque pigmentation. Metamorphosed larvae were characterized by large mouths, eyes and teeth, virtually straight intestines, poorly developed fins, air bladder and no scales, fin rays were evident, air bladders became fully functional, intestine became convoluted. Early juvenile coloration was first detected by the development of the pale translucent white head bar.

Survival percentage (Fig 4.2) of the larvae indicated that (T-1; CL*Na*R), *Nannochloropsis* fed rotifers showed the highest survival of around 90% followed by T-5; CLOER with 85% survival and T-2 CL*lg*R *Isochrysis* fed rotifers with 80% survival. Mortality of larvae in T-1 occurred mainly during day 2 and day 3. Mortality during day 7 and 8 was less than 5% during metamorphosis. Mortalities on day 2 and day 3 were mainly due to handling stress, genetically disorder; these larvae had, a characteristically vertical movement. In T-1 less than 10% mortality was recorded from day 4 to day 15. In T-5 more than 10% mortality was observed from day 4 to day 15. In T-2, 5% mortality was observed during day 2 to 5 and nearly 10% mortality was observed between days 8 and 15. The survival percentages in T-3 (CL*Cl*R) and T-4 (CLYR) were less than 60% and 50% respectively. In T-3 about 15% mortality was observed between day 5 and day 10, and about 30% of mortality was observed on day 11, day 12 and day 13, i.e., during metamorphosis. In T-4, wherein the larvae were poorly pigmented and very pale in appearance heavy mortality (50%) was observed between day 3 and day 15. About 40% of the mortality was observed during metamorphosis

from day 12 to day 15 (Table 4. 2 and Fig 4. 3). The majority of the larvae were very thin with a large head and tapering body down to the tail.

Table 4. 2. Growth and survival of the larval clown fish, fed on various enriched rotifers

Day after hatching	Rotifer enriched with	Wet weight gained (mg)	Survival rate (%)
15	*Nannochloropsis salina*	43.81± 1	90
15	*Chlorella marina*	24.2 ± 0.2	60
15	*Isochrysis galbana*	31.01 ± 0.04	80
15	*Saccharomyces cerevisiae*	12.07 ± 0.1	50
15	Shark liver oil emulsion	65.38 ± 1	85

Table 4. 3. Pigmentation and metamorphosis of larval clownfish, fed on various enriched rotifers

Experiment period	Rotifer enriched with	No. of days for 1st pigmentation of larvae	No. of days for metamorphosis of larvae
15	*Nannochloropsis salina*	4	8
15	*Chlorella marina*	6	12
15	*Isochrysis galbana*	4	10
15	*Saccharomyces cerevisiae*	9	15
15	Oil emulsion	5	7

Fig. 4. 1. Weight of larvae enriched with different diets

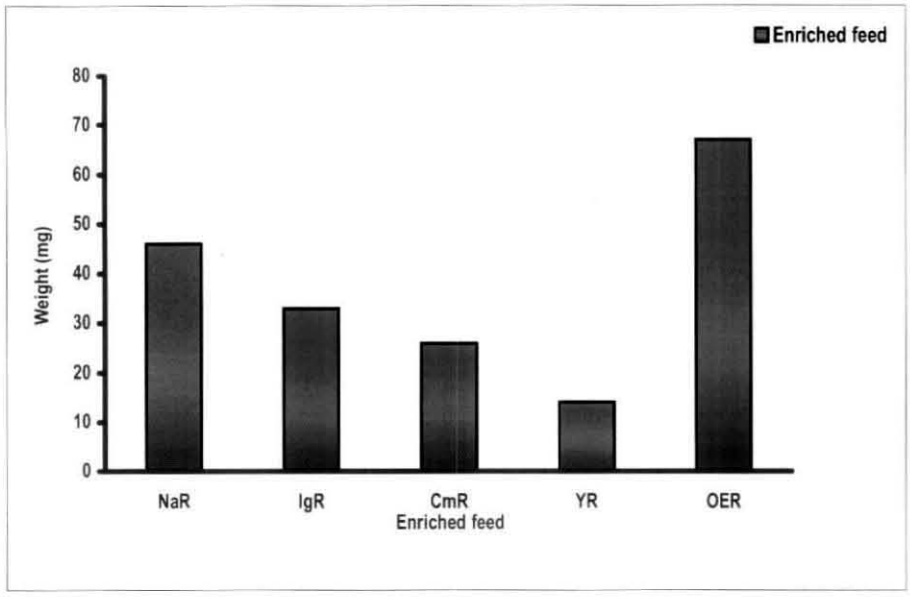


Fig. 4. 2. Survival of clown fish larvae enriched with different diets

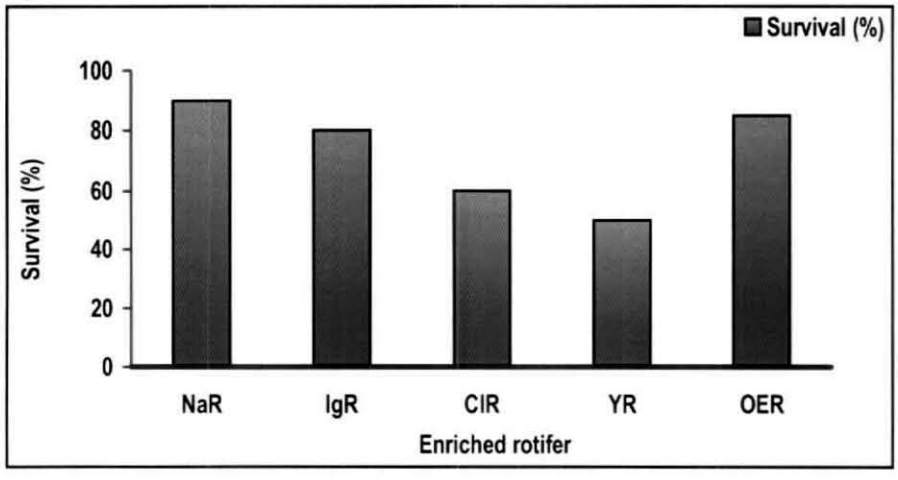
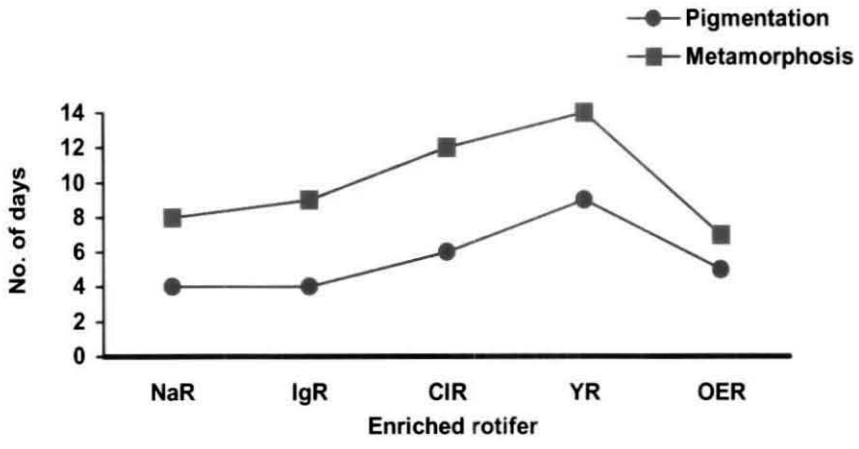


Fig. 4. 3. Pigmentation and metamorphosis of clown fish larvae enriched with different diets



Experiment- II

The initial average wet weight of the larvae on day-1 ranged between 0.838 ± 0.2 mg. The growth rate of the larvae on day 15 differed between treatments (Table 4. 4). Larvae fed on rotifers enriched with oil emulsion attained the maximum growth 64.92 ± 0.2 mg (CLOER; T-5) followed by T-1 with a weight gain of 40.747 ± 1 mg on day 15. Larvae fed with *Isochrysis galbana* enriched rotifers attained a maximum growth rate of 29.44 ± 3 mg on day 15 (T-2 CL*Ig*R). Larvae fed with *Chlorella marina* enriched rotifers attained a maximum growth of 21.813 ± 0.1 mg on day 15 (T-3; CL*C*lR) and larvae fed with yeast-enriched rotifer attained a maximum growth 10.53 ± 0.1 mg on day 15(T-4; CLYR) (Fig. 4. 4). Among the 5 different treatments the growth rate was very poor in T-4 CLYR.

Observations on number of days taken for first pigmentation (Fig. 4. 6) in the larvae of clown fish indicates that T-1 and T-2 had taken only 4-5

days followed by T-5 which took 6 days for first pigmentation. T-3 and T-4 had taken 8 days and 10 days respectively for first pigmentation (Table 4. 5).

Regarding the metamorphosis of the larvae T-5 had taken 9 days followed by T-1, which had taken 11 days. T-2 had taken 13 days for metamorphosis; T-3 and T-4 had taken 14 days and 18 days respectively for metamorphosis (Table 4. 5 and Fig. 4. 6).

Regarding the survival of the larvae 90% of the larvae survived in T-1 followed by T-5 and T-2 with 80% survival. Larvae of T-3 and T-4 had very poor survival with 65-70% and 40% respectively (Table 4. 4 and Fig. 4. 5).

Table 4. 4. Growth and survival of the larval clown fish, fed on various enriched rotifers

Day after hatching	Rotifer enriched with	Wet weight gained (mg)	Survival rate (%)
15	*Nannochloropsis salina*	40.74 ± 1	90
15	*Chlorella marina*	21.81 ± 3	66.6
15	*Isochrysis galbana*	29.44 ± 0.01	80
15	*Saccharomyces cerevisiae*	10.5 ± 0.1	40
15	Oil emulsion	64.9 ± 0.2	80

Table 4. 5. Pigmentation and metamorphosis of larval clown fish, fed on various enriched rotifers

Experiment period	Rotifer enriched with	No. of days for 1st pigmentation of larvae	No. of days for metamorphosis of larvae
15	*Nannochloropsis salina*	4.6	11
15	*Chlorella marina*	8	14
15	*Isochrysis galbana*	4.6	13
15	*Saccharomyces cerevisiae*	10	18
15	Oil emulsion	6	9

Fig. 4. 4. Weight of clown fish larvae enriched with different diets

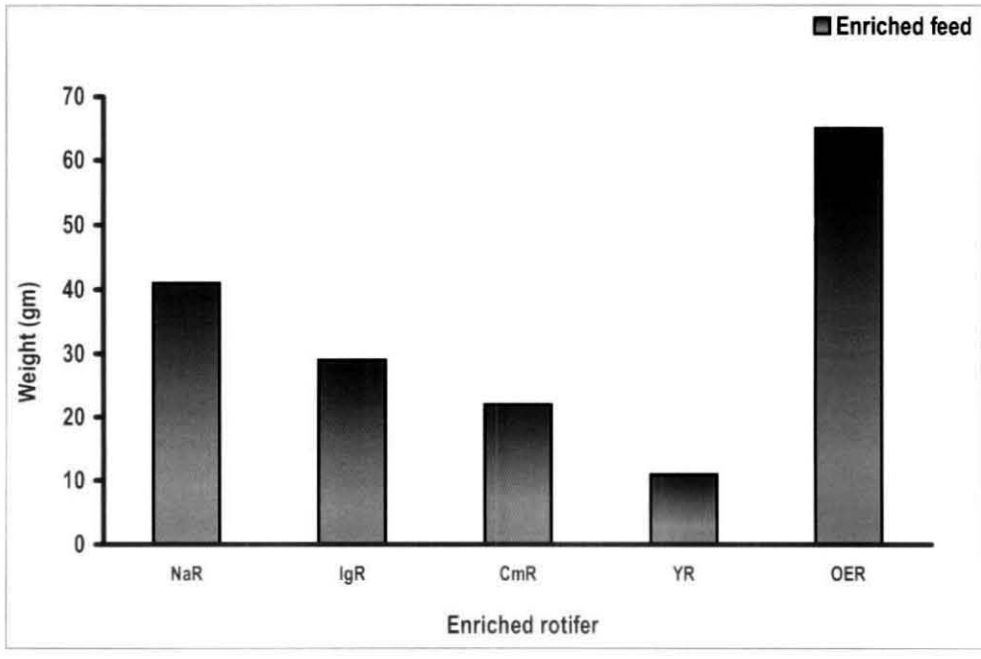


Fig. 4. 5. Survival of clown fish larvae enriched with different diets

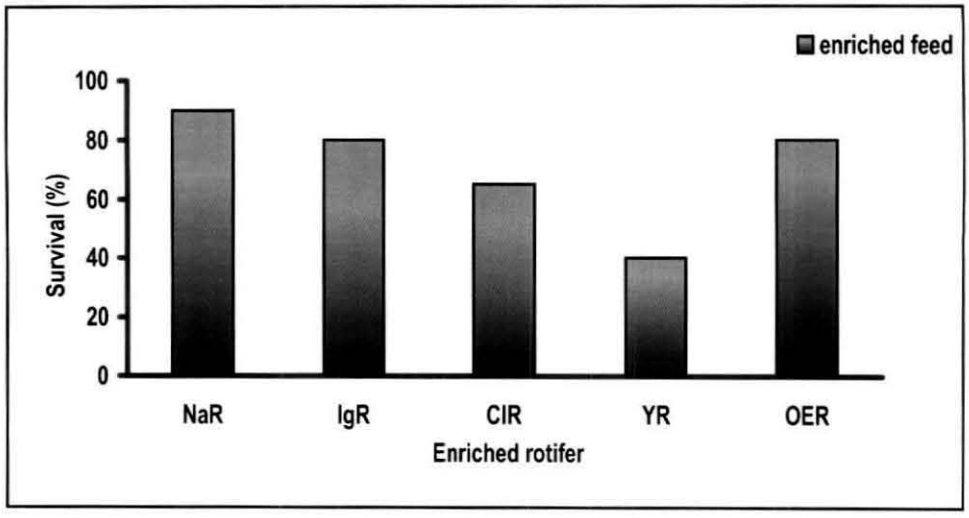
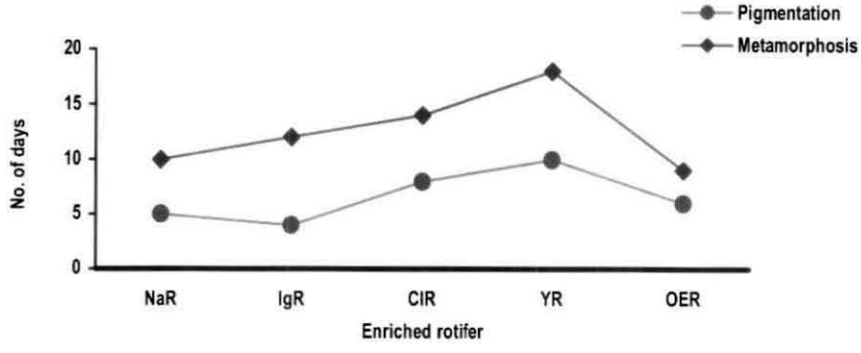


Fig. 4. 6. Pigmentation and metamorphosis of clown fish larvae enriched with different diets



Experiment III

The initial average weight of clown fish larvae just after hatching was 0.596 mg. Larvae fed with oil emulsion enriched rotifers gained maximum growth 55.6 ± 3 mg (CLOER; T-5) followed by T-1 larvae 38.46 ± 0.1 gm, (CL*Na*R) and T-2 larvae 19.41 ± 0.1 mg, (CL*Ig*R) after 15 days. Larvae reared in T-3 (CL*C*lR) and T-5 (CLYR) showed poor growth (Table 4. 6 and Fig. 4. 7).

Larvae reared in T-1, T-2 took 5 days and T-5, 6 days for the development of body pigmentation. T-3 and T-4 took 7 days and 9 days respectively for complete body pigmentation (Table 4. 7 and Fig 4. 9).

Regarding metamorphosis the larvae reared in T-5 took only 9 days for first metamorphosis followed by T-1, (9 days) while the larvae in T-2 took 12 days; larvae in T-3 and T-4 took 15 and 18 days (438 hrs) for first metamorphosis (Table 4. 7 and Fig. 4. 9).

After 15 days of rearing clown fish larvae a significantly higher survival rate was obtained in T-1 90% than T-5 80-85%. Larvae in T-2 with a survival rate of 80% followed by T-3, 60% and T-4 ñ 50% (Table 4. 6 and Fig. 4. 8).

Fig. 4. 7. Weight of clown fish larvae enriched with different diets

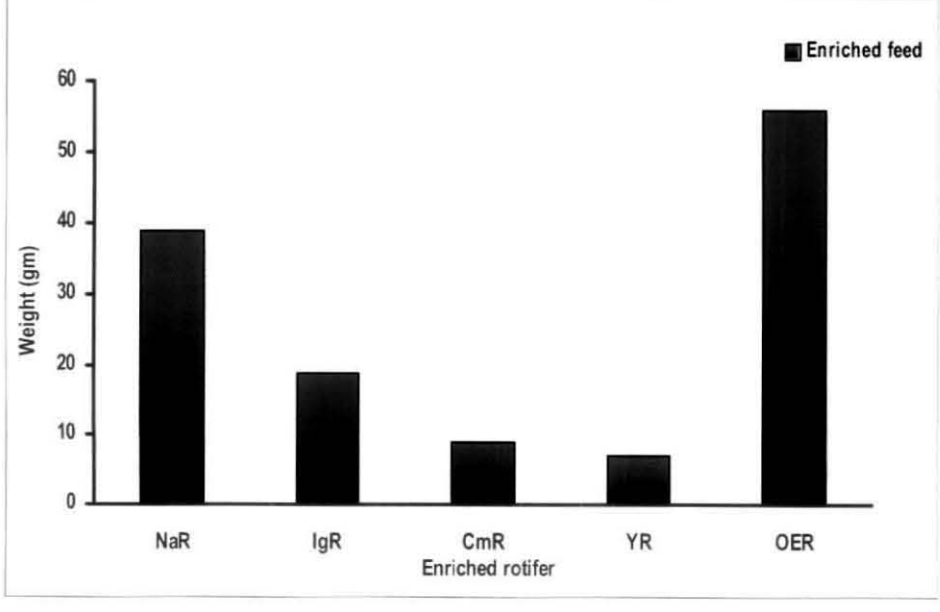


Fig. 4. 8. Survival of clown fish larvae enriched with different diets

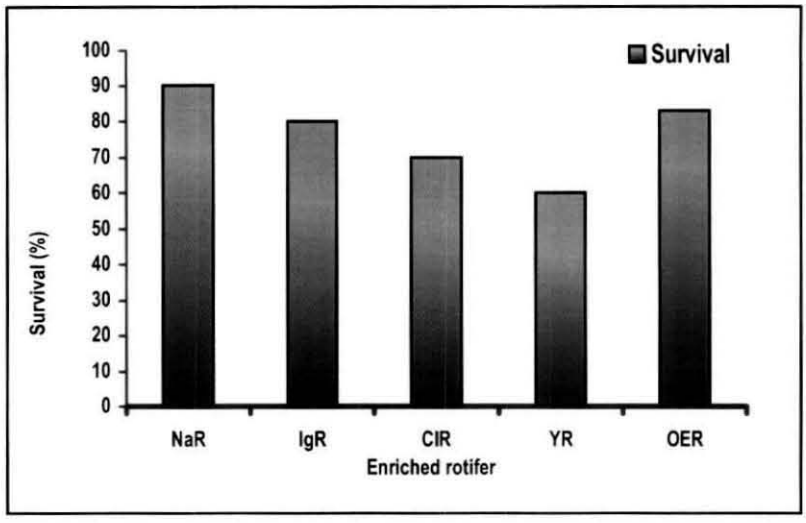


Fig. 4. 9. Pigmentation and metamorphosis of clown fish larvae enriched with different diets

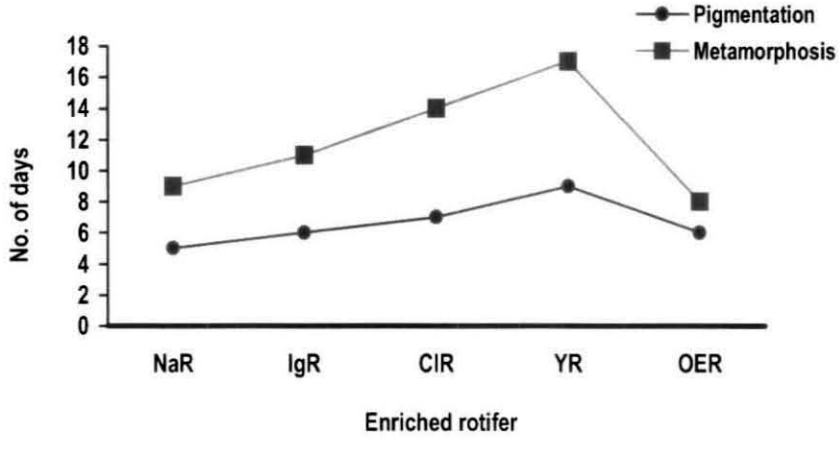


Table 4. 6. Growth and survival of the larval clown fish, fed on various enriched rotifers

Day after hatching	Rotifer enriched with	Wet weight gained (mg)	Survival rate (%)
15	*Nannochloropsis salina*	38.46 ± 0.1	90
15	*Chlorella marina*	8.66 ± 0.1	70
15	*Isochrysis galbana*	19.41 ± 0.1	80
15	*Saccharomyces cerevisiae*	7.36 ± 0.1	60
15	Oil emulsion	55.63 ± 3	82.5

Table 4. 7. Pigmentation and metamorphosis of larval clownfish, fed on various enriched rotifers

Experiment period	Rotifer enriched with	No. of days for 1st pigmentation of larvae	No. of days for metamorphosis of larvae
15	*Nannochloropsis salina*	5	9
15	*Chlorella marina*	7	15
15	*Isochrysis galbana*	5	12
15	*Saccharomyces cerevisiae*	9	18
15	Oil emulsion	6	9

4. 3. 3. Statistical analysis

a) The two factor ANOVA (with interaction) of the final weight of clown fish larvae is given in Table 4. 8. From the table, it is evident that the feeds explained more than 90% of the total variation and were obliviously significant. Though it is as an established fact that nutritional studies involve organisms from the same gene pool and hence their origin should not be contributing significantly to the variations (in the present experiments, larvae

from different mothers were used for three different experiments), the over dominance of the feed component in explaining the total variation had virtually camouflaged the intra group variation to such an extent that the origins were also significant in the strict statistical sense, though they explain only 4% of the total variation. Hence for all practical purposes, this significance of between experiments can be ignored. The DMRT performed on the final weight of larvae showed the existence of five homogenous groups among the five levels of enriched feeds. The groupings are (1) oil enriched rotifers (2) *N. salina* enriched rotifers (3) *I. galbana* enriched rotifers (4) *C. marina* enriched rotifers and (5) *S. cerevisiae* enriched rotifers. The mean values for the final growth of clown fish larvae enriched with oil emulsion was significantly higher. The DMRT for feed levels are given in the Table 4. 9.

Table 4. 8. ANOVA for growth rate of larval clown fish fed with enriched rotifers

Source of variation	Sum of squares	df	Mean square	F	Significance
Experiment	3719.346	2	1859.673	121.316	0.000
Feed	75704.65	4	18926.16	1234.65	0.000
Expt* Feed	863.433	8	107.929	7.041	0.000
Error	3219.123	21	15.329		

Table 4. 9. DMRT grouping for final growth of larval clown fish

Sl. No.	Name of the feed fed to rotifers	*Mean values of final growth of larvae
1	*Nannochlorpsis salina*	41.0067^{b}
2	*Isochrysis galbana*	26.6222^{c}
3	*Chlorella marina*	18.2244^{d}
4	*Saccharomyces cerevisiae*	9.9844^{e}
5	Shark liver oil emulsion	61.979^{a}

*Means within the row not bearing the same letter superscripts are significantly different

b) The two factor ANOVA (with interaction) of the transformed survival of clown fish larvae is given in the Table 4. 10. From the table, it can be reasoned that feeds were highly significant ($P < 0.01$). The over dominance of the feed component in explaining the total variation had virtually camouflaged the intra group variation to such an extent that the source were also statistically significant. The DMRT performed on the transformed survival percentage of larval clown fish showed the existence of five homogenous groups among the five levels of enriched feeds. The groupings are (1) *N. salina* enriched rotifers (2) oil enriched rotifers (3) *I. galbana* enriched rotifers (4) *C. marina* enriched rotifers and (5) *S. cerevisiae* enriched rotifers. The mean values for survival percentage of larval clown fish enriched with *N. salina* was higher than other enrichers. The DMRT for feed levels is given in the Table 4. 11.

Table 4. 10. ANOVA for survival percentage of larval clown fish fed with enriched rotifers.

Source of variation	Sum of squares	df	Mean square	F	Significance
Experiment	0.0244	2	0.012	76.313	0.000
Feed	1.195	4	0.299	1866.881	0.000
Expt* Feed	0.0603	8	0.008	47.090	0.000
Error	0.0048	30	0.000		

Table 4. 11. DMRT grouping for survival of larval clown fish

Sl. No.	Name of the feed fed to rotifers	*Mean (hours) of transformed values of larval survival
1	*Nannochlorpsis salina*	1.249^{b}
2	*Isochrysis galbana*	1.107^{c}
3	*Chlorella marina*	0.944^{d}
4	*Saccharomyces cerevisiae*	0.785^{e}
5	Shark liver oil emulsion	1.143^{a}

*Means within the row not bearing the same letter superscripts are significantly different

The two factor ANOVA (with interaction) of the pigmentation and metamorphosis of the larval duration (in hours) were given in the Table 4. 12 and 4. 14, both the cases, the feed factor was highly significant ($P<0.01$). The DMRT performed on the larval pigmentation showed the existence of five homogenous groups among the five levels of enriched feeds in the Table 4. 13. The groupings were (1) *N. salina* enriched rotifers (2) *I. galbana* enriched rotifers (3) oil emulsion enriched rotifers (4) *C. marina* enriched rotifers and (5) *S. cerevisiae* enriched rotifers. The DMRT performed on the

larval metamorphosis showed the existence of five homogenous groups among the five levels of enriched feeds in the Table 4. 15. The groupings were (1) oil emulsion enriched rotifers (2) *N. salina* enriched rotifers (3) *I. galbana* enriched rotifers (4) *C. marina* enriched rotifers and (5) *S. cerevisiae* enriched rotifers.

Table 4. 12. ANOVA for pigmentation of larval clown fish fed with enriched rotifers.

Source of variation	Sum of squares	df	Mean square	F	Significance
Experiment	3388.744	2	1694.372	88.074	0.000
Feed	58297.855	4	14574.464	757.584	0.000
Expt* Feed	5833.897	8	729.237	37.906	0.000
Error	538.667	28	19.238		

Table 4. 13. DMRT grouping for pigmentation of clown fish larvae

Sl. No.	Name of the feed fed to rotifers	*Mean values (hours) of larval pigmentation
1	*Nannochlorpsis salina*	102.888^{a}
2	*Isochrysis galbana*	111.222^{b}
3	*Chlorella marina*	162.222^{d}
4	*Saccharomyces cerevisiae*	221.285^{e}
5	Shark liver oil emulsion	136.444^{c}

*Means within the row not bearing the same letter superscripts are significantly different.

Table 4. 14. ANOVA for metamorphosis of larval clown fish fed with enriched rotifers

Source of variation	Sum of squares	df	Mean square	F	Significance
Experiment	30565.200	2	15282.600	100.676	0.000
Feed	249048.356	4	62262.089	410.159	0.000
Expt* Feed	6333.244	8	791.656	5.215	0.000
Error	4554.000	30	151.800		

Table 4. 15. DMRT grouping for metamorphosis of clown fish larvae

Sl. No.	Name of the feed fed to rotifers	*Mean values (hours) of larval metamorphosis
1	*Nannochlorpsis salina*	216.888[b]
2	*Isochrysis galbana*	258.444[c]
3	*Chlorella marina*	320.444[d]
4	*Saccharomyces cerevisiae*	391.777[e]
5	Shark liver oil emulsion	183.777[a]

*Means within the row not bearing the same letter superscripts are significantly different.

4. 4. Discussion

Larvae of clown fish (*A. sebae*) can be reared upto metamorphosis with enriched rotifers as the sole feed. Two main live feeds used for marine fish seed production, rotifers and brine shrimp nauplii, naturally lack *n*-3 HUFA, being rich in linolenic acid and to a lesser extent linoleic acid must be supplemented with *n*-3 HUFA's to ensure successful survival, growth and metamorphosis of the larvae. The major source by far, of *n*-3 HUFA for live feed supplementation has been fish oils whose contents of EPA and DHA

can vary substantially (Sargent *et.al.*, 1999). Rotifers constitute the most essential initial diet for most marine larvae. These are living packages or messengers of initial nutrition. Clown fish fry can be weaned onto a formulated dry feed from seven days after hatch with no significant reduction in survival, although the optimum time for weaning on a dry formulated feed was found to between 15 and 20 days after hatch. The yeast fed rotifer was lacking significantly in essential *n*-3 fatty acids (HUFA) such as EPA and DHA and also PUFA's like linolenic, linoleic and arachidonic acids (AA). The common practice is to feed micro algae 12-24 hours before harvest and products like Roti-Rich, Culture Selco, Super Selco are used to enrich rotifers before feeding. But the disadvantages are that algal maintenance is very labour intensive, unpredictable crash to the culture, and that the special enrichers are very costly.

Clown fish larvae fed on shark liver oil enriched rotifers, with a very good profile of *n*-3 and *n*-6 fatty acids, attained maximum growth rate and survival in the present study. Enrichment products like Selco, squid oil, fish oil etc. containing EFA such as 20:5*n*-3, 22:6*n*-3 and PUFA like 20:4*n*-6, certain vitamins such as B_{12}, C and A can also be utilized and easily incorporated into this regime. The long chain highly unsaturated fatty acids (HUFA's), particularly EPA and DHA are important in the nutrition of young marine fish (Kanazawa; 1985; Watanabe *et al.*, 1989). Various investigators have used DHA/EPA ratio as an index of the optimal level required for normal growth and development in fish larvae (Koven *et al.*, 1993; Mourente

et al., 1993; Rainuzzo *et al.*, 1994; Reitan *et al.*, 1994; Tocher *et al.*, 1997; Rodriguez *et al.*, 1998). Apart from DHA and EPA arachidonic acid (AA, 20:4*n*-6) has also been recognized an essential for marine fish (Castell *et al.*, 1994). AA is the main precursor of eicosanoids responsible for osmoregulation; cardiovascular functions, neural control and reproduction (Mustafa and Srivastava, 1989). In the present investigation, significant difference was observed in growth and survival of clown fish larvae fed with DHA enriched live food (high DHA/EPA ratio) i.e., fish oil enriched rotifer, *Nannochloropsis* enriched rotifers and *Isochrysis* enriched rotifers compared with *Chlorella* and yeast enriched rotifers. Fatty acid profile of the enriched rotifers is discussed in chapter 3.

Rainuzzo *et al* (1994), found a positive correlation between pigmentation success and the DHA/EPA ratio in the total and polar lipid fraction of the turbot larvae. Baker *et al* (1998) also noted a direct relation between normal pigmentation and levels of DHA in the diet of summer flounder larvae where the DHA/EPA ratios in the enriched live food is high. In the present study however pigmentation success was significantly better in clown fish larvae fed with *Nannochloropsis*, *Isochrysis* and shark liver oil enriched rotifers compared with *Chlorella* and yeast fed rotifers (Consistent for the three larval rearing trials).

Metamorphosis is the most traumatic event in the life of a clown fish or any fish. It is the "Jeckel and Hide" transformation (metamorphosis) from

simple larvae into a post-larvae (early stage juvenile). The number of days taken for metamorphosis of clown fish larvae was significantly better in larvae fed the shark liver oil enriched rotifers and *Nannochloropsis* enriched rotifers (consistent for three larval rearing trials). Larvae fed with *Isochrysis* enriched rotifers comes to the above mentioned diets. But yeast and *Chlorella* enriched treatment, larvae observed significant difference in the metamorphosis process. Clown fish larvae fed rotifers enriched with yeast and *Chlorella* exhibit poor performance especially in growth and survival. The larvae are not well pigmented the weight gained is very poor and develops pinhead syndrome mainly due to the lack of EFA in that diet. The kind and quantity of fatty acids required differ from species to species; especially between fresh water and sea water fish. The requirements of marine fin fishes range from 0.5 to 2.0% varying of course with the growth stages from the newly hatched larvae receiving live foods to brood stock for reproduction.

Live food enriched with EFA's, DHA and EPA, improved larval performance in striped bass and palmetto bass (Tuneer and Harrell, 1992), cod (Takeuchi *et al.*, 1994) red sea bream (Furuita *et al.*, 1996a), yellow tail (Furuita *et al.*, 1996b), striped jack (Takeuchi *et al*,. 1996) and summer flounder (Baker *et al.*, 1998). Mourente *et al*, (1993) reported the best growth rate in gilt head sea bream larvae fed HUFA-enriched rotifers with high DHA/EPA ratio. In milkfish the effects of DHA enriched live food on growth

may not be readily discussed over a short period (hatchery phase) but rather after extended rearing (nursery stage) (Gapasin and Duray, 2001).

Marine finfish require *n*-3 HUFA such as EPA and DHA as essential fatty acids (EPA) for their normal growth. But it remains unclear as to which of the *n*-3 HUFA, either EPA or DHA, was important. DHA must be present in the diet to maximize the survival of larvae of coral reef damselfish, *Acanthochromis polycanthus* (Southgate and Kavanagh, 1999) The developing eggs rapidly utilize DHA either for energy or for production of physiologically important substance like prostaglandins. This report reveals that in marine larval fish DHA is superior to EPA as EFA. Apart from the best growth and survival in an activity test for the larvae fed on DHA-rotifer, the incidence of hydrops seemed to be totally prevented dietetically by DHA. In all the species examined to date, DHA has been demonstrated to be superior to EPA in EFA value, especially in providing vitality to the larvae (Watanabe 1993). In the present study the fatty acid profile of shark liver oil and *N salina* enriched rotifers showed higher levels of DHA compared to other diets like *I galbana*, *C marina* and *S cerevisiae*. The same fatty acid profiles were also reflected when these enriched feeds were fed to clown fish larvae. Diets containing higher levels of DHA performed higher survival, growth rate and a shorter period for metamorphosis and pigmentation of larval clown fish.

Recent research has shown that the *n*-6 HUFA, arachidonic acid (20:4*n*-6 or AA) is also important for growth, survival and stress resistance

(Koven *et al.*, 2001). Due to the very fast embryo development in tropical species, which increases the degree of essentiality of AA, prostaglandins and other eicosanoids compared with cold/temperate species such as trout and salmonids which take a longer time, about one or two months, to hatch. Environmental stress like heavy rain and typhoon, further increase the necessity for eicosanoids (Sargent *et al.*, 1999). AA is nutritionally more important in tropical species than in temperate species. *Boops boops* contained relatively high levels (4.6 %) of AA (Thrush *et al.*, 1993). In the present study, the rotifer enriched with shark liver oil and *N. salina* satisfied the AA requirement of clown fish larvae.

Sargent *et al.*, (1999) have stated that both the amount and proportions of DHA, EPA and AA are important in marine fish nutrition and suggested that the optimum ratios may vary with species but would be in the range of 10:5:1 for DHA: EPA: AA. Rotifers grown on a mixed algal diet of *Isochrysis galbana, Tetraselmis succea, Nannochloropsis* spp. and *Pavlova lutheri* were able to satisfy the EFA requirements of haddock larvae, (Castell *et al.*, 2003).

In the present study survival rate was very poor (40-60%) when yeast enriched rotifers were fed to clown fish larvae. *S cerevisiae* is deficient in all the *n*-3 and *n*-6 PUFA's and HUFA's, which determine the growth and survival of marine fin fishes.

Low survival and growth of striped mullet larvae at day 15 post hatching indicates that rotifers fed only yeast were nutritionally deficient in fatty acids. The fatty acid requirements of mullet appeared to be satisfied with rotifers cultured on a combination of yeast and *Nannochloropsis oculata* (Tamaru *et al.*, 1991).

5. Larval rearing of *Penaeus monodon* (Fabricius) larvae with enriched rotifers

5. 1. Introduction

Variation in abundance and distribution of wild penaeid post larvae and the increasing world market for prawn especially tiger prawns has led to the rapid development of commercial larval culture systems for penaeids. Nutritional studies on lipids have demonstrated that crustaceans require essential fatty acids (EPA) for their normal growth (Deshimaru *et al.*, 1979; Kanazawa *et al.*, 1977; Sandifer and Joseph, 1976; Derresse *et al.*, 1990; Sorgeloos and Leger, 1992). Prawn fed diet containing 3% marine shrimp oil had increased accretion of eicosapentaenoic acid (EPA) and decosahexaenoic acid (DHA). (Sandifer and Joseph, 1976). Penaeids have a dietary requirement for linoleic acid, arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid (Merican and Shim, 1996; D'A bramo 1997; Glencross and Smith 1999, 2001). Marine fish and crustaceans, like all other animals that have been studied to date, lack the ability for *de novo* synthesis of *n*-6 and *n*-3 fatty acids while some aquatic animals, such as salmonids; can synthesize their own highly unsaturated fatty acids. Penaeids either do not have this ability, or it is insufficient for their apparent needs. These families of fatty acids perform essential biological and physiological functions and must be supplied in the diet (Castell *et al.*, 1972; Fujii and Yone; 1976; Kanazawa *et al.*, 1979a).

5. 2. Material and Methods

5. 2. 1. Larval rearing of *Penaeus monodon* with enriched rotifers

The experiment was conducted at Fisheries Research Laboratory of CMFRI at Thoppumpady, Cochin. *Penaeus monodon* (Fabricius) larvae (Plate XI c) were spawned at the Aquaplaza Hatchery at Cherai (Ernakulam Dist.) from wild caught, unablated spawners collected from Munambam harbour (Ernakulam Dist.). Larvae (Mysis-I) were transported in tightly sealed polythene bags, quarter filled with filtered seawater and inflated with oxygen.

Survival percentage and number of days for metamorphosis from Mysis-II to post larvae-I were determined from two trials, each with three replicates for five experimental diets enriched for rotifers. For each trial larvae from different mother prawns were used.

The experimental set up includes 4 litre capacity pearl pets (Plate XI a), which were thoroughly washed with sodium hypochlorite solutions filled with 3 litre of filtered seawater (33±2 ppt), gently aerated by 1-cm diameter air bubbles supplied by blower which is passed through a ciliate filter (Plate XI a). Temperature of the medium was maintained at (31 ± 1.5°C) and pH 8.2. 100 number of larvae (Mysis-II) was counted and introduced slowly into each containers, time in hours was noted and each treatments were named as follows: M*Na*R (M*Na*R, M*Na*R_2, M*Na*R_3); Mysis fed with rotifers enriched by *Nannochloropsis salina*, M*Cl*R (M*Cl*R_1, M*Cl*R_2, M*Cl*R_3); mysis fed with rotifers enriched by *Chlorella marina,* M*lg*R (M*lg*R_1, M*lg*R_2. M*lg*R_3); mysis fed

Plate – XI

a) **Experimental setup for rearing of *Penaeus monodon* larvae with enriched rotifers**

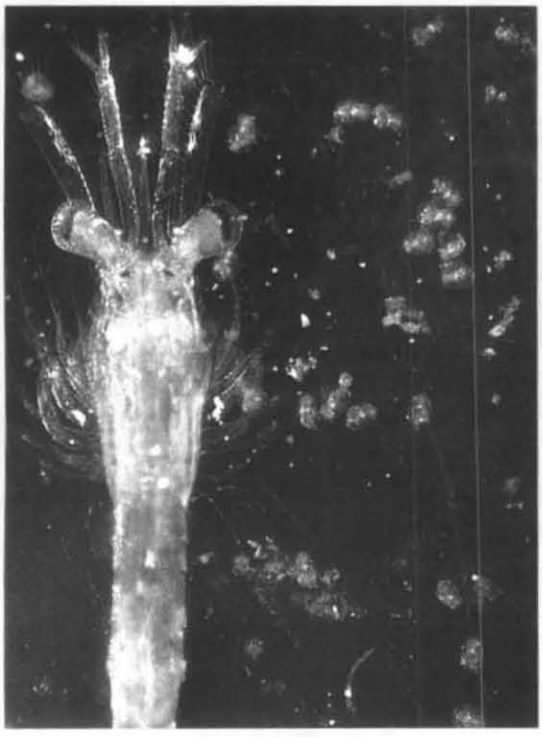
b) ***P. monodon* larvae feeding on enriched rotifers**

c) ***P. monodon* larvae gut full with rotifers**

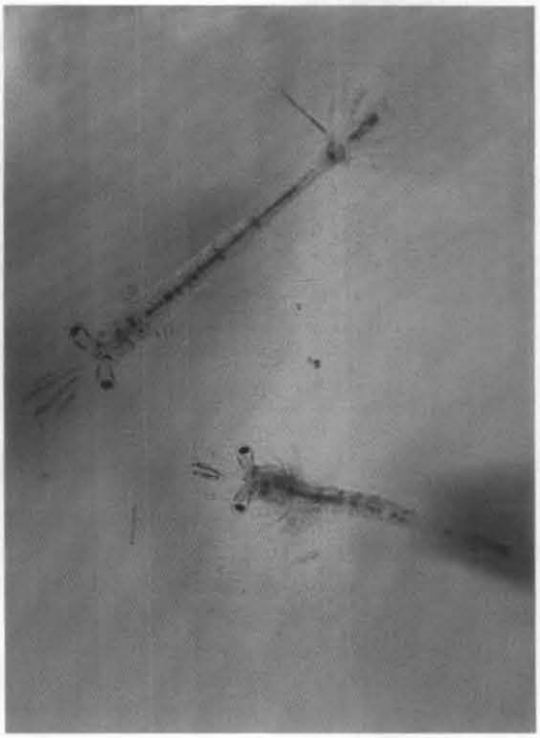
Plate XI

a

b



c



with rotifers enriched by *Isochrysis galbana*, MOER ($MOER_1$, $MOER_2$, $MOER_3$); mysis fed with rotifers enriched by shark liver oil emulsion. MYR (MYR_1, MYR_2, MYR_3) and mysis fed with rotifers enriched by yeast.

Rotifer enrichment was carried out in separate containers for duration of 12 hrs, same procedure followed in chapter-4. Enriched rotifers were fed to shrimp larvae in each containers and maintained at 75 nos/ml by feeding in the morning and evening hours (Plate XI b). Seawater was renewed every day morning (50% water exchange) and debris and waste were siphoned out using small tubes through a filter cloth to prevent the escape of live rotifers. Larval growth and survival was assessed every day. Time (hours) taken for the metamorphosis of Mysis-II to postlarvae1 and survival (%) in each treatment was studied. Experiment was repeated with same facility with larvae from different mother prawn is used (Experiment II).

5. 2. 2. Statistical analysis

The transformed survival rates of shrimp larvae were analysed using a two-factor interaction model with feed at five levels and experiments at two levels. The means of five levels of feeds were compared using Duncan's multiple range test, (DMRT). Analysis of variance (ANOVA) was applied on the metamorphosis data (in hours), with feed and experiment being the factors of a two-factor interaction model. The homogenous means among the five levels of feeds were grouped using Duncan's multiple range test (DMRT).

5. 3. Results

5. 3. 1. Larval rearing of *Penaeus monodon* with enriched rotifers

Experiment I

The mean survival and number of days taken for metamorphosis by Mysis II to postlarvae1 of *P. monodon* during the first experiment with different enriched rotifers are given in the Fig. 5. 1 and 5. 2. The highest percentage of survival was attained when *N. salina* was enriched to rotifers (83%) followed by *Isochrysis galbana* enriched rotifers (79%) (Plate XI c). The percentage of larval survival when rotifers fed with shark liver oil emulsion were lower (75.6%) compared with *Nannochloropsis* and *Isochrysis* fed rotifers. *C. marina* and *S. cerevisiae* enriched rotifers exhibited the poor survival rates in the experiments with (63.6%) and (42.5%) respectively. It was observed that the major mortality was during the transformation stage of Mysis III to post larvae. It was also observed that even after the transformation the post larvae became very lethargic and died ultimately. This type of mortality was more prevalent for *chlorella* and yeast enriched rotifers.

Regarding the metamorphosis of larvae from Mysis II to post larvae, larvae fed with rotifers enriched with *Nannochloropsis*, shark liver oil emulsion and *Isochrysis galbana* had taken only less than three days to transfer from Mysis II to Mysis III and to post larvae 1 (60 hrs, 50.6 hrs, 73.3 hrs). Mysis III had taken only less than 1.5 days to transform further to post

larvae in all the above three treatments with variation only in few hours. Larvae fed with yeast enriched rotifers taken more than 4 days (97.3 hrs) for the first transformed post larvae. In this treatment about 30% mortality was observed due to the delay of transformation of Mysis II to Mysis III. Mysis II had taken more than two days to transform to Mysis III and also taken more than two days to transform to post larvae. In the case of larvae fed with *Chlorella* enriched rotifers, Mysis II had transformed with in 4 days (95.3 hrs) to PL-I without heavy mortality but the transformation from Mysis III to post larvae was delayed to more than two days. During this transformation heavy mortality of larvae has observed in the rearing containers.

Fig. 5. 2. Metamorphosis of shrimp larvae fed with enriched rotifer (time in days)

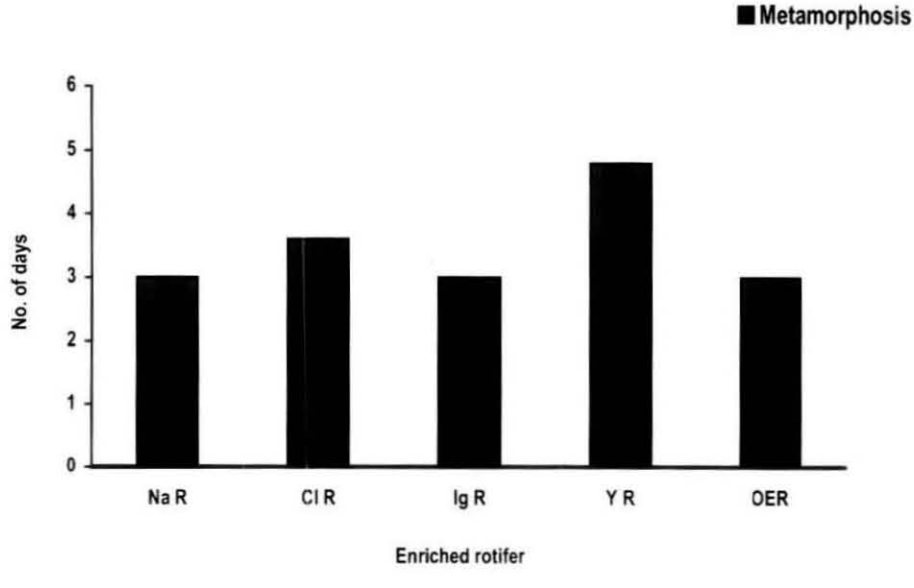
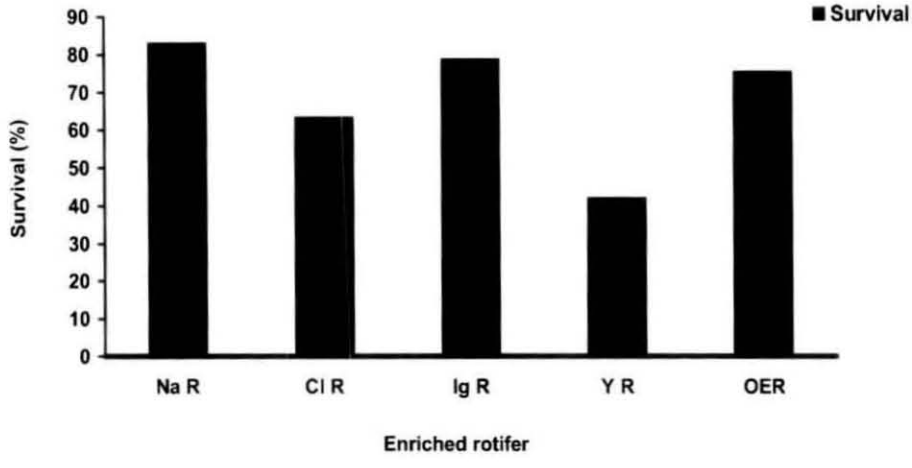


Fig. 5. 1. Survival of shrimp larvae fed with enriched rotifer



Experiment II

The mean survival and number of days for the transformation of Mysis II to post larva1 of *P. monodon* with various enriched rotifers are given in the Fig. 5. 3 and 5. 4. Larvae fed with *N. salina* and *I. galbana* enriched rotifers produced a better survival rate with a mean of 82% and 79% respectively compared with larvae fed on shark liver oil emulsion enriched rotifers which gave a mean survival rate of 71%. In all the above-mentioned treatments maximum mortality up to 30% was observed during the transformation from Mysis III to post larvae I. The larval survival for *Chlorella* and yeast enriched rotifers were poor with 60% and 40% respectively. In both the treatments more than 20 % mortality was observed even during the Mysis II to Mysis III transformation stage, but higher mortality observed during the transformation during Mysis III to post larvae I.

Regarding the metamorphosis of larvae from Mysis II to post larvae I larvae fed with *N. salina, I. galbana* and oil emulsion enriched rotifers had taken less than three days (66 hrs, 73.3 hrs, 52 hrs) for all the three treatments respectively. Larvae fed with *C. marina* and *S. cerevisiae* had taken 4 days (94 hrs,) and 5 days (96.6 hrs) respectively. Mysis II had taken less that 1.5 days for. *N. salina; I. galbana* and oil emulsion enriched rotifers to transform to Mysis III and further 1.5 days to post larvae in all the three treatments. In the treatments like yeast and *C. marina* fed rotifers the larval Mysis II had taken 4 days and 5 days respectively to transform into post larvae with 2 days each for first transformation i.e., into Mysis III and further the yeast fed rotifer larvae had taken 3 more days to post larvae I while *C. marina* fed rotifers had taken 2 more days to post larvae I. In both the treatments high mortality was observed during the Mysis III to post larvae I transformation period.

Fig. 5. 3. Survival of shrimp larvae fed with enriched rotifer

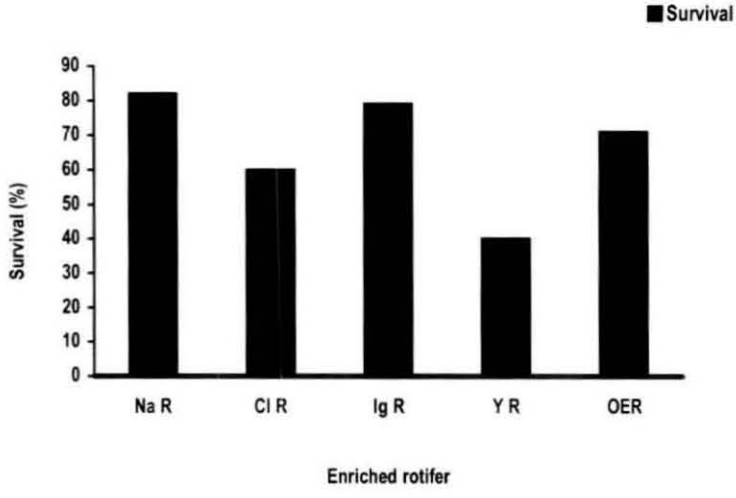
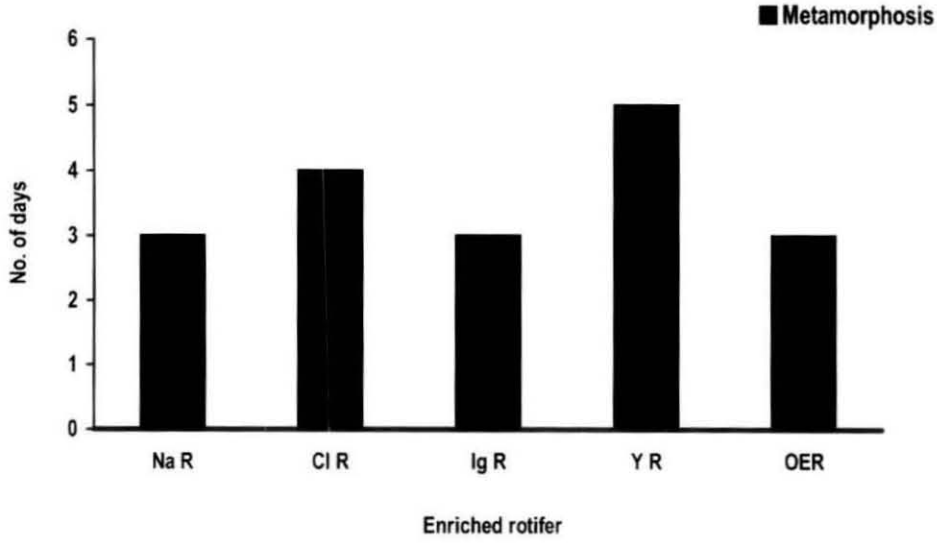


Fig. 5. 4. Metamorphosis of shrimp larvae fed with enriched rotifer



5. 3. 2. Statistical analysis

The two factor (with interaction) ANOVA of the transformed survival rate of shrimp larvae (post larvae) given in the Table 5.1. It can be observed that the different feed has a significant contribution ($P< 0.05$) to the total variation in the data whereas the experiment levels and their interaction with the different levels of feed were not significant. The post Hoc Duncan's multiple range test (DMRT) revealed the grouping of the five levels of feeds into four subsets. (Mean of the transformed values is given in the Table 5. 2.

Table 5. 1. ANOVA for transformed survival percentage of post larvae fed with enriched rotifers

Source of variation	Sum of squares	df	Mean square	F	Significance
Experiment	0.006	1	0.006	2.905	0.104
Feed	0.769	4	0.192	101.608	0.000
Exp*Feed	0.002	4	0.001	0.315	0.865
Error	0.038	20	0.002		

Table 5. 2. DMRT of transformed value for post larval survival

Sl.No.	Name of the feed fed to rotifers	*Mean of transformed value of post larval survival.
1	*N. salina*	1.143^{a}
2	*I. galbana*	1.095^{a}
3	*C. marina*	0.905^{d}
4	*S. cerevisiae*	0.696^{c}
5	Shark oil emulsion	1.03^{b}

* Means with similar superscripts do not differ significantly.

The two factor (with interaction) ANOVA of larval metamorphosis (time in hrs) is given in the table 5. 3. The table shows that feeds have a significant contribution ($P< 0.05$) to the total variation in the data. The post Hoc Duncan's multiple range test (DMRT) revealed the grouping of the five levels of feeds into four sub-sets (Mean values are given in the Table 5. 4). Here also the origins as well as their interaction with feed levels were not significant.

Table 5. 3. ANOVA for larval metamorphosis in hours with enriched rotifers

Source of variation	Sum of squares	df	Mean square	F	Significance
Experiment	8.533	1	8.533	2.133	0.160
Feed	9443.467	4	2360.867	590.217	0.000
Exp*Feed	51.467	4	12.867	3.217	0.034
Error	80.00	20	4.00		

Table 5. 4. DMRT of larval metamorphosis with enriched rotifers

Sl. No.	Name of the feed fed to rotifers	*Mean of larval metamorphosis (hrs)
1	*N. salina*	63.00[b]
2	*I. galbana*	73.33[c]
3	*C. marina*	94.66[d]
4	*S. cerevisiae*	97.00[d]
5	Shark oil emulsion	51.33[a]

* The means with similar superscripts does not differ significantly.

5. 4. Discussion

Rotifers are fed to a number of larval stages of penaeid prawn and it is an established fact that rotifers are given to protozoea II onwards were often seen to clutch and eat the rotifers. Although *Brachionus* was taken during PZ- II it was considered that the larva were unlikely to consume much until M1 when the pereopod endopods were formed (Emmerson, 1984). In the present study *Brachionus rotundoformis* is fed to M II to PL I without supplementing any other live or artificial feeds.

Studies on the nutritional requirements of marine fish and crustaceans have shown that fatty acids of the *n*-3 family have higher EFA values than the fatty acids of the *n*-6 family and have also demonstrated that marine fish and crustacean lack the ability for de novo synthesis of *n*-6 and *n*-3 fatty acids (Sargent *et al.*, 1990). Growth (moulting) and the survival by shrimps larvae fed with different enriched diets proved that particular dietary essential fatty acids have considerable growth promoting effects and the interaction dietary *n*-3 and the *n*-6 fatty acids also has considerable impact on the growth of the prawns. Kanazawa and Teshima (1977) demonstrated that penaeid shrimps are not capable of synthesizing PUFA's such as linolenic and (18:2*n*-6) linolenic acid (18:3*n*-3), Eicosapentaenoic acid (20:5*n*-3) and docosahexaenoic acid (22:6*n*-3). They inferred that these fatty acids are essential for shrimp and should be supplied through their diet. In the present experiment rotifers enriched with shark oil emulsion and *Nannochloropsis salina* with a very good profile of EFA's produced better survival and growth against other rotifers enrichment feeds like *Chlorella marina*, *Isochrysis*

galbana and yeast which having low EFA profile. The moulting of M II had shortened and survival percentage increased when HUFA enriched feeds are given to larvae. This is in conjunction with the findings made by Immanuel *et al.*, 2003. Apart from accelerating survival of candidate species, dietary lipids may also influence the somatic growth. Also the growth in turn determined by moulting which is an indispensable and an important phenomenon in crustaceans. The involvement of lipid during moulting has been well established by Forster (1976) and Read (1977). Briggs *et al.*, (1988) have reported that 9.5% of lipid in the diet registered 56% of survival and 12.5% of lipid in the diet showed only 45% of the survival rate in *P. monodon*. In the present study survival percentage had increased to 72-83% when feed containing all the n-3 HUFA's were fed to shrimp larvae i.e., *N salina*, shark liver oil and *I galbana* enriched rotifers.

Moulting is largely influenced by fatty acids of the *n*-3 family (Guary *et al.*, 1976). Glencross *et al.*, (2002) stated that prawns have some capacity for synthesis *n*-3 HUFA, inclusion of the *n*-3 HUFA in the diet doubt alleviates some of the resources required for the synthesis of HUFA and subsequently allows for improved growth potential. In the present study the moulting time between successive larval stages reduced when enriched rotifers especially shark liver oil and *N salina* was fed to larvae. This break through is a very important in the feeding protocols of shrimp hatcheries by reducing the high priced *Artemia* nauplii and some of the inert feeds which deteriorates the water quality. It has been documented that *P. monodon* cannot synthesis

either 18:2*n*-6 or 18:3*n*-3 (Kanazawa *et al.*, 1979 a, b). However, limited conversion of these shorter chains polyunsaturated (PUFA) to the longer HUFA (20:*n*-6, 20:5*n*-3 or 22:6*n*-3) was observed (Kanazawa *et al* 1979a, b). Elongation and desaturation of 18:3*n*-3 to HUFA *n*-3 (20:5*n*-3 and/or 22:6*n*-3) by *M. japonicus* was observed to be about 20% of that achieved by rainbow trout (Watanabe, 1982). Kanazawa *et al.*, (1985) observed that survival of *M. japionicus* increased with increasing n-3 HUFA levels from 0% to 1% but decreased at 2% suggesting a possible over done or contamination by oxidation product of HUFA. Xu *et al.*, (1994) suggested that the relatively high levels of HUFA such as AA, EPA and DHA in the body lipids of *F. chinensis* fed essential fatty acid free diets were probably. The result of preferential utilization of short and medium chained fatty acid as energy source for metabolism rather than on increase in absolute content of the HUFA.

The present study demonstrated this, as previously seen for other penaeid shrimp. The *n*-3 is essential for normal growth and survival of larval *Penaeus monodon*. It is clear from the experiments that feeds lacking HUFA's (yeast, *Chlorella marina*) had poor survival and growth. Rotifers enriched with shark liver oil emulsion, *Isochryisis galbana* and *Nannochloropsis salina*, had good survival and growth.

Summary

The present study evaluated the relative efficacy of selected micro algae and yeast for the mass culture of *B rotundiformis* and also to optimize the environmental factors like pH, salinity, and photoperiod. Fatty acid profiles of enriched rotifers were analysed and the enriched rotifers were used for the production of *A sebae and P monodon* juveniles by replacing *Artemia* nauplii.

The efficacy of micro algae like *Nannochloropsis salina*, *Chlorella marina*, *Isochrysis galbana* and Baker's yeast were evaluated at different concentrations as feed for mass production of rotifers. Experimental duration was six days for each experiment and at the end of the experiment samples were taken and counted under microscope and the growth rate of rotifers was calculated. The instantaneous growth rate (K), per day growth and doubling time of were also calculated.

The results of the experiments show that *Nannochloropsis salina* and *Isochrysis galbana* are the best feeds for mass production of *B. rotundiformis*. The instantaneous growth for rotifers fed with *N.salina* is higher (0.308±0.004) compared with other feeds. The per day growth was highest in rotifers fed *I. galbana* (40.18±1.97) and doubling time was reduced to 2.25 days when *N. salina* was fed to rotifers.

Results of experiments also revealed that pH range of 7 -7.2, salinity range of 20 to 25 ppt and photoperiod of 18 l: 6d were optimum for the mass production of *B.rotundiformis*.

Rotifers enriched with shark liver oil emulsion had a very good profile of the essential *n*-3 HUFA's, the DHA:EPA:AA ratio and the DHA/EPA, EPA/AA ratio were also high compared to all other treatments. Among various micro algae enriched rotifers, *Nannochloropsis* enriched rotifers had superior HUFA, levels, total *n*-3 fatty acids, and total unsaturated fatty acids. The DHA: EPA: AA ratio was 0.4:8.4:0.2.

The results showed that larvae of *A sebae* fed with shark liver oil enriched rotifers exhibited the best performance in terms of final weight, metamorphosis, followed by those fed with *Nannochloropsis* enriched rotifers. The percentage of survival was higher in case of *Nannochloropsis salina* and oil enriched rotifer. Pigmentation was observed on the fourth day, when *Nannochloropsis salina* enriched rotifer was fed to the fish larvae. Oil enriched rotifers fed fish larvae had taken seven to ten days for metamorphosis. This reflects the essentiality of HUFA's during early larval development of marine finfishes.

The feeding trials on shrimp larvae (*P.monodon*) showed, highest survival was attained with *Nannochloropsis salina* enriched rotifers was fed to

the larvae. Regarding metamorphosis, larvae fed with oil emulsion had taken only less than three days to transform into post larvae. Present study revealed the essentiality of fatty acids especially HUFA's for normal metabolism and growth of shrimp larvae.

This is the first attempt on the nutritional enrichment using shark liver oil, micro algae and yeast in rotifers for feeding marine fin fish and crustacean larvae, it is expected that the results of the present investigation would definitely be beneficial to researchers and aquaculturists around the world.

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