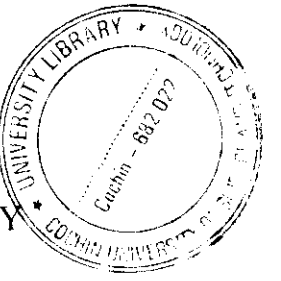
CULTURE AND NUTRITIONAL ENRICHMENT OF THE ROTIFER *BRACHIONUS ROTUNDIFORMIS* (TSCHUGUNOFF) FOR THE REARING OF MARINE FIN FISH AND SHRIMP LARVAE



THESIS SUBMITTED TO THE
COCHIN UNIVERSITY
OF SCIENCE AND TECHNOLOGY

IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

UNDER THE FACULTY OF MARINE SCIENCES

BY

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COCHIN

AUGUST 2004

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.

Dr. C.P. Gopinathan,
(Supervising Guide)
PrincipalScientist
CMFRI, Kochi.

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.

Acknowledgement

I acknowledge the abundant blessings and grace of the Almighty who enabled me to complete this research work for my Ph.D.

I am greatly indebted to Dr. C.P. Gopinathan, (supervising guide), Principal Scientist, FEMD, CMFRI, Kochi, for his guidance, constant encouragement, advice, criticism, and support during the course of my investigation and documentation.

I am extremely grateful to Prof. (Dr.) Mohan Joseph Modayil, Director, CMFRI, Kochi for extending all the facilities for successful completion of this research work.

I am also grateful towards former Directors of CMFRI, Dr. M. Devaraj and Dr. V.N. Pillai for their help during my research work.

I acknowledge my profound gratitude to Dr. V.C. George, Director, Centre for Aquaculture Studies, Sacred Heart College, Kochi, for his valuable guidance, critical comments and suggestions during the course of work and writing.

My very special thanks are due to Dr. R. Paul Raj, Head, PNPD and Officer-in-Charge, PGPM, for his support and guidance and for critical evaluation of the manuscript. My special thanks are due to Dr.G. Gopakumar, Principal Scientist, RC of CMFRI, Mandapam, Mr. P. Vijayagopal, Senior Scientist, RC of CMFRI, Mandapam, Dr. J. Jayasanker, Senior Scientist, FRAD, CMFRI for their help during the course of my work.

I express my sincere thanks to Dr. K. Devadasan, Director, CIFT, Dr. P.G.V. Nair, Principal Scientist and HOD and Dr.

T.V. Shankar, Senior Scientist, Biochemistry and Nutrition Division, C.S.F.T for their help rendered in the fatty acid analysis.

I am extremely thankful to Rev. Fr. John Pinadath, CMI, Manager, S. H. College, Rev. Fr. George Peter Pittapillil, CMI, Director, Mithradam, Rev.Fr. Saviance, CMI, Principal and Rev. Fr. Joseph Poonely, CMI (Former Principal), Rev. Fr. Vargheese Panthalookaran,CMI, of S.H. College for their help during the course of my work.

I express my thanks to Gopal Ji and Devan Ji for their support and encouragement during the course of the work.

I am thankful to Dr. P.K. Suresh Kumar, Scientist, TBGRI, Trivandrum, Dr. Satish Sahayak, Mr. R. Gireesh, Dr. K. J. Abraham, Mr. Binu Vargheese, Mr. U. Unnikrishnan, Mr. Joice Abraham, Mr. Mitchu Srinivas and Mr. R. Avinash and all other Research scholars of C.M.F.R.I., for their support in various stages of my work.

I take this opportunity to express my love and affection to my wife and relatives who have been motivating factor in all my endeavours.

I am grateful to all the staff and students of C.M.F.R.I., for their support rendered to me at various stages of my investigation.

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).

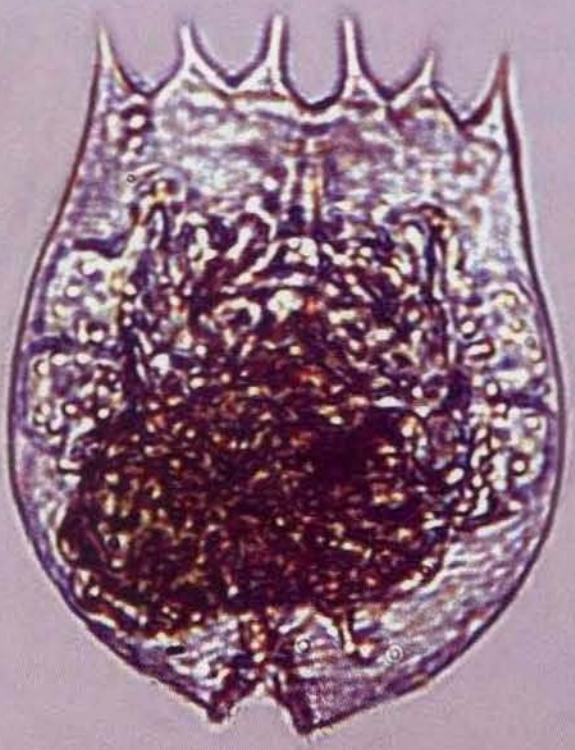
2. 2. Material and methods

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*

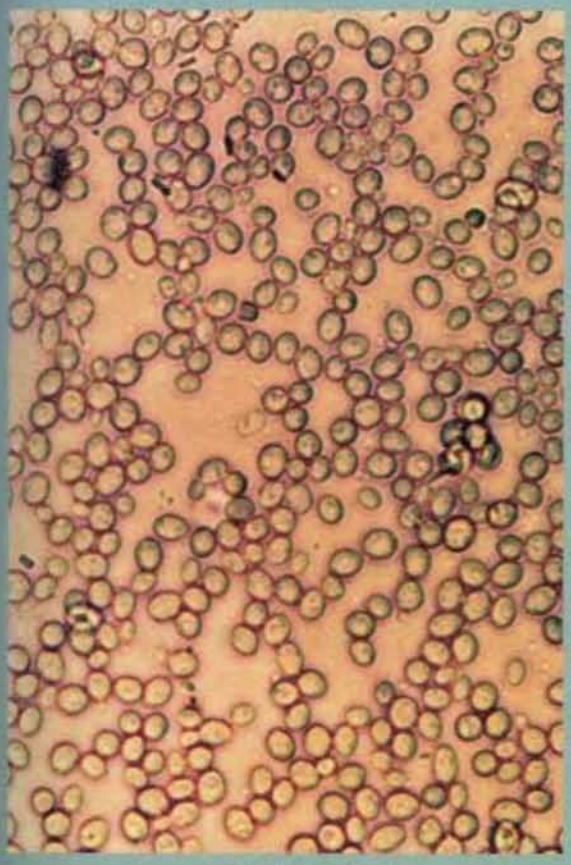
b) *Chlorella marina*

c) *Isochrysis galbana*

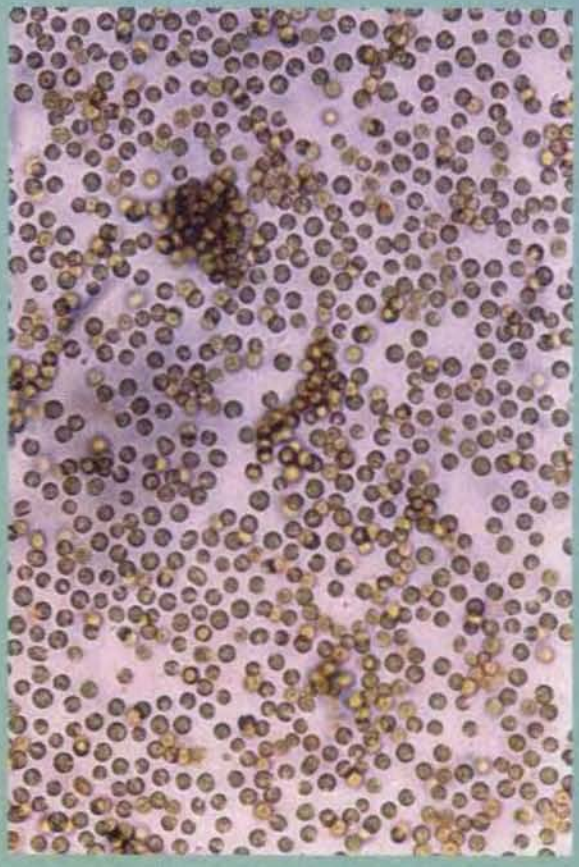
d) *Nannochloropsis salina*

Plate II

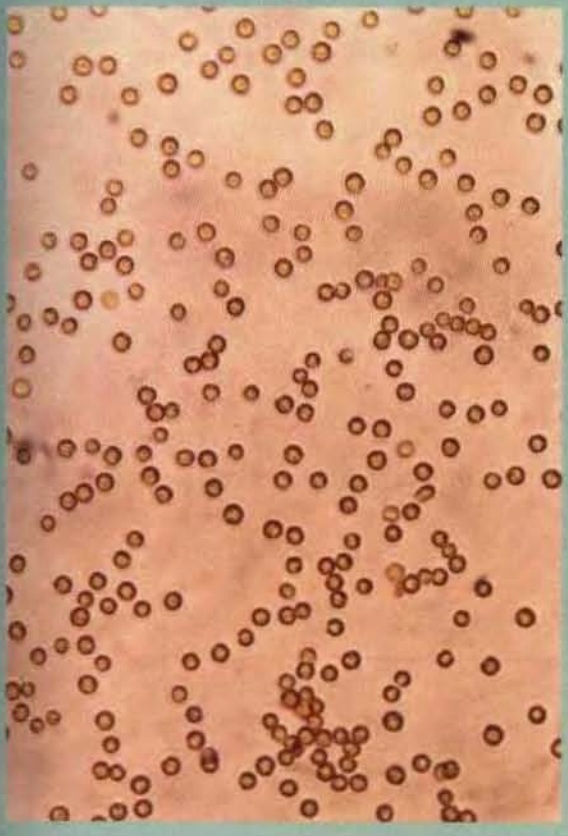
a



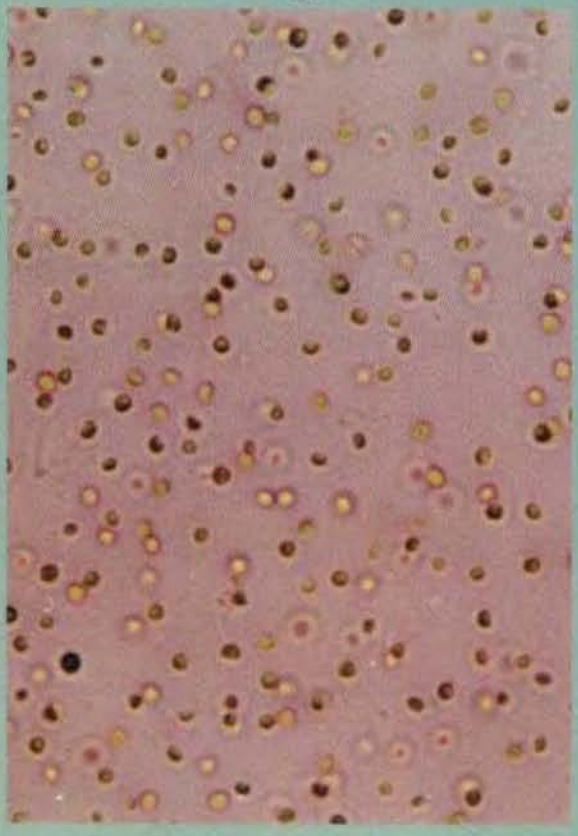
b



c



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.