NEXT GENERATION RESEARCH IN AQUACULTURE

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Challenges in Larval Fish Nutrition

Adnan Hussain Gora, Saima Rehman,Prabu D.L., Chandrasekar S, Sanal Ebeneezar, Vijayagopal P

Abstract

Nutrition of marine fish larvae is an important factor for minimizing losses during the hatchery phase. Different aspects of nutrition of marine fish larvae have been studied deeply to understand the intricacies of the problem of their lower survival. To assist with the growing body of knowledge in larval nutrition, this contribution provides a review of the major research breakthroughs that have significantly improved our knowledge and lead to major research leads in this field. This chapter explains in detail the five important aspects of marine fish larval nutrition: nutrient requirements, live foods, formulated feeds, cannibalism and probiotics. Specifically, we target researchers, who are new to the field of marine fish larval nutrition, and we offer simple, but comprehensive explanation of the challenges associated with it.

Keywords: Cannibalism, Larvae, Microdiets, Nutrition, Microbes

Introduction

The marine fish larvae are the smallest autonomously feeding vertebrates which are exposed to fluctuating and harsh environment (Weiser, 1995). The life cycle of fish is divided into following phases: embryo, hatchling, larva, juvenile, adult and senile. The end of the lecithotropic phase and ingestion of exogenous food marks the beginning of the larval phase of life. Alliot (1979) classified marine fish larvae as altricial larvae, as they need exogenous supply of food even before the digestive system is fully developed. This is in contrast to the higher vertebrates in which the young one spends this critical period of development inside the embryo. In fact, the fish larva is quite different from the higher vertebrates in terms of developmental process and hatches before the yolk is fully absorbed. The reason for this type of development in fish is not clearly explained. However, this may be attributed to ecological reasons making the larvae the ideal collectors and transfer organisms of biologically bound energy from zooplankton to the top of the food pyramid (Nellen, 1986). The small size of the marine fish larvae is compensated by high fecundity, but it leaves the larvae vulnerable affecting the survival in natural ecosystem and culture conditions (Anderson, 1988).

The basic principle of aquaculture is the rearing of aquatic organisms in a controlled biological system with different levels of interventions directing growth of the animal, reducing the cost of rearing and occurrence of diseases (Beveridge, 2008). Historically, mariculture depended on capture of wild fry from natural waters, whereas hatchery production of fish started only in the middle half of the nineteenth century. Mariculture in the previous decades has witnessed a period of great research and development especially involving the early life stages of fish. This served as the foundation for commercializing the culture of a number of marine fish species like salmonids, amberjacks, sea breams, sea bass, croakers, groupers, drums, mullets, turbot, other flatfishes, snappers, cobia, pompano, cods, puffers and tunas (FAO, 2014). However, this drive to find out new candidate species for mariculture has reduced the effort in optimization of larval production of already commercialized species (Conceição et al., 2003). Although, the survival rate in hatcheries for many species is much higher than what is observed in nature, the seed production of marine fish still remains a bottleneck for mariculture growth.

One of the main causes of the bottleneck in larval rearing of marine fishes is the nutritional aspect. Theoretically, there are four modes of nutrient acquisition in fish larvae: endogenous nutrition, integument absorption, mixed feeding and exogenous nutrition (Balon, 1986). In the hatcheries, there are two ways of sustaining the larvae: live foods and formulated microdiets. The nutritional bottleneck in the larval rearing comes from a number of research problems that were recognized almost 40 years ago. Despite significant

developments have been made, the problem of lower survival of marine fish larvae fed on microdiets still remains. The problems are mainly because of the notoriously small size of the larvae (Rønnestad et al., 2001). However, there are also other aspects of the problem of larval nutrition of marine fish, which must be understood in detail. These may be summarized under the following headings:

1. Nutrient requirements of larvae
2. Live food and its nutritional profile
3. Performance of larvae on formulated microdiets
4. Cannibalism in fish larvae
5. Gut microbes in fish larvae

Therefore, the main aim of this paper is to highlight the key research efforts made in these aspects of larval nutrition. This analysis will contribute to the understanding of the implications of research in fish larval nutrition while at the same time highlighting the developments made so far in the different areas of this field.

Table 1: The different strains of rotifers used in aquaculture.

Rotifer type	Temperature	Salinity	Size
B. plicatilis (L)	Temperate	Euryhaline	170-230u
B. rotundiformes (SM)	Tropical	Brackishwater	120-160 u
B. rotundformes (SS)	Tropical	Marine	90-120u

Table 2: Characteristic features of different copepod orders.

Characteristic Feature	Calanoids	Harpacticoids	Cyclopoids
Antennule length	Long, upto 27 segments	Antennule, upto 10 segments	Antennule extending upto cepthalothorax 6 to 17 segments
Antennae	Biramous	Biramous	Uniramous
Habitat	Pelagic, occurring at almost all depths	Benthic	Pelagic, bentic and also epibenthic
Feeding Habit	Filter feeding	Detrivorous, benthic grazers	Omnivory; filial cannibalism; parasitic

Nutrient Requirements of Marine Fish Larvae

Nutrient requirement is generally defined as the daily dietary intake needed to fulfill a physiological role. Nutrients at different dietary levels show different responses like maintenance of body condition, growth, optimization of feed conversion or even disease resistance (Pohlenz et al., 2012). However, for the larvae, the nutrient requirement is usually considered as the intake needed to provide the maximum survival. The knowledge of nutrient requirements in marine fish larvae is largely qualitative in nature rather than quantitative (Conceição et al., 1993). This lack of knowledge in precise nutritional requirements is probably one of the causes of high mortalities and quality problems commonly observed in mariculture (Bell et al., 2003).

In case of juvenile or adult fish, nutrient requirement studies may be carried out by standard methodology involving graded levels of nutrients and the use of purified or semi purified ingredients (Shearer, 2000; Encarnação et al., 2004). However for the larvae, this is not possible as purified and semipurified feed ingredients are not well acceptable, and are poorly digested and utilized (Kolkovski, 2001). Consequently, these diets do not favor optimum growth and survival in the fish larvae (Person-Le Ruyet et al., 1993). Therefore, the direct dose-effect trials are difficult to conduct in fish larvae and this has lead to research on standardizing alternative methodologies to determine the nutrient requirements of larvae. Also the extrapolation of the information of nutrient requirement in juvenile is not an acceptable methodology. This is because the larval stage of fish is different from the juvenile phase in terms of the growth rate and physiological changes (McCormick et al., 2002).

The biochemical composition of the marine fish eggs may give an idea about the nutrients required in the larval stage. During the early embryonic phase, marine fish larvae utilize egg yolk for its growth and development. Therefore, egg contains all the nutrients that are essential for the embryo and larval development up to the stage of yolk-sac absorption. However, a feed developed on the basis nutrient composition of the yolk will not take into account the bioavailability and digestive losses of nutrients in the larval gut. Also, this approach may give unreliable results because of the variability in the yolk due to maternal age and nutrition (Jerez et al., 2012; Caamal-Monsreal et al., 2015). These reasons make it difficult to quantitatively estimate the nutrient requirements of larva from the biochemical composition of eggs.

The body profile of some nutrients like amino acids remains more or less stable even if the larvae are fed at different levels (Fiogbe and Kestemont, 1995). Growth is characterized

by net accretion of dietary amino acids bearing a close similarity to the pattern in body tissue. This forms the basis of the determination of nutrient requirements through body composition. This concept was first established in farm animals (Cole, 1978) and introduced in fish by Arai (1981). The estimation of dietary amino acid requirements from the body profile has been proposed as a good index of the indispensable amino acid (IAA) requirements of larval fish (Watanabe and Kiron, 1994). According to this concept, the first limiting amino acid is determined as the amino acid with the lowest, statistically significant, relative difference between its contribution to the diet and the larval amino acid profiles (Conceição et al., 2003). This relative difference ($rIAA_i$) in the diet and larval profiles is calculated as:

$$rIAAi = [_{diet}(IAA_i/ IAA_t) - _{larval}(IAA_i/IAA_t) \times 100]/ _{larval}(IAA_i/IAA_t)$$

Where, IAA_i is the content (g/100g) of the amino acid under study; IAA_t is the content of the total indispensable amino acid.

The limiting amino acid also sets the limit of protein synthesis. However, the tissue amino acid profile does not always give correct estimate of the dietary requirement. Allometric growth in fish larvae may also cause change in the tissue amino acid profiles to the tune of more than 30%. There are other variables like selective absorption efficiency of amino acids (Jurss and Bastrop, 1995) and preferential use of particular amino acid for energy purposes or growth (Rønnestad and Fyhn, 1993). Also, the compensation in the imbalance of amino acid profile of diet due release of free amino acids from body proteins can be as high as 50% in the larvae (Conceição et al., 2002). Similar phenomenon is observed in case of lipid profile of the tissues as acyslases and transacylases that esterify fatty acids into phospholipids do not have absolute specificities for particular fatty acids. Therefore, fatty acid compositions of tissues are partly determined by the levels of fatty acids available from the diet. This is true in the case of PUFA where an excess of one dietary PUFA e.g., 20:5n-3, can lead to and elevation of that PUFA in tissue phospholipids at the expense of another PUFA present in much lower concentrations in the diet e.g., 22:6n-3. This effect has been established for phospholipids of fish brain.

All these variables are not accounted for while determining the nutrient requirement from body profile. Therefore, to determine the amino acid profile required by the fish larvae, a thorough understanding of the following aspects of nutrient metabolism is required:

1. The rates of absorption of nutrients from the diet.
2. The inevitable catabolism of some nutrients for energy.

3. The change in nutrient profile caused by growth.
4. Release of nutrients from body turnover.
5. The conversion of nutrients into secondary metabolites.

Rust et al. (1993) took the first major step in this direction. Using radioactive tracers, they compared the absorption of undigested protein, enzyme digested peptides and pure amino acid in larval *Morone saxatilis*. To calculate the absorption of amino acids, they fed the larva with radiolabelled diets then maintained it in a vial until the gut was emptied. After emptying of the gut, the larvae were removed from the vial and the scintillation counts of the larval body and water in the vial were calculated. The assimilation of the nutrients (A) was determined by using the formula:

$$A = [L/(L+W)] \times 100$$

Where L is Bq of the larva, W is Bq of the incubation water, Bq (unit for the measurement of radiation).

The results indicated that pure amino acid was absorbed with high efficiency in the gut of the larvae. Although the authors realized that stress during intubation of the larvae would have changed the absolute values of absorption, they claimed that relative values of absorption are more informative and would be unaffected during the experiment. However, this method did not reveal the fate of amino acids absorbed by the larvae, i.e., whether the absorbed amino acids remained in free form or incorporated into protein. To answer this question, Rønnestad et al. (2000) made a slight change of the protocol which helped them to differentiate the absorbed amino acids into two compartments: the amino acids retained in free form and the amino acids incorporated into body proteins. The larvae were treated with 6% TCA and then a tissue solubilizer, which separated the free amino acid pools from the protein bound pool by using the formulas:

$$AA_{Protein} = [L_{Protein}/ (L_{FAA} + L_{Protein} + W)] \times 100$$
$$AA_{Free} = [L_{FAA}/ (L_{Protein} + L_{FAA} + W)] \times 100$$

Where, $AA_{Protein}$ and AA_{Free} represent the retention of amino acids in protein bound and free form respectively; L_{FAA}, $L_{Protein}$ and W represent the disintegrations per minute of the free amino acid pool, protein bound pool and rearing water respectively.

Both these methods underestimated the absolute values of retention because the

studies could not differentiate between the two sources of amino acids in water: exogenous source (amino acids unabsorbed from food) and endogenous source (amino acids from the body protein catabolized for energy). The catabolism of amino acids is a vital component of the metabolism. The larval fish may use amino acids to the tune of 60% of their energy requirements. Therefore, to account for this loss, a further advancement was made by Rønnestad et al. (2000). They developed a method hypothesizing that tracer from unabsorbed amino acids is bound to the amino acids whereas the tracer from catabolized amino acids will be bound to CO_2. The tracer from CO_2 was trapped using a potassium hydroxide trap and then estimated by reducing the pH. By this method, it was possible to differentiate nutrients retained from different parts of the body by dissecting out the gut and liver from the carcass of the larvae. The retention in the different parts was recorded by using the formula:

$$Rx = [X/B+L+G+W] \times 100$$

Where R_X is the retention of compartment X, B is Bq (unit for the measurement of radiation) of the larval carcass/body, L is Bq of the liver, G is Bq of the gut and W is Bq of the incubation water.

These pioneering studies improved our understanding of the larval nutritional physiology, however, in all the studies, only one particular amino acid, methionine, was labeled. To what extent methionine may be considered as the representatives of all the other amino acids remains to be revealed. Also, these models were based on single meal of labeled diet and the analysis of tracer content in the different body compartments. One-time feeding of a labeled diet to the larvae followed by the evaluation of the metabolic activities is a method, far from ideal. This may erroneously estimate the utilization efficiency compared to the hatchery conditions where continuous feeding is the standard procedure. This is because the continuously fed larval gut already harbors the enzymes in the lumen and is activated for digestion. Furthermore a continuous feed intake will change the rate of evacuation of the gut compared to single feeding altering the digestion process. To avoid these problems the larvae may be fed continuously after intubation of a hot diet (diet in which the tracer has been incorporated), a technique known as cold chase. Cold chase approach assumes that the absorption of the hot diet is affected partly by the cold diet (diet without markers) that follows. However, even the cold chase approach suffers from the drawback that the catabolism cannot be evaluated and must be assumed to be constant (Conceição et al. 2007).

An alternative method of nutrient requirement studies of the larvae was developed by Conceiçáo et al., 2003 that involved the use of ^{13}C-NMR technique and ^{13}C labeled rotifers.

This method is based on the concept that bioavailabilities of individual amino acids that can be determined by the ratio of specific activities for each individual amino acid in the diet and the live food. The bioavailabity of the individual amino acid (B_i) was determined by the formula

$$B_i = [(t_i/T_i)/(t_{all}/T_{all})]_{larvae} / [(t_i/T_i)/(t_{all}/T_{all})]_{diet}$$

Where *i* stands for a given individual AA; t_i and *t* the ^{13}C contents obtained by ^{13}C-NMR spectroscopy of, respectively, AA_i and all AA considered in the present study; T_i and T_{all} the AA contents obtained by HPLC of, respectively, AA_i and all AA.

The only assumption that this technique makes is that the tracers are metabolized in the same way as the trace molecule. However, the authors stated that this study might be beneficial for only fast growing larvae. This technique, although accurate, is costly and requires strict control which makes it difficult to execute this kind of experiment in all settings. Also the combination of the amino acid profile studies with the bioavailability data, a dietary deficiency of certain amino acids could be revealed, which was otherwise not apparent when taking the amino acid profile data alone (Saavedra et al., 2007).

It is clear that the nutrient requirement studies in fish larvae will not be possible without clear understanding the digestive process and the metabolic regulations acting over it. Most of the progress made so far in this area is by the use of radioactive markers. Presently, a range of tracer methodologies is available which are instrumental to improve our understanding of the nutrient requirements of larval fish (Conceição et al., 2007). Although results obtained using tracer studies do not necessarily represent the digestive and metabolic performance of an undisturbed larvae feeding *ad libitum* in a culture system or in the open ocean, these methods are used in standardized conditions, and can serve as tools to assess and compare the performance of larvae under different conditions. However, Rønnestad and Conceição (2012) pointed out that since normally a number of body compartments are compared in a few time points with short periods of up to 24h, the expression of data becomes limited to relative percentage basis (%). Therefore, it was necessary to understand the kinetics of the nutrient fluxes in addition to compartmental distribution of the tracer AA with the help of mechanistic modeling. The first attempt in this area was by Conceição et al., (1993). Modeling studies incorporating the use of tracers will reveal more accurate and useful information about the nutritional physiology of the larval fish. Although some work has been initiated in mammals (Fouillet et al. 2009; Stoll and Burrin, 2006) such studies are lacking in fish larvae.

Live Food and its Nutritional Profile

In most of the hatcheries, the first food of the fish larvae is rotifer or artemia. Although none of them is the natural food for most of the first feeding larvae, their use presents economical benefits in the hatcheries. The importance of artemia in fish larviculture was found in the 1930's. *Artemia* has many species of which *Artemia salina* is one of the seven that reproduce bisexually. *Artemia salina* is now collected from 600 sites around the world. The common feature of all artemia biotopes around the world is their high salinity. An efficient osmoregulatory system, capacity to synthesize respiratory pigments and the ability to form dormant cysts helps the brine shrimp to cope with high saline conditions. The ability to form easily transportable cysts is also the main factor that led the wide use of artemia in the 1970's and present annoying overdependence of hatcheries on it.

The total protein content of artemia fulfills the protein requirement of the fish larvae. However, the free amino acid content of the artemia is much lower than copepods. Artemia does not represent a complete diet for the larvae and needs enrichment. The moult to the 2^{nd} naupliar stage occurs 8 hours after hatching and is the feeding stage of artemia nauplii in which it feeds on particles that are smaller than 25u in size. Artemia enrichment was primarily developed for improving the essential fatty acid content with successfully increasing the HUFA content from 10% to 35% of total fatty acids. This may have a negative effect on the protein content bringing it down from more than 40% to less than 35% (Evjemo, 2001). Also the modulation of DHA: EPA ratio by bio enrichment rarely increases to 2:1 whereas in the marine zooplankton the ratio is naturally 4:1 (Shields et al., 1999). One reason for the inability of artemia to maintain a high DHA to EPA ratio may be the retro conversion of DHA to EPA (Navarro et al., 1999). Upto 70% of the DHA obtained through bioenrichement is catabolized and converted to EPA. However, some species of artemia resist the retroconversion within a limited temperature range (Evjemo, 1997). Enrichment of artemia with traditional products also increases the amount of triacylglycerol fraction at the cost of phospholipid content (McEvoy et al., 1996) that is essential for a number of marine fish larvae (Coutteau et al., 1997). The higher content of linolenic acid in the phospholipid fraction of the artemia also restricts fish larvae in assimilating sufficient EPA and DHA. Also, there is no known strains of *artemia* contain significant levels of 22:6(n-3) making (n-3) HUFA enrichment necessary. Procedures for enrichment with emulsions of marine fish oils are well developed. Commercial products are readily available to achieve this objective. However, up gradation of current procedures in the light of recent knowledge of PUFA requirements is essential. Current problems in enrichment of live feed are – 1. 22:6(n-3) content is very small in triacylglycerol micelles generated in enrichment procedures and

are prone to autooxidation, especially under vigorous aeration. 2. Natural antioxidants such as α-tocopheryl acetate and scorbyl palmitate are not effective especially until hydrolysed in the intestinal tract and absorbed. Ethoxyquin and Butylated hydroxy anisole minimizes peroxidation. However, the level of these in enrichment emulsions is an area where there is no information. With the exception of ascorbic acid and thiamine, artemia contain quite high levels of vitamins. However, the bioenrichment by algae is known to even more reduce the ascorbic acid and cyanocobalamin levels in the artemia.

Besides the shortcomings in the nutritional value of the brine shrimp nauplii, another reason of concern is the highly variable supply of cysts. Almost 90% of the global artemia cyst supply comes from the Great Salt Lake of Utah, USA. Historically, the cyst harvest has been highly variable in the past decades varying from a peak of almost 12000 tonnes in 2002 to all time low of less than 2000 tonnes in 1999. Overdependence on artemia, which is costly and has irregular supply, is a challenge in fish nutrition research. Larval survival as well as profit margin of hatchery managers is, to a large extent, controlled by artemia supply. *Artemia* must be replaced by a live food, which is relatively cheaper and more stable in terms of supply.

Rotifers are another immensely important live food class that is now used in most hatcheries. Research on rotifers started in the 1960's in Japan after it was found that rotifers, previously considered as pests in culture ponds, have optimum size as first food for marine fish larvae (Hirata, 1979). This incidental discovery has made the larval rearing of a number of marine fish species possible, especially those whose mouth size was not big enough for *Artemia* feeding.

Rotifers are most extensively used in mass culture because of their size, which varies between 90 to 350 u (Table 1). However from the culture point of view, one of the most important reasons for the wide use of rotifers is their feeding habit. Rotifers are microphagous feeders with some sensory mechanism regulating food selection mainly based on prey size and also following a type-1 functional response (Holling, 1966). Rotifers can feed on a variety of food sources having the particle size of 0.5 to 20u. Microalgae mixed with baker's yeast and/or rice bran is the usual food of the rotifer in the hatcheries. However, this characteristic habit of feeding makes the rotifers highly variable in their nutritional profile. The protein content of rotifer varies from 28-63% depending on the food quality and availability. The lipid content also depends on the dietary lipid content and quality and varies between 9 to 28%. The highly variable nutritional profile is not a favorable characteristic for its use as live food.

Like artemia cysts, rotifers can also produce subitaneous eggs or cysts. However, the normal mode of reproduction of rotifers is female parthenogenesis and cyst production is not a common phenomenon. Unlike artemia, rotifers do not have restricted geographical distribution, therefore the supply of rotifer it is not a critical issue. Nevertheless, practical use of rotifer cysts in larval culture has been identified especially in the case of crash of cultures. But due to the high cost of production of resting eggs they are not presently used at a scale comparable to artemia cysts. Rotifers cultures are very prone to collapse mainly due to water deterioration and bacterial load. Therefore the amount of food provided to the culture must be carefully maintained and split into a number of meals. The ozonization of water has been described as a very efficient way of maintaining the water quality (Suantika et al., 2001). Even then, the rotifer culture needs to be regularly checked for certain warning signs indicative of future collapse. These include the egg ratio of less than 10%, swimming velocity (Snell and Hoff, 1988), ingestion rate (Juchelka & Snell, 1994), viscosity of the culture medium (Hagiwara et al., 1998), and disease occurrence especially birnavirus infection. The crash of culture is almost inevitable when the egg ratio is lower than 10%. Rotifer culture also requires large space and volume of microalgal culture (to the tune of 5 times the volume of rotifer culture) to sustain itself. This is a drawback of rotifer culture and attempts have been made to culture the rotifers on non-algal based diets like baker's yeast and capelin oil (Tamaru et al., 1993; Reitan and Olsen, 1994) but it almost invariably lead compromise on the nutritional quality. Also, the nutritional profile like EPA and DHA content in the rotifers is greatly affected due to extended periods of residence in the fish tanks. This is especially important in case of marine fish larvae as, they are not able to synthesize these essential fatty acids *de novo* (Tocher, 2010). Deterioration of the nutritional quality of the rotifers may be avoided by providing algae to the fish culture tanks that demands an extra economic investment.

Although artemia and rotifers are immensely important as live food in the hatchery environments, however, in nature the larvae have higher preference for copepods. In fact, copepods (Table 2) are the first vital link in the marine food chain leading from primary producers to fish (Stottrup, 2000). The three important characteristics of copepods determining their success as live food in the hatchery conditions are: nutritional quality, ease of culture, and ease of harvest and capture by the fish larvae.

The nutritional value of the copepods is, in general, far better than rotifer or artemia nauplii used in the hatcheries. The protein content may be as high as 71% of the dry matter with higher percentage of free amino acids. The ratio of DHA: EPA in most of the copepods varies between 2 to 4 which is higher than the ratio observed in the artemia nauplii or rotifer even after enrichment. Also the content of these essential fatty acids attached to the

polar lipids is much higher than in artemia and rotifers. Some copepod species are able to synthesize the DHA and EPA (Norsker and Strottup, 1994). They may therefore have few specific nutritional needs in culture and may be able to synthesize long-chained HUFAs regardless of their diet.

Although most copepods reproduce sexually, some harpacticoids have been known to exhibit parthenogenesis, resting eggs have been observed in only calanoids. The culture of calanoid copepods like *Acartia, Centropages, Eurytemora* and *Temora* has been successful but the density of culture remains a limiting factor. Most harpacticoid copepods are benthic for at least part of their life cycle and a substratum is required for culture in most species. This makes clean harvest of all developmental stages from mass culture extremely challenging. Also the non-swimming behavior of harpacticoid nauplii complicates the harvesting of the live food for use in larval feeding.

At present, the need to find a copepod species with a relatively small body size, but good swimming abilities in all developmental stages and with the ability to grow in the absence of substrate is the highest priority (Fleeger, 2005). Available information on the biology of *P. spinosus* indicates this species has potential for development into a live feed for finfish larval rearing. (Kajihara and Nakamura, 1985). *P. spinosus* is an obligate symbiont and its culture may require construction of a mussel bed. The whole process undoubtedly has cost implications. It remains to be proven that whether the added costs of maintaining such copepod cultures are offset by lower percentage of fish larvae with morphological or developmental defects (Støtrrup, 2000).

Formulated Microdiets Performance

Formulated microdiets are developed to overcome the drawbacks associated with the live foods. Live foods are difficult to sustain and require considerable space and expense. Formulated microdiets, although cheaper and stable in supply, have not proven very successful for rearing the marine fish larvae. Compared with live foods, fish larvae fed with microdiets almost invariably exhibit lower growth performance and survival. Microdiets are acceptable by the larvae only after a few weeks of hatching (the only exception till date is Wolf fish larvae which can accept microdiets immediately after mouth opening). This is because feed ingestion and utilization is hampered in the larvae when fed with microdiets.

Feed ingestion is the result of a complex process that is preceded by searching, detection and capture of feed. In the earliest stage of larvae, when the notochord inflexion has not taken place, the locomotive capacity and hence searching ability is compromised. At this

stage, the larva depends on the food that comes to it rather than actively searching for it. Post notochord inflexion, the larvae exhibit stronger movements and darting behavior which helps cover more distance and search for food. In the larval stage, most fishes depend on vision to feed (Boeuf and Le Bail, 1999) and this is also supported by the extraordinarily large size of the eyes in the head (an exception being African catfish larvae that can be reared in complete darkness even from the first feeding). Detection of food can be improved by providing different colors to the feed particles to which the fish is responsive. Also, tank background color and contrast is an important factor, which helps the larvae detect the feed immediately. Some pigments have been used in order to improve the feed detection by fish larvae (Cahu and Infante, 2001). Chemoreception of the larvae may be utilized for the better detection and ingestion of food. Poor ingestion rates of microdiets by fish larvae may be improved by the addition of specific chemical compounds, which act as feeding stimulants. Free amino acids like arginine, alanine and glycine; sodium salt of betaine have been evaluated and found promising (Kolkovski et al., 1997). Another study has confirmed that supplementation of phospholipids, especially phosphatidylcholine, stimulates feed intake (Koven et al., 2001). As the live feeds like artemia, copepods and rotifers have high phagostimulatory effects, their extracts may be mixed with microdiets in order to improve their ingestion. Although the bioactive properties of *Artemia* extracts have recently come to light (Deezagi et al., 2016), their use in microdiets have not been evaluated yet.

Ingestion is followed by digestion of food and absorption of nutrients released. The difficulty in weaning the larvae was attributed to the inability of microdiets to contribute to digestive capacity of larvae. It was thought that the live food somehow activates the digestive system of larvae and aids in digestion by providing exogenous enzymes. However it was found that the contribution of the live food towards the total protease activity was only 5% of the total protease activity (Cahu and Zambiano Infante, 1995; Kurokawa et al., 1998). Therefore the argument that live food carries with it the enzymes needed for its digestion has been now rejected. The contribution of the live food enzymes in digestion is thought to be not more than 1 percent of the total enzyme activity present in the gut of the larvae. The marine fish larva is altricial which means that the exogenous feeding begins before the stomach is fully developed. However, the pancreatic secretion of enzymes begins in the larva either before the exogenous feeding or at the same time and these enzymes are quite active. Theoretically, the absence of stomach hinders the digestion of the proteins as the efficiency of proteolysis is reduced when the substrate protein is undenatured. However, research shows that the absence of a functional stomach does not hinder the protein digestion in the fish larvae (Cahu and Zambiano-Infante, 1994) as pancreatic enzymes compensate the process. However, there is the possibility of an overall compromised protein digestion in the marine fish larvae.

The argument that protein digestion is compromised in the altricial larva is supported by the observation of higher digestibility of proteins from live foods, which have higher amount of free amino acids. After the pancreatic digestion, the food moves to the intestine, which exhibits two types of digestive enzymes- the cytosolic digestive enzymes and the brush border enzymes. Decrease in the cytosolic enzymes and increase in the brush border enzyme activity during the early development is considered as a general rule in animals and has been observed in the marine fish larvae as well. However, nutritionally inadequate feeds may prevent this genetically programmed sequence of intestinal maturation and may lead to mortality of the larvae (Cahu and Infante, 2001).

Protein solubility is another important determinant of digestibility of the proteins in the diet of larval fish (Carvalho et al., 2004). Tonheim et al. (2007) evaluated the *in vitro* digestibility of water-soluble and water-insoluble protein fractions of some common fish larval feeds and feed ingredients, and found that that digestibility is improved with protein solubility. But a diet having higher content of soluble proteins and free amino acids is also prone to loss of nutrients by leaching. Thus the challenge before larval fish nutritionists is the development of the diets having soluble proteins while reducing the leaching losses at the same time.

At present, it may be concluded that the digestive capacity cannot be claimed as the sole reason of the weaker performance of the microdiets. The larvae are naturally programmed to feed on live foods that are rich in soluble proteins and free amino acids. The inability of the marine fish larvae to grow and survive on microdiets is therefore due to a conglomerate of factors, which include the chemical characteristics of the feed as well as the larval digestive capacity each contributing little, but significantly towards the problem.

Cannibalism in Fish Larvae

Cannibalism is defined as the act of killing and consuming the whole, or major part, of an individual belonging to the same species, irrespective of its stage of development (Smith and Reay, 1991). This phenomenon is considered to be commonly present in a number of families and spread across the animal kingdom (Polis, 1981). In fishes, cannibalism has been studied extensively but its evolutionary advantage has not been conclusively explained. Dong and Polis (1992) linked evolution of cannibalism to oligotrophic environment and high acquisition efficiency of con-specific victim. Nishimura and Hoshina (1999) argued that cannibalism reduces the risk of mortality due to starvation in the animals and if a large mouth gape size in larvae promotes feeding efficiency of fish larvae, cannibalism tends to evolve. The occurrence of cannibalism has been now accepted as a general rule in fishes

(Dominey and Bloomer, 1984). Depending upon the developmental stage of prey, genetic relationship of cannibal to prey, and the age relationship of cannibal and prey, cannibalism is classified into different types (Figure 1). A conglomerate of factors that can be grouped into genetic (agonistic behavior and size variation), behavioral (social dominance) and environmental (food deprivation; stocking density) causes cannibalism in fish.

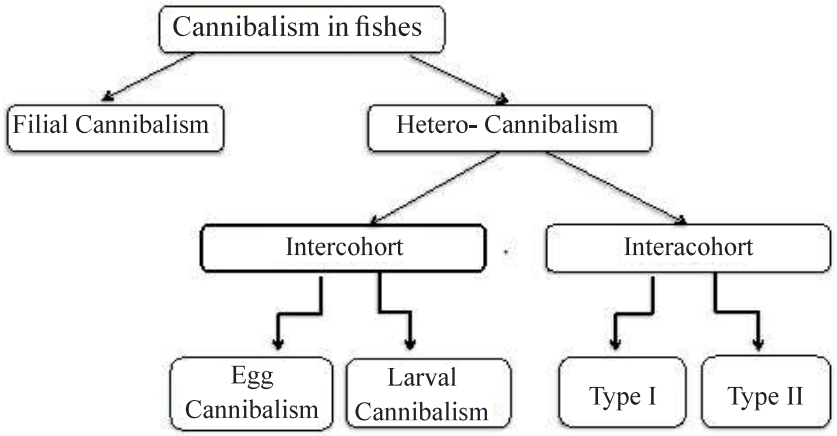

Fig. 1: Different types of cannibalism observed in fishes

Fig 2: An example of Type II cannibalism observed in Asian Sea bass (*Lates calcarifer*) larvae (22 days post hatch). A larger sized larva ingesting a prey head-first. The posterior part of the prey is visible in the pictures.

Cannibalism in fish can influence both aquaculture production and fisheries, although the effect is opposite. In natural populations, cannibalism was initially thought to be a detrimental phenomenon (Pfening, 1997), but its wide occurrence in the animal taxa suggested otherwise. It is now considered as a stabilizing mechanism, enhancing the fitness at the individual level (Bobisud, 1976) and exerting density-dependent regulation at the population level. A fish feeding on conspecifics exhibits increased robustness and vigor, develops faster due to

increased somatic growth rates (Cuff, 1977; Li and Mathias, 1982). Beneficial as they may appear at first glance, these advantages turn out to be counterproductive in a culture setting.

In fact, cannibalism is considered as one of the major causes of mortality in culture conditions. Fish production during culture is usually affected by intracohort cannibalism in case of intensive production system. The effect of intracohort cannibalism (Figure 2) is observed more during the larval stages because of higher feed requirements, higher growth rate and higher tendency towards depensatory growth. Additionally, hatchery environments are designed to give maximum output by rearing the larvae at high stocking densities with little chance to escape the cannibal cohorts. All these conditions make the problem of cannibalism more serious during the larval rearing phase of culture.

The general opinion of biologists is that complete eradication of cannibalism in the larviculture is impossible (Baras and Jobling, 2002). However, with a thorough understanding of the causative factors and the ontogeny of cannibalism, its mitigation is possible (Hecht & Pienaar, 1993). Different factors have been studied and reported to influence the intensity of cannibalism in aquaculture (Ribeiro and Qin, 2015; Kailasam et al., 2002). However, one of the most important factors, which can be used to mitigate cannibalism, is the food and feeding behavior of the larvae. Even in some cases, food and feeding associated factors are the principal cause of cannibalism (Hecht and Appelbaum, 1988). Additionally, many hatchery conditions (stocking density and temperature) are optimized for maximum output and cannot be tailored to mitigate cannibalism. Therefore, improving the acceptance of feeds by improvising the feeding strategies is a viable avenue to suppress cannibalism (Kubitza and Lovshin, 1999).

Starvation almost always induces cannibalism in fish whereas abundant and uniform distribution of food helps in mitigation of cannibalism by increasing the ratio of food particles to potential cohort prey (Damme et al., 1989). Generally, live foods have a mitigating effect on cannibalism (Hecht and Pienaar, 1993) whereas dry diets have a cannibalism inducing effect in larvae (Ehrlich et al., 1989). Starvation or poorly accepted micro-diets may either induce cannibalism directly or by development of growth depensity in fishes. Intra cohort growth depensity is a major cause of cannibalism in fish larvae (Hecht and Appelbaum, 1988), which is directly affected by food availability and feeding frequency. In contrast to quantitative food deprivation, temporal starvation has shown specific responses, showing a surge in cannibalism in some cases (Katavic et al., 1989) as well as showing no effect in others (Folkvord and Ottera, 1993). However, it has been historically accepted that the larvae must be provided with uniform and suitably sized feed, as the fish grows, to avoid depensity and cannibalism (Nakamura and Kasahara, 1956).

Failure to switch to microdiets is another cause of cannibalism. Different weaning strategies may be employed in fish to improve the acceptance of diets that are more profitable to the culturist. Kubitza and Lovshin (1999) classified weaning strategies as (1) one-step weaning; (2) gradual feed transition; and (3) gradual feed ingredient transition. In case of fish larvae, one-step weaning is risky and may lead to high mortalities. Gradual feed ingredient transition requires a number of weaning diets to be prepared. Therefore gradual feed transition is the most practical strategy of weaning which, may lead to least mortality and mitigate cannibalism.

Study of feeding behavior of fish larvae may provide leads that may be investigated in order to mitigate the cannibalism. The availability of the microdiets for the capture of larvae may be increased by manipulation of the specific gravity of the diets (Person-Le Ruyet and Noel, 1982). Coloured microdiets may be also used as they have shown to be preferred by the fish larvae (Clarke and Sutterlin, 1984). Specific attractants used in feeds may also help to mitigate cannibalism (Sveinsson and Hara, 1990). However, mitigation of cannibalism through nutrition demands the knowledge of the physiological factors, which induce cannibalism and determine the acceptance of feed. Generalizations may be formulated, but species specific feeds and feeding protocols for larval rearing must be developed in order to reduce cannibalism. This is particularly challenging as it involves the ethological study of acceptance of feeds along with the physiological effects of feeds in larvae.

Gut Microbes in Fish Larvae

The larval phase is a very sensitive period in the life of marine fish owing to under developed digestive system, immune system and osmoregulatory system. There is abundant evidence that one of the main causes of mortalities in the larvae is the detrimental host microbe interaction (Vadstein et al., 2013). The higher organic load in the hatchery due to the presence of feed, dead larvae, live food and the feces further stimulate the bacterial growth in a selective manner (Andrews & Harris, 1986). The situation is aggravated due to the fact that unlike farm animals the fish, feed and excretion products are present in the same matrix that is, the rearing water. All these factors make the microbial biota of the hatcheries perturbed and significantly different from the natural waters. Although, the bacteria that develop in the hatchery may be detrimental to the larvae there is evidence that the normal microbiota in the larvae provides health benefits and induces better development (Rawls et al., 2004). Therefore, it may be quite useful to direct the microbial community of the larvae in the hatchery to the maximum advantage of the larvae.

The first exposure of the larvae with the bacteria is through the egg microbiota, live

food and the intake water (Vadstein et al., 2013). After the hatching, the water intake by larvae helps the bacteria present in the rearing water, colonize the gut even before exogenous feeding commences (Olafsen, 2001). Older larvae may also ingest bacteria by grazing on suspended particles and egg debris (Olafsen and Hansen, 1992). Thus, during the first days after hatching, uptake of bacteria from the water is important, whereas uptake of bacteria associated with the food dominates once the exogenous feeding starts (Eddy and Jones, 2002).

The probiotic mechanism represents a monoculture or low diversity addition of beneficial bacteria. The addition of probiotic bacteria in the diet of the larva is one of main approaches of probiotic introduction in the larval gut (Makridis et al., 2001). A variety of opportunistic bacteria get established in the gut as soon as the exogenous feeding starts (Hansen & Olafsen, 1999). Therefore, early delivery of probiotic would possibly prevent the dominance of opportunistic bacteria in the gut. Once the probiotic is able to colonize the digestive tract of the host, the beneficial effect may occur through a number of mechanisms like stimulation of the host immune response, production of bacteriocins, competition for nutrients and adhesion sites and alteration of the gut physicochemical environment (McCracken and Lorenz, 2001). However, deciphering precisely the mechanism of action of the probiotics with respect to benefits incurred to the host remain a challenge to fish nutritionists.

One of the main reasons of this paucity of knowledge is over dependence on *in vitro* studies (Tinh et al., 2008), which leads to poor understanding of the complexities of microbial activity in the gut (Klaenhammer & Kullen, 1999). The *in vitro* studies cannot provide information about certain events like death of the microbe inside the gut (Vine et al., 2006) and failure of the probiont to maintain *in vitro* physiology (Tinh et al., 2008). Also, in *in vivo* conditions, a stationary phase of growth does not occur because of the continuous flushing in the gut. This may hinder the production of certain metabolites, which occur only during the stationary phase of growth. Furthermore, as the development of the larvae takes place, the maturation of the GI tract might result in modifications particularly in the hydrophobicity of the mucus (Lee and Puong, 2002) affecting the ability of probiont to attach. All these factors may not be accounted for in *in vitro* studies.

Although the role of bacteria in enhancing the digestive process has been acknowledged (Clements, 1997), whether there is any direct nutritive value of the bacteria remains to be confirmed. Bacteria may have upto 56% crude protein that may be digested and absorbed by the larvae (Brown *et al.,* 1996). Although, the digestion of the bacteria inside the larval gut could not be proved (Hansen and Olafsen, 1999; Ringø, 1999) the uptake of bacteria in enterocytes by endocytosis in cod and herring larvae has been confirmed (Olafsen and

Hansen, 1992) and this may have contribution towards nutrition or immuno stimulation. The production of some nutrients like vitamin B_{12} (Kashiwada et al., 1970) vitamin K (Poston, 1964) and beneficial short chain fatty acids (Clements, 1997) has been established but their uptake and utilization need in larval stages needs to be confirmed. Similarly the enzymatic contribution of the bacteria towards the digestion of the nutrients in the larval gut remains to be confirmed (Pollak & Montgomery, 1994).

Microbial intervention represents one of the promising approaches to improve the survival of larvae in marine hatcheries. However, due to the infancy of the research area, only a few technologies have reached commercial market. Larval microbiology is a challenging area of larval biology due to the inherent problems of bacterial identification. Also, the microbial interactions with the larval digestive and immune system represent an area that has not been investigated much and demands further research.

Conclusion and Future Directions

In the Kyoto Conference of 1976, nutrition of larval fish was recognized as a bottleneck for mariculture. Since then, hundreds of researchers have worked to study larval physiology. This led us to better understanding of the feeding behavior and nutritional physiology of the marine fish larvae. At present, although our knowledge of the fish larvae is immense and valuable but paradoxically, it has not lead to parallel increase in the survival of larvae in the hatcheries. This discrepancy implies that either the research effort is sporadic or there is drastic physiological difference across species of marine fishes in the larval stage. The second implication negates the possibility of using a model species for marine fish larval studies and demands dedicated research on each species of culture importance. In particular, the use of molecular tools and techniques in proper understanding of the digestive processes of the fish larvae will provide great impetus to improvement of the survival of larvae in the hatcheries. Careful examination of the changes in genes expression and other changes at the molecular scale could reveal the processes that are involved in the health and welfare of the fish larvae.

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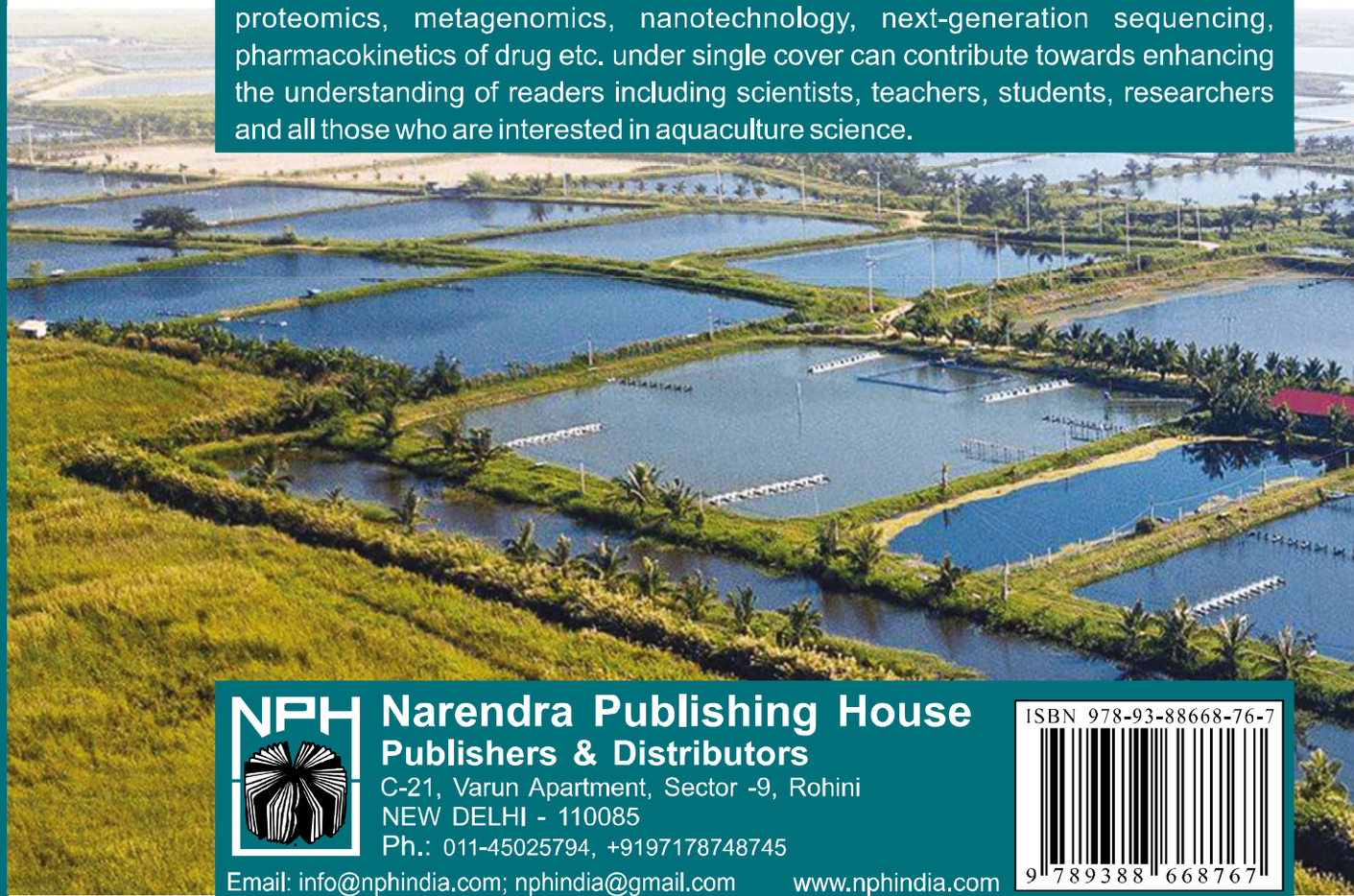
NEXT GENERATION RESEARCH IN AQUACULTURE

J K SUNDARAY • M A RATHER

Aquaculture has witnessed numerous transformations during the last few decades and established itself as a key food producing sector, providing nutritional security as well as livelihood support to huge mass around the globe. Aquaculture is a beautiful outcome of coordinated collation of several vital disciplines and being nourished through several cutting edge research findings and technologies. It has started as an extensive system and currently practiced at intensive and super intensive scale largely due to the development of several scientific inventions and innovations. This book (**Next Generation Research in Aquaculture**) is intended to throw light on next-gen aquaculture research comprising various advanced research approaches to generate answers and solutions for key aquaculture problems pertaining to different aspects such as feed, seed, disease management etc. Unique aggregation of a different advanced aspect of aquaculture research such as nutrigenomics, proteomics, metagenomics, nanotechnology, next-generation sequencing, pharmacokinetics of drug etc. under single cover can contribute towards enhancing the understanding of readers including scientists, teachers, students, researchers and all those who are interested in aquaculture science.



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