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Challenges and prospects of using live feed substitutes for larval fish

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Abstract

Larviculture of commercially important aquaculture species faced limitations associated to the incomplete understanding of larval nutrition and the inability to total replacement of live feeds by formulated diets at the early larval stage. The main challenges to alternatives of live feed in larval fish culture are related to the inherent behaviors of the larvae and the incomplete knowledge and practice leading to the inefficiency of using micro diets. Although significant achievement has been reached in the complete replacement of live feeds by formulated micro diets in freshwater species and marine shrimps, its success is far from complete in marine finfishes. However, recent progress in biotechnological advances in manufacturing process and advanced knowledge of the nutritional necessities of larvae indicated improvements in the field. A range of technologies in the manufacturing of micro diets for larval fish are in place currently. To this end, several achievements of substituting live feeds with formulated micro diets at later stages of larval development have been reported by various researchers providing a clue on the prospects for the future. Therefore, the objective of this review is to compile existing information on the challenges of substituting live feeds by formulated diets in the past and prospects for future development.

Keywords: Live feeds, Micro diet, Nutrition

Introduction

The success in the larviculture of shellfish and finfish of commercially important aquacultured species is hampered by several challenges including a knowledge gap in larval fish nutrition, heavy dependency on live feeds, and challenges in using formulated diets for larval fish (Hamre et al., 2013; Rønnestad et al., 2013).

Larval nutrition is linked to several factors apart from their inherent behaviors that make the understanding of larval fish nutrition incomplete. Amongst these extraneous factors, nutritional conditions of broodstock in which all aspects of reproduction staring from the onset of sexual maturity to gamete formation and production of viable eggs and sperms is the major one (Jobling, 2016). The quality of dietary lipids and proteins in broodstock conditioning periods determine the quality

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of the yolk reserves on which the larvae depend on before exogenous feeding. On the other hand, the success in the hatchery production of shellfish and finfish larvae is mainly reliant on the accessibility of suitable live-feed organisms like rotifers and *Artemia* during the first feeding stage. This is mainly due to the undeveloped digestive system larvae possess for several days post-hatching (DPH) (Fontaine & Revera, 1980).

Although convenient due to their small size and gentle swimming behavior, several challenges arise in the continuous application of these live feeds in the larviculture of aquacultured species. These challenges associated to live feeds for larval fish include deficiency and uncertainty of their nutritional composition (Hamre et al., 2013), uncertainty of their mass culture, high loads of pathogenic microorganisms associated with them and high production costs and labor.

Due to this fact, alternatives to live feeds in larviculture of commercially important aquaculture species have been an area of research for decades. Concerning this, development and study of dietary values of artificial micro diets for several larval fish species have been an area of investigation for the past three decades (Alam et al., 2015; Hauville et al., 2014; Liu et al., 2012).

Consequently, success to replace live feeds with formulated micro diets has been achieved in freshwater species while a complete replacement of live feeds by formulated micro diets in marine species is far from complete (Langdon & Barrows, 2011) except for marine shrimps. This is partly due to the biotechnological advances in the manufacturing of different micro diets and positive progress in larval fish nutrition research.

On the other hand, the application of formulated micro diets at the very beginning of larval feeding proved to be impossible especially in marine species due to several challenges related to both the larval fish behavior coupled with properties of micro diets. Larval fish-associated challenges such as the rudimentary digestive system of larvae coupled with very low enzymatic activity at first feeding and micro diet-associated challenges including physical form, feeding frequency, gut retention time, and culture system greatly influences the success of using larval fish substitutes in the sector.

Therefore, this review provides comprehensive information on the challenges and prospects of using live feed substitutes for larval fish reported so far in the literature.

Finfish Larval Nutrient Requirement

The requirement for a specific nutrient can be defined from a physiological aspect as the nutrient uptake needed to fulfill a physiological function. However, the preparation of feeds necessitates interpretation of the requirements into the nutrient content in the feed. Macro and micro nutrient necessities are usually given as dietary portions and stated in a way that requirements do not always rise under demanding conditions, such as metamorphosis and high growth rates (Kolkovski, 2013). Moreover, the dietary requirements of larvae are different from fingerlings and adults in which quantification and qualifications of both macro and micro nutrients for larval fish should be measured along the differentiation and functionality of the digestive system, feeding behavior, and the nutritional needs of the larvae at different developmental stages (Lazo et al., 2011). Therefore, it is in this regard that larval nutrient requirement studies during the past years are linked to the ontogenetic evolvement of the digestive system of larvae after the start of first feeding. However, it is believed that the optimum preparations for first-feeding larvae should mimic the yolk composition and somewhat disclose the nutrient requirements and metabolic capacities of yolk sac stage fish larvae (Jobling, 2016). To this end, the primary roles and requirements of macro nutrients (lipids and proteins) and micronutrients (minerals and vitamins) in both pre-feeding and post-feeding fish larvae have been a major area of research in recent years. Despite considerable progress, larval nutrition is among the most poorly understood areas of fish nutrition (Hamre et al., 2013). The influence of nutrition on larval fish starts from the broodstock conditioning periods and extends to the post-weaning stages (Fernández-Palacios et al., 2011). Nutrition has an impact on all sides of reproduction staring from the onset of sexual maturity to gamete formation and production of viable eggs and sperms (Jobling, 2016). This in turn affects the quality of yolk sack deposition for the larvae to depend on before exogenous feeding.

The nutritional modes of marine invertebrate larvae are categorized as either lecithotrophic or planktotrophic. The larvae of lecithotrophic species develop from comparatively large, energy-rich eggs and have a short larval period in which no or minimal external food is required. On the other hand, the larvae of planktotrophic species emerge from relatively small, energy-poor eggs and have a longer period of ontogeny in which external particulate feed is required to complete their metamorphosis. This indicates that the developing embryo and newly hatched larvae of finfish depend on the nutrient contents deposited in the oocyte by the mother during vitellogenesis for their survival and growth.

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Therefore, to enhance the quality of eggs and sperm produced, the lipid composition specifically n-3 HUFA manipulation in broodstock fish has been an area of research. Concerning this, feeds containing poor-quality lipids have resulted in poor quality eggs.

The other nutrient of importance in broodstock fish for better larval quality is the amount and quality of crude protein where feeds usually for broodstocks contain relatively higher protein content than grower diets. Amongst the amino acids, a good supply of tryptophan particularly is found to be important in reproduction as a precursor for serotonin and helps in gonad maturation in both sexes. On the other hand, the supplementation of taurine, a sulfur-containing amino acid-like compound also seems to be important for broodstock performance, especially in marine species. Apart from extraneous sources of difficulty mentioned above, the inherent behavior of the larvae of fishes by itself is an area of difficulty in understanding larval nutrition. Moreover, fish larvae growth rate in the first days of post-hatching is extremely fast resulting in rapidly changing nutrient requirements from one stage to another.

Another bottleneck in understanding larval fish requirements is the difficulty of manufacturing nutritionally complete artificial larval diets. Due to this fact, the first feeding of many larval fishes depends on live feed organisms like rotifers and *Artemia* which are not the natural foods of the species. The variable nutritional contents coupled with the own metabolism of live feeds make it difficult to quantify the nutrient requirements of most larvae of marine fishes (Hamre et al., 2013).

Lipid requirement in larval fish

Due to the importance of dietary lipid utilization for the success of larval rearing, increased attention has been given to different aspects of larval lipid nutrition such as digestion, absorption, transport and metabolism, which are frequently studied by different researchers. The dietary lipids play critical role as a source of essential fatty acids (EFAs) which are in turn the functional units for having physiological role in fish larvae (Izquierdo & Koven, 2011). Generally, the functions of EFAs in fish larvae are somehow similar with that of juveniles and adults, in which their roles being source of metabolic energy, building blocks of bioactive molecules and structural components in the phospholipids (PLs) of cellular membrane (Sargent et al., 1999). Particularly, long - chain polyunsaturated fatty acids (LCPUFAs) such as eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (ARA, 20:4n-6) have important physiological functions as critical structural components of the membrane PLs that facilitate key intra membrane reactions and processes, while, docosahexaenoic acid (DHA, 22:6n-3) is particularly important as a precursors for eicosanoids (Izquierdo & Koven, 2011). LCPUFAs function in larval fish by acting as ligands involved in gene transcription and expression and compete for acylation and incorporation into the membrane PLs of most cells as well as substrates for enzyme systems. Therefore, the overall impact of EFAs on fish larval physiology is directly related to the level and ratio of these compounds in the tissue PLs. This means that the ratio of EPA, DHA, and ARA in the diet largely determines the efficacy of many physiological processes during the rapid growth and dramatic development characterizing fish larviculture (Masoudi Asil et al., 2017).

To account for some roles of the different LCPUFAs in fish larval nutrition; attributes of DHA as a regulator of membrane fluidity which in turn influences weight gain related to its structural function in the PL bilayer of the cellular membrane has been reported (Wu et al., 2002). On another hand, dietary ARA incorporation during larval rearing has been shown to have a significant effect on juvenile pigmentation in flatfish where elevated ARA levels during pre or early metamorphic pigmentation period produces malpigmentation (Lund et al., 2008). Conversely, dietary ARA fed during metamorphosis (35 DPH) and after the specific metamorphosis period improved pigmentation compared with fish feeding on high DHA rotifers and *Artemia* (Campoverde & Estevez, 2017). Moreover, studies on the role of LCPUFAs for larval fish indicated the importance of these fatty acids for resistance to stressors such as handling, temperature and salinity (Liu et al., 2002). In addition, increased dietary n-3 LCPUFAs have been shown to enhance the immune system in fish (Montero & Izquierdo, 2011).

Concerning the requirements of EFAs, although fish are incapable of their *de novo* synthesis, freshwater fish seem to possess sufficient Δ6 - and Δ5 - desaturase and elongase capability to produce ARA, EPA, and DHA from their shorter - chain precursors linoleic acid (LA; 18:2n-6) and linolenic acid (LNA; 18:3n-3) if they are present in the diet. In contrast, marine species have very limited elongase and desaturase capability, requiring incorporation of fully formed EFAs in the diet for good growth and survival (Izquierdo, 1996). In general, n-3 LCPUFA requirements during larval development of several fish species vary from very low values in carp (*Cyprinus carpio*) (0.05%: dry weight bases to relatively higher values in *Dentex dentex* [4%, DW]).

As reviewed by Izquierdo & Koven (2011), the ranges of DHA, EPA and ARA requirements in both marine and freshwater larval fish ranged from 0.5%–2.5%, 0.7%–1.7% and 0.5%–2.5% on DW bases respectively, while, the ratios of EPA/ARA and DHA/EPA requirements were 3.5%–5% and 1.2%–8% DW respectively.

Copepods have been the excellent sources of EFAs for marine larval fish species in natural water bodies, while, the common live feeds (rotifers and *Artemia*) in larviculture of those species are deficient in EFAs especially DHA/EPA ratios. Therefore, EFA enrichment of those live feeds based on oil-based emulsions is being conducted to improve their n-3 LCPUFA as discussed in section (3).

Live Feeds and Their Nutritional Enrichment for Larval Fish

The success in the hatchery rearing of shellfish and finfish larvae is mainly dependent on the accessibility of suitable live feed organisms especially during the first few DPH (Fontaine & Revera, 1980). Despite the advancement in the formulation and application of micro diets as a substitute in recent years, larviculture of most species of economic importance in aquaculture depends heavily on live feeds during their early life stages.

In the past three decades, microalgae, rotifers, and *Artemia* have been the most applied live feeds in the aquaculture industry due to their convenience in mass culture and availability. Although convenient due to their small size and slow swimming behavior, these live feeds are generally deficient in nutritional composition and their dietary values are not always predictable. Moreover, those popular live feeds in the aquaculture industry are lacking in EFAs which are an indispensable part of the larval nutrient requirements (Hamre et al., 2013).

Amongst are the rotifers which are in most cases the first feeds for many if not for all marine fish larvae. Several kinds of research have indicated that the fatty acid profile of rotifers has been under the optimal requirements of most cultured larvae and the quantitative and qualitative lipid class and fatty acid profiles of rotifer diets determines the lipid and fatty acid composition of both long and short-term enriched rotifers (Theodorou, 2017).

In addition to the EFAs, a previous study by Hamre et al. (2008) indicated that micro elemental contents like vitamins A, E, C, and thiamine were found also below the levels found in copepods which are the standards for enrichment for marine larvae in a yeast-based diet. The same study also revealed that micronutrients manganese, copper, zinc, iodine, and selenium were too low in the rotifers cultured without supplementation.

Despite its inferior and variable nutritional quality compared to wild zooplankton, brine shrimp *Artemia* is the second most important live feed organism in the aquaculture industry. However, its high biomass production in contrast to other zooplankton and the capability to improve its nutritional profile through enrichment products ensures its continuous application in the larviculture industry. In line with this, Craig et al. (2012) reported a decreased growth rate and jaw dysmorphogenesis in Zebra fish fed un-enriched *Artemia*. Therefore, various types of live feed enrichment techniques are being applied to optimize the nutritional composition in line with the requirements of larvae (Samat et al., 2020).

Although marine microalgae are excellent sources of dietary fatty acids to enrich rotifers and *Artemia*, their large-scale application has been hampered due to the high cost of production and relatively complicated technologies applied to mass culture techniques. Due to this fact, alternatives to microalgae in the form of preserved algae pastes, yeast-based diets, micro-encapsulated diets, and lipid emulsions and liposomes are being utilized currently. On the other hand, bio-encapsulation of rotifers and *Artemia* by a variety of enrichment diets to manipulate their content in certain nutrients, especially n-3 HUFA and vitamins C, A, and E has been an established protocol in several hatcheries of the world (Drillet et al., 2011).

To account for some results that enriched live feeds have impacted the larviculture industry, several trials were made on different species in the past. Cho et al. (2001) reported that larvae of Korean rock fish *Sebastes schlegeli* fed with *Artemia nauplii* and *Brachionus plicatilis* nutritionally enriched with spirulina powder showed significantly higher survival than the larvae fed un-enriched *A. nauplii* and *B. plicatilis*. A recent study by Thépot et al. (2016) indicated that barramundi larvae fed rotifers enriched with a 50:50 mixture of *Nannochloropsis oculatato* and *Chlorella vulgaris* microalgae gave significantly higher eye diameter, larval length, and body depth at the 10th-day post-hatching and the trend was maintained for the whole growth period.

Challenges of Using Live Feed for Larval Fish

Although live feeds have been critical in the larviculture of

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aquaculture species especially marine fish, their successful application had never been achieved without challenges. These challenges are mainly related to poor nutrient composition, the uncertainty of their mass culture, high levels of pathogenic microorganisms associated with them (Vadstein et al., 2018).

Since the challenges related to the poor nutritional profiles of the most applied live feed organisms are discussed in section (3) of this document sufficiently, the challenges related to the uncertainty of mass culture, higher pathogenic microbial load, and production cost are detailed a bit in this section.

To this end, constraints related to the uncertainty of mass culture systems can be best exemplified by the inconveniencies caused due to sudden collapses in rotifer culture systems which make the rotifer culture supply unpredictable. One of the causes of these sudden culture crashes in rotifer culture systems is pathogenic bacterial proliferation due to the high organic matter load and high temperature in rotifer culture tanks (Vadstein et al., 2018). Among these bacteria, some of them can be pathogenic to rotifers directly while others pose a risk of causing disease for the larval fish feeding on the rotifers. In support of this, Yu et al. (1990) reported toxicity of *Vibrio alginolyticus* towards the survival of *B. plicatilis*. Moreover, *Vibrio anguillarum* TR27 was found to cause negative effects on rotifer reproduction under sub - optimal culture conditions. Although most bacteria have no detrimental effect on rotifers themselves, the proliferation should be avoided as the real risk will be on the host (larvae) feeding on the rotifers.

Contamination with ciliates is the second problem causing poor performance and sudden crash of rotifer culture systems. Ciliates belonging to the group Halotricha and Hypotricha are not desired in rotifer cultures since they compete for feed. Ciliates are especially a problem in batch cultures with lower rotifer density and higher culture volumes. Moreover, the competition for feed between rotifers and ciliates is correlated to the type of feed administered and the growth phase of rotifers. In a competition experiment done by Cheng et al. (2004), between the rotifer *Brachionus rotundiformis* and the ciliate *Euplotes vannus*, the growth of rotifers was suppressed by the ciliate when the feed administered was *Tetraselmis tetrathele*. Whereas, when *N. oculata* was administered as feed, the rotifer growth was not suppressed by the ciliate. The interference of the ciliate on rotifer growth was strong during the lag phase and stationary phase. Thirdly, rotifer birna virus and fungus have been reported to cause sudden crashes in rotifer cultures making the rotifer culture system vulnerable.

Although hatching of *Artemia* cysts seems to be simple compared to other live feeds, obtaining *Artemia* cyst-lots with good hatching synchrony and high hatching efficiency has been a challenge as considerable variation has been observed between cysts of various origins, and even between batches from the same strain. Moreover, the dependency of the hatchability of the cysts on several parameters like constant temperature (25℃–28℃), salinity (15–35 g/L), oxygen levels (close to saturation), and strong light (2,000 Lux at the water surface) affects the hatching rate and maximum output whereby elevating the production cost of the harvested *A. nauplii*. On the other hand, complex and costly mass production system requirements challenge the application of microalgae (Chauton et al., 2015) and copepods (Drillet et al., 2011) in the larviculture of commercially important aquaculture species.

Prospects of Using Live Feed Substitutes for Larval Fish

The usual practice of the early larval rearing of marine fish is that live feed like rotifers and *Artemia* are fed to the larvae followed by artificial diets for later stages of development (Langdon & Barrows, 2011). However, As discussed earlier, the quality of those conventional live feeds is inferior compared to natural prey such as copepods, and the mortality of larvae reared on these live feeds is reported to be high. On the other hand, the formulated feeds in the form of micro diets provide the opportunity to incorporate nutrients lacking in live feeds to the larvae. In addition, the development and application of formulated micro diets offer an opportunity to study the nutritional requirements of larval fish which has been difficult with live feeds (Alam et al., 2015) since micro diets have a relatively uniform nutritional composition which can be adjusted to the requirements of the species. In addition, the potentially predictable and constant nutritional content of micro diets coupled with the year-round availability without dedicating much culture space and time unlike live feeds makes the application of artificial micro diets feasible compared to the conventional live feeds.

Consequently, alternatives to live feeds in larviculture of most aquaculture species had been a long-term practice and its success has been significant in freshwater species. Complete substitution of live feeds by formulated diets has been successful for marine shrimps while the total replacement of live feeds by artificial diets in marine finfish species is far from complete (Langdon & Barrows, 2011). In support of this fact that live

feeds are still superior to artificial micro diets in marine fish larvae, a report by Curnow et al. (2006a) indicated that Barramundi (*Lates calcarifer*) larvae fed a co-feeding of micro diet plus rotifers at first feeding and a total micro diet at the *Artemia* feeding stage resulted in poor development of the larvae compared to a co-feeding regime of micro diet plus *Artemia*.

Despite this fact, People Le Ruyet et al. (1993) found that shortening the weaning process of European seabass (*Dicentrachus labrax*) from 35 to 20 DPH onto micro diets cuts up to 80% of the cost of live feeds. Due to this fact, dietary values of artificial micro diets for several larval fish species have been an area of investigation for the past three decades (Liu et al., 2012).

A study by Pedro Cañavate & Fernández-Díaz (1999) indicated that co-feeding of commercially formulated feed and rotifers and *Artemia* on larval weaning of Sole (*Solea senegalensis*) gave similar results in specific growth rate and survival compared to the larvae fed live feeds alone. However, larvae fed solely on commercial micro diets at the weaning stage showed significantly reduced growth rate and survival during later larval stages indicating the need for live feed supplementation. Another study by Blair et al. (2003) indicated that an attempt to wean larvae of Haddock (*Melanogrammus aeglefinus*) from 21 to 45 DPH using commercially prepared micro diets gave significantly inferior growth and survival compared to the larvae fed rotifers and *Artemia* as a live feed. Curnow et al. (2006b) on the other hand reported that Barramundi (*L. calcarifer*) larvae grew successfully from 20 to 33 DPH in a co-feeding regime of micro diet and live feeds in which commercially available micro diets Gemma Micro (Skretting, Australia) and Proton (INVE, Dendermonde, Belgium) were applied. Later on, Fletcher et al. (2007) reported that micro-particulate diets in association with live-feed *Artemia* provided significantly higher growth rates and survival compared to the larvae fed solely the micro particulate diets in larvae of Atlantic cod (*Gadus morhua*). Nhu et al. (2010) indicated that although the use of commercial micro diet Proton® (INVE) from eight DPH gave better growth on larvae of Cobia (*Rachycentron canadum*), survival and stress resistance was found to be poor in a salinity stress test. A study by Liu et al. (2012) indicated that weaning of the Chinese long snout catfish (*Leiocassis longirostris*, Günther) can be started as early as 6 DPH with co-feeding of live feed and formulated diets while with solely artificial diets the larvae can be weaned after 10 DPH. Successful early weaning (17 DPH) of Florida Pompano, *Trachinotus carolinus* by the use of micro diets and live feed in a co-feeding regime was reported by Hauville et al. (2014). A more recent study by Willer & Aldridge (2017) indicated that European flat oyster (*Ostrea edulis*) larvae fed a combination of microencapsulated diet and microalgae for 8 days resulted in a 46% higher increase in maximum size, 171% greater increase in minimum size, and 5% better survival than larvae fed microalgae only.

Fish oil has been a precursor in the formulations of micro diets to deliver better EFAs to the larvae. However, the cost of fish oil has been the major bottleneck although excellent results are obtained. Due to this fact, the replacement of fish oil ingredients with relatively cheaper materials has been proposed. In line with this, Eryalcin (2018) attempted to replace fish oil with microalgal products in preparation for a micro diet for the larval weaning of Gilthead sea bream (*Sparus aurata*) 20 DPH where the algal EFAs replaced fish oil ingredients without significant difference in growth survival and stress resistance. Overall, early weaning of fish larvae onto artificial micro diets even if total substitution of live feeds is not possible at this stage has a tremendous impact on the reduction of a substantial amount of money in the industry. Therefore, the positive factors for the partial success of the development and application of micro diets in fish larviculture are discussed in detail in the next sections.

Biotechnological advances in micro diet formulation

A micro diet may be referred to as formulated, inert, dry, or weaning diets prepared to feed larvae of commercially important aquaculture species in which the particle size ranges between 150–800 μm (Kolkovski, 2013). Several micro diet formulation methods are currently being used in the world ranging from micro-bound diets (MBD), micro coated diets (MCD), micro-encapsulated diets (MED), and micro-extrusion marumerization diets (MEM) (Kolkovski, 2013; Langdon, 2003) (Table 1). Apart from the forms described above, crushed and sieved micro diets are produced by extruders that produce large particles.

Advances in larval fish nutrition research

Understanding of the variation of the digestive system and accessory glands during larval development is critical to understand the digestive and nutritional physiology of larval fishes and synchronizing this physiological development with rearing protocols and feeding practices. This, in turn, provides an opportunity to overcome the partial or total substitution of live feeds by artificial inert diets which is the major bottleneck in fish hatcheries (Lazo et al., 2011). Furthermore, the main feature that dictates the end of the alteration from larvae to the

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Table 1. Comparison of micro diet types for delivery of nutrients to fish larvae

Micro diet type	Advantage	Disadvantage
MBD	• Inexpensive • Easy to produce • Binders can be nutritionally inert	• Poor retention of LMWS nutrients and possibly water-soluble proteins
Cross-linked protein walled capsules	• Possible to modify capsule wall properties • Digestible for some species of fish larvae	• Expensive • Use of organic solvents • Poor retention of LMWS nutrients
Lipid – walled capsules and lipid spray beads	• Inexpensive • Easy to produce • Better retention of LMWS nutrients compared with micro-bound particles and cross-linked protein-walled capsules	• Hard-lipid particles are not digestible by most species of fish larvae and depend on mechanical breakdown • Possible oxidation of unsaturated lipids during preparation and storage
Liposomes	• Better retention of LMWS nutrients compared with micro-bound particles and cross-linked protein-walled capsules • Digestible • Phospholipid wall material may contribute to larval nutrition	• Expensive • Use of organic solvents • Preparation involves several steps • Possible oxidation of unsaturated lipids during preparation and storage
MCD	• Digestible • Lower nutrient leaching	• Expensive
MEM	• Produce larger particle size (100–500 μm)	• Double step • Expensive
PARA	• Single step • Can produce smaller particles (50–500 μm)	• Expensive
MED	• The capsule wall helps maintain the integrity of the food particle until it is consumed • Lower leaching and degredation of nutrients in the water	• Restrict leaching of water-soluble dietary components and therefore reduce the larvae's attraction to the food particles • The capsule wall impairs digestion • Expensive

MBD, micro-bound diets; LMWS, low molecular water soluble nutrients; MCD, micro coated diets; MEM, micro-extrusion marumerization diets; PARA, particle-assisted rotational agglomeration; MED, micro encapsulated diets.

juvenile stage in finfish is the development of a complete, functional, and fully advanced digestive system. Due to this fact, the ontogeny of the digestive system of fish larvae has been the focus of many investigations for the past three decades.

Evaluating the epigenetic and ontogenetic alterations in the morpho-anatomy and histological arrangements of the digestive organs through microscopy and assessing the activity of digestive enzymes employing biochemical quantification have been the approaches of these studies in the past. Recently, molecular techniques that give insight into temporal and spatial gene expression patterns involved in the development and functionality of the digestive system during early ontogeny have been a complement to those studies. Contrary to what sued, these studies have demonstrated that the digestive system of fish larvae is very efficient in providing the larvae with energy and all nutrients needed for routine maintenance and growth to improve their survival and transform into juvenile fish (Lazo et al., 2011).

Evaluating the nutritional situation of fish larvae is of vital in aquaculture where the development of reliable and sustainable larval rearing techniques necessitates better awareness of the essential aspects of larval nutrition concerning the differentiation of the digestive system as well as creating the limits for initiation of exogenous feeding. Larval development depends on proper nutrition obtained from diets in addition to optimal biotic and abiotic factors once exogenous feeding is started. At this stage fish larvae are subtle to poor feeding conditions and sub-optimal nutritional supplementations since most organs and tissues are under intense and progressive development and larvae do not have sufficient reserves stored to resist starvation (Gisbert et al., 2008). To this end, a recent progress in evaluating the nutritional conditions of fish larvae using different tools has been discussed in detail in the next sections.

Histological biomarkers

It has been shown that diet quality and quantity dictate the cellular mechanisms that different organs and digestive systems

employ in invertebrates. Hence the use of intestinal and digestive accessory glands in the nutritional and physiological status of fish larvae is well recognized and is moderately standardized. Gisbert et al. (2008) reviewed the use of this technique in fish larvae in which the histochemical properties and histological organizations of the liver, intestine, and exocrine pancreas as tools on regular bases as targets to reveal the effect of different dietary administrations or nutrients on larval nutrition, physiology, and early development. Amongst several features, enterocyte height has been the most widely used technique as a valuable histological indicator of sub-optimal feeding or starvation in several fish species. Lipid and protein depositions in enterocytes are also used as a biomarker in fish larval nutrition studies in which central nuclei are observed in livers containing low lipid inclusion in the diet while peripheral nuclei are observed in livers with higher levels of lipid deposition (Gisbert et al., 2008).

Digestive enzymes

Enzymes are better indicators of the nutritional conditions of larvae due to their critical role in metabolic reactions, sensitivity, short latency, and species and age specificity. Hence, different enzymes ranging from intestinal brush border and cystosolic enzymes to proteolytic pancreatic enzymes are being employed currently. The secretion and synthesis of pancreatic enzymes in fish larvae appeared to be associated with food deficiency and dietary composition in which lower pancreatic secretions have been attributed to the poor diet composition for early larvae (Lazo et al., 2011).

On the other hand, the maturation and morpho-anatomical differentiation of the intestine is characterized by a decrease in the activity of cytosolic enzymes and accompanied increase in the activities of the brush border enzymes from the enterocytes is nutrient-sensitive in which significant deviation of the composition of the diet from the requirements of the larvae may prevent or delay the genetically programmed intestinal development sequence.

Gene expression

Genes coding for digestive enzymes are being used as markers for fish larval development since their ontogeny of expression is genetically programmed and their expression patterns are stage-specific (Lazo et al., 2011). Therefore, results from digestive enzyme gene expression analysis studies on fish larvae suggested that including the molecular level as the fourth organization category (organ, tissue, cellular, and molecular) in the list of markers for fish nutritional conditions study is possible. Information obtained about the nutritional condition of fish larvae through enzymatic indicators can be complemented by the knowledge of gene expression patterns and amount of digestive enzyme precursors which is critical in the aquaculture industry where the nutritional requirement of fish larvae needs to be adjusted and the source of the sub-optimized larval growth performance is derived from food supply is usually unidentified.

In a study by Darias et al. (2005), variances in the number of transcripts (amylase) found in starved larvae of Red porgy (*Pagrus pagrus*) and Gilthead seabream (*S. aurata*) compared to the fed ones as a result of triggered physiological mechanisms as an adaptation of the energetic balance to the different nutritional status provides an indication that the nutritional status of fish larvae could be reflected in the gene expression patterns of digestive enzymes during ontogenesis.

Indirect methods

It is well understood that not only digestive system development is influenced by the nutrient status of larvae but also skeletogenesis. Recent study by Darias et al. (2010) indicated a correlation between ossification status and osteocalcin gene expression in which its expression levels can be again associated with the dietary levels of several nutrients giving suitable molecular markers for larval nutritional status studies.

Use of tracers in fish larval nutrition

The inability of larvae of fishes to grow well on predefined micro diets and difficulties to determine feed intake and the digestibility of those diets have contributed to the development and intensive use of tracers in larval nutrition (Hamre et al., 2013). Currently, a range of tracer methodologies are available to perform larval nutrition studies. The delivery of tracers for fish larvae can be achieved using several methodologies including uptake from water, micro diet labeling (Conceição et al., 2007), labeling of live feed and tube feeding (Guthrie et al., 2000). Radioactive tracers and stable isotopic tracers have been applied in the past but the use of the later ones is largely recommended as the radioactive tracers can be hazardous to both human and animal metabolism. However, lower detection sensitivity, expensiveness, and relatively long time consumption limit the use of stable isotopes in fish larval nutrition studies. Therefore, with the necessary precautions in place, the use of radioactive tracers seems acceptable (Conceição et al., 2007). Some of the radioactive tracers are ^{14}C, ^{35}S, and ^{3}H while the most frequently used

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stable isotope is ^{15}N.

Generally, the principle of tracers in larval fish nutritional studies relies on the tube feeding of radiolabeled nutrients followed by quantification of the tracer present in feces, retained in tissues, and catabolized, after some hours to assess the digestion/absorption capacity for different amino acids, fatty acids and lipid classes. In general, the above-mentioned methods and further advancements in larval nutrition will contribute to the definitive goal of understanding the digestive ontogeny of fish larvae which in turn can lead to effective weaning to micro diets.

Challenges of Using Live Feed Substitutes in Larval Fish

Challenges associated with larval fish

It is generally accepted that rearing of the Altricial type of fish larvae is the major 'bottleneck' in the aquaculture of many important species. According to fish biologists, fish larvae belonging to the altricial type are characterized by having a relatively smaller yolk reserve at the time of hatching. Those larvae when the yolk reserve is fully absorbed remain under-developed in having a functional digestive system. The digestive system remains vegetative, lacking stomach and protein digestion takes place mainly towards the hindgut epithelial cells (Fontaine & Revera, 1980). Therefore, with such kind of stomach, larvae are not capable of processing artificial diets sufficiently and the larval mortality is high compared to precocial type larvae. Therefore, in most cases, larvae need live feeds during their early life stages (Conceição et al., 2010).

In addition, enzymatic activity in altricial type larvae at first feeding is generally low and each enzyme develops independently during ontogenesis (Kolkovski, 2001). Due to this fact, external enzymes from live feeds consumed are assumed to provide the necessary initiation for digestion in the larvae although destroyed soon after (Fontaine & Revera, 1980). In support of this, recent efforts with the culture of *Macrobrachium rosenbergii* larvae using formulated diets containing egg albumin as a protein source were unsuccessful. Although the diets were readily consumed and the guts were full, growth and survival were significantly lower than achieved in live feeds. This suggests that egg albumin despite possessing an excellent amino acid profile is not efficiently utilized. This, in turn, indicates that the presence of a sufficient quantity of enzymes does not guarantee the successful digestion of proteins as the enzymes are highly specific to certain protein sources. Therefore, an approach of attempting to mimic the ingredient composition of larval diets with that of successful diets for juvenile culture may not prove possible.

Moreover, marine fish species have a long and complex life cycle with changing feed requirements. In addition, the suitable size for the small mouth gapes of most finfish larvae and their ability to stimulate the feeding response through movement makes live feeds an indispensable part of the larviculture of the lateral type finfishes compared to artificial micro diets.

Challenges associated with the micro diet

Physical form of the diet

The suitability of a micro diet for larval fish is critically dependent on its physical form. The physical forms of a micro diet that is important for increasing its detection and attractiveness for fish larvae include shape, size color, movement, smell, taste, and texture. In line with this, Guthrie et al. (2000) demonstrated that consumption rates of larvae of Walleye (*Stizostedion vitreum*) were not similar for diets formulated with the same composition but different forms strengthening the conclusion that the physical form of a diet is detrimental.

Amongst the physical properties, particle size is particularly important to the early-feeding larvae and adjustments should be made based on the ontogenetic stages of the species. The size of diets formulated for fish and crustacean larvae are usually small enough to be entirely ingested without the assistance of the mouth parts. Concerning this, particles of 60%–80% of the width of the mouth size have been successfully ingested by the larvae of Sea bream (Fernández-Diaz et al., 1994).

Another important aspect of the physical form of a micro diet is the color of the diet itself and the rearing system. This is partly the reason why most hatcheries use the green water technique in which microalgae are added (Tredici et al., 2009). The green water system is beneficial for visually feeding larvae by increasing contrast to the background of the rearing system. As an example, larvae of Red drum and Cobia (*R. canadum*) preferred reddish brown to orange-colored diets in their larviculture. Another aspect of the physical form of the feed is its moisture content in which diets having low moisture content are relatively preferable as the rate of leaching of water-soluble nutrients may be high with higher moisture content diets.

One of the problems of a micro diet is the lack of visual stimuli for visually feeding predator fish larvae, unlike live feeds. Micro diets need to remain slightly below the surface of the water and have a motion that is fast enough to catch the attention of the larvae which is quite difficult to accomplish of course.

Eventually, most micro diets currently available on the market sink to the bottom adding to the water quality deterioration and few species feed on the bottom at their early larval stages.

Chemical attractiveness is another aspect of the physical form of micro diets in which larvae live in a chemically enriched environment with highly developed chemoreceptors to detect several nutrients like amino acids, nucleotides, organic acids, and bile salts. On the other hand, Kolkovski (2013) reported that live feeds (copepods) contain large amounts of free amino acids (glycine, arginine, and betaine) which are shown to be strong inducers of feeding behavior in larval fish.

Nutrient leaching is another important aspect of the physical form of micro diets where there is an apparent difference between live feeds and micro diets in the degree of losing of small molecular weight water-soluble nutrients (LMWS) which are crucial in the nutrition of fish larvae (Langdon & Barrows, 2011). LMWS are maintained in live feeds in their tissues while they are alive but later lose them when homeostasis stops on the death of live feeds which is unlikely to replicate this in the manufacturing of micro diets.

Accompanied by leaching, the sinking rate of a micro diet is a detrimental aspect of the composition of feed the larvae eat. Generally slower sinking feeds have the advantage of being easier to catch, available for longer periods on the water column, and less build-up at the bottom of the culture system. However, diets that are floating on the surface for a longer time will already start leaching compromising the nutritional quality of the diet before it is ingested by the larvae.

Feeding frequency

Frequency of feeding is an important aspect of the successful application of micro diets as live feed substitutes. In contrast to the perceived belief of better efficiency with frequent feeding, more rapid consumption of food and efficient use of nutrients may be achieved through a reduction in the frequency of feeding per day. This in turn reduces fouling thereby reducing the nutrient leaching of diets by reducing the time when feed remains suspended in the water column. This high residence time of micro diets in the water further leads to settlement, adherence, and clumping of particles on to the surface which again leads to a substantial loss of feed.

Gut retention time

According to Kurmaly et al. (1990), movement through the digestive system decreases during ontogeny where by the average time of food retention increases as well in larval lobsters. This attribute then can be used to develop micro diets for different stages of larval fishes in which the absorption of nutrients through a critical time in a diet having low digestibility will be realized if the food rapidly passes through the gut. In this case, the lack of high digestibility of the diet will be compensated by the consumption of a relatively large volume of feed per unit of time (D´Abramo, 2002). On the other hand, in larvae of fish and crustaceans having long gut retention time, an adequate supply of essential nutrients for survival and growth per unit of time is challenging as the diet has to be highly digestible while passing through the gut. Furthermore, achieving better digestibility of micro diets for larvae of fish and crustaceans is a challenge despite the presence of sufficient enzyme activity in the primordial gut. This is partly due to the limited quality and specificity of enzymes (Rønnestad et al., 2013).

Culture system

For fish larvae that are not filter-feeding, the design of the culture vessel can seemingly exert a marked effect on the success of food acquisition, specifically for larval forms. Consumption of diet may not always be primarily determined by the chemical characteristics, physical properties, or attractant value, but rather by the environment into which the formulated diet is applied (D´Abramo, 2002). As an example, the shape of the container or the magnitude of the movement of water within the container may be important factors contributing to the success of the larval culture.

Conclusions and Recommendations

Despite progresses in the biotechnological advancement of formulated diet manufacturing and substantial knowledge of the nutritional requirement of fish larvae, it can be concluded that the complete replacement of live feeds with micro diets for marine finfish larvae has not yet been achieved without reduced survival and growth.

Several challenges have been documented for the lack of success from the several studies conducted so far. Those challenges can be broadly classified into larvae and micro-diet-related challenges in which larval related challenges are mainly geared to the ontogeny of the digestive system and enzyme activity at first feeding while the later challenges are related to several factors such as physical form, gut retention time, frequency of feeding and of the micro diet and culture system to which the

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diets are applied.

However, despite this fact, significant progress has been achieved to shorten the weaning period of several marine fish species to micro diets with the possibility of cutting a substantial amount of live feed production cost in hatcheries. Moreover, the technological advancement in the development of micro diets has shown a tremendous impact on the study of larval nutritional requirements.

Therefore, future research must be directed to manufacturing and commercial scale applications of micro diets with proper nutritional and physical properties such as attractively, stability, availability, and digestibility. To achieve this, revisions of the current manufacturing techniques and optimizing culture system hydrodynamics are recommended. In addition, the larval nutritional requirement must be up scaled using the few successful micro diet application experiences to expand to other species of economic importance.

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