Histol Histopathol (2008) 23: 1525-1539

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Review

Nutritional cellular biomarkers in early life stages of fish

E. Gisbert[1], J.B. Ortiz-Delgado[2] and C. Sarasquete[2]

[1]Institut de Recerca i Tecnologia Agroalimentàries, IRTA Sant Carles de la Ràpita, Tarragona and

[2]Andalusian Institute of Marine Sciences, CSIC. Campus Universitario Rio San Pedro, Puerto Real, Cádiz, Spain

Summary. Histological (tissular and cellular) indices have a tradition of determining the nutritional condition of fish both in the laboratory and in the wild. The assessment of condition by means of microscopical methods is probably the mos accurate indicator of nutritional status during the early life stages of fish. This success is partly attributable large amount of information that can be derived from their study and because they are thought to be the only true starvation indices. The technique usually consists of the examination of cells and organs and the establishment of a grading system based on the presence/absence of standardised biomarkers. Each organ is examined, and the cellular aspect or tissular cohesion is evaluated qualitatively and even quantitatively in order to obtain a measure of the general condition of a larva. The literature indicates that there are certain tissular and cellular responses to food availability and quality, particularly in the digestive and muscular tissues, which are 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, the exocrine pancreas and the muscular fibers, 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.

Key words: Fish, Early life stages, Nutritional condition, Cellular biomarkers

Offprint requests to: Dr. Carmen Sarasquete, Andalusian Institute of Marine Sciences, CSIC, Campus Universitario Rio San Pedro, Apdo. Oficial 11510, Puerto Real, Cadiz, Spain. email: Carmen.sarasquete@icman.csic.es

Introduction

Teleost fish larvae are considered to be the smallest vertebrates on earth (Wieser, 1995). Generally, marine fish larvae hatch much earlier in their development than other vertebrates, suggesting that the spatial and temporal sequences of larval development in teleosts are quite different from those of higher vertebrates. Consequently, during the first weeks of life, marine fish larvae undergo significant morphological, structural, and physiological modifications to acquire all the juvenile/adult features by the end of the larval period and metamorphosis. Thus, the early life stages (ELSs) of fish represent a transitional ontogenetic period of simultaneous growth and differentiation, during which fish undergo the transition from endogenous to exogenous feeding, i.e. from yolk consumption to ingestion of external food. This transition implies profound anatomical and physiological alterations in different structures and systems of the organism, including the functional differentiation of the sensory organs, neuroendocrine and locomotory systems, and feeding apparatus. Whereas these processes are almost completed for most of the species at the onset of exogenous feeding, additional physiological changes occur during the larval period until metamorphosis of the juvenile fish, which include the reorganization of trunk musculature (differentiation in aerobic red and anaerobic white fibres), differentiation of gills (transition from cutaneous to branchial respiration) and skeletal tissue, development of a functional stomach and establishment of acidic digestion, and complete development of the excretory and thyroid systems (Sarasquete et al., 1993, 1995, 1996, 1998; Segner et al., 1994; Ribeiro et al., 1999; Ortiz-Delgado et al., 2003a,b; 2006; Elbal et al., 2004; Santamaria et al., 2004; Falk-Petersen, 2005; Sanchez-Amaya et al., 2006; Darias et al., 2007; Zambonino et al., 2008, among others).

According to the above-mentioned considerations, the ELSs may be considered the most critical stages of development from fertilization until metamorphosis, and

consequently, nutritional and environmental stress factors are of great relevance during these ontogenetic phases. Therefore, fish larvae allow us to study a wide range of developmental processes influenced by biotic, abiotic, and xenobiotic factors that are involved in the early stages of teleost development and affect growth performance, survival and recruitment.

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. After the onset of exogenous feeding, development and differentiation depend on the proper nutrient input provided by diet, in addition to optimal environmental conditions. In this sense, short periods of food deprivation after yolk resorption can result in abnormal behaviour and morphological development, degeneration of the alimentary tract and trunk musculature, and reductions in food utilization efficiency and feeding activity. Dietary imbalances during larval development might also have serious effects upon brain formation, neuroanatomical differentiation, and neuroendocrine action. During early ontogeny of fish species, larvae are especially sensitive to non-optimal feeding conditions or nutritional stressors, because most tissues and organs are under progressive and intense differentiation and development. For example, it has been suggested that fish respond to lack of food or an unsuitable diet by biliary dysfunctions, inducing mitochondrial alterations, depleted glycogen reserves, hepatic cholestasis, and general hepatocyte and pancreocyte degenerations (Yúfera et al., 1993, 1996, 2000; Diaz et al., 1997; Gisbert et al., 2004 a,b; Segner et al., 2004; Falk-Petersen, 2005; Fernández-Diaz et al., 2006), among other noticeable histopathological alterations in different target organs.

Histological assessment of condition is probably the most accurate indicator of nutritional status during ELSs (Ferron and Leggett, 1994). Histological observations can be made from several tissues from a single specimen, which respond at different rates to food deprivation and diets, enabling a more precise description of the nutritional state of an individual. Condition indices are usually divided into three different categories according to the main organisation levels: organismal, tissular, and cellular. These indices operate at different time scales. Typically, the higher the organisation level of the index, the longer it takes to respond to an environmental change for a given developmental stage, species, set of environmental conditions and specific nutritional status of the individual at the moment of study. Some indices can serve directly to estimate mortality (typically necrotic tissues, e.g. McFadzen et al., 1997), whereas others are a reflection of growth or immediate feeding status (e.g. some biochemical indices).

As Catalan (2003) reviewed, condition indices at the organismal level are typically studied through morphometrics for their integrative character (changes in cellular properties cause histological variations that may end up provoking shape changes). In fish larvae, morphometrics have been used to detect the effects of suboptimal feeding for many years (Theilacker, 1978). The use of these indices is based on the premise that, in response to food deficiency, some body features (e.g. body depth) are modified with respect to some less starvation-sensitive parts or organs (e.g. eye diameter or body length). Much of the success of these indices is attributable to their short processing time, low cost, and easiness of obtainability. The use of morphometric indices for assessing the nutritional condition of fish larvae have been criticised for having low sensitivity to short-term events (less than a week). Other criticisms are based on their sensitivity to shrinkage (due to both preservatives and sample collection), size and age dependence, the high differences between calibration data vs wild specimens, or high species specificity.

However, histological (tissular and cellular) indices have a tradition of determining condition both in the laboratory and in the wild (Theilacker, 1978; Oozeki et al., 1989; Theilacker and Watanabe, 1989; Ferron and Leggett, 1994; Bisbal and Bengston, 1995; McFadzen et al., 1997; Catalan and Olivar, 2002; Gisbert et al., 2002). The success of histological indices is partly attributable to the large amount of information that can be derived from their study and because they are thought to be the only true starvation indices (Suthers, 1998). The technique usually consists of the examination of cells and organs and the establishment of a grading system based on the presence/absence of standardised biomarkers. Each organ is examined, and the cellular aspect or tissular cohesion is evaluated in order to obtain a measure of the general condition of a larva. An important advantage of histological indices measured through multiple grading is that the general pattern of tissular degradation is relatively independent of size and, to an extent, species (Ferron and Leggett, 1994). Although biomarkers have been used for more than 30 years in pollution research, at present, biomarkers of effect, which are defined as measurable biochemical, physiological, cellular, or other alteration within tissues or body fluids of an organism that can be recognized as associated with an established or possible health impairment or disease (van der Oost et al., 2003), can be easily applied to assess the nutritional condition and health status of fish larvae from laboratory or field studies.

Finally, nutritional condition indices based on cellular biomarkers are conducted by means of biochemical (e.g. RNA/DNA) and some histological indices. From the reviewed literature, it seems that suboptimally fed young larvae soon deplete liver glycogen stores and endogenous lipids, and rapidly rely on catabolism of muscle proteins, which are the main energy source of starved young larvae (Catalan, 2003). As the larvae grow bigger, lipid and glycogen stores can cope with starvation for a longer time before muscle proteins are utilised. The establishment of biochemical

condition indices in specimens of unknown age (typically wild-collected), needs to relate the variation of a particular starvation-sensitive variable to a less-sensitive one.

In this review, special attention is given to the use of biomarkers of effect in order to evaluate the nutritional condition (non-optimal feeding protocol conditions, unbalanced diets, starvation…) in fish ELSs. For this purpose, we evaluate and discuss, in different early fish stages, the use of different tissular, cellular and molecular biomarkers and the presence of malformations and/or histopathological alterations in target organs, tissues, or cells derived from unsuitable nutritional conditions.

Nutritional stress biomarkers

In vertebrates, different segments of the gastrointestinal tract 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. The intestine is involved in important physiological digestive functions, being the primary site of food digestion and nutrient uptake, while the liver is the central metabolic organ of the body with a predominant role in the intermediary metabolism, with important functions in lipid storage and digestive and detoxification processes. The optimum utilization of dietary nutrients ultimately depends on the effectiveness of functions in the intestine and liver and, consequently, the structural alteration of the histomorphological organization of these organs can provide useful information about the quality of the diet, metabolism, and the nutritional status of the fish. Besides the above-mentioned target organs, several studies have satisfactorily used other tissues, such as the muscle and cartilage, as indicators of the nutritional condition of starved fish larvae (Catalan and Olivar, 2002).

Cellular and molecular approaches may serve as good biomarkers of effect to analyze different physiological and/or pathological responses to some nutritional factor deficiencies (e.g. unbalanced diets), as well as to extended periods of food deprivation in fish during ELSs. The information obtained might be of great value to assess the nutritional condition of fish larvae. The ability to distinguish starving from feeding fish larvae is useful in studies of both natural (e.g. recruitment studies) and cultured populations. Histological indices have the disadvantage of their large dependence on experimental rearing and dietary conditions, with the 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 in laboratory conditions (Catalan and Olivar, 2002). In particular, studies on the occurrence or frequency of starved larvae in either field populations or aquaculture operations require detailed experimental investigation, in which specific starvation indicators and biomarkers are described, standardised, and validated for fishes of known nutritional history (Bisbal and Bengtson, 1995).

Many methods have been described to characterize the nutritional condition of fish larvae. The physical deterioration of larvae resulting from experimentally induced food deprivation has been described and interpreted by means of morphometric and gravimetric methods (Hempel and Blaxter, 1963; Ehrlich et al., 1976; Powell and Chester, 1985; Bisbal and Bengtson, 1995; Mookerji and Rao, 1999), biochemical methods (RNA/DNA ratios, Robinson and Ware, 1988; Clemmensen, 1993; Suneetha et al., 1999; Gwak and Tanaka, 2001), histological criteria (Watanabe, 1985; Theilacker, 1986; Theilacker and Watanabe, 1989; Margulies, 1993; Yúfera et al., 1993, 1996; Navarro and Sarasquete, 1998; Green and McCormick, 1999), or various combinations (Bisbal and Bengtson, 1995; Gwak et al., 1999; Gisbert et al., 2004a,b). Since fasting-induced changes in body morphometrics are species-specific, there is not a universal morphometric index to evaluate the nutritional status of fish larvae. Consequently, the optimal experimental design for studies on starvation might use several morphological criteria combined with a smaller sub-sample of fish which should be evaluated histologically (Theilacker, 1986).

Regarding histological criteria, the liver, the exocrine pancreas, the intestine, the muscular fibers and cartilaginous tissue organization have been used on a regular basis as histological targets to analyse the nutritional condition of fish larvae and elucidate the effects of different dietary regimes or nutrients on larval physiology, nutrition and early development. Their general use arises from the fact that during ontogeny fish ELSs are especially sensitive to non-optimal feeding conditions or nutritional stress. This is because most tissues and organs are under progressive development, and they respond rapidly and sensitively to nutritional disorders.

Accessory digestive organs: Liver and pancreas

The liver is of significant importance for the nutrition and homeostasis of fish. The early development and functionality of this accessory gland supports its vital role in developing fish. This organ is the central metabolic organ of the body, with a predominant role in the intermediary metabolism, and important functions in lipid and glycogen storage and digestive and detoxification processes. The histomorphological organization of the liver accurately reflects any physiological disorder originated from a nutritionally unbalanced diet or feed deprivation. Observed dietary effects on the liver may be seen as intra- or extracellular structural changes, of which resorption of glycogen and lipids and changes in mitochondria appearance are the earliest signs of change (Hoehne-Reitan and Kjørsvik,

2004).

The basic mechanisms of organ development are similar in all teleosts, while there are considerable differences regarding the relative timing of their differentiation (see review in Hoehne-Reitan and Kjørsvik, 2004). The macroscopical organization of the fish liver in two or more distinct lobes, depending on the species, is achieved at the end of larval metamorphosis and during early juvenile stages. One criterion of a functional liver is the ability to synthesize, store, and mobilize carbohydrates and lipids. The contribution of the liver to the storage and mobilization of the above-mentioned nutrients during early life stages has only been studied in detail in a few species. During the lecitotrophic stage, the liver of gilthead seabream (*Sparus aurata*) larvae mainly accumulates glycogen, while during the transition from endogenous to exogenous feeding this glycogen is reabsorbed, being reconstituted after the depletion of yolk sac reserves. In other species, such as Atlantic cod (*Gadus morhua*) and Atlantic halibut (*Hippoglossus hippoglossus*), glycogen deposition in the liver is not apparent during the lecitotrophic stage, although a significant increase is observed after yolk sac absorption. Differences between species in hepatic glycogen content during the endogenous feeding stage might possibly be related to species-specific differences in larval energy metabolism and differential yolk lipid content at this stage (Hoehne-Reitan and Kjørsvik, 2004).

After the onset of exogenous feeding, the storage of reserves in the hepatocytes increases the vacuolization level of their cytoplasm. Glycogen (eosinophilic PAS-positive and Diastase-PAS negative granules) and neutral lipids (colourless, PAS-negative and Sudan black-positive vacuoles) are stored in the hepatocytes, while proteins are more evident in the hepatic vascular system. The position of the nucleus in the hepatocyte cytoplasm depends on the degree of accumulation of nutrient reserves. 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 (Deplano et al., 1991; Sarasquete et al., 1995; Ortiz-Delgado et al., 2003a,b; Gisbert et al., 2005; Sanchez-Amaya et al., 2006; Darias et al., 2007; Zambonino et al., 2008).

Hepatic energy stores respond sensitively to nutritional changes in the deficient diets (Segner et al., 1994). Under food deprivation conditions, liver glycogen and lipids are the first energy sources to be mobilized (Fig. 1). The mobilization of these nutrients under continued fasting conditions results in the reduction of energy available to larvae (Green and McCormick, 1999). Thus, once yolk sac and oil globule reserves are exhausted, the histological organization of different regions of the digestive tract and accessory organs progressively deteriorates as a consequence of food deprivation. Histopathological changes in food-deprived larvae are similar amongst different species and include

Table 1. Cellular criteria used to grade tissues and assess the nutritional condition in teleost larvae.

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 hepatocites 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.
Midgut epithelium	Mucosal cell height reduced by >50% in heigth; 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.
Trunk musculature	Pronounced fiber separations; intermuscular tissue dark, glassy and reduced by >50%; nuclei dark and indistinct.	Muscle fibers slightly separated; myofibrils with some loss of striation and separation; intermuscular tissue reduced by 25 to 50%; nuclear staining variable, often indistinct.	Muscle fibers compact; myofibrils striated and compact; abundant intermuscular tissue; distinct intermuscular nuclei.
Cartilage	Condensed nuclei; cytoplasm often absent or severely reduced; chondrocytes shrunk.	Some nuclei condensed at several degrees; cytoplasm frequently reduced, occupying only a small portion of the capsular space; chondrocytes moderately shrunk.	Large nuclei; cytoplasm occupying most of the capsular space.

Data rewritten from Margulies (1993), Catalan (2003) and Gisbert et al. (2002b).

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 (Margulies, 1993; Yúfera et al., 1993, 1996; Diaz et al., 1998; Green and McCormick, 1999; Crespo et al., 2001; Gisbert and Doroshov, 2003, 2004b; Carriquiriborde et al., 2007). In general, starvation results in a linear change of some histological and histochemical parameters, i.e. cell size and volume, hepatic glycogen and lipid content, whereas other biochemical, morphometric, and gravimetric parameters, such as the condition factor, hepatosomatic index, hepatic protein content, RNA/DNA, and the activity of gluconeogenic and glycogenolytic enzymes, revealed a non-linear response under starving conditions. This fact suggests the existence of two phases in the metabolic adaptation of starved fish larvae: a first phase of metabolic disturbance, followed by a second phase, the establishment of a new homeostasis for maintaining liver metabolic integrity (Moon and Johnston, 1980).

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 (Hoehne-Reitan and Kjørsvik, 2004). In well-fed larvae, glycogen and lipids tend to accumulate in the liver at varying degrees. The liver volume generally increases, and the rough endoplasmic reticulum and Golgi apparatus increase as the larva develops. In contrast, in larvae fed unbalanced diets, the above-mentioned cellular organelles are generally poorly developed (Segner et al., 1993). Problems associated with dietary lipids seem to be among the most serious issues in adult and fish larvae nutrition (Hoehne-Reitan and Kjørsvik, 2004), especially those related to a deficiency of the highly unsaturated essential fatty acids (HUFA), in particular of the n-3 and n-6 series, or an imbalance between them (Fontagné et al., 1998; Izquierdo et al., 2000; Sargent et al., 1999, 2002; Morais et al., 2004; Gisbert et al., 2005). Microscopically, the structural modifications of the hepatocytes might be useful biomarkers of effect to reflect a nutritional pathology. The hepatic vacuolization (neutral lipids) observed in fish larvae fed with Artemia nauplii has been indicative of adequate nutrient absorption and lipid metabolism (Segner et al., 1994; Sarasquete al., 1995; Gisbert et al., 2004a). However, 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 (Segner and Braunbeck, 1988; Strüssmann and Takashima, 1990; Caballero et al., 1999; Mobin et al., 2000). Dietary fatty acid deficiency is also linked to a swollen, pale liver with a severe lipid infiltration of the hepatocytes. In gilthead seabream larvae, a deficiency of n-3 HUFA results in increased lipid content in the liver, while in African catfish (*Clarias gariepinus*) larvae fed live prey with low n-3 HUFA levels exhibited a higher glycogen content and less lipids in the liver than those fed high n-3 HUFA levels (Hoehne-Reitan and Kjørsvik, 2004). Besides the level of dietary HUFA, the form of the HUFA supply (phospholipids or neutral lipids) also has a direct effect on fat storage in the liver and its histomorphological appearance (Gisbert et al., 2005). Thus, European sea bass larvae (*Dicentrarchus labrax*) fed compound diets containing high levels of n-3 HUFA in the neutral lipid fraction showed higher levels of lipid accumulation in the liver than those fed similar or even higher levels of n-3 HUFA in the form of phospholipids (Fig. 2). 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 with unbalanced diets (Segner et al., 1994; Mobin et al., 2000; Sarasquete and Gutiérrez, 2005).

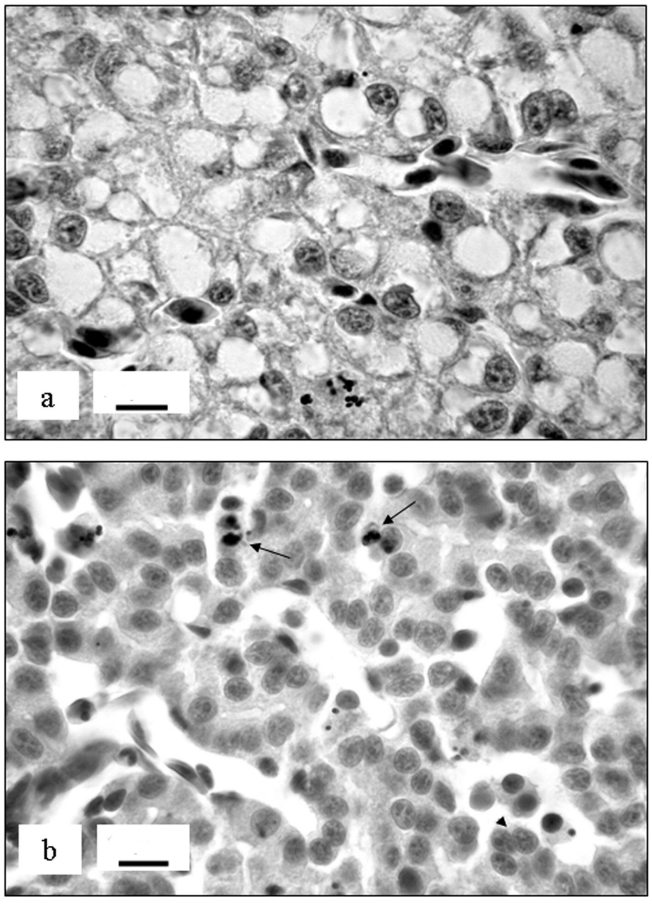


Fig. 1. a. Liver of a feeding green sturgeon (*Acipenser medirostris*) larva at 25 days post hatching, with polygonal hepatocytes arranged along the sinusoids and containing large lipid vacuoles. **b.** Liver of a green sturgeon larva starved for 12 days (27 days post hatching); note the absence of cytoplasmatic lipidic inclusions, large intercellular spaces, collapsed cytoplasm (arrow head) and pycnotic nuclei of hepatocytes (arrows). Staining technique: Haematoxylin-eosin. Scale bar: 30 µm.

A functional exocrine pancreas is characterized by differentiated organ morphology (acinar gland), including developed excretory ducts and the presence of acidophilic zymogen granules (pancreatic enzyme precursors) (see Hoehne-Reitan and Kjørsvik, 2004 for a detailed description of the organogenesis). Histological techniques, as well as immunohistochemical and molecular procedures employed to detect pancreatic enzymes or their precursors, have revealed that the larval exocrine pancreas appears histologically differentiated and functional at hatching or mouth opening, depending on the species. This early differentiation and morphogenesis of the exocrine pancreas facilitates its use at a cytological and biochemical level as a good biomarker of the nutritional condition of the larva. Food deprivation induces degeneration of the exocrine pancreas (Yúfera et al., 1993; Crespo et al., 2001). 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 content and activity of pancreatic enzymes change with the food substrate. This alteration is also valid for fish larvae, but variations in timing during development exist

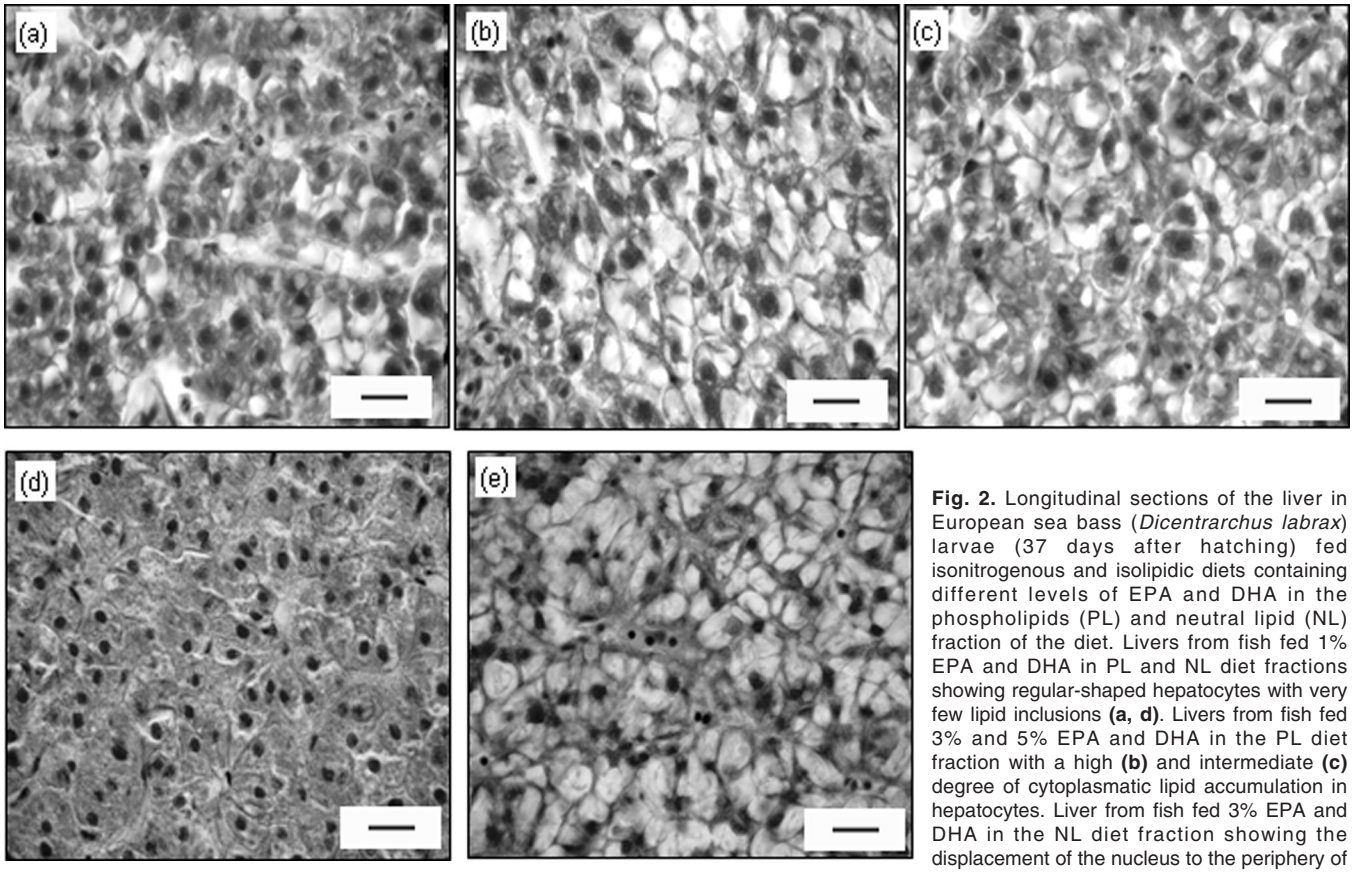
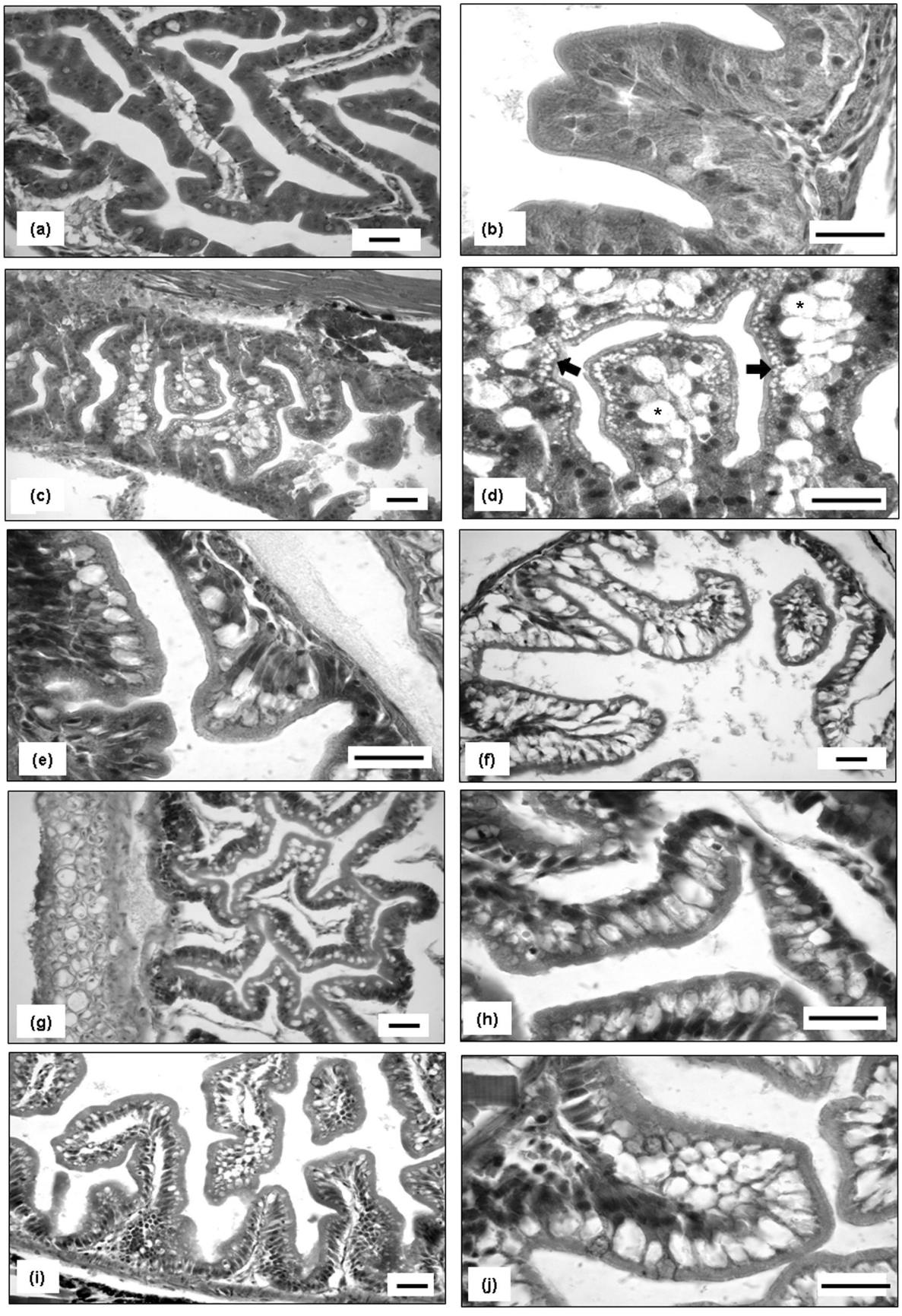


Fig. 2. Longitudinal sections of the liver in European sea bass (*Dicentrarchus labrax*) larvae (37 days after hatching) fed isonitrogenous and isolipidic diets containing different levels of EPA and DHA in the phospholipids (PL) and neutral lipid (NL) fraction of the diet. Livers from fish fed 1% EPA and DHA in PL and NL diet fractions showing regular-shaped hepatocytes with very few lipid inclusions **(a, d)**. Livers from fish fed 3% and 5% EPA and DHA in the PL diet fraction with a high **(b)** and intermediate **(c)** degree of cytoplasmatic lipid accumulation in hepatocytes. Liver from fish fed 3% EPA and DHA in the NL diet fraction showing the displacement of the nucleus to the periphery of the hepatocytes and the presence of numerous varying-size cytoplasmatic lipid vacuoles **(e)**. Haematoxylin-eosin. Scale bar: 25 µm.

Fig. 3. Longitudinal paraffin sections of the postvalvular intestine in 37 days post hatching European sea bass (Dicentrarchus labtrax) larvae fed diets containing different levels of EPA and DHA in the phospholipids (PL) and neutral lipid (NL) fraction of the diet. **a, b.** Intestinal mucosa of a larva fed 1% EPA and DHA in the PL fraction of the diet showing the mucosa lined by a simple columnar epithelium with centrally located nuclei and not containing lipid accumulations in cytoplasmatic vacuoles. **c, d.** Intestinal mucosa of larvae fed 3% EPA and DHA in PL fraction of the diet showing the presence of small apical lipid droplets (arrow), the displacement of nuclei to a basal position, and large intercellular lipid vacuoles between enterocytes (asterisk). Postvalvular intestine of larvae showing different signs of intestinal steatosis depending on the level of EPA and DHA in the phospholipids (PL) and neutral lipid (NL) fraction of the diet [1% PL (e), 3% PL **(f),** 5% PL **(g, h)**, 1% NL **(i)**, 3% **(j)**. Note the lower accumulation of lipids in the enterocytes of fish 3% EFA and DHA in the NL fraction of the diet in comparison to those fed the same quantity of EPA and DHA but in the PL fraction of the diet. Haematoxylin-eosin. Scale bar: 100 µm.


(a)
(b)
(c)
(d)
*
*
(e)
(f)
(g)
(h)
(i)
(j)

for the different pancreatic enzymes, and species-specific differences can also be found, which can be partially related to natural feeding habits (Hoehne-Reitan and Kjørsvik, 2004).

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, revealing the coexistence of chemical and neural mechanisms controlling secretion in larvae. Under feed deprivation conditions, Gwak et al. (1999) reported a decline of trypsin and amylase activities to very low levels in Japanese flounder (*P. olivaceus*), which was associated with a reduction of pancreatic volume and partial necrosis of the exocrine pancreas. In other species, such as California halibut (*Paralichthys californicus)* (Gisbert et al., 2004b) or gilthead seabream (Yúfera et al., 1993), while the exocrine pancreas degenerated due to starvation, zymogen granules were still present in starved larvae. Mobin et al. (2000) reported in Japanese flounder (*Paralichthys olivaceus*) a feeding-level-dependent atrophy of the pancreatic acinar cells, which might stem from the hyperfunctioning of the pancreas as a result of digesting an excessive quantity of food.

Intestine

The intestine is the longest portion of the digestive tract and one of the first digestive organs to differentiate. Three different regions can be distinguished along the intestine according to their histological organization. The antero-median segment (pre-valvular intestine), which receives the pancreatic and biliary secretions, is histologically characterized by a columnar epithelium with prominent microvilli composed of a high number of goblet cells, especially abundant close to the pyloric sphincter. This region of the intestine is the main site for lipid absorption (Diaz et al., 1997; Olsen et al., 2000), while proteins are absorbed in the posterior intestine (Deplano et al., 1991). However, other studies have reported that lipid digestion and absorption continue in the posterior and rectal regions of the intestine (García-Hernández et al., 2001; Gisbert et al., 2005). The post-valvular intestine is histologically similar to the anterior-median region except for the number and size of mucosal folds, which are longer, deeper and more numerous in the prevalvular intestine. The intestine terminates in a short rectal zone that, depending on the species, can be either lined by a simple or columnar epithelium with few goblet cells or by a cuboidal epithelium.

The development of a functional intestine involves different maturational and morphological events that are very well conserved among vertebrates (Henning et al., 1994). From a cytological point of view, the presence of lipid inclusions in the enterocytes of fish is a common phenomenon in fish larvae and juveniles. Their size and ultrastructural characteristics are valuable biomarkers to evaluate lipid absorption and metabolism during ELSs. 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). Changes in the size and type of lipid

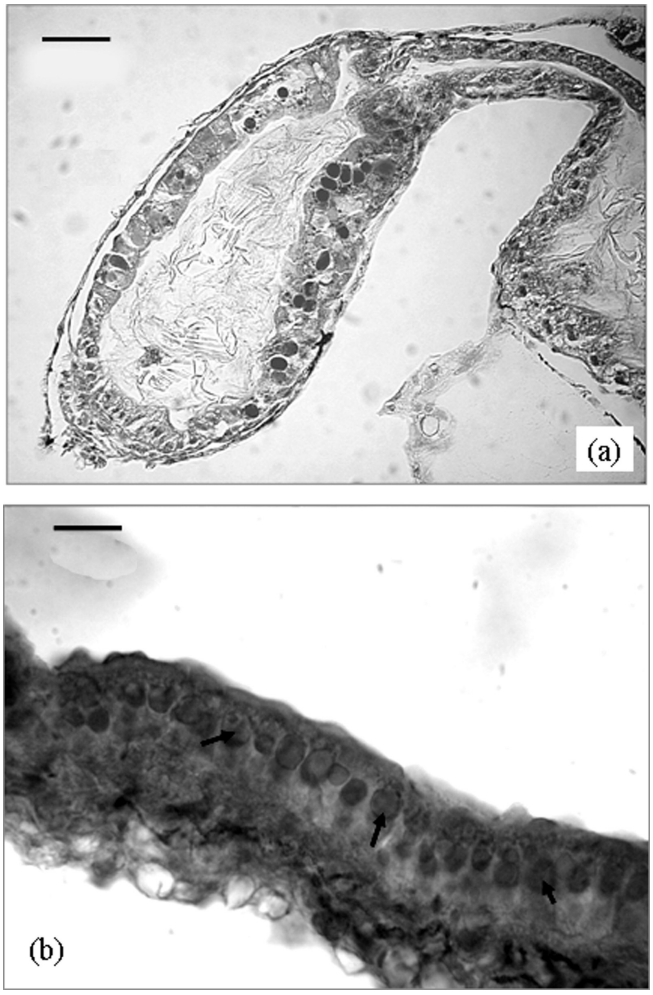


Fig 4. Posterior (post-valvular) intestine with the epithelium filled with acidophilic supranuclear bodies (dark cytoplasmatic inclusions) in a 3-day-old larva of California halibut (*Paralichthys californicus*) **(a);** Scale bar: 100 µm. Detail of the post-valvular intestine epithelium in a 10-day-old California halibut larva. Note the presence supranuclear inclusions (arrow) occupying most of the cytoplasmatic space in the enterocytes **(b)**. Haematoxylin-eosin. Scale bar: 30 µm.

inclusions may be dietary-dependent. Thus, the size of lipoprotein particles increases with the fat content of feed and the degree of unsaturation of the lipids ingested.

In fish larvae, lipid droplets are not considered part of the endoplasmic reticulum or the Golgi apparatus of the enterocytes, and in many cases these inclusions do not appear to be enclosed by any membrane. This has led to the suggestion that intestinal lipid inclusions are a temporary storage form of re-esterified fatty acids in cases when the rate of lipid absorption exceeds the rate of lipoprotein synthesis (Sheridan, 1988), or because of an inability to metabolize lipids (Kjørsvik et al., 1991). Under normal conditions, the rapid development of the intestinal enterocytes during larval growth is combined with increasingly effective lipoprotein synthesis, which is accompanied by a considerable decrease in the number of large lipid vacuoles in the enterocytes, as well as an important increase in the number of small lipid particles in the intercellular spaces (Deplano et al., 1991; Sarasquete et al., 1995). However, the excessive abundance of lipid inclusions of varying size in the enterocytes could be the result of a failure in the lipoprotein synthesis mechanism. This phenomenon may be a result of the immaturity of the enterocytes, as has been suggested for mammals (Snipes, 1977).

The formation of large lipoproteins and lipid droplets is closely related to an excess of fats in immature enterocytes caused by the high fatty acid contents of live preys and compound diets. In some cases, large accumulation of lipids in the enterocytes, the so-called intestinal steatosis, 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; Mobin et al., 2000). Differences in

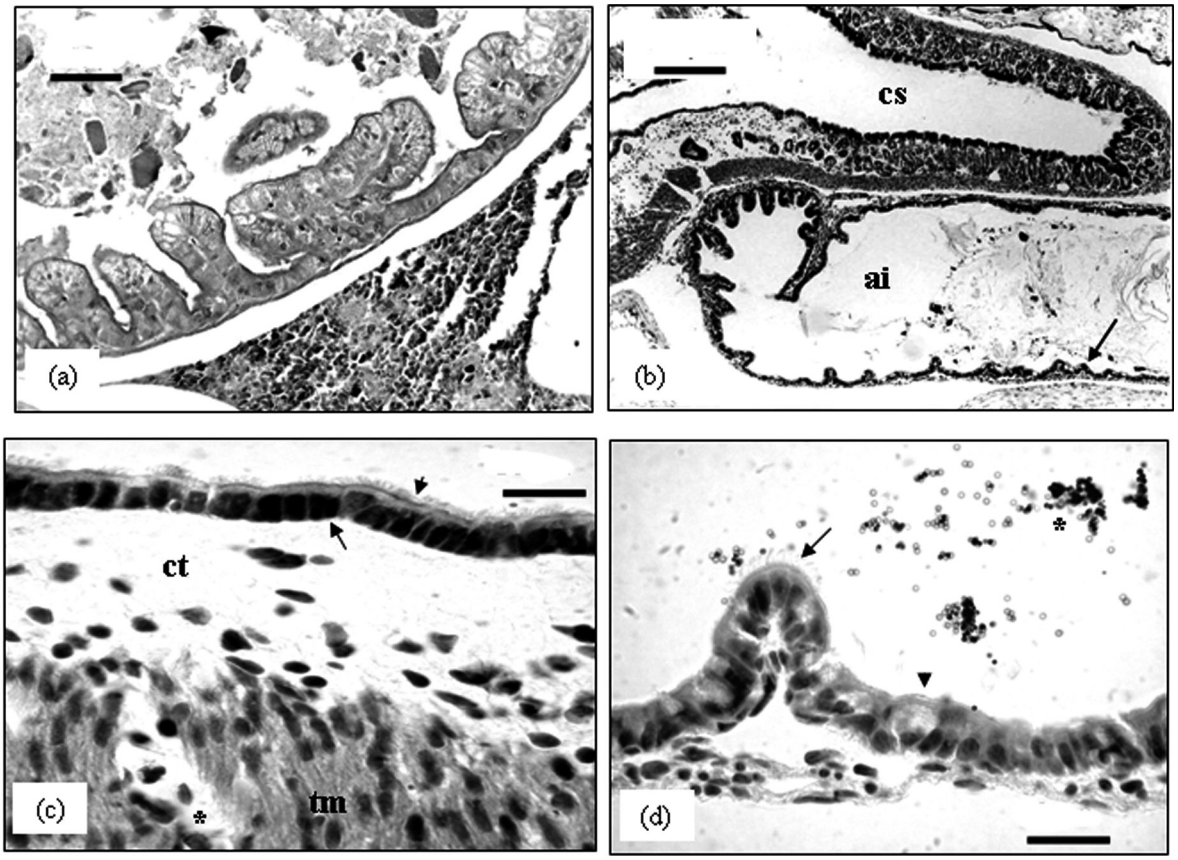


Fig. 5. a. Detail of the anterior intestine mucosa of a green sturgeon (*A. medirostris*) larva fasted for 13 days (28 dph); note the presence of goblet cells (arrow head), the disarrangement of the enterocytes' brush border (arrow) and the presence of melanin granules (asterisk). **b.** Spiral valve of a green sturgeon larva starved for 12 days (27 dph); note the presence of the melanin plug (mp), the thinning of the mucosa and reduction in folding. **c.** Detail of the pyloric stomach of a green sturgeon larva starved for 12 days (27 days post hatching, dph) showing the enterocytes with a collapsed cytoplasm (arrow) and disarrangement of their brush border (arrow head). Note the onset of separation of muscle fibres (asterisk) in the tunica muscularis (tm) and the lax connective tissue (ct). **d.** Detail of the anterior intestine mucosa of a green sturgeon larva fasted for 13 d (28 dph); note the presence of goblet cells (arrow head), the disarrangement of the enterocytes' brush border (arrow) and the presence of melanin granules (asterisk) resulting from the digestion of the yolk reserves. Haematoxylin-eosin. Scale bars: a, 50 µm; b, 100 µm; c, d, 30 µm.

lipid absorption and accumulation in different regions of the intestinal mucosa are also influenced by dietary lipid classes, their levels, and the n-6/n-3 ratio, as has been recently reported in European sea bass larvae (Gisbert et al., 2005). Larvae fed high levels of neutral lipids (11%) showed important intracellular and intercellular accumulation of lipids in the anterior intestine, while the anterior intestinal mucosa of fish fed low and moderate levels of phospholipids (13-11%) and neutral lipids (3-6%) had a normal appearance and organization. Similarly, lipid deposition in the posterior intestine depended on the dietary lipid class (Fig. 3), since larvae fed different levels of phospholipids showed large lipid deposits, whereas fish fed triglycerides had a lower lipid accumulation in this region. This result probably revealed a specialization of the posterior intestine in the absorption and transport of phospholipids (Gisbert et al., 2005).

Besides lipids, protein inclusions may also be used as a biomarker in fish larval nutrition and digestive physiology studies. During the transition from endogenous to exogenous feeding, the post-valvular intestine has a basic nutritional role assuring protein absorption in macromolecular form, but it is also involved in the assimilation of simple molecules. Thus, the presence of acidophilic supranuclear inclusions (so-called supranuclear bodies) is a typical feature (Fig. 4) of the posterior intestine of fish larvae during ELSs (Govoni et al., 1986; Kjørsvik et al., 1991; Sarasquete et al., 1993, 1995; Ribeiro et al., 1999; Hamlin et al., 2000; Santamaría et al., 2004; Sanchez-Amaya et al., 2006; Darias et al., 2007). Electron microscopy studies revealed the presence of a scarcely developed apical tubulo-vesicular system with medium or high electron-dense content and several vacuoles in the enterocytes, which indicates a reduced capacity for absorption by pinocytosis. In parallel, histochemical methods show that the acid phosphatase activity, characteristic of lysosomal systems, is low, suggesting that the capacity for intracellular digestion is also low. After the transition

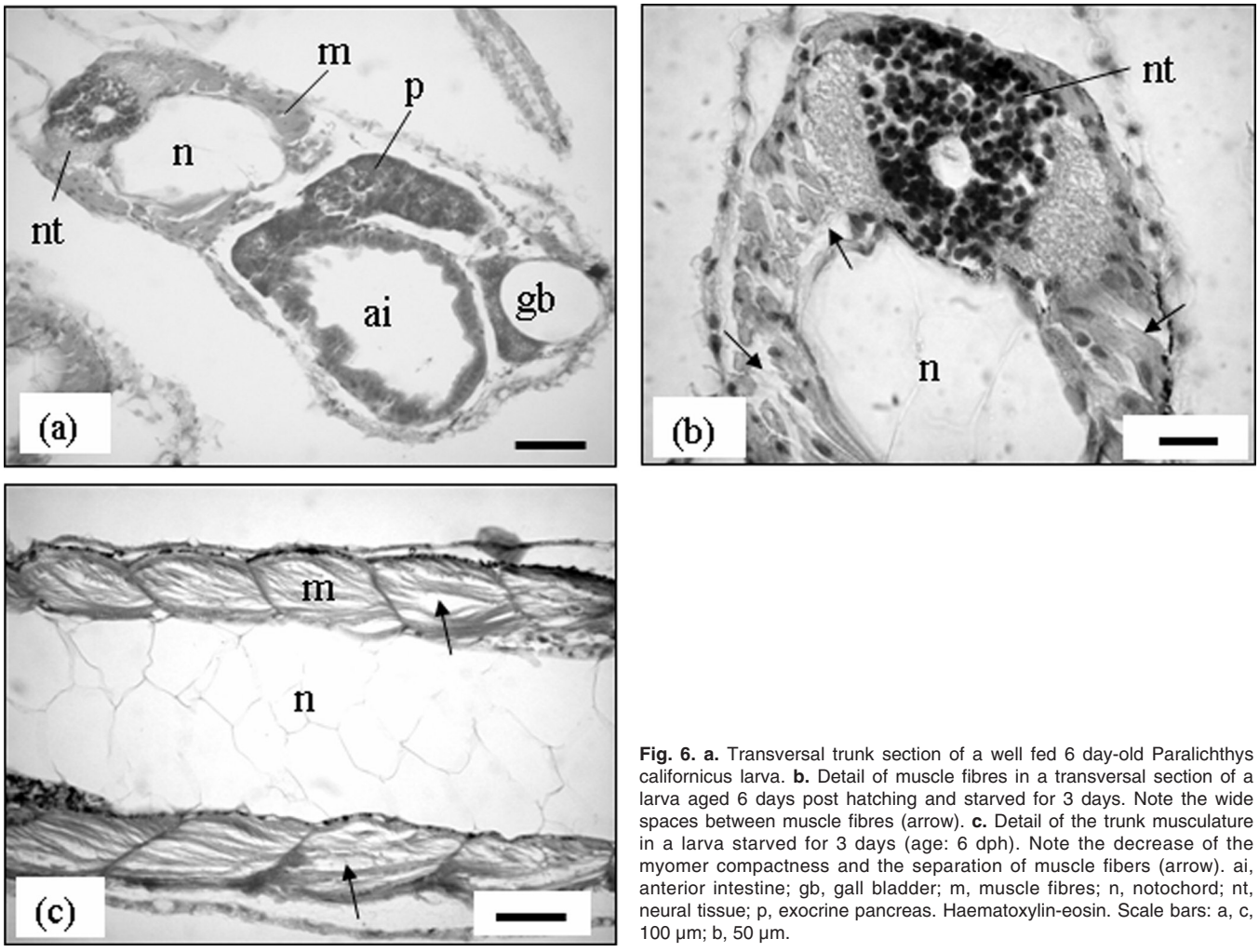


Fig. 6. a. Transversal trunk section of a well fed 6 day-old Paralichthys californicus larva. **b.** Detail of muscle fibres in a transversal section of a larva aged 6 days post hatching and starved for 3 days. Note the wide spaces between muscle fibres (arrow). **c.** Detail of the trunk musculature in a larva starved for 3 days (age: 6 dph). Note the decrease of the myomer compactness and the separation of muscle fibers (arrow). ai, anterior intestine; gb, gall bladder; m, muscle fibres; n, notochord; nt, neural tissue; p, exocrine pancreas. Haematoxylin-eosin. Scale bars: a, c, 100 µm; b, 50 µm.

to exogenous feeding, enterocyte maturation continues, and their tubulo-vesicular system greatly develops, increasing their capacity for protein absorption, which appears to be much greater than in adults (Deplano et al., 1991; García-Hernández et al., 2001). These inclusions point to the absorption of protein macromolecules by pinocytosis as an alternative pathway until the development of the stomach and acid digestion takes place. In most studied species, supranuclear bodies are observed throughout the larval period, although their number and size decreased as the stomach differentiated and extracellular digestion took place. The reduction of supranuclear bodies in the post-valvular intestine after the acquisition of the gastric function may result from a change in protein digestion mechanisms, as the secretion of hydrochloric acid and enzymes produced in the gastric glands may reduce pinocytotic activity and intracellular digestion by cytosolic enzymes, leading to extracellular digestion processes (Cahu and Zambonino-Infante, 2001). Changes in the 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 ELSs.

The histological organization of the intestine, like that of the liver, is particularly sensitive to food deprivation and starvation (Fig. 5). Major alterations include the reduction in the height of the enterocytes and the number and size of folds of the intestinal mucosa (Ooezeki et al., 1989; Bisbal and Bengtson, 1995; Theilacker and Porter, 1995; Gwak et al., 1999; Gisbert et al., 2004b; Rodríguez et al., 2005). These changes seem to be a consequence of modifications to cell protein synthesis through the regulation of ribosomal biogenesis (Carriquiriborde et al., 2007). Indeed, proteolysis of the intestinal mucosa is a common response to starvation; thus, enterocyte degeneration involves a reduction of the nutrient absorption surface area, compromising the digestive capabilities of re-feeding larvae and directly affecting their growth and survival. For these reasons, the criterion of enterocyte height has been widely used as a valuable biomarker of sub-optimal feeding or starvation in several fish species (Theilacker and Watanabe, 1989; Theilacker and Porter, 1995; Bisbal and Bengtson, 1995; Theilacker et al., 1996; Green and McCormick, 1999; Gisbert et al., 2004b). However, Catalan and Olivar (2002) reported that hindgut cell heights in *D. labrax* larvae were less useful to distinguish different feeding treatments than other quantititative measurements (e.g. hepatocyte maximum diameter, muscle fiber separation). Consequently, for any selected species, any current or putative nutritional condition index or biomarker should be previously tested and validated under laboratory-controlled conditions.

Marine fish larvae exhibit specific digestive features during the first weeks after hatching. Some metabolic pathways in fish larvae are different from those of juvenile or adult fish. These differences lessen throughout the larval period with the maturation of some tissues and organs, in particular with the onset, decrease or increase in activity of a wide range of digestive enzymes (Zambonino-Infante and Cahu, 2007). Although the use of digestive enzyme activities to assess the nutritional condition in fish ELSs is not the objective of the present review, measurements on metabolic enzyme activities are a valuable biochemical and metabolic biomarker in nutritional and ecological studies (see reviews in Cahu and Zambonino-Infante, 2001; Dahlhoff, 2004; Zambonino-Infante and Cahu, 2007). In particular, as in the pancreas, the activity of several intestinal enzymes may be used as an indicator of the stage of development of the specimen or its nutritional condition, induced by either dietary conditions or feed deprivation. Thus, the onset of digestion at the brush border membrane of the enterocytes, concurrent with the decline of cytosolic digestion, is a crucial ontogenetic event in larval digestion, and it is currently used as an indicator of the functional maturation of the digestive tract in larvae (Zambonino-Infante et al., 1997; Zambonino-Infante and Cahu, 1999).

Muscular tissue

The teleost myotome is composed of two main fiber types, which in many species are arranged into anatomically discrete regions. The bulk of the muscle mass is made up of white fibers, while the remainder, consisting of aerobic red fibers, forms a thin strip lying just underneath the lateral line. These muscle types differ not only in morphology and ultrastructure, but also in metabolism (Johnston, 1999). In this sense, the particular metabolic strategy employed by the muscle as a response to starvation appears to be related, in part, to the amount and sites of storage of body lipids. Thus, "fatty fish", such as herring (*Clupea harengus*) and mackerel (*Scomber scombrus)*, have high concentrations of lipid in the muscle tissue. In these species, there is little protein breakdown even after a long period of starvation. In contrast, species with less extensive lipid reserves (the so-called "non-fatty fish" such as Gadidae), where the principal source of stored lipid is in the liver, tend to maintain carbohydrate stores at the expense of peripheral protein by means of active gluconeogenesis (Johnston and Goldspink, 1973; Moon and Johnston, 1980).

Muscle cellularity shows considerable plasticity with respect to exercise, feeding, and environmental factors, although the responses observed vary with developmental stage and species (Johnston, 1999). Some characteristics of muscle cellularity and muscle fiber growth patterns have been pointed out as possible indicators of the nutritional condition of specimens. In particular, during severe starvation in American plaice (*Hippoglossoides platessoides*) and plaice (*Pleuronectes platessa*), a major part of white muscle contractile protein becomes utilised, resulting in water contents in excess of 95%. Degradation of myofibrillar proteins

occurs to a greater extent in white than in red muscle fibres (Johnston and Goldspink, 1973; Johnston, 1981). The mechanism of protein degradation would appear to involve a uniform decrease in myofibril diameter, rather than the selective destruction of complete myofibrils. Ultrastructurally, changes in severely atrophied muscle fibers due to starvation included an increased number of lysosomes, a reduction in myofibrillar diameter and in euchromatin material in the nuclei of both red and white fibres, swollen terminal cysternae and alterations in the structure of Z-discs and M-lines (Beardall and Johnston, 1983; Johnston, 1981). From a microscopical point of view, the trunk musculature in fish larvae is striated, closely packed, and composed of abundant interfibrillar substances and parallel myofibrils over the lateral surfaces of the notochord. However, in starved fish larvae, myomere compactness progressively decreases, and muscle fibers appear slightlly or largely separated, depending on the degree of starvation (Margulies, 1993; Bisbal and Bengston, 1995; Green and McCormick, 1999; Gisbert et al., 2004b). In this sense, Catalan and Olivar (2002) suggested using the level of muscle fiber separation (MFS) as a biomarker to assess the state of malnourishement of fish larvae. According to these authors, MFS should be measured on the central part of the trunk, in sections where myotomes and notochord would be clearly visible. Due to the variability of the parameter for microscopical areas comprising less than three myomeres, those authors recommended analysing regions of trunk musculature comprising a minimum of three myotomes. Laboratory studies have allowed correlating severe muscle degradation with the maximum observed larval mortality rate, which is important when it comes to the interpretation of the different indices for survival. In short-term starvation experiments, the muscle responded as fast as the liver to food deprivation, which indicated its reliance as a target tissue for assessing the nutritional condition of fish larvae.

Histological grading system for assessing the nutritional condition of ELSs

Histological analysis of body tissues has been used to assess the nutritional condtion of larvae and juveniles. As previously described, the literature indicates that there are certain tissular and cellular responses to food availability, particularly in the digestive and muscular tissues, which are common to most teleost fish larvae. These responses, which are independent of water temperature, can be used for assesing the nutritional condition of fish larvae (Watanabe, 1985; Theilacker, 1986; Margulies, 1993; Green and McCormick, 1999; Catalan and Olivar, 2002). These methods require the standardisation of the nutritional condition of fish larvae under laboratory conditions that allow the correlation of certain cellular criteria to a well known and described nutritional condition of fish larvae (e.g. days without feed) and to the mortality rate. Specifically, each organ is examined, and the cellular aspect or tissular cohesion is evaluated in order to obtain a measurement of the general condition of a larva. A crucial advantage of histological indices measured through multiple grading is that the general pattern of tissular degradation is relatively independent of size and, to an extent, of the species (Ferron and Leggett 1994; Catalan, 2003).

In practice, each tissue is assigned a grade from 3 (healthy) to 1 (degraded) based on a variety of cellular criteria from different target tissues and organs, which may change according to the study, authors, and age of larvae (Margulies, 1993). In some other cases, due to the increase in complexity of the target tissues in juveniles in comparison to that in smaller larvae and to the higher variability in cellular responses to starvation, the above-mentioned grading is assigned from 5 (healthy) to 1 (degraded). Considering some of the previously mentioned histological alterations, several authors have developed a quantitative scoring system to describe the nutritional condition of fish larvae (Tabe 1). In addition, the general condition of fish larvae has to be assessed considering the grading values assigned for each selected histological criteria. In this sense, multivariate analyses allow the discrimination of which of the target tissues and/or organs better defines the nutritional condition of larvae (Green and McCormick, 1999; Catalan and Olivar, 2002; Gisbert et al., 2004b). However, the literature review indicated that there is not a universal histological criterion to assess the nutritional condition of fish larvae. For instance, MFS index was the variable that best discriminated the nutritional condition of European seabass larvae (Catalan and Olivar, 2002), while according to Green and McCormik (1999) and Gisbert et al. (2004b), the height of enterocytes in the intestinal mucosa was the most appropriate index for the examination of the importance of starvation in *Amphiprion melanopus* and *P. californicus* larvae. In all cases, the best performance of each of the histological criteria was reflected in the percentage of correct classification of individuals to their age-feeding group (stepwise canonical discrimination analysis) and in the speed of response to food deprivation or recovery from fast.

As Catalan (2003) extensively reviewed, the use of histological methods in the determination of larval nutritional condition has at least two unresolved problems. One regards the low objectivity of some methods, since the measures are mainly qualitative. Indeed, a large number of studies used qualitative or semi-qualitative measurements and relied on the experience of the histologist (McFadzen et al., 1997). To date, quantitative data have been restricted to the measurement of cell heights of 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. Thus, the use of criteria related to the histological organization of muscular fibers represents a valuable

tool for solving the above-mentioned limitiation. In addition, digital image analysis offers versatile tools that permit the study of trends (areas, volumes, colour-related differences etc.) whose calculation was tedious some years ago. Johnston (1993) measured organelle areas in the muscle of *Clupea harengus* larvae using an image analyser; McFadzen et al. (1994) quantified starvation in *Scophtalmus maximus* using the ratio perimeter/area of the foregut; and Catalan and Olivar (2002) the degree of separation of muscular fibers in European sea bass larvae. However, there are not enough studies in which multiple quantitative and qualitative measurements of several organs taken from the same individuals can be compared. The second main problem of histological indices (extendable to any condition index) is the large dependence of condition on the experimental rearing parameters, with the 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.

Conclusions

The assessment of condition by means of microscopical methods at a tissular and cellular level is probably the most accurate indicator of nutritional status during early life stages of fish. This success is partly attributable to the large amount of information that can be obtained from only one single specimen. These techniques usually consist of the examination of target cells and organs and the establishment of a grading system based on the presence/absence of standardised biomarkers. The microscopical organization of the liver hepatocytes, the intestinal mucosa, the exocrine pancreas, and the muscular fibers are generally used as target tissues and organs to assess the nutritional conditon of fish larvae. However, their use, in both natural and cultured fish larvae populations, requires detailed experimental investigation in which specific nutritional and starvation indicators and biomarkers are described, standardised, and validated for fish of known nutritional history.

Acknowledgements. The Ministry of Education and Science, Spain (Projects MCYT/AGL2003-03558; AGL2006-13777-C03-02 and AGL2005-02778) supported this work. Many thanks to Department of Histology and Histopathology (ICMAN.CSIC). EG and JBOD are supported by the Programa Ramón y Cajal (MEC, Spain).

References

Beardall C.H. and Johnston I.A. (1983). Muscle atrophy during starvation in a marine teleost. Eur. J. Cell Biol. 29, 209-217.

Bisbal G.A. and Bengtson D.S. (1995). Development of the digestive tract in larval summer flounder. J. Fish Biol. 47, 277-291.

Caballero M.J., López-Calero G., Socorro J., Roo F.J., Izquierdo M.S. and Fernández A.J. (1999). Combined effect of lipid level and fish meal quality on liver histology of gilthead seabream (*Sparus aurata*). Aquaculture 179, 277-290.

Cahu C. and Zambonino-Infante J.L. (1994). Early weaning of sea bass (*Dicentrarchus labrax*) larvae with a compound diet: effect on digestive enzymes. Comp. Biochem. Physiol. 109A, 213-222.

Cahu C. and Zambonino-Infante J.L. (2001). Substitution of live food by formulated diets in marine fish larvae. Aquaculture 200, 161-180.

Carriquiriborde P., De Luca J.C., Dulout F.N. and Ronco A.E. (2007). Nucleolar variation in response to nutritional condition in juvenile pejerrey *Odontesthes bonariensis* (Valenciennes). J. Fish Biol. 70, 947-958.

Catalan I.A. (2003). Condition indices and their relationship with environmental factors in fish larvae. PhD Thesis, University of Barcelona.

Catalán I.A. and Olivar M.P. (2002). Quantification of muscle condition using digital image analysis in Dicentrarchus labrax larvae, and relationship with survival. J. Mar Biol. Ass. UK 82, 649-654.

Clemmensen C. (1993). Improvements in the fluorimetric determination of the RNA and DNA content of individual marine fish larvae. Mar. Ecol. Prog. Ser. 100, 177-183.

Crespo S., Marín de Mateo M., Santamaría C.A., Sala R., Grau A. and Pastor E. (2001). Histopathological observations during larval rearing of common dentex *Dentex dentex* L. (Sparidae). Aquaculture 192, 121–132.

Dahlfoff E.P. (2004). Biochemical indicators of stress and metabolism: applications for marine and ecological studies. Annu. Rev. Physiol. 66, 183-207.

Darias M.J., Ortiz-Delgado J.B., Sarasquete C., Martínez-Rodríguez G. and Yúfera M. (2007). Larval organogenesis of *Pagrus pagrus* L., 1758 with special attention to the digestive system development. Histol. Histopathol. 22, 753-768.

Deplano M., Connes R., Díaz, J.P. and Paris J. (1989). Intestinal steatosis in the farm-reared sea bass *Dicentrarchus labrax* L. Dis. Aquat. Org. 6, 121-130.

Deplano M., Díaz J.P., Connes R., Kentouri-Dinavach M. and Cavalier F. (1991). Appearance of lipid absorption capacities in larvae of the sea bass *Dicentrarchus labrax* L. during transition to the exotrophic phase. Mar. Biol. 108, 361-381.

Diaz J.P., Guyot E., Vigier S. and Connes R. (1997). First events in lipid absorption during post-embryonic development of the anterior intestine in gilthead sea bream. J. Fish Biol. 51, 180-192.

Diaz J.P., Mani-Ponset E., Guyot E. and Connes R. (1998). Hepatic cholestasis during the post-emrbyonic development of fish larvae. J. Exp. Zool. 280, 277-287.

Ehrlich K.F., Blaxter J.H.S. and Pemberton R. (1976). Morphological and histological changes during growth and starvation of herring and plaice larvae. Mar. Biol. 35, 105-118.

Elbal M.T., García-Hernández M.P., Lozano M.P. and Agulleiro B. (2004). Development of the digestive tract of gilthead seabream (*Sparus aurata* L). Light and electron microscopic studies. Aquaculture 234, 215-238.

Falk-Petersen I.B. (2005). Comparative organ differentiation during early life stages on marine fish. Fish Shellfish Immunol. 19, 397-412.

Fernández-Diaz C., Kopecka J., Cañavate J.P., Sarasquete C. and Solé M. (2006). Variations on development and stress defences in Solea senegalensis larvae fed on live and microencapsuled diets. Aquaculture 251, 573-584.

Ferron A. and Leggett W.C. (1994). An appraisal of condition measures for marine fish larvae. Adv. Mar. Biol. 30, 217-303.

Fontagné S., Geurden I., Escaffre A.M. and Bergot P. (1998). Histological changes induced by dietary phospholipids in intestine and liver of common carp (*Cyprinus carpio* L.) larvae. Aquaculture 161, 213-223.

García-Hernández, M.P., Lozano M.T., Elbal M.T. and Agulleiro B. (2001). Development of the digestive tract of sea bass (*Dicentrarchus labrax* L.). Light and electron microscopic studies. Anat. Embryol. 204, 39-57.

Gisbert E. and Doroshov S.I. (2003). Histology of the developing digestive system and the effect of food deprivation in larval green sturgeon (*Acipenser medirostris*). Aquat. Liv. Res. 16, 77-89.

Gisbert E., Piedrahita R.H. and Conklin D.E. (2004a). Ontogenetic development of the digestive system in California halibut (*Paralichthys californicus)* with notes on feeding practices. Aquaculture 232, 455-470.

Gisbert E., Piedrahita R.H. and Conklin D.E. (2004b). Effects of delayed first feeding on the nutritional condition and mortality of California halibut larvae. J. Fish Biol. 64, 116-132.

Gisbert E., Villeneuve L., Zambonino-Infante J.L., Quazuguel P. and Cahu C.L. (2005). Dietary phospholipids are more efficient than neutral lipids for long chain polyunsaturated fatty acid supply in European sea bass *Dicentrarchus labrax* larval development. Lipids 40, 609-618.

Govoni J.J., Boehlert G.W. and Watanabe Y. (1986). The physiology of digestion in fish larvae. Envir. Biol Fish. 16, 59-77.

Green B.S. and McCormick M.I. (1999). Influence of larval feeding history on the body condition of *Amphiprion melanopus*. J. Fish Biol. 55, 1273–1289.

Gwak W.S. and Tanaka M. (2001). Developmental change in RNA:DNA ratios of fed and starved laboratory-reared Japanese flounder larvae and juveniles, and its application to assessment of nutritional condition for wild fish. J. Fish Biol. 59, 902-915.

Gwak W.S., Seikai T. and Tanaka M. (1999). Evaluation of starvation status of laboratory-reared Japanese flounder *Paralichthys olivaceus* larvae and juveniles based on morphological and histological characteristics. Fish. Sci. 65, 339-346.

Hempel G. and Blaxter J.H.S. (1963). On the condition of herring larvae. Rapp. P. V. Reun. Cons. Int. Explor. Mer 154, 35-40.

Hamlin H.J., Hunt von Herbing I. and Kling L.J. (2000). Histological and morphological evaluations of the digestive tract and associated organs of haddock throughout post-hatching ontogeny. J. Fish Biol. 57, 716-732.

Henning S.J., Rubin D.C. and Shulman R.J. (1994). Ontogeny of the intestinal mucous. In: Physiology of the gastrointestinal tract. 3rd ed. Johnson L.R. (ed). Raven Press, New York. NY pp 571-610.

Hoehne-Reitan K. and Kjørsvik E. (2004). Functional development of the liver and exocrine pancreas. Am. Fish. Soc. 40, 9-36.

Izquierdo M.S., Socorro J., Arantzamendi L. and Hernández-Cruz C.M. (2000). Recent advances in nutrition in fish larvae. Fish Physiol. Biochem. 22, 97-107.

Johnston I. (1981). Quantitative analysis of muscle breakdown during starvation in the marine flatfish *Pleuronectes platessa*. Cell Tissue Res. 214, 369-386.

Johnston I.A. (1993) Temperature influences muscle differentiation and the relative timing of organogenesis in herring (*Clupea harengus*) larvae. Mar. Biol. 116, 363-379.

Johnston I.A. (1999). Muscle development and growth: potential implications for flesh quality in fish. Aquaculture 177, 99-115.

Johnston I.A. and Goldspink G. (1973). Some effects of prolonged starvation on the metabolism of the red and white myotomal muscles of the plaice *Pleuronectes platessa.* Mar. Biol. 19, 348-353.

Kjørsvik E., van der Meeren T., Kryvi H., Arnfinnson J. and Kvenseth P.G. (1991). Early development of the digestive tract of cod larvae, *Gadus morhua* L., during start-feeding and starvation. J. Fish Biol. 38, 1-15.

Margulies D. (1993). Assessment of the nutritional condition of larval and early juvenile tuna and Spanish mackerel (Pisces: Scombridae) in the Panamá Bight. Mar. Biol. 115, 317–330.

McFadzen I.R.B., Coombs S.H. and Halliday N.C. (1997). Histological indices of the nutritional condition of sardine *Sardina pilchardus* (Walbaum) larvae off the north coast of Spain. J. Exp. Mar. Biol. Ecol. 212, 239-258.

Mobin S.M.A., Kanai K. and Yoshikoshi K. (2000). Histopathological alterations in the digestive system of larval and juvenile Japanese flounder *Paralichthys olivaceus* reared on four feeding levels. J. Aquat. Anim. Health 12, 196-208.

Mookerji N. and Rao R. (1999). Rates of yolk utilization and effects of delayed initial feeding in the larvae of the freshwater fishes rohu and singhi. Aquacult. Int. 7, 45–56.

Moon T.W. and Johnston I.A. (1980). Starvation and the activities of glycolytic and gluconeogenetic enzymes in skeletal muscles and liver of plaice, *Pleuronectes platessa*. J. Comp. Physiol. 136, 31-38.

Morais S., Cahu C., Zambonino-Infante J.L., Robin J., Rønnestad I., Dinis M.T. and Conceição L.E.C. (2004). Dietary triacylglycerol source and level affects performance and lipase expression in larval seabass (*Dicentrarchus labrax*). Lipids 39, 449-458.

Navarro N. and Sarasquete C. (1998). Use of freeze-dried microalgae for rearing gilthead seabream, *Sparus aurata*, larvae: I. Growth, histology and water quality. Aquaculture, 167, 179-193.

Olsen R.E., Myklebust R., Ringo E. and Mayhew T.M. (2000). The influences of dietary linseed oil and saturated fatty acids on caecal enterocytes in Artic charr (*Salvelinus alpinus* L.): a quantitative ultrastructural study. Fish Physiol. Biochem. 22, 207-216.

Oozeki Y., Ishii T. and Hirano R. (1989). Histological study of the effects of starvation on reared and wild-caught larval stone flounder *Kareius bicoloratus*. Marine Biol. 100, 269-276.

Ortiz-Delgado J.M., Darias M., Yúfera M. and Sarasquete C. (2003a). Organogenesis of the sargo, *Diplodus sargo*. Histological and Histochemical Approaches. Histol. Histopathol. 18, 1141-1154.

Ortiz-Delgado J.B., Darias M., Cañavate J.P., Yúfera M. and Sarasquete C. (2003b). Organogenesis of the digestive tract in the white seabream, *Diplodus sargus*. Histological and histochemical approaches. Histol. Histopathol. 18, 1141-1154.

Ortiz-Delgado J.B., Ruane N.M., Pousào-Ferreira P., Dinis M.T. and Sarasquete C. (2006). Thyroid gland development in Senegales sole, *Solea senegalensis* Kaup 1858) during early life stages: a histochemical and immunohistochemical approach. Aquaculture 260, 346-356.

Pedersen B.H. and Andersen K.P. (1992). Induction of trypsinogen secretion in herring larvae (*Clupea harengus*). Mar. Biol. 112, 559-565.

Powell A.B. and Chester A.J. (1985). Morphometric indices of nutritional condition and sensitivity to starvation of spot larvae. Trans. Am Fish. Soc. 114, 338–347.

Ribeiro L., Sarasquete C. and Dinis M.T. (1999). Histological and histochemical development of the digestive system of *Solea senegalensis* (Kaup, 1858) larvae. Aquaculture 171, 293-308.

Robinson S.M.C. and Ware D.M. (1988). Ontogenetic development of

growth rates in larval Pacific herring, Clupea harengus pallasi, measured with RNA–DNA ratios in the Strait of Georgia, British Columbia. Can. J. Fish. Aquat. Sci. 45, 1422–1429.

Rodríguez A., Gisbert E. and Castelló-Orvay F. (2005). Nutritional condition of *Anguilla anguilla* starved at various salinities during the elver phase. J. Fish Biol. 67, 521-534.

Sánchez-Amaya M.I., Ortiz-Delgado J.B., García-López, A., Cárdenas S. and Sarasquete C. (2006). Larval ontogeny of redbanded seabream *Pagrus auriga* Valenciennes, 1843 with special reference to the digestive system. A histological and histochemical approach. Aquaculture 263, 259-279.

Santamaría Rojas C.A., Marín de Mateo M., Traveset R., Sala R., Grau A., Pastor E., Sarasquete M.C. and Crespo S. (2004). Organogenesis in larval common *Dentex dentex* L., (Sparidae): histological and histochemical aspects. Aquaculture 237, 207-228.

Sarasquete C. and Gutiérrez M. (2005). New tetrachromic VOF stain (Type III-G.S) for normal and pathological fish tissues. Eur. J. Histochem. 49, 105-114.

Sarasquete C., Polo A. and González-Canales M.L. (1993). A histochemical and immunohistochemical study of the digestive enzymes and hormones during the larval development of the sea bream, *Sparus aurata* L. Histochem. J. 25, 430-437.

Sarasquete C., Polo A. and Yúfera M. (1995). Histology and histochemistry of the development of the digestive system of larval gilthead seabream, *Sparus aurata* L. Aquaculture 130, 79-92.

Sarasquete C., Gonzalez de Canales M.L. Arellano J.M., Muñoz-Cueto J.A., Ribeiro L. and Dinis M.T. (1996). Histochemical aspects of the yolk-sac and digestive tract of the Senegal sole, *Solea senegalensis*. Histol. Histopathol. 11, 881-888.

Sarasquete C., González de Canales M.L., Arellano J., Muñoz Cueto J.A., Ribeiro L. and Dinis M.T. (1998). Histochemical study of skin and gills of Senegal sole, *Solea senegalensis* larvae and adults. Histol. Histopathol. 13, 727-735.

Sargent J.R., McEvoy L., Estévez A., Bell G., Bell M., Henderson J. and Tocher D. (1999). Lipid nutrition in marine fish during early development: current status and future directions. Aquaculture 79, 217-230.

Sargent J.R., Tocher D.R. and Bell J.B. (2002). The lipids. In: Fish nutrition. Third edition. Halver J.E. and Hardy R.W. (eds). Academic Press. San Diego. pp 182-257.

Segner H. and Braunbeck T. (1988). Hepatocellular adaptation to extreme nutritional conditions in ide, *Leuciscus idus melanotus* L. (Cyprinidae). A morphofunctional analysis. Fish Physiol. Biochem. 5, 79-97.

Segner H., Rösch R., Verreth J. and Witt U. (1993). Larval nutritional physiology: studies with *Clarias gariepinus*, *Coregonus lavaretus* L. World Aquac. 21, 121-134.

Segner H., Storch V., Reinecke M., Kloas W. and Hanke W. (1994). The development of functional digestive and metabolic organs in turbot, *Scophthalmus maximus*. Mar. Biol. 119, 471-486.

Sheridan M.A. (1988). Lipid dynamics in fish: aspects of absorption, transportation, deposition and mobilization. Comp. Biochem. Physiol. 90B, 679–690.

Strüssmann C.A. and Takashima F. (1990). Hepatocite nuclear size and nutritional condition of larval pejerrey, *Odontesthes bonariensis* (Cuvier et Valenciennes). J. Fish Biol. 36, 59–65.

Suneetha K.B., Folkvord A. and Johannessen A. (1999). Responsiveness of selected condition measures of herring, *Clupea harengus*, larvae to starvation in relation to ontogeny and temperature. Env. Biol. Fish. 54, 191-204.

Suthers I.M. (1998). Bigger? Fatter? Or is faster growth better? Considerations on condition in larval and juvenile coral-reef fish. Aust. J. Ecol. 23, 265-273.

Theilacker G.H. (1978). Rearing container size affects morphology and nutritional condition of larval jack mackerel, *Trachurus symmetricus*. Fishery Bulletin 78, 789-791.

Theilacker G.H. (1986). Starvation-induced mortality of young sea-caught jack mackerel, *Trachurus symmetricus*, determined with histological and morphological methods. Fish. Bull. 84, 1-17.

Theilacker G.H. and Porter S.M. (1995). Condition of larval walleye pollock *Theragra chalcogramma*, in the Western Gulf of Alaska, assessed with histological and shrinkage indices. Fish. Bull. 93, 333–344.

Theilacker G.H. and Watanabe Y. (1989). Midgut cell height defines nutritional status of laboratory-raised larval northern anchovy, *Engraulis mordax*. Fish. Bull. 87, 457-469.

van de Oost R., Beyer J. and Vermeulen P.E. (2003). Fish bioaccumulatoin and biomarkers in enviromental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57-149.

Watanabe Y. (1985). Histological changes in the liver and intestine of freshwater goby larvae during short-term starvation. Bull. Jap. Soc. Sci. Fish. 51, 707-709.

Wieser W. (1995) Energetics of fish larvae, the smallest vertebrates. Acta Physiol. Scand. 154, 279-290.

Yúfera M., Pascual E., Polo A. and Sarasquete M.C. (1993). Effect of starvation on the feeding ability of gilthead seabream (*Sparus aurata* L.) larvae at first feeding. J. Exp. Mar. Biol. Ecol. 169, 259–272.

Yúfera M., Sarasquete C. and Fernández-Diaz C. (1996). Testing protein-walled microcapsules for the rearing of first-feeding gilthead seabream, *Sparus aurata* L. Mar. Fresh Water Res. 47, 211-216.

Yúfera M., Fernández-Diaz C., Pascual E., Sarasquete C., Moyano F.J., Diaz M., Alarcon F.J., Garcia-Gallego M. and Parra G. (2000). Towards an inert diet for first-feeding gilthead seabream (*Sparus aurata* L) larvae. Aqua. Nutr., 6, 143-152

Zambonino-Infante J.L. and Cahu C.L. (1994). Influence of diet on pepsin and some pancreatic enzymes in sea bass (*Dicentrarchus labrax*) larvae. Comp. Biochem. Physiol. 109A, 209-212.

Zambonino-Infante J.L. and Cahu C.L. (1999). High dietary lipid levels enhance digestive tract maturation and improve *Dicentrarchus labrax* larval development. J. Nutr. 129, 1195-1200.

Zambonino-Infante J.L. and Cahu C.L. (2001). Ontogeny of the gastrointestinal tract of marine fish larvae. Comp. Biochem. Physiol. 130C, 477-487.

Zambonino-Infante J.L. and Cahu C.L. (2007). Dietary modulation of some digestive enzymes and metabolic processes in developing marine fish, applications to diet formulation. Aquaculture 268, 98-105.

Zambonino-Infante J.L., Cahu C.L. and Peres A. (1997). Partial substitution of di- and tripeptides for native proteins in sea bass diet improves *Dicentrarchus labrax* development. J. Nutr. 127, 608-614.

Zambonino-Infante J., Gisber E., Sarasquete C., Navarro I., Gutiérrez J. and Cahu C.L. (2008). Ontogeny and physiology of the digestive system of marine fish larvae. In: Feeding and digestive functions of fish. Cyrino J.E.O., Bureau D. and Kapoor B.G. (eds). Science Publishers. Inc, Enfield, USA pp 277-344.

Accepted May 19, 2008