

# Chapter 7

## Nutritional Modulation of Marine Fish Larvae Performance

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**Abstract** Nutrition shapes the individual physiological pathways prior to hatching, resulting in long-term effects on postnatal growth and physiological functions. The environment, including nutrition, determines the rate of myogenesis, the number and size of muscle fibres, the composition of sub-cellular organelles, the patterns of gene expression, influences protein turnover and the efficiency of protein deposition, among others. Moreover, protein retention efficiency and fish adaptive fitness have been said to be negatively correlated. In addition, high mortalities are normally observed in the marine larval stages in optimized farming conditions (70–80%) affecting fish production. The consequences and implications of early nutrition for aquaculture production is an important challenge for the future. Likewise, optimized feeds need to be provided in sufficient quantities and in a manner adequate to feeding behaviour, to fully express growth potential, while avoiding deterioration of water quality or disease problems caused by excessive feeding. Understanding the mechanisms that control early development and growth and their relation with nutrition are critical for the identification of time windows in development that introduce growth variation, impact growth potential, and affect viability and quality of juveniles.

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## 7.1 The Role of Nutrients in Promoting Larval Growth

### 7.1.1 Protein and Amino Acids Larval Nutrition— Modulating Growth Performance

Growth is essentially muscle protein deposition and, dependent of the balance between protein synthesis and degradation (Carter and Houlihan 2001). Since protein synthesis requires that all indispensable amino acids (IAA) occur at an optimal ratio with respect to each other, dietary amino acids imbalances will compromise protein deposition and consequently affect negatively the high growth potential of fish larvae. Due to the methodological difficulties in determining amino acid requirements in fish larvae, indispensable amino acid profiles of whole-larvae or egg protein have been used as an indicator (Conceição et al. 2011). Using this approach, several studies have shown that live feeds used in marine larviculture present imbalanced amino acid profiles (e.g. Aragão et al. 2004; Hamre 2016; Saavedra et al. 2015). Therefore, the use of microdiets designed for a species according to the whole-larvae amino acid profile of the species has been suggested to enhance growth performance.

However, studies performed on large yellow croaker *Larimichthys crocea* and Senegalese sole *Solea senegalensis* larvae did not entirely support this hypothesis. Li et al. (2013) used a Control diet formulated using intact protein (a mixture of white fish meal, shrimp meal and squid meal) as the only protein source and experimental microdiets where crystalline amino acids replaced 40% of fish meal protein-bound nitrogen, and simulated the overall amino acid pattern of white fish meal (WFM) or of large yellow croaker egg, whole-larvae or muscle protein. Both Control and WFM microdiets resulted in higher larval growth and higher protein content than the other microdiets. Therefore, microdiets formulated according to egg or larval amino acid profile were less suitable for large yellow croaker larvae compared with microdiets based on white fish meal amino acid pattern (Li et al. 2013). Studies in Senegalese sole larvae also showed that microdiets with a balanced amino acid profile do not necessarily translate enhanced larval growth performances (Canada et al. 2016a, b). By feeding Senegalese sole with a practical microdiet supplemented with encapsulated crystalline amino acids in order to balance the dietary amino acid profile, a positive impact on the larvae capacity to retain either 1.0 or 6.8 kDa peptides during the metamorphosis climax was observed. However, the positive short-term impact on protein retention observed at metamorphosis did not translate into increased growth (Canada et al. 2016b). A similar study using higher levels of crystalline amino acids to balance the dietary amino acid profile resulted in reduced growth performances of Senegalese sole

larvae fed the Balanced diet (Canada et al. 2016a). In the latter study, the expression pattern of key genes regulating myogenesis was affected by the microdiet amino acid profile at metamorphosis, with the expression of myogenin and myosin heavy chain transcript levels being significantly reduced in larvae fed the Balanced diet.

These results were surprising, but several hypotheses have been raised. Besides methodological difficulties in obtaining an effectively amino acid balanced microdiet using crystalline amino acids due to leaching losses, the different rates of absorption between free amino acids (FAA) and proteins (Rønnestad et al. 2000) was suggested to impair results using amino acid balanced microdiets (Canada et al. 2016a, b). However, Li et al. (2013) showed that there were no significant differences on growth and survival of large yellow croaker larvae when fed microdiets with an amino acid profile resembling that of fish meal either using supplementation with crystalline amino acids or intact protein. Another possible explanation raised, is that the whole-larvae amino acid profile changes during ontogeny (Aragão et al. 2004; Saavedra et al. 2006, 2015), thus using a diet which referenced the whole-larvae amino acid composition in a certain period, may not meet the amino acid requirements over the entire larval stage (Li et al. 2013). An increase in voluntary feed intake to compensate the dietary amino acid imbalances has also been proposed (Canada et al. 2016a, b). Another alternative explanation is that even when apparently the amino acid requirements for growth are being covered by amino acid balanced microdiets, these may be insufficient to cover metabolic processes other than growth and energy supply.

Although the major fate of amino acids is towards protein synthesis, it has been recognized that amino acids regulate key metabolic pathways important not only for growth (Li et al. 2009). Saavedra et al. (2010) showed that further supplementation of amino acid balanced microdiets (based on the larvae whole-body amino acid profile) with aromatic amino acids did not increase growth or survival of white seabream *Diplodus sargus* larvae, but reduced skeletal deformities and mortalities caused by stress. The importance of aromatic amino acids for physiological processes other than growth has also been demonstrated by Pinto et al. (2009) using nutrient flux trials with  $^{14}\text{C}$ -phenylalanine and  $^{14}\text{C}$ -tyrosine. The authors found that aromatic amino acids were highly retained by Senegalese sole (a species with a very marked metamorphosis process) during pre-metamorphosis and metamorphosis climax, while no significant differences were found for gilthead seabream *Sparus aurata* larvae along its smooth metamorphosis process. Further nutrient flux trials with  $^{14}\text{C}$ -tyrosine indicated potential benefits of dietary aromatic amino acid supplementation during metamorphosis climax of Senegalese sole, while no apparent benefit was found for gilthead seabream along the metamorphosis process (Pinto et al. 2010b). Furthermore, even for some dispensable amino acids (DAA), the regulatory role in key metabolic pathways may be translated in growth enhancement. Microdiets supplemented with glutamine promoted antioxidant status and stress resistance capacities of half-smooth tongue sole *Cynoglossus semilaevis* post-larvae through modulation of activities and mRNA expression of antioxidant enzymes (such as glutathione peroxidase and catalase), decreased malondialdehyde contents, and increased mRNA levels of the 70 kDa heat shock

protein (*Hsp70*) expression after hypoxia stress. The enhanced antioxidant abilities and increased hypoxia stress resistance were ultimately translated in better survival and growth performance (Liu et al. 2015).

Amino acids that are not incorporated in proteins are often not considered in feed formulations. Taurine, a  $\beta$ -sulfonic-amino acid that only exists in free form, is the most abundant amino acid in the FAA pool from animal blood and tissues. In the recent past, taurine has been considered a required nutrient for normal growth of marine fish (NRC 2011). Several studies have demonstrated that dietary taurine supplementation improved growth performance of several species of marine fish larvae, such as Senegalese sole (Pinto et al. 2010a), northern rock sole *Lepidopsetta polyxystra* (Hawkyard et al. 2014, 2015), red seabream *Pagrus major* (Kim et al. 2016), cobia *Rachycentron canadum* (Salze et al. 2011), greater amberjack *Seriola dumerili* (Matsunari et al. 2013), California yellowtail *Seriola lalandi* (Hawkyard et al. 2016; Rotman et al. 2017), Pacific bluefin tuna *Thunnus orientalis* and yellowfin tuna *Thunnus albacares* (Katagiri et al. 2017). However, for some marine species, dietary taurine supplementation did not improve growth performance, as is the case of gilthead seabream (Pinto et al. 2013) and white seabass *Atractoscion nobilis* (Rotman et al. 2017). This is not entirely surprisingly, since the capacity to biosynthesize taurine has been shown to be highly species-specific (Goto et al. 2003; Yokoyama et al. 2001) and even stage-dependent (Kim et al. 2008).

Taurine is not used by the cells for protein synthesis and the mechanisms by which dietary supplementation may improve larval growth are still largely unknown. Studies using nutrient flux trials showed an increase in amino acid retention concomitant with a higher body taurine content in Senegalese sole larvae fed taurine supplemented microdiets (Pinto et al. 2010a), which may explain the increased growth performance. The larval growth improvement by dietary taurine supplementation was also suggested to be due to an increase in protein synthesis efficiency (Katagiri et al. 2017). Furthermore, Salze et al. (2012) showed that dietary taurine supplementation directed protein translation towards digestive enzymes in cobia larvae during the first two weeks after hatching. The authors suggested that the heightened enzymatic activities may lead to enhanced nutrient availability, thus providing some explanation for the growth improvement in taurine-supplemented larvae.

In line with the improved growth performance, dietary taurine supplements also increased development rates and enhanced metamorphosis of flatfish species (Hawkyard et al. 2014, 2015; Pinto et al. 2010a). This effect may be due to the correlation between metamorphosis and larval size, though Hawkyard et al. (2014) suggested some degree of developmental plasticity associated with dietary taurine concentrations. Dietary taurine supplementation has been shown to affect more than larval growth. Nutrient flux studies showed a higher retention of  $^{14}\text{C}$ -methionine in gilthead seabream larvae receiving a taurine supplement (Pinto et al. 2013). These results indicated that although increased methionine retention was not translated into higher growth performance, dietary taurine supplementation may ultimately affect larval metabolism by increasing methionine availability for several important physiological purposes.

For most altricial fish species it has been generally assumed that early-stage larvae have a limited capacity to digest and absorb the native protein sources commonly used in commercial fish feed formulations. Since dietary protein is mainly absorbed as FAA and di- or tri-peptides (Conceição et al. 2011), pre-digested proteins have been introduced in larval feed formulations in order to ease the dietary protein digestion, with the expectation of promoting absorption and further protein synthesis. This formulation strategy has been tested since the 90s, but even in more recent years the results are still unsatisfactory (Cai et al. 2015; Gisbert et al. 2012; Srichanun et al. 2014). A moderate inclusions of hydrolysed protein (<25% inclusion on protein basis) in larval microdiets is still the advisable strategy in order to promote growth and survival. In more recent years the formulations that have included higher levels of proteins hydrolysates presented similar results to previous studies regarding larval performance, like in Atlantic halibut *Hippoglossus hippoglossus* (Kvåle et al. 2009), Asian sea bass *Lates calcarifer* (Srichanun et al. 2014), gilthead seabream (de Vareilles Sommières 2013), large yellow croaker (Cai et al. 2015), spotted wolffish *Anarhichas minor* (Savoie et al. 2011), and white seabream (de Vareilles et al. 2012). This lower larval performance when fed microdiets containing protein hydrolysates has been attributed to a saturation of the peptide transport system in the intestinal brush-border membrane due to overloading of short peptides, and/or to impaired utilization of the fast absorbed FAA and/or di or tri-peptides, with these being used for energy production rather than growth. However, growth of larval Senegalese sole was augmented by a diet with high inclusion level of a protein hydrolysate with a moderate hydrolysis, but not with a diet with a high degree of protein hydrolysis (Canada et al. 2017). Moreover, this diet with moderate protein hydrolysis lead to decreased growth in post-larvae in comparison to intact protein. Therefore, the degree of protein hydrolysis is important, and the optimal dietary protein complexity will likely change throughout larval development.

The impact of dietary protein complexity on the efficiency of protein utilization by Senegalese sole has been studied at short-term in a nutrient flux study (Richard et al. 2015). In this study it was shown that, whereas 1.0 kDa oligopeptides are highly digestible and its retention efficiency is constant throughout development, the digestibility and body retention of larger polypeptides (6.8 kDa) are low in pre-metamorphic larvae, but improve throughout development. Also in Senegalese sole the nutrient flux method was used to assess the microdiet fed larvae digestive capacity to utilize polypeptides with different molecular weight (MW), 1.0 and 7.2 kDa. The PartH microdiet (target peptide MW 5–70 kDa) stimulated growth in metamorphosing larvae, whereas the Intact microdiet (target peptide molecular weight (MW) > 70 kDa) stimulated growth after weaning. The Intact microdiet stimulated the larvae absorption capacity for 1.0 kDa peptides at metamorphosis climax stage, which may have contributed for enhanced growth in later stages (Canada et al. 2017). The conclusion seems quite similar among the different experiments and fish species: a moderate rate of inclusion of protein hydrolysates is advisable, but a higher inclusion may be beneficial when hydrolysates with a lower degree of hydrolysis are used. A more complex protein should be incorporated into

the larval microdiets as the maturation of the digestive system progresses and a higher proteolytic capacity is acquired by the fish.

### 7.1.2 Improving Lipid Utilization

Most research on larval lipid nutrition has been centred on essential fatty acid requirements, since its importance to larval growth and quality has been long-recognized. However, recent studies have shown that total lipid content in microdiets should also be considered to understand larval nutritional requirements. The effects of dietary lipid level on growth and some lipid metabolism related genes were investigated in orange-spotted grouper larvae *Epinephelus coioides* and half-smooth tongue sole, using isonitrogenous microdiets with graded contents of lipid (6–22% dry weight approximately) and fish oil as main lipid source (Li et al. 2016; Yuan et al. 2017). On both studies, microdiets with lipid contents close to the estimated requirement (13.56 and 15.99% dry weight, respectively for sole and grouper) improved larval growth performances. Moreover, dietary lipid content influenced both de novo lipogenesis and lipolysis at transcriptional level. It was shown that larvae may cope with high dietary lipid contents mainly through down-regulating lipogenesis-related gene expression of fatty acid synthesis (FAS) and acetyl-CoA carboxylase beta (ACC2) (Li et al. 2016; Yuan et al. 2017). Furthermore, in orange-spotted grouper larvae, the expression of lipolysis-related genes, lipoprotein lipase (LPL) and hormone sensitive lipase (HSL), significantly increased first and then decreased in relation to the dietary lipid content (Li et al. 2016).

Through genomic approaches it has also been demonstrated that Senegalese sole larvae modulate metabolism to manage dietary lipid levels and obtain the energy and molecules that warrant optimal growth rates (Hachero-Cruzado et al. 2014; Román-Padilla et al. 2017). Senegalese sole larvae were fed live prey enriched with different emulsions resulting in high and low triacylglycerols (TAG) levels (Hachero-Cruzado et al. 2014). Larvae fed high TAG microdiets activated co-ordinately the transcription of apolipoproteins and other related transcripts involved in chylomicron formation, likely to facilitate proper lipid absorption and delivery. In contrast, larvae fed low TAG microdiets showed higher mRNA levels of several pancreatic enzymes and appetite modulators and some intra- and extra-cellular lipases. Senegalese sole fed live preys enriched in oil emulsions differing in fatty acid composition showed also different expression of genes involved in lipid metabolism, absorption and transport (Bonacic et al. 2016). Live preys were enriched in cod liver oil (rich in long-chain polyunsaturated fatty acids, LC-PUFA), linseed oil (rich in *n*-3 PUFA), soybean oil (rich in *n*-6 PUFA), and olive oil (rich in monounsaturated fatty acids). Larval performance was higher when larvae was fed preys containing higher levels of LC-PUFA and *n*-3 PUFA and this was partly explained by an up-regulation of phospholipid metabolism and apolipoprotein

synthesis, which resulted in enhanced lipid transport and mobilization, as well as tissue growth and remodelling (Bonacic et al. 2016).

Betancor et al. (2017) used a molecular approach to evaluate lipid metabolism in first-feeding Atlantic bluefin tuna *Thunnus thynnus* larvae fed enriched rotifers (*Brachionus plicatilis*) and copepod nauplii (*Acartia* sp.). These authors suggest that the absolute docosahexaenoic acid (DHA) level may be important for the survival of Atlantic bluefin tuna larvae but that the DHA:EPA (eicosapentaenoic acid) ratio may be relatively more important for larval growth. An up-regulation in peroxisome proliferator-activated receptor gamma (*ppar $\gamma$* ), fatty acid binding protein 4 (*fabp4*) and acyl coA oxidase (*aco*) expression in rotifer-fed larvae were associated with a compensatory response to reduced growth, whereas lower expression of lipoprotein lipase (*lpl*) denoted reduced lipid utilization.

Recent studies on the LC-PUFA biosynthesis pathway, based on the expression of fatty acyl desaturases and elongases, suggest that Atlantic bluefin tuna (Morais et al. 2011) and Senegalese sole (Morais et al. 2012) larvae are able to biosynthesize DHA from EPA or, in the case of the later, DHA from  $\alpha$ -linolenic acid (Navarro-Guillén et al. 2014). It has also been shown that the dietary DHA:EPA ratio may modulate the expression of LC-PUFA biosynthetic pathway genes, namely delta-6 fatty acyl desaturase (*fads2d6*) (Betancor et al. 2017). However, studies by Pinto et al. (2016) testing microdiets with two lipid and two DHA levels concluded that it is not advisable to include low DHA (<5% total fatty acids) and lipid (<7% dry matter) levels in weaning diets for Senegalese sole post-larvae, as a reduction of these levels may compromise post-larval growth performance.

Dietary lipids are a major source of energy and provide essential fatty acids and phospholipids, widely acknowledged as critical success factors for larval fish rearing. The importance of phospholipids in marine larval rearing has been the subject of many recent studies. The results from these studies will not be reviewed in this Chapter, since this subject is addressed in Chap. 5. Concerning the essential fatty acids, arachidonic acid (ARA) has been traditionally the less studied, but its potential to affect growth, survival and stress resistance has been previously acknowledged (Bell and Sargent 2003) and recent studies have provided some knowledge on the molecular mechanisms involved. Marine fish larvae seem to tolerate a wide range of dietary ARA:EPA (up to 3.0) ratios (Alves Martins et al. 2011, 2012). The adaptation to dietary ARA levels in marine fish larvae has been shown to involve the modulation of the expression of genes related to eicosanoid synthesis, lipid metabolism and stress response (Alves Martins et al. 2012; Montero et al. 2015). Nevertheless, despite growth and survival were not affected by the range of dietary ARA:EPA ratios, acute stress coping response seem to be more efficient in Senegalese sole post-larvae fed low (0.7) than high (3.0) ARA:EPA ratios (Alves Martins et al. 2011).

## 7.2 Larval Muscle Plasticity to Nutrition

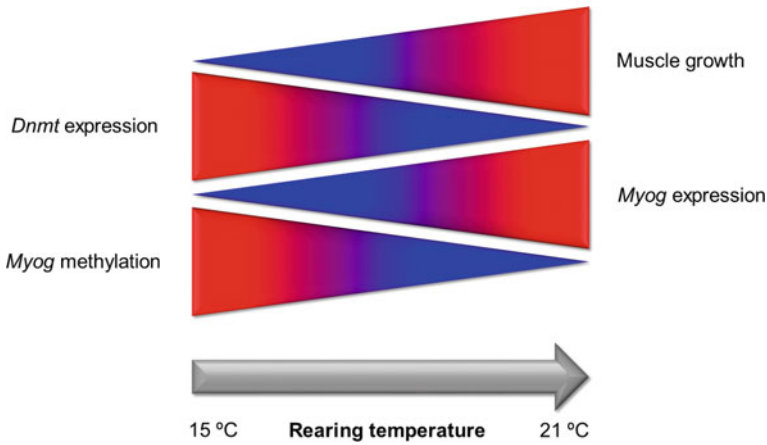
Muscle tissue comprises 40–60% of the total body mass in most fish and is the major determinant of fish growth. The axial muscle of most teleost species mainly consists of a deep bulk of fast twitch fibres with glycolytic metabolism and rapidly fatigue, and a superficial thin strip of slow-twitch fibres that are fatigue-resistant and have an oxidative metabolism (Sanger and Stoiber 2001). The skeletal muscle characteristics and the way muscle tissue grows are major factors influencing overall growth capacity in fish, as in mammals and poultry (Johnston 1999; Rehfeldt et al. 2011). In teleost, not only skeletal muscle fibre size change after birth in response to environmental factors and physiological conditions, but new fibres may also continue to be recruited into adulthood determining the maximum size attained by a species (Valente et al. 2013; Vélez et al. 2017). Muscle growth is very plastic in fish and there is increasing evidence that early events imprint an individual physiological memory (Campos et al. 2013b, c, 2014), resulting in long-term effects on postnatal growth and physiological function, irreversibly affecting growth potential and final size attained.

The importance of nutritional status in regulating protein metabolism and muscle growth is widely accepted, and the role of macronutrients is clearly implicated in vertebrates developmental programming (McMillen et al. 2008). In mammal models, studies have highlighted a link between nutritional conditioning during early life stages and survival, growth, learning process, lipid and glucose synthesis in later life, suggesting that developmental programming may have an epigenetic component as epigenetic marks such as DNA methylation or histone tail modifications could provide a persistent memory of earlier nutritional states (Lucas 1998; Sharples et al. 2016; Vickers 2014).

In fact, studies on the epigenetic modulation of DNA methylation are a recent trend in fish and up to now no published literature is available concerning nutritional conditioning of muscle growth, and just a few reports related to early temperature exposure. Campos et al. (2014) have suggested that an epigenetic mechanism could promote differential gene expression and modulate Senegalese sole muscle growth in response to different thermal conditions. Different rearing temperatures during the pelagic phase induced changes in the methylation status of the myogenin putative promoter, its mRNA transcript levels and in the expression of *dnmt1* and *dnmt3b* DNA methyltransferases, which catalyse the methylation of CpG dinucleotides, silencing gene expression (Campos et al. 2013b; Fig. 7.1). These changes resulted in alterations in the fast twitch muscle cellularity of Senegalese sole during metamorphosis climax, and influenced subsequent somatic growth in later stages (Campos et al. 2013a). Campos et al. (2014) suggested the pelagic phase in Senegalese sole as a critical developmental time window prone to epigenetic modifications with long-lasting effects on the regulation of myogenesis and subsequent influence on the potential for growth.

The impact of nutritional factors on genetic pathways regulating muscle fibre determination and growth has been poorly studied in fish and the few studies





**Fig. 7.1** Temperature-induced phenotypic plasticity model of muscle growth in Senegalese sole. A higher rearing temperature lead to lower methylation levels of the myog, which correlated with a decrease in *dnmt1* and *dnmt3b* and an increase in *myog* expression and muscle growth. Blue and red indicate lower and higher levels of methylation, gene expression and muscle growth, respectively. Original figure in Campos et al. (2013b)

focused on juveniles (Alami-Durante et al. 2010; Campos et al. 2010; Lopes et al. 2017; Valente et al. 2016). In marine fish larvae, Canada (2017) have recently hypothesized that manipulating the formulation of dietary protein might lead to changes in the dietary protein absorption and body retention, and subsequently modify the post-prandial availability of methyl-donors, such methionine, with possible consequences on the DNA methylation and regulation of gene expression in different tissues. In fact, the transcript levels of key genes regulating myogenesis changed between Senegalese sole fed a practical microdiet supplemented with encapsulated crystalline amino acids, during the metamorphosis climax and at the 51 days after hatching (Canada et al. 2016a). The group fed the supplemented diet had lower *dnmt3b* mRNA levels compared to the control group. A lower post-prandial availability of methyl-donors as a result of a lower protein intake might explain the reduced expression of *dnmt*'s in those fish. Whether this would also imply an overall DNA hypomethylation in skeletal muscle remains to be clarified. The dietary protein complexity was also shown to induce changes in protein utilization (Canada et al. 2017) and the regulation of myogenesis in Senegalese sole larvae (Canada 2017). Microdiets mostly based on highly hydrolysed protein sources were associated with reduced muscle growth potential and the up-regulation in the transcript levels of genes encoding for de novo DNA methyltransferases. These results support the hypothesis that nutrition may induce changes in post-prandial availability of methyl-donors that might alter the expression of *dnmt*'s, with putative consequences on DNA methylation.

In conclusion, the molecular and epigenetic mechanisms underlying skeletal muscle memory in response to early life nutrition are far from being understood in

fish. Further research is required to help understanding the ‘epi’-memory of skeletal muscle enabling nutritionists to tailor microdiets able to enhance muscle growth.

### 7.3 New Formulations for Nutritional Modulation in Fish Larvae

Larvae of most marine fish species, and even many freshwater species, rely for a period ranging from one week to one or more months on live prey to grow. Considerable progress was done in microdiet technology in recent years, nevertheless for most marine fish larvae the feeding regime is still based on live prey (rotifers and/or *Artemia* sp.). The progress was delayed until recent years by several factors: low attractiveness of microdiets and consequent low ingestion rates; poor digestibility of microdiets by the larva; high leaching losses of soluble molecules such as FAA, peptides, vitamins and minerals in the microdiets; and difficulties to formulate complete and well balanced microdiets due to lack of knowledge on larval nutritional requirements (Hamre et al. 2013). Larval nutritional requirements are still largely undetermined for marine fish species.

To overcome this limitation on understand of nutritional requirements, larval microdiets are normally formulated by excess of the different essential nutrients, including amino acids, fatty acids, phospholipids, vitamins and minerals. In addition to a formulation that fulfill the nutritional requirements, microdiets for fish larvae need to comply with several structural and functional characteristics: (1) be stable enough to prevent disintegration after the immersion in water, and to avoid major losses of water-soluble nutrients as amino acids, vitamins and minerals—given the small size of the feed particles, with a high surface/volume ratio that reduces the diffusion distance from the core to the surface; (2) have a high floatability so to be accessible to larval fish in the water column for a period as long as possible (3) be attractive to larvae and have an appropriate diameter; and (4) be easily digestible by the larval digestive system. Meeting simultaneously all these requisites is a technological challenge, in particular to balance between stability to prevent excessive leaching of nutrients and high digestibility.

Larval diet formulations rely on protein hydrolysates to improve diet attractiveness, accelerate maturation of the digestive system and improve digestibility. Several diet formulations strategies for marine larvae using protein hydrolysates have been tested with different degrees of success: increasing incorporation rates (de Vareilles Sommières 2013; Savoie et al. 2011; Srichanun et al. 2014); different degree of hydrolysis (Cai et al. 2015; Canada et al. 2017; de Vareilles et al. 2012; Srichanun et al. 2014); different raw material for the protein hydrolysates, from vegetable (Canada et al. 2017), to marine (Cai et al. 2015; de Vareilles et al. 2012; Delcroix et al. 2015; Srichanun et al. 2014), and from animal by-products to yeast (Gisbert et al. 2012; Skalli et al. 2014). In general, as commented in Sect. 7.1.1, moderate inclusion levels of protein hydrolysates give best results, independent of

the base raw material used. For instance, the substitution of fish hydrolysates in microdiets for gilthead seabream (Gisbert et al. 2012) and European seabass *Dicentrarchus labrax* (Skalli et al. 2014) by animal by-products or yeast (6–12% in seabream and 9–12% in seabass) showed that is feasible to incorporate alternative sources in larval microdiets at moderate levels. Still, the incorporation success may be species-specific.

The quality of the oils and protein-rich ingredients used in larval microdiets is also of paramount importance. Typically, larval microdiets use the highest quality ingredients available, and nowadays mostly cold-extrusion is used to prepare microdiets so to preserve to the maximum their nutritional value. Marine ingredients (i.e., fish meal, fish oil, squid meal, krill meal, krill oil) are usually the main sources of protein and oils for larval microdiets, but yeast, microalgae, soy lecithin, and vegetable protein concentrates (e.g., wheat gluten, pea protein concentrates, soy isolates/concentrates) are also commonly used.

Larvae of different fish species seem to perform better on different protein sources. When testing a very high quality fish meal, squid meal and a mix of vegetable protein concentrates as main ingredients for gilthead seabream (Conceição et al. 2015), squid meal was the best protein source in what concerns growth performance. However, fish meal seemed to make seabream larvae more robust. This may be due to the presence of some micronutrient(s) in fish meal, and absent in squid meal and the vegetable mixture. Gilthead seabream larvae did not perform well with the mixture of vegetable protein concentrates and both digestive capacity and lipid metabolism were negatively affected, be it due to the presence of an antinutritional factor, and/or to a deficiency in one or more micronutrients present in marine ingredients (Conceição et al. 2015). However, Senegalese sole performed well with a microdiet having the same mixture of vegetable protein concentrates as the main protein source (Pinto et al. 2016). In a study with Ballan wrasse *Labrus bergylta* larvae, a combination of cod muscle meal and shrimp meal performed better than microdiets using fish meal alone, or fish meal in combination with krill hydrolysate or shrimp meal (Kousoulaki et al. 2015).

The larval requirements for *n*-3 HUFAs (highly unsaturated fatty acids) is normally satisfied by DHA-rich fish oils, but it has been shown that at least for gilthead seabream fish oil may be fully replaced by different microalgal products rich in essential fatty acids (Eryalcin et al. 2013). Even though fish meal contains phospholipids, normally these are not sufficient to meet fish larval requirements, even when fish meal is incorporated at high levels. Normally used phospholipids sources are krill oil and soybean lecithin, mainly the latter due to price. When the two were compared in gilthead seabream larvae, krill oil was shown to have a higher effectiveness in promoting survival, growth and skeletal mineralization in comparison with soybean lecithin (Saleh et al. 2015).

Micronutrients as vitamin and minerals are typically supplied as premixes in larval microdiets, which most likely should be different from premixes for juveniles. Studies with gilthead seabream (Atalah et al. 2012), European seabass (Betancor et al. 2013) and meagre *Argyrosomus regius* (El Kertaoui et al. 2017), have pointed out to the importance of having high dietary levels (compared to

accepted requirements for juvenile fish) of vitamin E and C to protect DHA and other essential fatty acids from oxidation, in larval microdiets which are typically rich in these highly unsaturated fatty acids. In this regard, the high efficiency of selenium as an antioxidant factor for early weaning diets for gilthead seabream has also been demonstrated (Saleh et al. 2014). Increasing attention has recently been paid to levels and sources of the oxidative stress-related minerals selenium, zinc and manganese that should be supplied in microdiets for marine fish larvae, but if in excess are toxic to larvae. A recent study showed the need to supplement gilthead seabream microdiets based on squid meal and krill oil with one or more of these antioxidant minerals, to promote larval growth and bone mineralization and to prevent skeleton anomalies (Izquierdo et al. 2017). This study also showed that organic minerals are more effective than inorganic forms and nanometals in promoting mineralization and stress resistance.

## 7.4 Nutritional Programming

Lucas (1998) named the concept programming when during a “critical window” an early stimulus or insult may re-set some physiological pathways with consequences in the long-term. For Aquaculture purposes a “critical window” is usually during egg or larva stage, or during the spawning season (broodstock). The yolk-modification through glucose microinjection in zebrafish *Danio rerio* (Rocha et al. 2014, 2015), prey enrichment with glucose at mouth opening in gilthead seabream (Rocha et al. 2016b), microdiet at early stage formulated to change lipid metabolism in European seabass (Vagner et al. 2007a, b), or protein metabolism in Senegalese sole (Canada et al. 2016a) are different strategies to identify “critical windows” in larvae at early stage of development.

The concept of nutritional programming in fish, is a relative “new” research area in Aquaculture. The first insights in marine fish were in European seabass larvae aiming to improve lipid utilization during juvenile stage (Vagner et al. 2007a, b, 2009). During 39 days seabass larvae were fed with a Low or a High HUFA microdiet, and after 3 months in order to test the concept fish was fed a HUFA experimental deprived diet. The results were auspicious since the relative expression of the delta-6 desaturase was higher in juveniles that during larval stage were fed the Low HUFA diet when compared to juveniles originated from larvae fed the High HUFA diet. Despite this first success experiments regarding nutritional programming in marine fish larvae are still scarce. The data that appear in the following years was acquired using freshwater species as a model (Balasubramanian et al. 2016; Fang et al. 2014; Geurden et al. 2013, 2014; Rocha et al. 2015). Only more recently new data started to be published in gilthead seabream, regarding carbohydrate (Rocha et al. 2016a, b) or lipid utilization (Izquierdo et al. 2015; Turkmen et al. 2017), and in Senegalese sole regarding protein utilization (Canada et al. 2016a).

Nutritional programming as a strategy to enhance nutrient utilization in animal production may lead to higher biological efficiency, meaning a higher production at lower production cost, and minimal environmental impacts. Worldwide aquaculture production is moving to a sustainable production. Meaning that is imperative that marine fish species start to be fed with microdiets containing none or very low amounts of marine ingredients like fish meal and fish oil. Programming carnivorous fish to better utilize dietary carbohydrates would give an advantage to the sector, since the inclusion of carbohydrates in the diet may improve protein and lipid retention by replacing the energy substrate (Kamalam et al. 2017). In gilthead seabream a glucose incorporation during live prey feeding period was able to enhance digestion of carbohydrates and hepatic lipogenesis, suggesting that the early glucose stimuli may alter carbohydrate utilization in seabream juveniles (Rocha et al. 2016a). Enhancing lipid utilization to improve aquaculture sustainability is based on the complete or very low incorporation of fish oil in fish microdiets. For this the nutritional programming should modify the pathways of fatty acid synthesis and the gene expression of key molecular markers such as  $\Delta$ -6 desaturase. Besides the experiment in European seabass, one experiment was done with gilthead seabream broodstock (Izquierdo et al. 2015). Gilthead seabream broodstock were fed four diets with different replacement levels of fish oil by linseed oil during six months and the progeny was fed microdiets containing low levels of fish meal and fish oil, and high levels of vegetable oil and vegetable meal. A 60% replacement of fish oil by linseed oil in the broodstock diets produced juveniles with a better ability to utilize diet formulated with low fish meal and fish oil and higher growth (Izquierdo et al. 2015).

DNA methylation is mediated by de novo DNA methyltransferases (*dnmt*; *dnmt1*, *dnmt3a* and *dnmt3b*) that are involved in the regulation of gene expression, by repressing transcription. *dnmt3a* and *dnmt3b* are responsible for addition of methyl group de novo, whereas *dnmt1* is responsible for maintenance of DNA methylation patterns during cell replication (Zhang 2015). Epigenetic modifications, such DNA methylation are labile in response to nutritional cues. Very few studies in fish studied how a nutritional cue could change gene regulation during development and juvenile or adult stage (Beaver et al. 2017; Canada 2017; Canada et al. 2016a). Fish larvae have high protein requirements and high amino acid losses for energy production (Conceição et al. 2011), suggesting that dietary IAA levels may be a limiting factor for growth. Senegalese sole larvae were fed with a microdiet where the dietary protein quality was changed by increasing the IAA/DAA ratio, to improve the larvae capacity to retain dietary protein by promoting protein accretion in skeletal muscle. Although *dnmt3b* expression was reduced in the larvae fed the IAA:DAA corrected diet, no correlation was established with the regulation of myogenesis (Canada et al. 2016a). Also in Senegalese sole, post-larvae that were fed a diet mostly based on highly hydrolysed protein (high content of 5 kDa peptides) displayed higher transcript levels of *dnmt3a* and *dnmt3b*, which was attributed to increased dietary methionine content (Canada 2017). There are several evidences that nutritional programming is possible in several species, in fish the first results indicate that nutrition change the *dnmt*'s expression in the short-term. Future

epigenetics studies are needed to identify “critical windows” and the type of stimulus that will re-set the pathways in the long-term.

## 7.5 Conclusions

Broodstock and early larval nutrition shapes the individual physiological pathways, resulting in long-term effects on postnatal growth and physiological functions. The importance of nutrition in regulating protein metabolism and muscle growth is widely accepted, and the role of macronutrients in vertebrates developmental programming has been clearly demonstrated. Growth is essentially muscle protein deposition and, dependent of the balance between protein synthesis and degradation. Due to the methodological difficulties in determining amino acid requirements in fish larvae, indispensable amino acid profiles of whole-larvae or egg protein have been used as an indicator. Consequently, the use of microdiets designed for a species according to the whole-larvae amino acid profile of the species has been suggested to enhance growth performance. However, during the formulation process one should keep in mind that even when apparently the amino acid requirements for growth are being covered by amino acid balanced microdiets, these may be insufficient to cover metabolic processes other than growth and energy supply. Regarding protein hydrolysates, a moderate rate of inclusion of protein hydrolysates is advisable in larval microdiets, but a higher inclusion may be beneficial only when hydrolysates with a lower degree of hydrolysis is used, and possibly only for the early larval stages. A more complex protein should be incorporated into the larval microdiets as the maturation of the digestive system progresses and a higher proteolytic capacity is acquired by the fish. Most research on larval lipid nutrition has been centred on essential fatty acid and phospholipid requirements and ratios, since its importance to larval growth and quality. However, recent studies have shown that total lipid content, and ratios between phospholipids and triacylglycerols, in microdiets should also be considered to understand larval nutritional requirements. The impact of nutritional factors on genetic pathways regulating muscle fibre determination and growth has been poorly studied in fish, and the few existing studies focused on juveniles. Recent data regarding a possible consequence on the DNA methylation and regulation of gene expression in muscle tissue in marine larvae supports the hypothesis that nutrition may manipulate fish ‘epi’-memory. The thorough study of long-term consequences of early nutrition for aquaculture production is an important challenge for the future.

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