CMFRI

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**Recent Advances in Breeding and Larviculture
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CURRENT STATUS OF LIVE FEED PRODUCTION FOR LARVICULTURE

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Introduction

The major expansion of marine finfish and crustacean aquaculture since 1980s around the world can be attributed to the development of standard mass production techniques of live feeds. Eventhough most farmed marine animals are either carnivorous or omnivorous from their post larval stages, micro algae are required for larval nutrition during a brief period either for direct consumption or indirectly as food for live prey fed to small larvae. The hatchery production of penaeid shrimp post larvae depends on the use of live diatoms for the early stages and *Artemia* for later stages. The hatchery production of juveniles of marine finfish is achieved globally by the use of rotifers and *Artemia.* Microalgae are also routinely used in the 'greenwater technique' employed for marine finfish larviculture.

Most marine finfishes have altricial larvae and when yolk sac is exhausted, they remain in an undeveloped state. The digestive system is rudimentary, lacking a stomach and much of the protein digestion takes place in the hindgut epithelial cells. Altricial larvae cannot digest formulated feeds and hence live feed is vital for their survival. Live feeds are able to swim in water column and are thus constantly available to the larvae. The movement of live feed in water stimulates larval feeding responses.

Micro algae

Micro algae play a critical role in the marine aquaculture of molluscs, shrimps and fish. Nearly 16 genera of microalgae are commonly employed for aquaculture purposes. They are generally free living, pelagic and in the nannoplankton range (2-20µm).

Typically culture units consist of vertical cylindrical tanks (100 – 1000 litres) made of transparent plastic, which are flat or conical at the bottom. Fluorescent tubes placed around the tank of culture provide artificial lighting. Injection of compressed air at the bottom of each tank produces bubbles that rise to this surface at a rate of 0.1 and 3.0 volume per culture volume and per hour. Rectangular tanks (0.5 – 1.0 m depth) are also used with surface lighting. These tanks are easier to clean than transparent vertical cylindrical tanks.

Precautions for maintaining culture aseptic and to prevent contamination are very much needed. All fluids and surface that come into contact with the culture must be sterilized. Natural water used for the culture should be free of pollution and stable in quality. A sand filter reduces the sizes of suspended particles to 10-20 µm, thereby eliminating most of the zooplankton. Additional filtration by cartridges or sieves reduces particle size to 1 µm. Chemical sterilization such as chlorination – dechlorination and by activated carbon can also be practiced. Natural filtered water is enriched by the addition of mineral salts required for photosynthesis, i.e. metabolisable nitrogen, phosphorus and trace elements including iron and silicate for diatoms. A chelating agent EDTA is often added to prevent precipitation of ferric hydroxide. Vitamins such as thiamine or biotin should be added with due caution because of their rapid degradation due to heat. The most commonly used enrichment media is f/2 medium (Gillard and Ryther, 1962) and the medium of Conway (Walne, 1966). Temperature is often controlled between 18 and 25ºC, but this should be fine tuned to each species.

Batch cultures are generally run according to production cycles of 3-7 days. Once illuminated tanks have been cleaned and filled with filtered sterilized water, enrichment medium is added and aeration is provided, and an inoculum is introduced. The algal strains are provided in few milliliters of culture in a test tube. Starting from this sample successive volumes of increasing size are inoculated in order to prepare the biomass required to reach inoculum

concentration in the production tanks. The cultures obtained in hatcheries seldom exceed a density of 6×10^6 cells ml^{-1} at the end of 5 days. The costs of producing microalgae in hatcheries include labour (90%) amortisation (6%), energy (3%) and miscellaneous expenses (1%). In Industrial facilities specialized in the production of microalgae and exploiting production system in controlled conditions such as photobioreactrors, the cost of production can be reduced considerably.

Microalgae have been shown to play a significant role in larviculture of marine finfish. When phytoplankton was included in larval rearing tanks, the survival, growth and food conversion index of many marine finfish species were better than in clear water condition. The green water technique (larviculture in an endogenous bloom of phytoplankton and rotifers) and the 'pseudo green water technique' (larviculture in a tank supplemented daily with exogenous phytoplankton and rotifers) have much commercial application in marine finfish larviculture. Micro algae can also influence live feed and larval microbiology. It has been found that exudates of some algal species can either enhance or inhibit the feeding activity of copepods in cultures .These substances are also involved in the settlement of micro flora required in the gut of fish larvae to prevent intestinal opportunistic bacteria from causing disease. Bacteria associated with live feed can be transmitted to larval fish during feeding. As live prey actively ingest bacteria, it is possible to introduce favourable bacteria as probiotic. In the 'green water technique' of larviculture micro algae contribute to maintaining the nutritional quality of live food and also positively influence on the settlement of a healthy intestinal micro flora in fish larvae Micro algae can also possibly influence the endotrophic stages (egg and pre–larvae) and early exotrophic stages. Micro algal background has an important effect on the timing and intensity of first zooplanktonic feeding. Micro algae also play a role in intestinal transit and gut repletion. Improvement in the survival at first feeding is the main result of larviculture with micro algae. Improvement in growth efficiency during rotifer period is another result of micro algal background in larval tanks. Early enhancement of digestive and assimilative functions improves the survival and growth of fish larvae and favours the transition to exotrophy. The use of micro algae in tanks increases the production of pancreatic and intestinal digestive enzymes and improves the quality of gut flora. Even after the endo-exotrophic phase, micro algae have a positive effect on larviculture and may increase the resistance of larvae to further stressing or adaptive conditions. The indirect effects of micro algae on larvae are mainly related to water quality, luminosity, the bacteriology of water and the quality and accessibility of rotifers. It is thus evident that strategic use of micro algae in hatcheries during the very early life of marine fish improves the success of first feeding, a prerequisite for efficient survival, growth and quality in fish larviculture.

Rotifers

Rotifers have been used as live feed for cultured marine fish, since four decades. It is well known that a continuous, stable and reliable supply of nutritionally adequate rotifers is the key to the larviculture of marine finfish. Rotifers of the species *Brachiounus rotundiformis and B. plicatilis* are almost indispensable for larval rearing of most marine finfish .

The success of rotifer cultivation is dependent on selecting the most suitable rotifer species or strain for local culture conditions, maintaining water quality in culture tanks and choosing the most appropriate culture technique. Size, the type of reproduction and reproductive rates are species or strain specific. Culture temperatures, salinities, type of food and its quality - all influence the type of reproduction and its rates. Mass production of rotifers is achieved by encouraging rotifers to reproduce asexually, since sexual reproduction results in males and resting eggs. The amount of food that has to be supplied daily to each tank depends on the reproductive rate of rotifers. Usually 1-4 g of baker's yeast is supplied per million rotifers per day.

The optimal range of pH for culturing rotifers is 7.5 – 8.5 and the pH affects the percentage of unionized ammonia in the water. The pH of cultures play an important role since the toxicity of NH_3-N is a function of pH, temperature and salinity. The optimal level for ammonia is <1 mg/L and the acceptable level of ammonia and nitrite levels is 6-10 mg/L. Rotifer cultures require aeration and the dissolved oxygen level should be maintained above 4 ppm. Surplus food is one of the major factors for the deterioration of water quality. This can be avoided by dividing the daily food ration into four to six meals a day or by continuous feeding using a peristaltic pump.

Stock cultures of rotifers are maintained for long periods which facilitate their availability to mass culture wherever they are needed. Natural seawater should be filtered through a 0.2 μm membrane filter and heat sterilized at 100^0C

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at atmospheric pressure for 30 minutes to avoid fermentation of insoluble precipitates. The cool sterile seawater can be employed for stock culture. Erlenmeyer flasks (100 ml in volume) or 50 ml sterile disposable tubes can be used for culture. Each heat sterilized flask is filled with 10 – 20 ml sterile sea water and 40 – 60 rotifers are added. Usually, a salinity of 30ppt is suitable for most strains. A drop of concentrated algae is added to each culture and the flasks or tubes are incubated at temperatures ranging from 20 – 35^{0}c. The cultures are fed *ad libitum* every 2 days with concentrated algae. Cultures are renewed every 7-10 days. Culture of *Nannochloropsis sp* was found to be the most convenient source of food for rotifer cultures. As in the case of microalgae, mass cultures are done by batch, semi continuous and continuous culture methods.

Evaluating the physiological state of rotifer culture is very important in hatcheries since larval production depends on a predictable and reliable supply of rotifers. Six parameters *viz* egg ratio, swimming velocity, ingestion rate, viscosity, enzyme activity and diseases are employed for assessing the state of health of rotifer cultures.

The nutritional quality of rotifers is improved by enrichment, in which rotifers are collected or harvested from culture tanks into containers where they are kept at very high densities and incubated for 8 – 20 hours with enrichment dietary components like HUFA. In addition to nutritional enrichment, rotifers can be enriched with antibiotics or with probiotic bacteria. The nutritional value of rotifers depends on their dry weight, caloric value and chemical composition .The number of rotifers consumed by the larvae determines the quantity of food reaching their gut. In red sea bream, the number of rotifers consumed daily increases with size or age of the larva, 55 – 72 rotifers per 3.9 mm length larvae to 4700 per 11.4mm length larva.

Various methods of storing rotifers have been studied. Frozen rotifers are not usually adequate as feed because of leaching of nutrients. Live *B .plicatilis* can be stored at 4^{0}C at relatively high densities for at least one month . Rotifers can be kept at -1^0 C without feeding or water exchange for about 2 weeks .*B. rotundiformis* strains are less tolerant to 4^0 C than *B .plicatilis* rotifer strains and the strains known as SS type are most susceptible and showed lowest survival. Amictic eggs of rotifers can be preserved by cryopreservation in liquid nitrogen after they have been impregnated with cryoprotective agents like dimethyl sulfoxide (DMSO) .This method ensures full preservation of genetic traits of importance to aquaculture. Cryopreservation is not a suitable method for preservation of large numbers of rotifers for direct use as feed.

Artificially produced rotifer eggs have been tried as an alternative to daily production of rotifers. The production of these eggs can be manipulated by environmental factors, such as salinity, food quality and quantity, rotifer culture density, exchange of culture media and temperature and varies between *B. plicatilis* and *B. rotundiformis*. The cost of producing resting eggs is very high and therefore not yet been extensively adapted in hatcheries.

It is evident that rotifer cultures will continue to be indispensable in marine finfish hatcheries. Current methodologies of producing and enriching rotifers are meeting the requirements of the industry. The current need to have very small sized rotifers is difficult to achieve, although several super small strains have been found and cultured .Improved methods for predicting the health of cultured rotifers may be useful in preventing culture crashes). Using preserved rotifers may eliminate the dependence on daily production of rotifers. Cheaper methods of resting egg production are another field which requires research attention in future.

Copepods

Copepods are a major component of the natural diet of marine fish larvae. The advantages of copepods over rotifers are that copepods have wide range of body sizes both within and between species. The early stage nauplii and copepodites can be extremely useful as initial prey for species that have very small larvae with small mouth gape at first feeding.

In extensive methods copepods are collected from nature and inoculated into outdoor tanks to mass produce for fish larval rearing. The larvae are then transferred at densities of 0.01 to 0.321/L. Additional prey may be added during the larval rearing when necessary to maintain prey densities in the range of 200 – 500/L. By this method from

1986 to1994 a total of around 2 million juvenile cod was produced .Disadvantages of this system include the inability to control production and thus food levels and predators. Lack of food results in differential growth in fish larvae.

Outdoor production of copepods in ponds or large tanks of 350 – 5000m^3 is carried out in Europe and Asia for cod, grouper and flatfish. Filtered seawater by using filters of around 20 – 40 μm is generally used in these systems. Phytoplankton bloom can be induced by application of commercial fertilizers. Filtering devices that allow for selective sieving are used to collect primarily nauplii (80 – 250 μm) and copepodite stages (80 – 600 μm) to inoculate the rearing tanks.

In Asia, a mesh size of 400 – 600 μm was used to inoculate outdoor tanks for grouper rearing with copepodites and adult stages 3 days before stocking the newly hatched fish larvae at densities of 5 m^{-3} (Toledo *et al* 1999). In this system, using wild harvested copepods, an average survival of 3.4% at harvest correspond to an average production of 0.17 grouper *Epinephelus coioides* juveniles" m^{-3}. Regular monitoring of densities of live prey in these outdoor systems is important for the successful rearing of marine fish larvae. An advantage of outdoor ponds over the extensive systems that rely on the local production of zooplankton is the possibility of culturing the zooplankton over one generation before using them as food.   Moreover, feeding wild plankton directly to the fish increases the risk of infections.

Several attempts to mass culture copepods in intensive systems have been undertaken with varying success. Species with relatively short generation at ambient temperatures are best suited for aquaculture purposes. Species inhabiting coastal environments are normally more tolerant to variations in salinity and temperature and have a wider thermal and salinity tolerance. The most frequently cultured calanoid species belong to the genera found in coastal waters, such as those of genera *Acartia, Centropages, Eurytemora* and *Temora.* These copepods are small, with relatively short generation time and a wide thermal and salinity tolerance and are easily adaptable to laboratory conditions.

Aeration is required to maintain phytoplankton in suspension and to create small turbulence which helps to distribute copepods within the culture tanks. Most calanoids require large volumes and the adult density rarely exceeds 100/L. Successful hatch culture of the calanoid *Acartia* sp was achieved in 1000 L polyethylene tanks, 1.3m in diameter with a conical base. The tanks are emptied after the 8 day hatch cycle and cleaned and a new batch culture was started . Contamination of copepod culture by bacterial blooms, ciliate infection, other copepods or rotifers may pose a problem. In commercial facilities, contamination by rotifers is most likely to cause the collapse of copepod culture, since the rotifers with their higher reproductive rate would quickly out compete the copepods. Hence these cultures should be strictly kept apart.

Ciliates are utilized by copepods and in periods of low phytoplankton concentration constitute the major dietary source. Ciliates are often an indication of overfeeding and if ciliates are noted in cultures it is advisable to empty the culture using a 60 or 80 μm mesh, which retains the adult copepods, but allows the ciliates to be washed out.

Harpacticoid copepods have several advantages for culturing .They include(i)High tolerance to a wide range of environmental conditions. (ii) Ability to feed on a wide range of live or inert diets. (iii)High reproduction capacity. (iv)Relatively short life cycles. (v)Ability to be cultured in high densities.(vi) Requirement for surface area rather than volume (vii) Planktonic naupliar stages.(viii) Can be used as tank cleaners in rotifer cultures, other copepod culture or larval tanks.

Filtered seawater can be used for harpacticoid culture and most feeds are acceptable to many harpacticoid species. Algae which quickly sediment are also good feed because bacteria colonise these cells, and the mixture of algae and bacteria form a good dietary combination for harpacticoids. Photoperiod influence offspring production and sex ratio. A photoperiod of 12 L / 12 D was shown to be most favourable for offspring production. Many harpacticoids have wide thermal and salinity tolerances. Ciliates and rotifers in the culture tanks compete for food and may lead to crash of copepod culture.

Improved growth, survival and / or rates of normal pigmentation have been documented for several marine fish species fed copepods alone or as a supplement to other traditional live feeds. The improvements in larval growth,

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survival and normal pigmentation are generally attributed to the levels of DHA, EPA and / or arachidonic acid (ARA) in the diet and in particular to the DHA: EPA ratio in the diet. Copepods which constitute the major diet for marine fish larvae in nature contain high levels of DHA and other PUFA. DHA levels in wild copepods can be more than 10 times higher than in enriched *Artemia* .

The interest in copepod culture as live feed is gaining momentum in recent years for the rearing of altricial larvae. A few of the culture methods developed to date can be adapted in commercial hatcheries. However there is a need to evolve intensive culture methods for copepods in future. It is felt that the future expansion of mariculture especially of marine finfish depends largely on the development production of resting eggs of copepods on commercial scale.

Artemia

Artemia is widely used in the mass culture of different sea bream species, sea bass species, wolf fish, cod, turbot, halibut, flounder species, milk fish, surgeon and many shrimps, prawn, crabs and lobsters. Nauplii in instar I and II stages are the most widely used forms of Artemia in aquaculture. They are the earliest and easiest live feed, being obtained directly from the cysts.

Several factors are critical for the successful hatching of *Artemia* cysts. Optimal hatching conditions are constant temperature, 15-35gl^{-1} salinity, pH around 8.0, Minimum oxygen levels of 2 mgl^{-1}, preferably 5 mg l $^{-1}$, maximum cyst densities of 2 gl^{-1} , and strong illumination (2000 lux). Best hatching results are achieved in containers with conical bottom, aerated from the bottom. Transparent or translucent containers will facilitate inspection of hatching, especially when harvesting.

Strong illumination (above 2000 lux at the water surface) is essential, atleast during the few hours after complete hydration, to trigger the start of embryonic development. It is advisable to keep the hatching tanks indoors and to provide artificial illumination, so as to ensure good standardization of the hatching process. When hatching large quantity of cyst, bacterial load rapidly develops. Reducing bacterial development during hatching will improve the hygienic status of nauplii and may result in better hatching. It can be achieved through simple disinfection of the cyst using the liquid bleach solution, through decapsulation. Attention should be paid to the selection of *Artemia* cyst batches with good hatching synchrony (less than 7h between hatching of first and last nauplii) and high hatching efficiency (more than 2 lakhs nauplii per gram).

After hatching and before feeding to fish larvae, the nauplii should be separated from the hatching wastes (empty cyst shells, unhatched cyst, debris, microorganism and hatching metabolites). Decapsulation of cysts results in disinfection of the cysts and also eliminates the introduction of cyst shells to culture tanks.

Most marine fish larvae cannot synthesize DHA, EPA or Arachidonic acid from shorter chain precursors and they must be provided in the larval diet, hence artemia is enriched for enhancing the nutritional value for using as a live feed. Although artemia is often an inferior food source for fish larvae compared with wild zooplankton, the ability to produce any amount of biomass within 24 hrs, and the constant improvement of enrichment products ensure its continued use in marine fish larviculture. It is quite possible that artemia will gradually replaced by formulated diets; it is obvious that the use of nauplii will continue in hatcheries at least for a few more years.

Prospects

It is quite obvious that at the present status of technology, live feeds cannot be dispensed in marine larviculture. The major challenge for the future is in the development of commercial artificial feeds for larvae. The immediate research thrust should be on technologies for high density and stable production of high quality live feeds. The identification of causes for sudden crashes in culture and remedial measures for the same requires priority attention. Genetic manipulations for the production of super small strains of rotifers and the commercial production of rotifer cysts are also major areas for intensified research. The production of high density microalgae in photobioreactors and by fermentation techniques are potential areas for future development. The copepod culture techniques, which is the key to the success of rearing of altricial larvae of marine finfish still remains to be standardized. Hence this should receive more focused research than the better studied traditional live feeds.

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