New Approaches to Assess the Nutritional Condition of Marine Fish Larvae

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Abstract

The typical approach to assessing the digestive capacity of marine fish larvae involves the histomorphological description and characterization of the digestive system and associated organs in relation to the developmental stage, as well as the quantification of digestive enzyme activities with biochemical and histochemical techniques. Although the biochemical quantification of digestive enzymes has been a very widespread and useful tool to assess the digestive capabilities of fish larvae, molecular tools have gained importance in the last decade to study the ontogeny of digestive enzymes gene expression, allowing the understanding of the mechanisms underlying the digestive physiology of young fish. The general patterns of the morphohistological development of the digestive tract and accessory glands might be also used as biomarkers of nutritional stress, since the digestive system is very sensitive to changes in diet quantity and quality. The literature indicates that there are certain tissular and cellular responses to food availability and quality, particularly in the digestive tissue, which is common to most teleost fish larvae. These responses, which are independent of water temperature, can be used for assessing fish larvae nutritional condition. In this regard, the microscopical organization of the liver hepatocytes, the intestinal mucosa and the exocrine pancreas, which are generally used as target tissues and organs to assess the nutritional condition of fish larvae, is deeply reviewed. The advantages and disadvantages of the use of different cellular biomarkers of effect are discussed considering different conditions.

Keywords: fish larvae, ontogeny, digestive system, digestive enzymes, nutritional biomarkers, weaning

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Assessing the nutritional condition of fish larvae is of vital importance in ecological studies, since the physical and physiological condition of larval fishes throughout their development influences their growth performance and survival, and ultimately contributes to recruitment to the adult population. These studies require that accurate, objective and quantitative criteria be used to characterize the nutritional condition of fish larvae. This approach can also be applied in aquaculture where the development of dependable and sustainable fish larval rearing techniques requires a deep knowledge of the critical aspects of larvae nutrition in relation to the development of the digestive and metabolic systems, as well as establishing the limits for initiating exogenous feeding.

Once exogenous feeding is established, larval development depends on the proper nutrient input provided by the diet, in addition to optimal biotic and abiotic conditions. Periods of food deprivation after the completion of yolk reserves can lead to abnormal behavior and morphological development, degeneration of the alimentary tract and trunk musculature, and reductions in food utilization efficiency and feeding activity. Fish larvae are especially sensitive to non-optimal feeding conditions or nutritional stressors (dietary imbalances), because most tissues and organs are under progressive and intense differentiation and development, and larvae do not have enough reserves stored to withstand starvation (Ferron & Leggett, 1994; Catalan, 2003; Gisbert *et al*., 2008).

The effect of feeding restriction or nutritional imbalance on aquatic organisms is routinely assessed by a number of indicators commonly named “condition indices” used to characterize nutritional condition of fish larvae. Condition indices were extensively reviewed by Ferron and Leggett (1994) and Catalan (2003) in terms or reliability, sensitivity, time response, size and age specificity, field vs laboratory estimates, processing time, costs and requirements. The former authors divided condition indices into three main categories according to the main organization levels: cell, tissue and organism. In this sense, the physical deterioration of fish larvae resulting from food deprivation or dietary imbalance has been assessed and interpreted by means of morphometric and gravimetric measurements (shape and weight changes), biochemical methods(RNA:DNA ratios,

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digestive and metabolic enzyme activities), histological criteria or various combinations of the above-mentioned methods (Ferron & Leggett, 1994; Catalan, 2003; Gisbert *et al*., 2008). Although there are a wide variety of nutritional condition indices, this section will only cover those related to the digestive system organization (histological biomarkers) and function (pancreatic and intestinal enzyme activities).

In vertebrates, different organs of the digestive system have been shown to employ different cellular mechanisms in response to diet quantity and quality. Thus, the use of the intestine and digestive accessory glands as target organs of the nutritional and physiological status in fish is well known, and up to a certain limit standardized. The use of **histological biomarkers** for assessing the nutritional condition of fish larvae has been recently reviewed by Gisbert *et al*. (2008).The histological organization and histochemical properties of the liver, exocrine pancreas and intestine have been used on a regular basis as targets to elucidate the effects of different dietary regimes or nutrients and starvation levels on larval physiology, nutrition and early development (Table 1).

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Table 1. Cellular criteria used to grade tissues and assess the nutritional condition in teleost larvae. Data rewritten from Margulies (1993), Catalan (2003) and Gisbert *et al.* (2004b)

Tissue	**Grade (condition)**		
	1 (Degraded)	2 (Average)	3 (Healthy)
Liver hepatocytes	Nearly all nuclei pycnotic and dark with clumped chromatin; cytoplasm lacks texture; intracellular vacuoles absent; cells small and indistinct.	At least 50% of cell nuclei with dark granules and situated medially; nearly 50% of cytoplasm granular; intracellular vacuoles reduced or absent; boundaries of most hepatocytes visible.	Nuclei distinct and often displaced laterally; cytoplasm lightly stained with abundant intracellular vacuoles containing lipids and glycogen; boundaries of hepatocytes prominent.
Exocrine pancreas	No acinar symmetry remaining; all nuclei dark (pycnotic) and indistinct.	Acinar symmetry reduced by 50%; 50% of nuclei dark and indistinct; moderate amounts of zymogen.	Cells formed in distinct, circular acini; all nuclei clear and distinct in basal position; abundant zymogen granules.
Intestinal epithelium	Mucosal cell height reduced by >50% in height; some loss of striations in bordering microvilli; supranuclear vacuoles reduced or absent.	Mucosal cells reduced by 25 to 50% in height; some loss of striations in bordering microvilli; supranuclear vacuoles reduced or absent.	Mucosa deeply convoluted and mosaic; mucosal cells compact, pronounced in height, with distinct nuclei; prominent supranuclear acidophilic inclusions and vacuoles.

The histological organization of the intestine, like that of the liver, is particularly sensitive to food deprivation and starvation. Major alterations of the intestinal mucosa include the reduction in the height of the enterocytes and the number and size of epithelial folds. Proteolysis of the intestinal mucosa is a common response to severe starvation, which involves a reduction of the nutrient absorption surface area, and compromises the digestive capabilities of refeeding larvae. For these reasons, the criterion of enterocyte height has been widely used as a valuable histological index of sub-optimal feeding or starvation in several fish species (Ferron & Leggett, 1994; Catalan, 2003; Gisbert *et al*., 2008). However, Catalan and Olivar (2002) reported that cell heights of the posterior intestine in

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European sea bass larvae were less useful to distinguish different feeding treatments than other quantitative measurements (e.g. hepatocyte maximum diameter, muscle fiber separation). Consequently, for any selected species, any current or putative nutritional condition index should be tested and validated under laboratory controlled conditions.

Lipid and protein inclusions in enterocytes may also be used as a biomarker in fish larval nutrition and digestive physiology studies (Gisbert *et al*., 2008).The presence of acidophilic supranuclear inclusions is a typical feature of the posterior intestine in fish larvae. These inclusions are due to the absorption of protein macromolecules by pinocytosis. In most studied species, supranuclear bodies are observed throughout the larval period, although their number and size decrease as the stomach differentiates and extracellular digestion takes place. Thus, variations in the normal pattern of accumulation of these inclusions may be indicative of changes in the nutritional physiology of the larva and, therefore, be used in developmental or nutritional studies dealing with larval early stages of development. The presence of lipid inclusions in the enterocytes of fish larvae is a common feature during their early development. The type and size of lipid inclusions varies depending on the fat content of feed and the degree of unsaturation of the lipids ingested. As a result, changes in the size and type of lipid inclusions may be dietary-dependent and may be useful for assessing the nutritional condition of a fish larva. Three types of inclusions can be distinguished in fish enterocytes according to their size: particles (20-70 nm in diameter) resembling mammalian VLDL; lipoprotein particles (70-500 nm in diameter) considered as chylomicrons; and large inclusions of triglycerides measuring up to 6 μm and described as lipid droplets (Diaz *et al*., 1997). In addition, the formation of large lipoproteins and lipid droplets is closely related to an excess of fats in enterocytes caused by the high fatty acid contents of diets. This large accumulation of lipids in the enterocytes may cause some pathological damage since large lipid inclusions produce epithelial abrasion, cellular necrosis, and/or inflammatory reactions along the intestinal mucosa (Deplano *et al*., 1989) that may affect nutrient absorption and reduce digestive efficiency.

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The histological organization of the liver accurately reflects any physiological disorder originated from a nutritionally unbalanced diet or feed deprivation episodes, since hepatic energy stores respond sensitively to nutritional changes (Table 1). Under food deprivation conditions, liver glycogen and lipids are the first energy sources to be mobilized. As reviewed by Gisbert *et al*. (2008), large central nuclei are observed in livers containing few lipid inclusions, while peripheral nuclei are detected in livers of larvae showing high levels of lipid deposition. Histopathological changes in food-deprived larvae are similar amongst different species and include changes in liver organization (shrinkage of the nucleolar volume, swollen and deformed mitochondria, dilated sinusoids, large intercellular spaces, vascularization, increase in lysosomes, cytoplasmatic necrosis, and hypertrophy of the bile canaliculi and the gall bladder) and a decrease in glycogen and lipid deposits stored in the hepatocytes. The liver is also a good biomarker for nutritional effects of different dietary composition and feeding regimes because the hepatic energy stores respond sensitively and rapidly to nutritional changes in fish larvae. In addition, alterations in fatty acid metabolism derived from unbalanced diets have resulted in modifications of the nuclei shape and size, chromatin density, and cytoplasmatic lipid deposition in hepatocytes (Caballero *et al*., 1999; Mobin *et al*., 2000). Disorders in glycogen and protein synthesis and/or their utilization may also result in an increased level of basophilia in the cytoplasm of the hepatocytes of larvae fed unbalanced diets (Segner *et al*., 1994; Mobin *et al*., 2000).

The earlier differentiation and morphogenesis of the exocrine pancreas in comparison to that of the liver or intestine facilitates its use as a histological index for assessing the condition of the larva as soon as it emerges from the egg envelope. Food deprivation induces degeneration of the exocrine pancreas, which may be summarized as a disruption of the acinar symmetry and organization of the pancreas, a reduction in size of secretory cells and an increase of pycnotic nuclei (Table 1).

Catalan (2003) extensively reviewed the use of histological methods in the determination of larval nutritional condition and suggested this has at least two unresolved limitations. One regards the low objectivity of some methods, since the measures are mainly qualitative and

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rely on the experience of the observer. To date, quantitative data have been restricted to the measurement of cell heights of a few tissues, mainly gut and liver, and have proved useful for early larval stages of some species. However, some of these measurements are only obtainable from species with an elongated digestive duct, or have been restricted to particular larval stages. The second main problem with histological indices (extendable to any condition index) is the large dependence of condition on the experimental rearing parameters, with subsequent poor applicability to field studies. Until further evidence is supplied, there is a need to establish a relationship between survival and each condition measurement under laboratory conditions.

Due to their essential role in metabolic reactions, enzymes can be good indicators for the condition of an organism. For fish larvae, the activity level of **digestive enzymes** is well suited as a biochemical indicator of the feeding activity. In addition, digestive enzymes are considered to be reliable indicators of the nutritional state of the individuals, due to their species and age specificity, sensitivity and short latency. Different digestive enzymes are used for this purpose, ranging from proteolytic pancreatic enzymes (Ueberschär & Clemmesen, 1992; Lamarre *et al*., 2004; Cara *et al*., 2007) to intestinal brush border and cytosolic enzymes (Zambonino-Infante & Cahu, 2007; Zambonino-Infante *et al*., 2008; Darias *et al*., 2009).

Pancreatic enzyme synthesis and secretion appear to be particularly sensitive to food deprivation and dietary composition in teleost larvae, and consequently, the pancreatic enzyme activity provides a reliable biochemical marker of larval fish development and condition (Zambonino-Infante and Cahu, 2001). The pancreatic secretory process matures during the first three or four weeks after hatching in temperate marine fish larvae. This maturational process can be disrupted when larvae are fed diets that do not meet their specific needs (Cahu and Zambonino-Infante, 1994): the earlier the feeding with such inadequate diets, the lower the pancreatic secretion level. On the other hand, some dietary components, i.e. free amino acids (Zambonino-Infante and Cahu, 1994) or some non-biodegradable particles (Pedersen and Andersen, 1992, can enhance pancreatic secretion,

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revealing the coexistence of chemical and neural mechanisms controlling secretion in larvae. Because protein is one of the major components of the fish larval diet, the activity levels of pancreatic proteolytic enzymes, e.g. trypsin and chymotrypsin, are well suited as indicators of the nutritional condition of the organism. Secretion rate of pancreatic enzymes is related to feed intake, the stomach filling and nutrient composition (Rønnestad & Morais, 2008); thus, starvation, reduced feed intake or an unbalanced diet in terms of free amino acids or protein content may result in a decrease in secretion and consequently, activity of trypsin and chymotrypsin (Pedersen *et al*., 1987; Ueberschär, 1995; Applebaum & Holt, 2003; Cara *et al*., 2007). In addition, some authors have suggested using the trypsin/chymotrypsin ratio as a better indicator of the larval nutritional condition, since it might indicate to what extent chymotrypsin is activated by trypsin, and this in turn may indicate growth potential of the fish (Cara et al., 2007). The higher the trypsin/chymotrypsin ratio, the higher the absorption rate of essential amino acids for protein synthesis and growth potential.

The morphoanatomical development and maturation of the intestine is characterized by a decrease in activity of the cytosolic enzyme activity of leucin-alanine peptidase which is accompanied by an increase in activity of the brush border enzymes from the enterocytes. This maturation process is known to be nutrient-sensitive; and consequently, disparity between diet composition and larvae digestive features may delay or prevent the genetically programmed sequence of intestinal development (Zambonino-Infante & Cahu, 2001). In this sense, intestinal maturation is often assessed by the alkaline phosphatase/leucine-alanine peptidase or aminopeptidase/leucine-alanine peptidase ratios (Zambonino-Infante & Cahu, 1994). These can be considered as nutritional condition indices for evaluating the switch from a primary or early to an adult mode of digestion. In any case independently of the digestive enzyme activity considered, reference values for each species, developmental stage and nutritional condition need to be standardized under laboratory controlled conditions, since the development of the digestive function varies among species, as do their basal levels of digestive enzyme activities, and it may turn out that some enzymes may be more informative than others.

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Results of digestive enzymes **gene expression** analyses from recent studies on fish larvae (Darias, 2005; Geurden *et al*., 2007; Sánchez-Amaya *et al.*, 2009) suggest the possibility of including the molecular level as the fourth organization category (organism, tissue, cellular and molecular) in the list of markers for nutritional conditions in fish. Knowledge of gene expression amount and pattern of digestive enzyme precursors constitute a valuable tool that complements the information about the nutritional condition of an organism obtained through enzymatic indicators. This is particularly interesting in aquaculture where nutritional requirements for fish larvae need to be optimized and the origin of the suboptimal larval growth and performance derived from food supply is often unknown. In this sense, the study of the molecular mechanisms underlying digestive system ontogeny and digestion would expand knowledge of larval physiology and facilitate finding solutions to nutritional problems by localizing the molecular pathways that have being disrupted. However, since gene expression does not always necessarily culminate in protein synthesis, both molecular and cellular indicators should be considered in order to obtain more comprehensive information about the physiological status of fish larvae.

The ontogeny of digestive enzymes gene expression is genetically programmed and their expression patterns are stage-specific. Therefore, genes coding for digestive enzymes could be used as markers for fish larval development. For instance, the development of pepsinogen gene expression reveals the attainment of complete functionality of the gastric glands, hence constituting a suitable indicator of the transition from larval to juvenile stage (Segner *et al.*, 1994; Darias *et al.*, 2005). Besides, the nutritional condition of fish larvae could be reflected in the gene expression patterns of some digestive enzymes during ontogenesis. The simplest example is provided by differences in amount of transcripts (i.e., amylase) found in starved larvae compared with fed ones as a result of triggered physiological mechanisms necessary to adapt the energetic balance to the different nutritional status (Darias, 2005; Sánchez-Amaya *et al.*, 2009). Furthermore, digestive enzymes gene expression can be modulated depending on diet composition, at least during late larval stages. For instance, dietary protein amount and nature modulates trypsin mRNA

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transcription and translation in European sea bass larvae (Péres *et al.*, 1998). Wang *et al.* (2006) also found that dietary protein level significantly affects trypsin mRNA level in Yellow catfish (*Pelteobagrus fulvidraco*) larvae. Digestive enzyme gene expression can be modulated even during the early larval development. Geurden *et al*. (2007) reported higher levels of α-amylase, maltase and glucokinase gene expression during the yolk-sac period of rainbow trout (*Oncorhynchus mykkis*) fed a hyperglucidic diet compared to a commercial diet. This indicates a very quick adaptation of this carnivorous species to the utilization of exogenous glucose and therefore could be suitable indicators of the larval nutritional condition.

The nutritional condition of a fish larva could also be indirectly determined. It is well known that nutrients can influence not only digestive system development, and hence survival and growth, but also skeletogenesis (Cahu *et al.*, 2003; Lall & Lewis-McCrea, 2007). Recent studies have demonstrated that the fish larval ossification degree is influenced by the diet and represents an adequate indicator of the larval quality. The ossification status has been shown to be correlated with osteocalcin gene expression (Mazurais *et al.*, 2008, Darias et al, 20010a). This gene is specifically localized in bone and constitutes the most specific marker for bone mineralization (Lian and Stein, 1995). Moreover, its expression level could be correlated with dietary levels of several nutrients, providing thus an eligible molecular marker for larval nutritional condition (Darias *et al.*, 2010b). From nutritional studies using molecular approaches (Villeneuve *et al.*, 2006, Mazurais *et al.*, 2008, 2009, Darias *et al.*, 2010b), other genes emerge as suitable markers for larval quality. For instance, TRPV-6 (Transient Receptor Potential cation channel-subfamily V, member 6) expression, which codes for the most important intestinal Ca^{2+} transporter, can be modulated by dietary vitamin D_3 levels, consequently affecting the intestinal maturation and, therefore, larval development (Darias *et al.*, 2010b). Low levels of vitamin mix has been shown to induce skeletal malformations correlated with the modulation of genes involved in osteoblast determination and differentiation such as BMP-4 (Bone Morphogenetic Protein 4), IGF-1 (Insulin Growth Factor-1) and PPARγ (Peroxisome Proliferator-Activated Receptor γ) (Mazurais *et al.*, 2008). Similarly,

inadequate dietary retinol levels alter morphogenesis through the modulation of Hoxd-9 (Homeobox protein Hox-D9) and RARγ (Retinoic acid Receptor γ) gene expression, provoking a variety of skeletal deformities (Villeneuve *et al.*, 2006; Mazurais *et al.*, 2009). Continuing in this line, genomic research technologies such as microarrays appear to be a useful tool not only for studying mechanistic to explain phenotypes but for exploratory interest, which is useful in the search for markers.

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