Larval Fish Nutrition

Section 1

Digestive Development and Nutrient Requirements

Chapter 1

Ontogeny of the digestive tract

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1.1 Introduction

Fishes, like other organisms, require an energy source to fuel their body systems and processes, including growth, metabolism, and reproduction. Different fish species have evolved feeding structures and digestive mechanisms that allow them to exploit a vast array of vegetal and animal food sources; consequently, the digestive tract of fishes has incorporated numerous adaptations for the efficient breakdown and absorption of essential nutrients, including appropriate digestive enzymes and absorptive surface areas (Moyle and Cech 2000). Since the dietary requirements of fish larvae are different from those of juveniles or adults, larval nutrition should always be considered along with the organization and functionality of the digestive system, nutritional needs, and the behavior of larvae at different stages of development. In addition to being the site of nutrient digestion and absorption, the digestive organs provide a barrier to environmental toxins, confer essential immune function, and have important roles in metabolism and salt and water absorption (Wallace et al. 2005).

Knowledge of differentiation of the digestive tract and accessory glands during larval development is essential for understanding the digestive and nutritional physiology of larval fishes, and synchronizing the physiological stage of development with feeding practices and rearing protocols. Thus, one of the main features that determines the end of the transformation from larvae to juvenile stages in teleosts is the development of a complete, functional, fully developed digestive system. Knowledge of the developmental stage will facilitate overcoming one of the major bottlenecks in fish hatcheries, the partial or complete replacement of live prey with a compound inert diet. In this sense, the ontogeny of the digestive tract of fish larvae has been the subject of many studies for the last 25 years, although most of the abovementioned effort has been focused on salmonids and marine finfish species due to their important commercial value for the aquaculture industry.

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Many studies have centered on evaluating the ontogenetic and epigenetic changes in the morphoanatomy and histological organization of the digestive organs by means of microscopy, as well as assessing the activity of different digestive enzymes from the pancreas, stomach, and intestine by means of biochemical quantification. Recently, those approaches have been complemented by molecular biological techniques that provide insight into both temporal and spatial expression patterns of genes involved in the development and functionality of the digestive tract during early ontogeny. Contrary to what was originally claimed, these studies have clearly demonstrated that fish larvae are not challenged with physiological or digestive deficiencies, although they hatch with very immature organs and systems compared with juveniles. In this sense, the digestive system of a fish larva should be considered as a very efficient system that provides the larva with all the nutrients and energy needed for routine maintenance metabolism, swimming, and growth in order to enhance its survival, growth performance, and transformation into a juvenile. Although fishes as a group show a remarkable diversity of structure and function of their nutritional physiology, the basic mechanisms of organ and system development are similar in all teleosts, even though there exist considerable interspecific differences in the relative timing of their differentiation, development, and functionality during early ontogeny. The timing of development of organ and physiological function is affected by the general life history and reproductive strategy of each species and by a variety of abiotic and biotic factors, including water quality, mainly temperature, and food availability and composition.

This chapter reviews the available information on the subject of the digestive physiology of marine and freshwater fish larvae, with special emphasis on the organization and functionality of the digestive tract and accessory organs during early ontogeny. We also focus on the use of different histological and biochemical markers to assess the nutritional condition of fish larvae under different nutritional and rearing conditions. Consequently, the chapter has been divided in three parts: first, a section devoted to describing the histomorphological development of the digestive tract and accessory glands in order to achieve a better understanding of their organization and functionality during early ontogeny, followed by a section focused on the functionality of the digestive organs based on the activity of specific digestive enzymes, and last but not the least, a review of the different biochemical and histological parameters that can be used for assessing and describing the nutritional condition of fish larvae. Throughout this chapter, the focus is on those dietary-induced changes in digestive tract functionality during larval ontogeny in species of aquacultural interest, although information is also included regarding other species of interest for biomedical studies (e.g., zebrafish).

1.2 Organogenesis of the digestive system

In most described species, the alimentary canal at hatching appears histologically as an undifferentiated straight tube lying dorsally to the yolk sac. However, during the lecithotrophic larval stage, the larva undergoes rapid developmental changes leading to the differentiation of several regions and organs of the digestive system, namely buccopharynx, esophagus, intestine, pancreas, and liver, whereas the morphogenesis of the stomach depends on the species. There are important morphological and functional differences between marine and freshwater fish species regarding the developmental events involved in the differentiation and functionality of the digestive tract, even between closely related species. In fact, although both groups of fishes hatch with a simple digestive tract appearing as a straight and undifferentiated tube located

dorsal to the yolk sac, closed to the exterior at both extremities (mouth and anus), and lined by a single layer of columnar epithelial cells (future enterocytes) with basal or central nuclei, there exist important morphoanatomical differences at the onset of exogenous feeding. It is generally accepted that in the case of marine fish species, the appearance of gastric glands and the onset of acidic digestion does not take place until metamorphosis far beyond the onset of exogenous feeding (Govoni et al. 1986; Zambonino-Infante and Cahu 2007), whereas in freshwater species with large- and medium-sized eggs, this process takes place during the transition to exogenous feeding, for example, cichlids (Lingling and Qianru 1981; Balon 1985; Fishelson 1995; Morrison et al. 2001; Alvarez-González et al. 2008), acipenserids (Gisbert et al. 1998; Gisbert and Doroshov 2003), salmonids (Sarieyyüpoglu et al. 2000; Rust 2002), or siluriformes (Verreth et al. 1992; Kozarić et al. 2008; de Amorim et al. 2009). However, this rapid development of the digestive system is not a generalized feature among other freshwater fish species, such as coregonids (Loewe and Eckmann 1988; Segner et al. 1993), percids (Ostaszewska 2005), cyprinids (Smallwood and Smallwood 1931; Wallace et al. 2005), or characids (Atencio García et al. 2007), which are characterized by small-sized eggs.

However, generalities normally have their own exceptions; for example, in the case of cichlids there exist species-specific differences in the time of differentiation of different digestive structures depending on the reproductive strategy of each species. Thus, substrate-spawning cichlids, such as the Nile tilapia *Tilapia nilotica* (Lingling and Qianru 1981; Morrison et al. 2001), common bay snook *Petenia splendida* (Alvarez-González et al. 2008), or *Cichlasoma dimerus* (Meijide and Guerrero 2000), tend to develop faster than mouthbrooding cichlids (Balon 1985; Fishelson 1995; Osse and van den Boogart 2004). In this sense, the reproductive strategy (parental care) of different cichlid species has a direct effect on the ontogenetic development of the larva, and consequently, on the sequence of development of its digestive tract. In particular, substrate spawners might need to develop faster in order to achieve full functionality of all their organs and systems, consequently maximizing their chances of survival to respond to environmental constraints. In contrast, those larvae receiving parental protection during their early ontogeny (mouthbrooding) might maximize energy for growth and tissue differentiation and minimize expenditures for other purposes (e.g., avoidance of predators), and consequently, these larvae might develop more slowly.

In the following sections, information about the histological differentiation and histochemical properties of different regions of the digestive tract and accessory organs is considered. Since this chapter is mainly devoted to describing the morphological organization and functionality of the digestive tract in relation to nutrient digestion and assimilation processes, the description of the buccopharynx has been deliberately omitted since this organ is more involved in prey capture than nutrition, and it has been recently reviewed by Zambonino-Infante et al. (2008).

1.3 Histological structure of the digestive tract and accessory glands

The digestive tract of fishes is composed of four basic histological layers, such as the mucosa, submucosa, muscularis, and serosa (Figure 1.1). However, the structure of these different segments of the alimentary canal varies considerably, and some of its parts or their constituents may be lacking depending on the species and/or the stage of development considered (Takashima and Hibiya 1995). In general, the wall of the digestive tract is formed by the serosa, muscularis, submucosa, and mucosa from the esophagus to

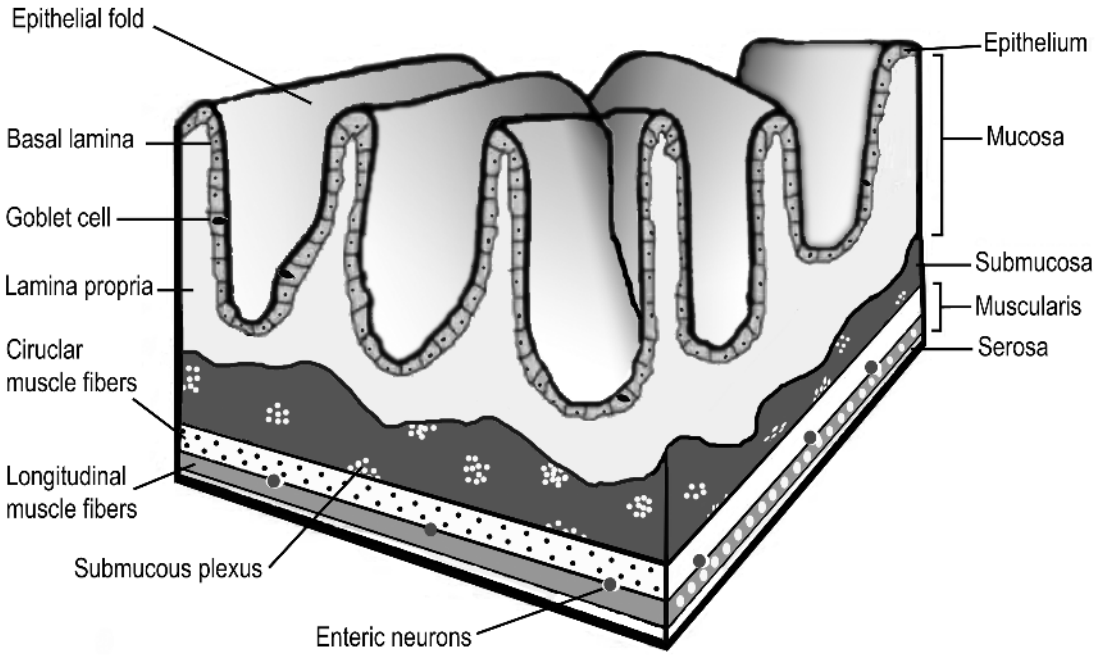


Figure 1.1 General organization of the wall of the digestive tract. Note that this organization in different layers and size of mucosal folds may change depending on the region of the gut considered, esophagus, stomach, or intestine (see details in the text).

the posterior intestine and rectum, whereas the buccal cavity and pharynx lack the serosa (Hossain and Dutta 1996). As the general reader is not normally familiar with the histological characteristics of the above-mentioned layers, and as these may change depending on the species, the stage of development, and the region of the digestive tract considered, we have decided to describe them briefly below (Figure 1.1) since their different histological organization depends on their specific functions.

The **mucosa** is the innermost layer of the digestive tube. Among the rest of layers that compose the digestive tube, the mucosa is the most variable in structure and function, endowing the tube with an ability to perform diverse and specialized digestive tasks along its length. Thus, the mucosal epithelium is regionally differentiated along the digestive tract to pursue multiple specialized functions, such as protection of the inner layers, secretion of the digestive juice, absorption, osmoregulation, and metabolism of nutrients among others. Thus, epithelial mucosal cells may differentiate into absorptive cells such as those of the intestine and pyloric ceca, in gastric and intestinal secretory cells, or in the goblet (mucous) cells found along the entire length of the digestive tract. Other noticeable types of cells that can be observed in the digestive mucosa include the foreign migrant cells (granulocytes, lymphocytes, and macrophages) that are part of the immune system. Under the basal part of the mucosal epithelium, there is the basal lamina, which can be recognized as a thin, dense, and continuous band formed by a complex layer of collagen fibers and polysaccharides. The lamina propria, a thin layer of connective tissue with blood capillaries, underlies the mucosal epithelium. Under the lamina propria, a thin layer of smooth musculature, the muscularis mucosa, may also be found, although it is

often poorly developed and commonly absent, in which case, a clear distinction between the connective tissue of the lamina propria and that of the submucosa is not possible.

The **submucosa** of teleosts is composed of one or more layers of connective tissue. This part of the wall of the gut shows some interspecific variability, with some fish species having a single-layered and loose submucosa, and many other species possessing a multilayered submucosa consisting of a stratum compactum, stratum granulosum, and in some cases, an extra layer of loose connective tissue beneath the stratum compactum. The submucosa also contains the submucous plexus, which provides nervous control to the mucosa.

Surrounding the submucosa is the **muscularis** formed by smooth or striated muscle layers depending on the region of the digestive canal. This layer may be organized into concentric inner circular and outer longitudinal muscle fibers. Enteric neurons within plexuses between the two muscle layers endow the digestive tube with an ability to be motile by means of peristaltic movements, which is of special importance in the esophagus and intestine.

The serous membrane, so-called **serosa**, is composed of a simple flat epithelium. This membrane is an extension of the mesentery and covers the external surface of the alimentary canal.

Up to now we have described the general structural characteristics of the wall of the alimentary canal; what follows is the particular histological organization of the different visceral regions of the digestive tract (esophagus, stomach, intestine, and pyloric ceca) and accessory glands (liver, pancreas, and gallbladder). However, due to the intrinsic differences between marine and freshwater species in their phylogenetic status, mode of reproduction, egg size, and larval feeding habits among others, we will describe, for comparative purposes, the ontogeny of the digestive system in freshwater and marine species separately.

1.3.1 Esophagus

At hatching, the esophagus in fish larvae is not a morphoanatomically differentiated region of the digestive tract and its morphogenesis takes place at later stages of development, just before the onset of exogenous feeding. At this stage, the esophagus appears as a short and rudimentary duct that connects the posterior region of the pharynx from the last gill arch with the anterior intestine since the stomach is neither differentiated nor formed in most of the described gastric species during the differentiation of this part of the alimentary canal. Once differentiated, the esophagus in fish is generally short, wide, and straight, and the most distinctive histological features that characterize its differentiation are the histological organization of the esophageal mucosa that varies among species, its longitudinal folding, and the appearance of goblet (mucus-secreting) cells (Figure 1.2a).

The epithelium that lines the esophageal mucosa in fish larvae is similar to that in adults. In general terms, the esophagus of freshwater fish species is lined by a multilayered squamous epithelium with large numbers of goblet cells (Figure 1.2b), whereas that of marine fish species is lined by a columnar epithelium with fewer mucous cells and highly vascularized mucosal folds (Figure 1.2c) (Stevens and Hume 2005). In some species, a ciliated epithelium is found in the esophagus of newly hatched larvae and adult cyclostomes, perch, and some elasmobranches, where this feature may be considered as a plesiomorphic character. However, there exist some exceptions regarding the histological organization of the esophageal epithelium in larvae from different fish species, as pointed out recently by Zambonino-Infante et al. (2008).

Another histological feature that characterizes the differentiation and functionality of the esophagus is the appearance of goblet cells and their histochemical characteristics (Figure 1.2d). The appearance of the first

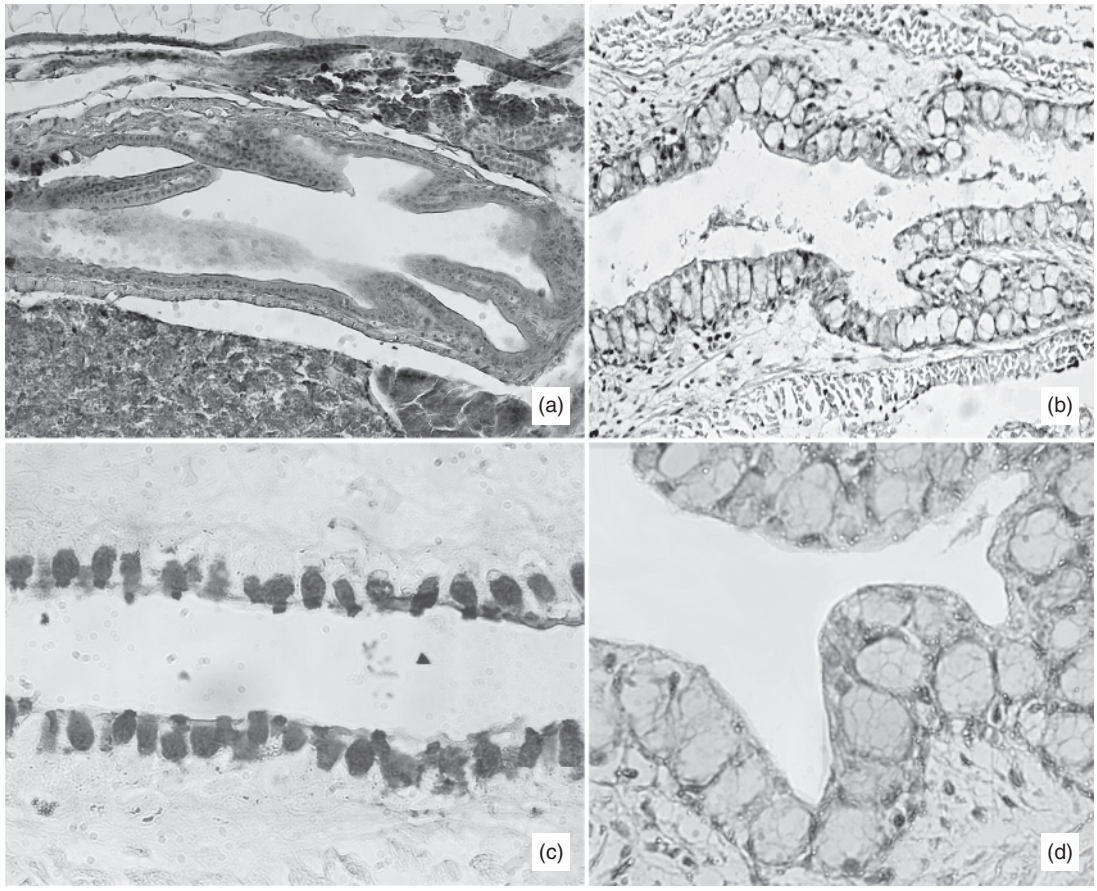


Figure 1.2 Longitudinal paraffin sections of the esophagus from different finfish larvae. (a) Esophagus of a California halibut (*Paralichthys californicus*) larvae aged 15 days posthatch (dph). Note the presence of longitudinal mucosal folds (magnification: 200×; hematoxylin-eosin [H-E] stain). (b) Detail of the esophagus from a bay snook (*Petenia splendida*) larva (24 dph) with abundant and large goblet cells (magnification: 200×; H-E stain). (c) Goblet cells containing neutral glycoconjugates (PAS-positive staining) in the esophageal epithelium of a 17-dph *Paralichthys californicus* larva (magnification: 400×; periodic acid–Schiff [PAS] stain). (d) Detail of goblet cells in the esophageal epithelium of *Petenia splendida* larva aged 13 dph. Note the hyaline content of these cells, indicating the presence of a mixture of secretory mucopolysaccharides (magnification: 400×; H-E stain). (Photographs by E. Gisbert.)

functional goblet cells along the esophageal epithelium varies among species: In Dover sole *Solea solea* (Boulhic and Gabaudan 1992), Senegal sole *Solea senegalensis* (Ribeiro et al. 1999), yellowtail flounder *Limanda ferruginea* (Baglole et al. 1997), large yellow croaker *Pseudosciaena crocea* (Mai et al. 2005), anemonefish *Amphiprion melanopus* (Green and McCormick 2001), Siberian sturgeon *Acipenser baerii* (Gisbert et al. 1999), green sturgeon *Acipenser medirostris* (Gisbert and Doroshov 2003), pike perch *Sander lucioperca* (Ostaszewska 2005), European catfish *Silurus glanis* (Kozarić et al. 2008), and common bay snook (Alvarez-González et al. 2008), goblet cells are detected coinciding with mouth-opening or just before the onset of exogenous feeding, whereas in other species such as the turbot *Scophthalmus maximus* (Segner et al. 1994), brill *Scophthalmus rhombus* (Hachero-Cruzado et al. 2009), California halibut *Paralichthys californicus*

(Gisbert et al. 2004a), gilthead sea bream *Sparus aurata* (Sarasquete et al. 1995), white bream *Diplodus sargus* (Ortiz-Delgado et al. 2003), red porgy *Pagrus pagrus* (Darias et al. 2005), common pandora *Pagellus erythrinus* (Micale et al. 2006), European sea bass *Dicentrarchus labrax* (García-Hernández et al. 2001), cobia *Rachycentron canadum* (Faulk et al. 2007), Atlantic cod *Gadus morhua* (Morrison 1993), and haddock *Melanogrammus aeglefinus* (Hamlin et al. 2000), goblet cells are detected at later stages of development. In haddock, special attention might be required in tanks during the first days of larval rearing, just before the appearance of functional esophageal goblet cells, since the ingestion of live prey may result in desquamation and abrasion of the esophageal epithelium due to the absence of mucus protecting this area. This might lead to significant larval mortality if high water quality is not maintained and bacteria proliferate (Gisbert et al. 2004a).

The species-specific differences in the ontogeny of the goblet cells in the esophageal mucosa of marine fish larvae were recently reviewed by Zambonino-Infante et al. (2008). Goblet cells secrete different types of mucosubstances that differ in their histochemical characteristics. In most of the described species, the mucous cells of the distal esophageal region secrete large amounts of neutral glycoconjugates (Figure 1.2c,d), while those from the anterior region produce, in addition to a minor component of neutral mucosubstances, a major quantity of carboxylated and sulfated acidic glycoconjugates with sialic acid residues. The large amount of mucous secreted by esophageal goblet cells may serve as a lubricant since fish do not have the salivary glands found in higher vertebrates. Mucosubstances produced by goblet cells may have the same functions as mammalian saliva in protecting the mucosa of the entire alimentary canal (Scocco et al. 1998). The presence of sialic acid residues in mucus prevents viruses from recognizing their receptor determinants and also preserves the mucosa from attack by the sialidase produced by bacteria (Zimmer et al. 1992). In addition, the secretion of this combination of mucosubstances has been described by several authors as a mechanism to allow the alimentary canal of young fish to respond to changes in environmental conditions and maintain osmotic balance (Domeneghini et al. 1998; Sarasquete et al. 2001). Ultrastructural studies have revealed that the esophageal epithelium is also involved in iono- and osmoregulation in seawater and freshwater environments. Thus, under hyperosmotic conditions, the ingested water is desalinated in the esophagus by the passive and active removal of Na^+ and Cl^-, although this segment of the digestive tract has low permeability to water and other ions. This results in a reduced osmolality of the water, facilitating its absorption in the intestine (Allen et al. 2009). Furthermore, neutral glycoconjugates secreted by esophageal goblet cells are considered to cooperate in the digestion of food and its transformation into chyme, as well as in the absorption of easily digested substances, such as disaccharides and short-chain fatty acids (Sarasquete et al. 2001).

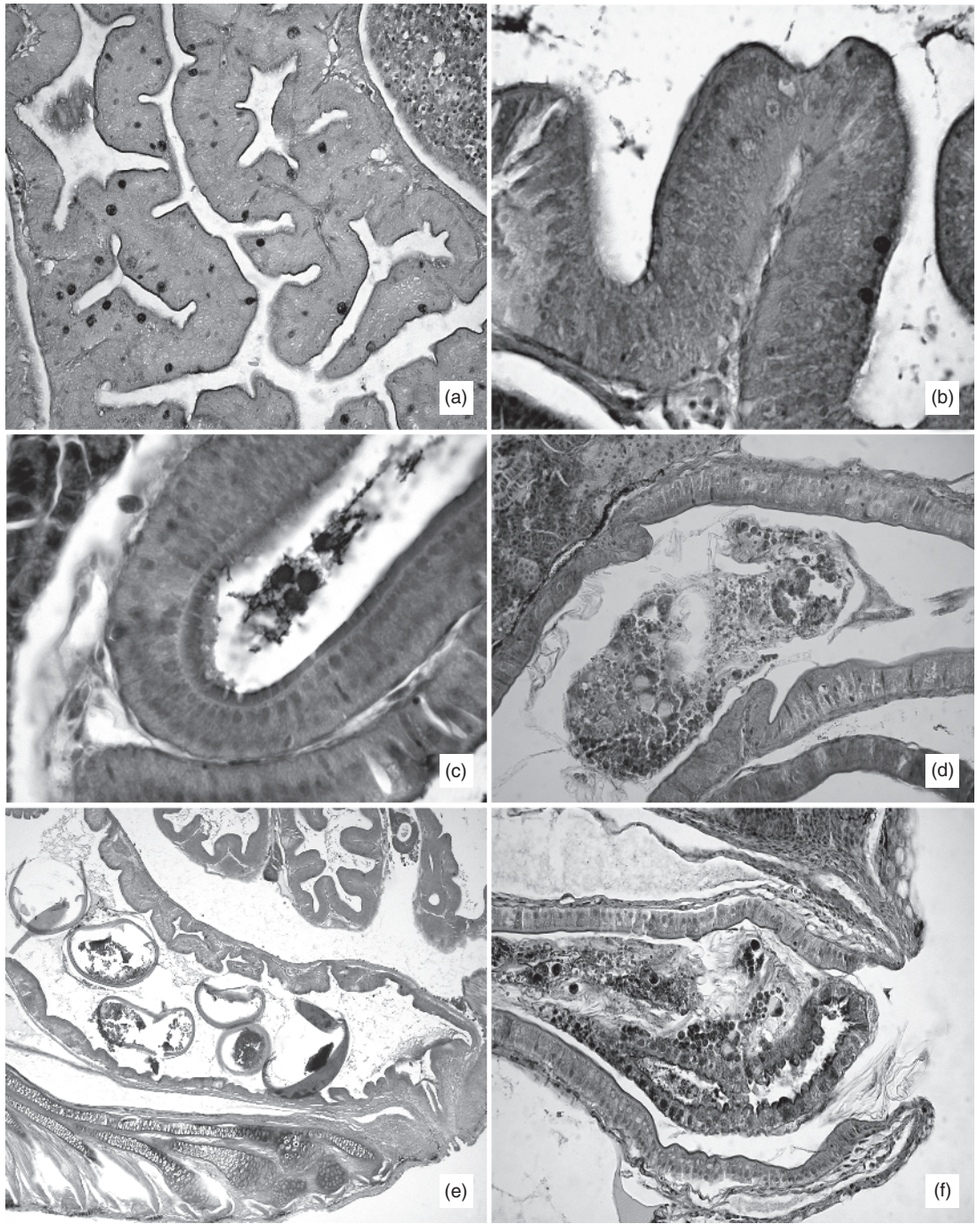
1.3.2 Intestine

The intestine is the longest portion of the digestive tract, occupying most of the abdominal cavity, and one of the first digestive organs to differentiate. The intestinal mucosa is a very dynamic and active tissue and is the main site of the digestion and absorption of nutrients, as well as being directly involved in the hormonal and nervous activation of enzyme and bile synthesis and their subsequent secretion from the pancreas and liver. At hatching, the intestine is an undifferentiated straight tube with a smooth lumen. During the lecithotrophic (endogenous feeding) phase, the posterior region of the intestine bends and the intestinal valve or

ileorectal valve forms as a constriction of the intestinal mucosa, dividing the intestine into two regions: the prevalvular (anterior) (Figure 1.3a) and postvalvular (posterior) intestine (Figure 1.3d). The intestinal mucosa is mostly rectilinear with several short folds. In most species, no histological differences are observed between the pre- and postvalvular intestine; both regions are lined by a simple columnar epithelium with basal nuclei, basophilic cytoplasm, and prominent microvilli (Figure 1.3b).

As the development of the intestine proceeds, the folding of the mucosa increases and the intestine coils, occupying most of the abdominal area. These morphoanatomical changes take place in order to accommodate the increasing length of the digestive tract inside the reduced abdominal cavity, and to increase the digestive and absorptive intestinal surface to cope with the increasing quantities of ingested food. However, not all fish species have a coiled intestine, for example, ayu *Plecoglossus altivelis* (Nakagawa et al. 2002) or zebrafish *Danio rerio* (Wallace et al. 2005); in those cases, the increase in absorptive surface of the intestine is achieved by only incrementing the folding level of the intestinal mucosa (Figure 1.3e). At this point, three different regions can be distinguished along the intestine according to their histological organization and characteristics. The anteromedian segment (prevalvular 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 (Figure 1.3a). This region of the intestine is the main site for lipid absorption (Diaz et al. 1997b; 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 postvalvular intestine is histologically similar to the anteromedian region except for the number and size of mucosal folds, which are longer, deeper, and more numerous in the prevalvular intestine (Figure 1.3e). In some fish species, such as sturgeons, the distal intestinal region prior to the rectum is modified soon after hatching into the spiral valve, which greatly increases the absorptive area of this region of the posterior intestinal mucosa (Buddington and Doroshov 1986; Gisbert et al. 1998). The intestine terminates in a short rectal zone that, depending on the species, can be lined either by a simple or columnar epithelium with few goblet cells or by a cubical epithelium (Figure 1.3f). Although some differences may be found among species, the organization of the intestinal mucosa throughout its length is quite conserved among teleosts and

Figure 1.3 Longitudinal sections of the intestine from California halibut (*Paralichthys californicus*) larvae. (a) Prevalvular intestine from a 25-dph larva. Note the presence of abundant secretory goblet cells containing neutral mucosubstances (PAS-positive staining) (magnification: 200×; periodic acid–Schiff [PAS] stain). (b) Detail of a mucosal fold of the intestine in a 25-dph larva showing the presence of prominent microvilli and the organization of the columnar epithelium (magnification: 600×; hematoxylin-eosin [H-E] stain). (c) Detail of the posterior intestine in a 15-dph larva showing the presence of eosinophilic supranuclear bodies in the enterocyte cytoplasm and the presence of prominent eosinophilic brush borders (magnification: 400×; H-E stain). (d) Detail of the ileorectal valve forming a constriction of the intestinal mucosa and dividing the intestine into two regions, the prevalvular (anterior) and postvalvular (posterior) intestine. The intestinal lumen appears filled with predigested *Artemia* nauplii (magnification: 400×; H-E stain). (e) General view of the postvalvular (posterior) intestine in a 35-dph larva. Note the reduction in mucosal folding in contrast to Figure 1.3a and the low presence of goblet cells (PAS-positive staining) in the intestinal epithelium (magnification: 100×; PAS stain). (f) Details of the rectum in a 35-dph larva. Note the flattening of columnar cells at the end of the rectum and the presence of incompletely digested *Artemia* nauplii in the intestinal lumen (magnification: 400×; H-E stain). (Photographs by E. Gisbert.)


(a)
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typically characterized into four distinct layers. In particular, zebrafish lack the submucosa layer of the intestine and instead possess an epithelium and lamina propria surrounded by circular and longitudinal smooth muscle layers. Another distinctive feature of the intestinal mucosa of the zebrafish is that the connective tissue found in the mucosa is less complex than in other cyprinid species (Wallace et al. 2005). In the literature, there are only a few reports of crypts in the intestinal mucosa of fish species, and as far as is known, only in cod, common wolffish *Anarhichas lupus*, and burbot *Lota lota* have these structures been described. The crypts comprise epithelial cells that differ morphologically from the general absorptive epithelium of the mucosal surface. Based on the presence of mitotic structures (proliferating cell nuclear antigen [PCNA]-staining nuclei) and less differentiated cells, crypts are considered a place for epithelial cell proliferation and regeneration, which might be similar to that observed in the mammalian intestine where intestinal epithelial cells divide in the lower part of the Lieberkühn crypts and differentiate as they migrate upward to the luminal surface. There exist differences in the distribution and morphology of intestinal crypts among the described fish species, although in all studied species they appear soon after hatching. In common wolffish, crypts are shallower and their openings regularly distributed over the whole mucosal surface of mucosal folds (primary and secondary), in contrast to cod, which exhibit groups of deep crypts with the openings at the base of mucosal primary folds. In burbot, crypts are restricted throughout the proximal intestine, including the pyloric ceca, while in common wolffish, crypts are observed in all parts of the intestine (Hellberg and Bjerkås 2005).

Four different types of cells can be identified along the intestinal epithelium. These include enterocytes, single enteroendocrine cells, rodlet cells, and goblet cells. **Enterocytes** are the most abundant epithelial cell type in the intestine, and they are involved in nutrient absorption, intracellular digestion, and osmoregulation. Enterocytes in the anterior intestine are responsible for the absorption of lipids and amino acids by diffusion, whereas enterocytes in the distal intestine are specialized for the uptake of protein macromolecules by pinocytosis. Intestinal **goblet cells** are the second most abundant cell type and are scattered along the intestinal epithelium. These cells are well known for the production of a physical barrier between the epithelium and the content of the lumen. Goblet-cell-secreted mucins are central to the establishment of this complex mucopolysaccharide barrier. The differentiation of these mucus-secreting cells follows two different patterns according to the larval stage of development at which they differentiate. For example, in wolffish, bay snook, Siberian and green sturgeons, Dover sole, yellowtail flounder, or spotted sand bass, goblet cells appear in the intestinal mucosa, before first feeding and/or coincide with the onset of exogenous feeding, while in other species, such as pike perch, gilthead sea bream, California halibut, Senegal sole, common pandora, kelp grouper, common dentex, cod, or haddock, goblet cells differentiate at latter stages of development (see review in Zambonino-Infante et al. 2008). Intestinal goblet cells contain a mixture of neutral and acid glycoproteins and the histochemical pattern of their content does not change through larval and juvenile periods to adult ages. Mucosubstances produced by rectal and distal postvalvular intestine goblet cells may serve to lubricate the feces, while in other regions of the intestine they protect the digestive mucosa and facilitate the absorption of nutrients. The presence of sulfated acidic glycoproteins produced by goblet cells may regulate the transfer of proteins or protein fragments into enterocytes where these compounds will be digested via pinocytosis (Domeneghini et al. 1998).

Enteroendocrine cells produce and secrete peptide hormones that, in collaboration with

the nervous system, control and coordinate the muscular and secretory activities of the gastrointestinal tract. These cells have a pyramidal or spindle shape with a narrow extension to the gut lumen, and their distribution and time of differentiation vary among species. In fish species with a convoluted intestine, enteroendocrine cells are rarely found beyond the anterior intestine, as described for Japanese flounder *Paralichthys olivaceus*, Atlantic halibut *Hippoglossus hippoglossus*, Pacific bluefin tuna *Thunnus orientalis* (Kamisaka et al. 2003), and zebrafish (Wallace et al. 2005), whereas in species with a straight digestive tract, such as the ayu, they are scattered along the entire intestine with the exception of the rectum (Kamisaka et al. 2003). As the former authors suggested, these cells seem to be located in regions where the chyme is retained, and consequently, they can easily receive chemical signals from the food and the digestive process in order to control the release of the digestive hormones. In species that hatch with a well-differentiated digestive system, such as the ayu, enteroendocrine cells are found just after hatching (Kamisaka et al. 2003), whereas in pelagic fish species with a less developed digestive tract, these cells may appear at latter stages of development just before the onset of exogenous feeding (Kurokawa et al. 2000). Intestinal **rodlet cells** have been considered as regulatory elements related to special functions such as osmoregulation, ion transportation, and nonspecific immune response (see review in Manera and Dezfuli 2004).

1.3.3 Pyloric ceca

Pyloric ceca are considered as an adaptation for increasing intestinal surface area without increasing the length or thickness of the intestine itself. These intestinal appendages are also involved in osmoregulatory processes and especially in water uptake in fish exposed to hyperosmotic environments (Allen et al. 2009). Among vertebrates, only teleost fish species have appendages such as the pyloric ceca at the gastrointestinal junction, which are entirely different from those found in birds and mammals, which have fermentation functions (Buddington and Diamond 1987). Pyloric ceca are present in 60% of the known fish species, and their number is highly variable, ranging from none (absent) to numerous (>1,000) depending on the fish group and species. The presence of ceca in some fish and absence in others, as well as variations in the number of ceca between and within species, are regarded as adaptations in the fish digestive system to different feeding habits and morphoanatomical characteristics of their digestive tract (Hossain and Dutta 1996). In addition, the phylogenetic relationships of the pyloric ceca between different fish species and their differences among different teleosts are reviewed in Hossain and Dutta (1996).

These fingerlike projections that form part of the anteriormost region of the intestine increase the surface area for absorption. In addition, pyloric ceca appear to be sites of digestion with the contribution of pancreatic enzymes, and some authors consider them to be accessory food reservoirs and breeding places for gut flora (Buddington and Diamond 1987; Hossain and Dutta 1996). Pyloric ceca have also been shown to neutralize the acid bolus entering the intestine from the stomach, which is supported by the absence of these structures in fishes lacking a stomach (Rust 2002). Hossain and Dutta (1996) reported that the greater the cecum size, the better its functional efficiency; thus, fish species with shorter intestines should have either more or larger pyloric ceca, as described for some detritivorous species. However, this relationship is not consistent for omnivorous species (Albrecht et al. 2001). Drewe et al. (2004) concluded that the relationship between diet and the structure and function of the pyloric ceca is complex and still poorly understood.

The histological organization of pyloric ceca closely resembles that of the intestine;

the four basic histological layers of the intestinal tract (serosa, muscularis, submucosa, and mucosa) are present in all ceca, although the cecal mucosa is more complex compared with that of the intestine. The main differences between these two regions of the digestive tract are the relative thickness of the cecal layers, which are several-fold smaller compared with those of the intestine, and the number of goblet cells secreting a combination of neutral and acidic mucosubstances that are less abundant in the ceca than in the intestine. In addition, the relative thickness of these layers and the lumen space varies within a specimen. In general, a large cecum has more muscle, mucosa, and lumen space than a small cecum. This close resemblance of the histology of ceca and intestine indicates that ceca not only are linked ontogenetically but also are connected functionally with the intestine (Hossain and Dutta 1996).

The development of the pyloric ceca and the stomach are the last steps in the differentiation of the alimentary canal, as well as being the anatomical digestive features that characterize the end of the larval phase and the beginning of the juvenile stage. However, there exist some differences among species. For instance, in some species, such as European sea bass (García-Hernández et al. 2001), spotted sand bass *Paralabrax maculatofasciatus* (Peña et al. 2003), haddock (Hamlin et al. 2000), or pike perch (Ostaszewska 2005), the morphogenesis of the pyloric ceca is contemporaneous with the differentiation of the gastric glands, whereas in cobia (Faulk et al. 2007) or chum salmon *Oncorhynchus keta* (Dabrowski 1984), pyloric ceca develop later than the stomach. The above-mentioned changes in the ontogeny of differentiation of the pyloric ceca might be linked to the species-specific differences in digestive requirements, especially those related to an increasing demand for digestive and absorptive intestinal surfaces coupled with an increase in length of the digestive tract as development proceeds.

1.3.4 Stomach

The main functions of the vertebrate stomach are to store ingested food, to secrete pepsinogen and hydrochloric acid (HCl), and to mix food and the gastric secretions mechanically through the action of muscles that allow for the distension and movement of the organ (Stevens and Hume 2005). The form of the stomach is very diverse, from a single bulge converted to an elongated pouch when it is full, to a well-differentiated sac. It might be bent to form a Y, V, or J shape and is usually separated from the intestine by a pyloric sphincter or valve. It is capable of considerable distension in carnivorous fish species that swallow whole prey, whereas microphagous species have much smaller stomachs. Despite these anatomical variations, all stomachs are relatively homogeneous in their histological structure and differ little, at least in function, from those of higher vertebrates (Guillaume et al. 2001). The stomach is generally divided into three regions, the cardiac (anterior), fundic, and pyloric (posterior) portions, that exhibit specific anatomical and histological adaptations for separate digestive functions. The mucosal epithelium of each region is single layered and folded. The folds of the cardiac portion are normally shallow, but those of the fundic and pyloric portions are deeper. Generally, epithelial cells of the cardiac region are cubical in shape, and those of the rest of the stomach epithelium are columnar. In some fish species, such as sturgeons, the walls (tunica muscularis) of the pyloric region are hypertrophied, especially the dorsal and ventral walls. Because of the presence of such a thickened smooth muscle layer, the pyloric region of the stomach has sometimes been referred to as the gizzard (Buddington and Doroshov 1986; Gisbert et al. 1998) in this group of chondrosteans, and it has a triturative function that may compensate for their lack of dentition (mandibular, oral, or pharyngeal). In some herbivorous species, such as mullets, the pyloric region of

the stomach forms a true differentiated organ with toughened walls, surrounded by a very thin circular musculature, following a stomach that has lost its secretory functions. Similarly to sturgeons, this region of the stomach is also called a gizzard and performs a purely grinding function (Guillaume et al. 2001).

The formation of the stomach varies depending on the type of egg cleavage. In fish with holoblastic (complete) cleavage such as sturgeons, where the yolk endoderm participates in the formation of the alimentary canal (Dettlaff et al. 1993), the stomach starts to differentiate in the anterior ventral region of the yolk sac from a fold of stratified epithelium that previously divided the yolk into two compartments; the anterior wall of the furrow becomes the ventral lining of the stomach, while its posterior wall lined with a columnar epithelium becomes the dorsal lining of the intestine. As a consequence of the holoblastic cleavage, epithelial cells lining the alimentary tract and the stomach are filled with eosinophilic yolk granules that gradually disappear as stomach morphogenesis proceeds (Gisbert and Doroshov 2003; Zambonino-Infante et al. 2008). In contrast, in fish with meroblastic (incomplete or partial) egg cleavage, which include the vast majority of finfish species, the digestive system and particularly the stomach differentiate independently of the extraembryonic yolk sac. In the latter fish species, the stomach appears as a slight enlargement, dilatation, or pouch of the esophagus that is accompanied with a notable thickening in the mucosa and narrowing of the lumen demarking the transition to the anterior intestine. This area of the alimentary canal is lined with a cubical epithelium that further differentiates into a columnar one, as has been described in European sea bass (García-Hernández et al. 2001), common pandora (Micale et al. 2006), cobia (Faulk et al. 2007), and Siberian sturgeon (Gisbert et al. 1999) among other species (see review in Zambonino-Infante et al. 2008). Further in development, a sphincter, formed by thickened layers of connective tissue and circular bundles of smooth muscle cells surrounding the digestive epithelium at the level of the constriction, develops at either end of the stomach, separating the gastric region of the alimentary canal from the esophagus and anterior intestine, respectively (Figure 1.4a).

As morphogenesis proceeds, the epithelium of the cardiac and fundic regions of the stomach folds transversally and forms the gastric pits, where the first formed gastric glands open into the gastric lumen. Gastric glands, which are tubular in shape, are generally situated in the lamina propria and surrounded by a loose connective tissue. The secretory cells present in these glands are responsible for the production of both HCl and pepsinogen, and they are called oxynticopeptic cells (Figure 1.4b,c). Ultrastructural characteristics of the secretory cells from undifferentiated and differentiated gastric glands are described in detail in García-Hernández et al. (2001). In short, glandular cells initially have numerous free ribosomes and clear vesicles in the apical zone. As they differentiate and become functional, an apical tubule–vesicular network, a very developed endoplasmatic reticulum, and zymogen granules appear. Histochemically, gastric glands contain glycogen, neutral and especially carboxylated glycoconjugates, and proteins rich in different amino acids, especially proteins rich in arginine, tyrosine, and tryptophan. These amino acids are involved in the synthesis and secretion of enzymatic precursors, that is, pepsinogen (Ortiz-Delgado et al. 2003). Along the epithelial cells of the stomach, goblet cells secreting neutral mucosubstances are found in the lumenally exposed gastric epithelium. The main role of the neutral mucosubstances present in the stomach is to protect the epithelium of the stomach from autodigestion processes caused by HCl and enzymes (e.g., pepsin) produced in gastric glands. In addition, several authors have pointed out that the periodic acid–Schiff (PAS)-positive reaction observed in the gastric epithelial cell

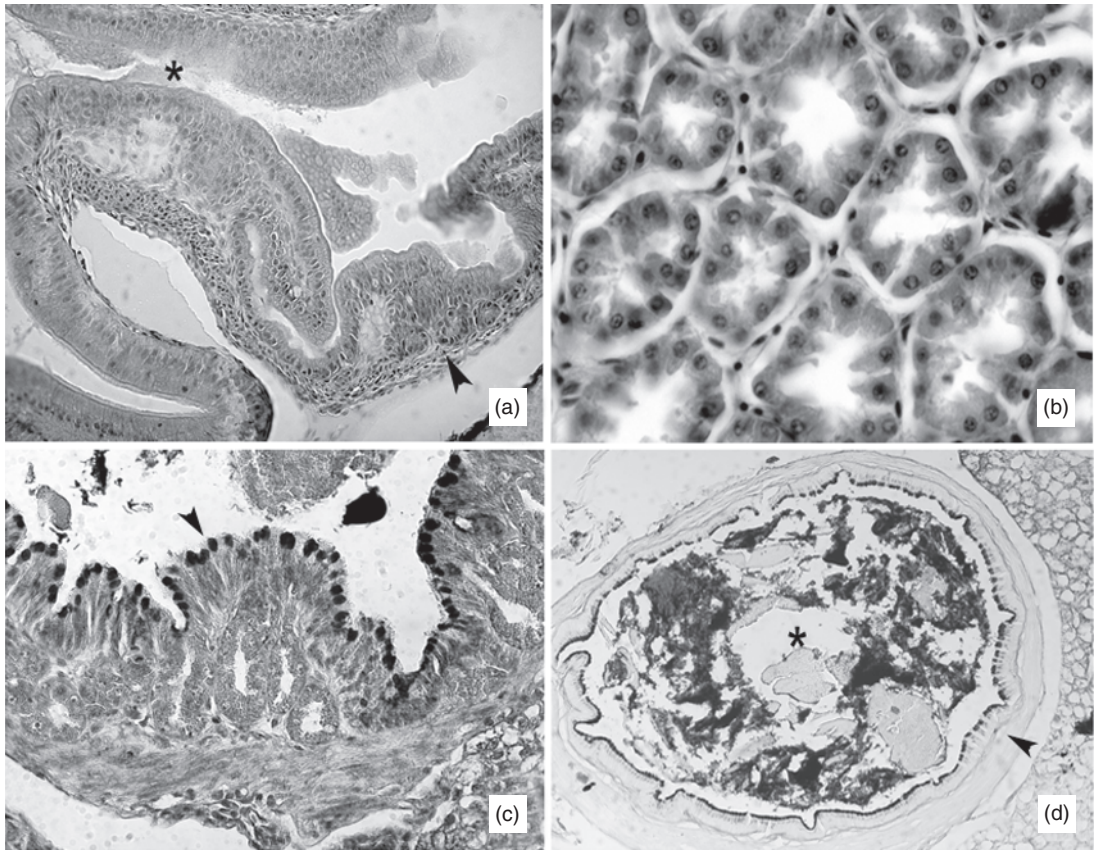

Figure 1.4 Different histological sections of the stomach from larvae of different fish species. (a) Onset of formation of the glandular stomach in a 24-dph California halibut *Paralichthys californicus* larva. Note the transition from the esophagus into the stomach in differentiation (asterisk) and the formation of first gastric glands (arrowhead) (magnification: 200×; hematoxylin-eosin [H-E] stain). (b) Detail of the gastric glands in *Paralichthys californicus* (magnification: 600×; H-E stain). (c) Multicellular tubular gastric glands in Siberian sturgeon *Acipenser baerii* (larva aged 16 dph) composed of a single secretory cell type observed in the cardiac stomach (magnification: 400×; hematoxylin–light green-orange G-acid fuchsin [VOF] stain). (d) Transverse section of the nonglandular stomach in *Acipenser baerii* (larva aged 25 dph). Note the PAS-positive staining of mucous cells lining the epithelial lumen of the stomach and the thick layer of musculature (arrowhead). The asterisk denotes the lumen of the nonglandular stomach filled with chyme (magnification: 400×; PAS stain). (Photographs by E. Gisbert.)

surface resembles that observed in the striated border of intestinal enterocytes, which may indicate nutrient absorption of easily digestible substances such as disaccharides and short-chain fatty acids occurring in this region of the alimentary canal, as previously reported in the esophagus.

The morphogenesis of gastric glands and the achievement of an adultlike digestion, characterized by low pH and gastric proteases, that is, pepsinogen, are achieved at different stages of development depending on the fish species and water rearing temperature. In this sense, there exists a wide variety of ontogenetic stages where gastric glands differentiate (Zambonino-Infante et al. 2008); for instance, salmonids and wolffish possess a functional stomach at the time of first feeding, whereas in others, gastric glands appear later in development, ranging from as early as 10 days after hatching (dah) in turbot (Cousin and Baudin-Laurencin 1985) to as

late as 90 dah in Atlantic halibut (Luizi et al. 1999). However, the development of gastric glands is not necessarily accompanied by the onset of stomach activity since morphology does not always mean functionality. An asynchrony between the morphological development of the gastric glands and their functionality has been reported for several species: around a week in summer flounder *Paralichthys dentatus* (Huang et al. 1998), 10 days in red porgy (Darias et al. 2005) and pike perch *Sander lucioperca* (Ostaszewska 2005), and several weeks in common whitefish *Coregonus lavaretus* (Mähr et al. 1983).

In higher vertebrates, gastric glands are mainly located in the fundic region of the stomach (Stevens and Hume 2005), whereas in fishes, several authors have reported the existence of interspecific differences in the localization of gastric glands. In some species, such as turbot (Segner et al. 1994), yellowtail flounder (Baglole et al. 1997), Dover sole (Veggetti et al. 1999), common pandora (Micale et al. 2006), and South American catfish *Rhamdia quelen* (Hernández et al. 2009), gastric glands are only found in the fundic region. In contrast, in gilthead sea bream (Elbal and Agulleiro 1986), white sea bream (Ortiz-Delgado et al. 2003), amberjack *Seriola dumerili* (Grau et al. 1992), European sea bass (García-Hernández et al. 2001), spotted sand bass (Peña et al. 2003), and white, Siberian, and green sturgeons (Gawlicka et al. 1995, Gisbert et al. 1998; Gisbert and Doroshov 2003), gastric glands are observed in the cardiac region of the stomach. In pike perch (Ostaszewska 2005), gastric glands are located in the cardiac and fundic regions, whereas in Senegal sole (Arellano et al. 2001) and cobia (Faulk et al. 2007), gastric glands are observed along the mucosa of fundic and pyloric stomach regions. On the other hand, the stomachs of Atlantic halibut (Murray et al. 1994), tilapia *Tilapia* spp. (Gargiulo et al. 1997), and bay snook (Alvarez-González et al. 2008) are entirely glandular, suggesting that the stomach in these species is mainly involved in chemical rather than a combination of mechanical and chemical digestion of food items, as it is in other fish that show regional differentiation of the stomach (Zambonino-Infante et al. 2008).

1.3.5 Accessory digestive glands

The accessory digestive glands, the liver, pancreas, and gallbladder, are of significant importance for the nutrition and homeostasis of fish (Hoehne-Reitan and Kjørsvik 2004). The liver is the central digestive organ not only for nutrient metabolism, conversion, and transfer to peripheral tissues but also for the production of bile and detoxification of toxins from both endogenous (metabolites) and exogenous sources. The pancreas consists of an exocrine portion that secretes pancreatic juices (digestive enzymes) that are involved in the intestinal digestion of nutrients, and an endocrine portion, the so-called islets of Langerhans, that secretes hormones such as insulin, somatostatin, pancreatic polypeptide, and/or glucagon. A final organ associated with digestion is the gallbladder, which secretes bile produced by the liver and aids in the emulsification of ingested food (i.e., lipids) and increases intestinal pH.

Histologically, the **liver** is composed of the liver *lobuli* (Figure 1.5b,c). In vertebrates, the liver has a primary array based on hepatocytes, bile canaliculi, and sinusoids, and structural differences occur among species in the organization of the stroma and parenchyma. In teleost fish, hepatocytes are arranged in anastomosed laminae around the central vein. Using electron microscopy, different cell types may be identified in the hepatic tissue based on their cellular organelles, stored substances, and cell surface specializations (Takashima and Hibiya 1995). The main cell type in the liver is the parenchymal hepatocyte (often shortened to hepatocyte), while endothelial cells, fat-storing cells, Kupffer cells, mesothelial (serosa) cells,

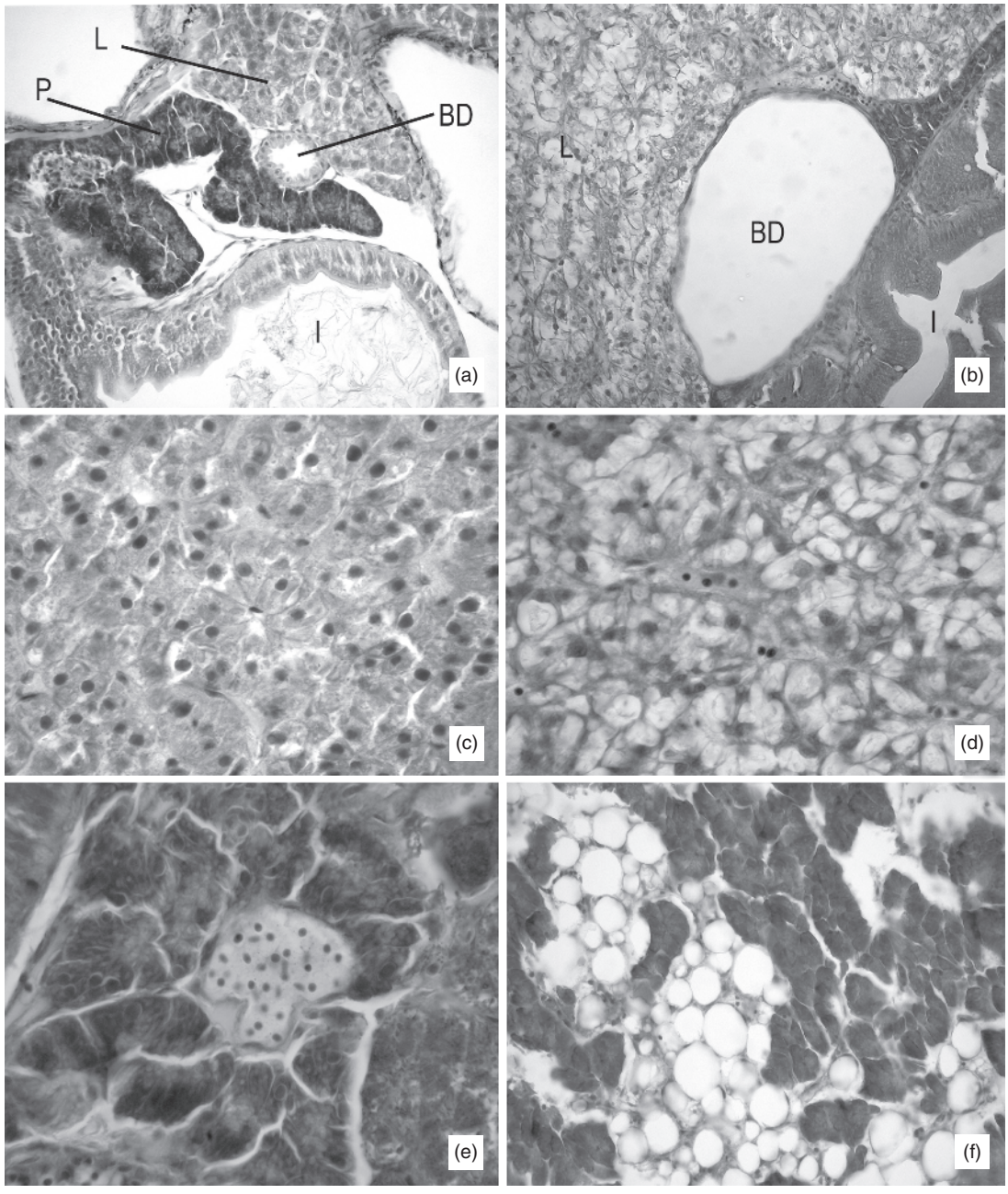

Figure 1.5 Histological sections of the liver and pancreas from California halibut (*Paralichthys californicus*) larvae. (a) Accessory digestive glands, liver and pancreas, in a 5-dph larva. Note the presence of the bile duct lined by a simple cuboidal epithelium (magnification: 200×; hematoxylin-eosin [H-E] stain). (b) Detail of the biliary duct in a 55-dph early juvenile. Note the increase in size of the biliary duct and the flattening of the epithelium (magnification: 200×; H-E stain). (c) Liver of a 35-dph larva with no lipidic inclusions. The liver appears as a compact tissue with basophilic polyhedral hepatocytes with centrally located nuclei (magnification: 400×; H-E stain). (d) Details of the liver of a 35-dph larva showing the large accumulation of lipids (unstained vacuoles) inside the hepatocytes that displaced nuclei to the periphery of the cell (magnification: 400×; H-E stain). (e) Endocrine pancreas (islet of Langerhans) surrounded by exocrine pancreatic tissue (magnification: 400×; H-E stain). (f) Exocrine pancreas with an infiltration of adipose tissue. Note the round shape of the adipocytes (unstained with H-E stain) and the peripheral position of their flattened nuclei (magnification: 400×; H-E stain). BD = biliary duct, I = intestine, L = liver, P = pancreas. (Photographs by E. Gisbert.)

and fibroblasts complement the basic liver architecture. The main stored substances in the fish liver are glycogen and, to a lesser extent, lipids. Eosinophilic and PAS-positive glycogen granules may be found scattered in the cytoplasm or aggregated forming large concentrations, and using electron microscopy they can be identified as rosette-like α particles and single β particles. For a detailed description of the ultrastructural characteristics of the above-mentioned cell types in adult fish, see Takashima and Hibiya (1995).

Timing of liver differentiation varies among species, and is mainly related to their general life history traits (Hoehne-Reitan and Kjørsvik 2004). As these authors stated, the timing of liver development clearly reflects the developmental state at hatching for different species. For example, the liver is already differentiated at hatching in Atlantic cod (Morrison 1993), haddock (Hamlin et al. 2000), common wolffish (Hoehne-Reitan and Kjørsvik 2004), white sea bream (Ortiz-Delgado et al. 2003), percula clownfish *Amphiprion percula* (Önal et al. 2008), tilapia (Morrison et al. 2001), and bay snook (Alvarez-González et al. 2008). In contrast, in some marine species, such as gilthead sea bream (Sarasquete et al. 1995), anemonefish (Green and McCormick 2001), California halibut (Gisbert et al. 2004a), common dentex (Santamaría Rojas et al. 2004), Atlantic halibut (Hoehne-Reitan and Kjørsvik 2004), common pandora (Micale et al. 2006), spotted sand bass (Peña et al. 2003), and the kelp grouper *Epinephelus bruneus* (Kato et al. 2004), and in freshwater species such as green sturgeon (Gisbert and Doroshov 2003), pike perch (Ostaszewska 2005), and European catfish (Kozarić et al. 2008), the liver develops after the larva emerges from the egg envelope during the endogenous feeding phase (Figure 1.5a).

Bile is secreted by the hepatic cells and is discharged into the extracellular bile canaliculi (Figure 1.5b). Bile canaliculi join to form the bile ducts, which subsequently converge into the hepatic duct. The latter leaves the liver and opens into the anterior intestine. In many fishes, the hepatic duct has a branch, the *ductus cysticus*, leading into the gallbladder, which stores bile juice. The walls of the bile ducts consist of a single layer of cuboidal to columnar cells over an underlying layer of connective tissue. The histological organization of the hepatic duct is similar but it includes a layer of smooth muscle (Takashima and Hibiya 1995). The histological development of the liver and the bile transport system develops concomitantly with the gradual maturation of hepatocytes, as well as their functional ability to synthesize, store, and mobilize carbohydrates and lipids (see review in Hoehne-Reitan and Kjørsvik 2004).

The exocrine part of the **pancreas** in teleosts is a diffuse organ, spread throughout the mesentery surrounding the digestive tract and other organs, and interspersed with adipose tissue (Figure 1.5e,f). Portions of it might also be distributed around major blood vessels within the liver of some species forming the hepatopancreas such as in cyprinids, characids, or some siluriformes among others (Takashima and Hibiya 1995; Petcoff et al. 2006), whereas some other species, such as anguillid eels, northern pike, or Japanese catfish, have a distinctive pancreas as is found in higher vertebrates (Hoehne-Reitan and Kjørsvik 2004). A functional exocrine pancreas is characterized by differentiated organ morphology, including developed excretory ducts and the presence of zymogen granulae for all major digestive enzymes.

Histologically, the arrangement of the pancreatic tissue is essentially similar and, in some aspects, resembles the basic architecture of the hepatic chords. Pancreatic secretory cells grouped into acini are deposited around blood vessels and form secretory functional units by the juxtaposition of adjacent cells. The secretory cells generally have a prismatic form with basal nuclei and peripheral heterochromatin and a prominent nucleolus. In light microscopic slides, the cytoplasm of secretory pancreatic cells is strongly basophilic, providing

a sharp contrast with the round, intensively acidophilic and eosinophilic zymogen granules. The ultrastructural characteristics of this type of secretory cell are described in detail by Takashima and Hibiya (1995). There is very little information on the histological development of the exocrine pancreas in fish larvae. According to Beccaria et al. (1991), the organogenesis of the exocrine pancreas can be divided into three distinct phases: appearance of a primordium at hatching in the form of a dorsal bud on the digestive tract; differentiation of the exocrine cells and appearance of the excretory ducts and blood vessels before mouth-opening; and growth of the organ during the larval and juvenile period. The quantitative growth after differentiation includes tissue size, an increase in the relative frequency of zymogen granules, and an increase in enzyme synthesis and secretion, while no new structural elements develop. In most of the described species, the exocrine pancreas is histologically differentiated at mouth-opening, as has been reported in Japanese flounder, Atlantic halibut, Atlantic cod, Senegal sole, bay snook, turbot, whitefish, and Siberian sturgeon, among others (Hoehne-Reitan and Kjørsvik 2004; Zambonino-Infante et al. 2008).

The fish **gallbladder** is an accessory digestive organ that stores and secretes concentrated bile. The bile has several functions, such as facilitating several digestive functions, eliminating conjugated metabolites in the liver (including xenobiotics), and participating in the enterohepatic bile circulation. The morphological interrelationship between the liver, the biliary system, and the gallbladder was extensively reviewed by Gilloteaux et al. (1996) and is not covered in the present review. Histologically, three layers are distinguished in the gallbladder of adult fishes: the inner layer, which is composed of a simple epithelium of columnar cells and connective tissue; the intermediate layer, which consists of smooth muscle; and the outer layer, which is the serous membrane (Takashima and Hibiya 1995). These layers are also distinguishable in fish larvae, although there are some ontogenetic differences regarding the type of epithelium lining the inner layer of the gallbladder and the level of development of the smooth muscle fibers that regulate the contraction of the organ. At early stages of development, the inner layer of the gallbladder is lined by a simple squamous epithelium that becomes cubical and columnar with age (Hamlin et al. 2000; Micale et al. 2006; Hachero-Cruzado et al. 2009).

Rodlet cells have been reported in the epithelium of the gallbladder and biliary ducts of some freshwater and marine teleosts. Different studies have shown that the presence of rodlet cells within the teleost gallbladder is species specific and may not necessarily depend on environmental conditions (e.g., pollutants) (see review in Hrubec and Caceci 2001). Considering the function of the liver in detoxification processes, Kramer et al. (2005) hypothesized that the abundance of rodlet cells within the gallbladder epithelium of fish exposed to environmental contamination indicates that this organ could serve as a storage or recruitment site for these cells and provide a portal through which rodlet cell secretions are deposited into the bile and carried away.

1.4 Ontogeny of the digestive enzymes

The development of adequate compound microdiets to replace live foods in the culture of marine fish larvae requires a thorough understanding of the digestion processes occurring during ontogeny (Cahu and Zambonino-Infante 1997; Lazo et al. 2000a). This knowledge is required for reducing the use of live feeds in the rearing of marine fish larvae. The lack of success in completely replacing live foods with compound microdiets from the onset of first feeding has been historically attributed to the presence of an

undeveloped digestive system at the time of hatching and consequent low digestive capacity (Lauf and Hoffer 1984; Munilla-Moran et al. 1990; Holt 1993), although most research to date indicates that marine fish larvae have a very defined and specific digestive physiology that merits the development of specific diets and weaning protocols, and that they possess a differentiated and effective digestive system early in development (Sarasquete et al. 1995; Ribeiro et al. 1999; Lazo et al. 2000a; Zambonino-Infante and Cahu 2001).

The conventional approach used for assessing digestive capacity in marine fish larvae has typically involved characterizing the morphological development of the digestive system and associated organs while also quantifying digestive enzyme activities using biochemical, histochemical, and molecular techniques (for an excellent review, see Zambonino-Infante et al. 2008). The morphological and functional development of the digestive system of fish larvae was first reviewed by Tanaka (1973) and Govoni et al. (1986), and more recently by Hoehne-Reitan and Kjørsvik (2004) and Zambonino-Infante et al. (2008). At hatching, the stomach is typically undifferentiated and nonfunctional. Acid digestion and pepsin expression are lacking, and the proton pump used to secrete HCl into the stomach lumen is not functional (Rust 2002; Gawlicka et al. 2001; Darias et al. 2005; Rønnestad et al. 2007). Most species also lack functional mouths and jaws, and the eyes are not yet pigmented. Early larvae typically possess a simple tubelike alimentary canal that is closed at both ends and lined with columnar epithelium. The alimentary canal undergoes rapid transformations during the transition to exogenous feeding. By the onset of first feeding, the alimentary canal has already developed into its different functional regions, but it is still less complex than in juveniles. However, the liver, pancreas, and gallbladder are usually present and functional (Hoehne-Reitan and Kjørsvik 2004). Digestion occurs in the midgut and hindgut, and nutrient absorption takes place through the apical region of the epithelium of each region, which is characterized by columnar cells (i.e., enterocytes). Alkaline proteases play a major role in digestion during the first days of feeding, while acid proteases become increasingly important toward the end of the larval period, concomitant with the appearance of a functional stomach (Lauf and Hoffer 1984; Lazo et al. 2007). As the developmental process progresses, oxynticopeptic cells in the gastric glands become functional, as suggested by the production of HCl through a functional proton pump, the expression of pepsinogen, and its activation to pepsin (Gawlicka et al. 2001). From the perspective of the digestion system, the transformation to the juvenile stage is complete once the stomach is fully differentiated.

High specific activity of digestive enzymes has been observed before the initiation of exogenous feeding in most species studied to date (Zambonino-Infante and Cahu 2001). This suggests the process of enzyme production is initiated by underlying genetic mechanisms (Buddington and Diamond 1989) rather than induced by the diet (Cahu and Zambonino-Infante 1994; Lazo et al. 2000a). While it appears that during the early stages of development digestive enzyme activities are controlled by gene expression rather than by feeding activity, diet composition can influence the maturation of the digestive system by triggering an onset or increase in the activity of some digestive enzymes (Zambonino-Infante and Cahu 2001). Feeding nutritionally unbalanced microdiets to marine fish larvae can disrupt the normal maturation process; the earlier the weaning onto unbalanced microdiets, the more negative the observed effect on maturation (Cahu and Zambonino-Infante 1994; Lazo et al. 2000a). In contrast, some nutrients, such as polyamines, can enhance the maturation and differentiation of the enterocytes involved in nutrient absorption. For example, sea bass (*Dicentrarchus labrax*) larvae fed a diet containing 0.33%

dry weight of the polyamine spermine displayed faster enterocyte maturation compared with fish fed a similar diet lacking in polyamine (Peres et al. 1997). Likewise, Tovar-Ramirez et al. (2002) included the polyamine-producing yeast (*Debaryomyces hansenii* HF1) in the diet of sea bass larvae and observed an increase in digestive enzyme secretion and earlier maturation of the enterocytes that were mediated by spermine and spermidine.

While most species can be effectively weaned onto microdiets before completion of metamorphosis, successful weaning during the early larval stages has proven more challenging (Kolkovski 2001). Only a handful of species can be reared on microdiets from the time of mouth-opening (i.e., red drum *Scieaenops ocellatus* and sea bass). Most species cultured to date require the use of rotifers or *Artemia* at some point during development (Cahu and Zambonino-Infante 2001).

As previously mentioned, early research suggested that problems associated with early weaning were due to low digestive enzyme activity or to the importance of live prey for aiding or triggering the digestive process. In contrast, recent studies indicate that enzymatic activity is high in early larvae, and that the potential contribution of digestive enzymes from the prey is negligible. Typically, enzymes for the luminal digestion of proteins (trypsin, chymotrypsin, and elastase, among others), lipids (lipases and phospholipases), and carbohydrates (amylases and maltases) are present in larvae before exogenous feeding commences or shortly thereafter. Their activity increases with age and length, although there are some exceptions (Alliot et al. 1980; Baragi and Lovell 1986; Cousin et al. 1987; Moyano et al. 1996; Baglole et al. 1998; Izquierdo et al. 2000; Zambonino-Infante and Cahu 2001; Lazo et al. 2007). Intracellular enterocyte digestive enzymes such as tri- and dipeptidases exhibit high levels of activity during the early larval stage and decrease as development progresses (Cahu and Zambonino-Infante 1995; Lazo et al. 2007). In contrast, the activity of intestinal brush border membrane enzymes such as aminopeptidases and alkaline phosphatases are lowest at first feeding and subsequently increase with age. A decrease in intracellular peptidase activity concurrent to an increase in brush border peptidase activity is indicative of the full intestinal maturity of marine fish larvae (Cahu and Zambonino-Infante 1994). The ratio of these two enzymes can be used as an indicator of the maturation of the digestive system in marine fish larvae and will be further described in the last section of this chapter. Thus, although not as complex as the juvenile digestive system, marine fish larvae possess a wide range of digestive enzymes that support the efficient digestion of nutrients if adequate feeds are provided (i.e., larvae can achieve very high growth rates in the wild and under culture conditions).

It has been proposed that exogenous enzymes from live prey could directly aid in larval digestion or activate the zymogens present in larval gut, thus increasing digestion and growth rates (Dabrowski 1979; Lauf and Hoffer 1984). The mechanisms through which exogenous enzymes could aid or stimulate the digestive process are not clearly understood. Moreover, the addition of exogenous enzymes to compound microdiets in the rearing of marine fish larvae has been shown to be beneficial only for sea bass larvae (for a review, see Kolkovski 2001). However, its benefits have not been conclusively demonstrated for other species. Moreover, several authors have reported a lack of significant differences in levels of pancreatic and intestinal enzymes in fish larvae reared with live prey or microdiets (Baragi and Lovell 1986; Cahu and Zambonino-Infante 1997; Lazo et al. 2000b), which indicates the ingestion of live prey does not stimulate enzyme production or secretion into the gut lumen. Kurokawa et al. (1998) estimated the relative contribution of exogenous enzymes to digestion in

Japanese sardine larvae (*Sardinops melanotictus*) and determined that it was only 0.6% of the total protease activity in the intestine, and therefore concluded that the contribution of the prey's enzymes to digestion was minimal. Similarly, Diaz et al. (1997a), using substrate-sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) to estimate protease activity in larval sea bream (*Sparus aurata*) and their live prey (rotifers), failed to detect proteases from the prey within the digestive tract. They suggested that the contribution of exogenous enzymes was limited to an autolytic process of the prey in the larval gut. Based on this data, it appears that the contribution of exogenous digestive enzymes to the total digestive capacity of the larvae is negligible in most species.

Since the lack of weaning success at an early date cannot be attributed solely to the absence of a functional stomach and lower digestive enzyme production, other factors have been conjectured to explain the lower performance on microdiets. These include low ingestion rates of the microdiets (Lazo et al. 2002) or the failure of microdiets to effectively stimulate digestive enzyme secretion (Cahu and Zambonino-Infante 2001). The latter would lead to low levels of enzymes in the lumen to digest feed particles. In combination with the relatively fast gut transit time typical of marine fish larvae (Govoni et al. 1986), this would effectively reduce the ability of the larvae to absorb the dietary nutrients necessary for meeting the requirements for normal growth. Recent research has begun to shape a more comprehensive understanding of the development of the digestive system by focusing on the study of the hormonal mechanisms controlling the expression and secretion of digestive enzymes and their modulation through dietary nutrients (recently reviewed by Rønnestad et al. 2007). For example, many compounds present in live feeds have the potential for influencing digestive enzyme activity in fish larvae. Polyamides, algal growth regulators that play multiple roles in stabilizing the intracellular conformation of nucleic acids and membranes (Mathews and van Holde 1990; García-Jimenez et al. 1998), have been shown to stimulate gut hormone (cholecystokinin [CCK]) release in rats, which in turn mediates the release of pancreatic enzymes (Fioramonti et al. 1994). Most formulated diets designed for marine fish larvae contain large amounts of fish meal, which is naturally low in the polyamide spermine (Bardocz et al. 1993). The addition of spermine to microdiets fed to sea bass larvae has been shown to increase pancreatic enzyme secretion and induce earlier intestinal maturation (Peres et al. 1997). In addition, amino acids may increase the secretion of certain hormones, such as somatostatin and bombasin, which also stimulate the secretion of pancreatic enzymes (Chey 1993; Kolkovski et al. 1997). Live feeds contain large amounts of free amino acids, which may stimulate the secretion of trypsin (Dortch 1987; Fyhn 1993). For example, Cahu and Zambonino-Infante (1995) reported increased trypsin secretion in sea bass larvae fed a mixture of free amino acids in their diets.

Both neural and hormonal processes are involved in regulating the secretion of pancreatic enzymes (Fange and Grove 1979) and is discussed in detail in Chapter 9, but a brief description is presented here. The sight, smell, or presence of food triggers a nervous control mediated by the vagus nerve that results in the induction of pancreatic secretion. Hjelmeland et al. (1988) induced secretion of trypsinogen from pancreatic tissue into the intestine of herring larvae (*Clupea harengus*) by feeding polystyrene spheres with no nutritional value. Similarly, Pedersen and Andersen (1992) were able to enhance the secretion of pancreatic enzymes by increasing the size of the inert particles fed to herring larvae. Additionally, gastrointestinal hormones, such as CCK, play an important role not only in the stimulation of pancreatic enzyme secretion but also in gallbladder

contraction, intestinal peristalsis, and gut transit time in fish larvae (Rønnestad et al. 2007), all of which are important factors regulating the digestion process. In first feeding larvae, CCK production seems to be genetically hardwired, but in older larvae it can also be regulated by dietary factors such as protein levels and chain length (Cahu et al. 2004). However, distension of the gut wall is not a factor that triggers CCK production (Koven et al. 2002). This indicates that the secretion of pancreatic enzymes is regulated by mechanisms in addition to CCK production and requires further research.

1.5 Expression of digestive enzyme genes

Even though numerous studies have characterized the ontogeny of the digestive system of marine and freshwater fish species, knowledge of the nutritional requirements during the larval period still needs to be improved in order to formulate adequate microdiets for optimal larval rearing. In the last decade, new molecular tools have been used to complement the morphological, histological, histochemical, and biochemical approaches commonly used, allowing researchers to expand the knowledge of the mechanisms underlying the digestive physiology of fish larvae.

The ontogeny of the digestive system is a species-specific and genetically programmed process where digestive enzymes follow a spatiotemporal pattern of gene expression during the larval development. These processes can be influenced by the diet and directly impact nutrient digestion and absorption, and consequently, larval performance and growth. For these reasons, the first studies on digestive enzyme gene expression in larvae were made on species reared under standard conditions using live prey in order to provide the reference gene expression patterns of digestive enzymes for future nutritional experiments.

The expression pattern of digestive enzyme precursors is intimately associated with the degree of development of the organs that produce them. For instance, in the red porgy (*Pagrus pagrus*), the first signs of amylase, lipase, and trypsinogen gene expression were detected in newly hatched larvae, indicating that the enzymatic equipment of the exocrine pancreas is ready to produce the required enzymes for food digestion at the beginning of the exogenous feeding period (Darias et al. 2006, 2007a, 2007b, 2007c). In the next section, we review the most studied digestive enzyme genes during development of marine fish larvae.

1.5.1 Amylase

Alpha (α) amylase (4-α-D-glucan glucanohydrolase, EC 3.2.1.1) is classified as family 13 of the glycosyl hydrolases and is responsible for the hydrolysis of the α-1,4 glycoside bonds in glycogen and starch, and related polysaccharides and oligosaccharides containing three or more (1→4)-α-linked D-glucose units. The main function of the digested and assimilated products is to supply energy to the organism. Alpha amylase is produced as a zymogen granule by pancreatic acinar cells, and their presence is considered to be an indicator of the exocrine pancreas maturation in fish larvae (Cahu and Zambonino-Infante 1994; Cahu et al. 2004). The appearance of acidophilic zymogen granules has been detected after the first exogenous feeding in some species (*Sparus aurata*, Sarasquete et al. 1995; *Solea senegalensis*, Sarasquete et al. 1996; *Paralichthys dentatus*, Bisbal and Bengtson 1995; *Paralichthys olivaceus*, Kurokawa and Suzuki 1996; *Pagrus pagrus*, Darias et al. 2007a), before mouth-opening in others (*Dicentrarchus labrax*, Beccaria et al. 1991; *Sciaenops ocellatus*, Lazo et al. 2000a), or even from hatching (*Melanogrammus aeglefinus*, Hamlin et al. 2000). Zymogen granule detection coincides

with the first amylase activity detected in *Solea senegalensis* (Ribeiro et al. 1999), *Diplodus sargus* (Cara et al. 2003), *Sparus aurata* (Moyano et al. 1996), and *Dicentrarchus labrax* (Zambonino-Infante and Cahu 1994a). Additionally, *in situ* hybridization technique has revealed that α-amylase gene expression occurs in the exocrine pancreas (Darias et al. 2006). Although only a few studies have evaluated amylase expression during fish larvae development, it is possible to appreciate differences in the pattern of gene expression among species. For example, in sea bass larvae (*Lates calcarifer*), amylase expression increases early in development (i.e., 5 days posthatch [dph]) to subsequently decrease in later stages (Péres et al. 1996; Ma et al. 2004), while in winter flounder (*Pseudopleuronectes americanus*), amylase expression did not decrease until after metamorphosis (Douglas et al. 2000), and in the red porgy (*Pagrus pagrus*), a constant level of amylase gene expression was observed until 30 dph and subsequently decreased thereafter (Darias et al. 2006). In any case, a relatively elevated level of amylase expression during the first stages of larval development has been observed in most species studied to date, but the main physiological function of this activity has not been completely elucidated.

It has been suggested that the expression of amylase is genetically programmed and regulated at a transcriptional level during the early developmental stages (Péres et al. 1996; Ma et al. 2001; Zambonino-Infante and Cahu 2001). The detection of amylase expression from hatching in *Lates calcarifer* and *Pagrus pagrus* (Ma et al. 2001; Darias et al. 2006) supports the existence of a hereditary component in this process and also indicates that the predisposition to synthesize amylase before the commencement of the exogenous feeding phase is independent of the external diet. This could be a programmed mechanism to ensure sufficient levels of this enzyme to be ready for digestion at the beginning of exogenous feeding.

However, once exogenous feeding commences, amylase expression can be modulated by the quantity and quality of the food (Péres et al. 1996). The different patterns of amylase expression observed in most fish species studied (Péres et al. 1996; Douglas et al. 2000; Ma et al. 2001; Darias et al. 2006) suggest that the variations are mainly due to the rearing conditions, including diet composition, quantity of diet offered, and sampling time during development.

1.5.2 Bile salt–activated lipase (BAL)

BAL is considered one of the most important lipases in fish (Patton et al. 1977; Murray et al. 2003) since it acts on a wide range of substrates of wax esters and triacylglycerols rich in polyunsaturated fatty acids (PUFAs). These substrates are more resistant to hydrolysis by other pancreatic lipases (Chen et al. 1990).

For lipid hydrolysis, pancreatic BAL is secreted to the intestinal lumen and activated by bile salts. Subsequently, the intestine can absorb the resulting substances. Diaz et al. (2002) observed adequate levels of lipase activity, bile function, and intestinal absorption at the beginning of exogenous feeding in three fish species. BAL activity was detected at hatching in several fish species, suggesting adequate enzymatic equipment for lipid digestion at first feeding (Hoehne-Reitan et al. 2001a; Murray et al. 2003; Pérez-Casanova et al. 2004). However, differences in patterns and activity levels have been observed among species.

Several studies evaluated the activity of different lipases in fish, including phospholipases, pancreatic lipases, nonspecific lipases, and BAL (Izquierdo et al. 2000; Hoehne-Reitan et al. 2001a, 2001b; Cahu et al. 2003). Gjellesvik et al. (1992) and Iijima et al. (1998) purified and characterized the BAL of *Gadus morhua* and *Pagrus major*, respectively, and

four other studies describe the ontogeny of BAL expression during larval development (Hoehne-Reitan et al. 2001a; Murray et al. 2003; Pérez-Casanova et al. 2004; Darias et al. 2007a).

Similar to trypsinogen and α-amylase gene expression, BAL expression is specifically localized in the exocrine pancreas (Figure 1.6b). BAL expression was detected from hatching in *Melanogrammus aeglefinus* and *Pagrus pagrus* (Pérez-Casanova et al. 2004; Darias et al. 2007a), while from mouth-opening in *Pleuronectes americanus*, progressively increasing during larval development (Murray et al. 2003). In other fish species, BAL expression was shown to be sensitive to changes in diet composition (Pérez-Casanova et al. 2004; Darias et al. 2007a). Variation of BAL activity during larval development of *Psetta maxima,* evaluated by enzyme-linked immunosorbent assay (ELISA), showed a similar pattern to the expression pattern of BAL in *Pagrus pagrus* (Hoehne-Reitan et al. 2001a; Darias et al. 2007a).

The effect of dietary lipid quantity on BAL activity has been demonstrated by Hoehne-Reitan et al. (2001b). They demonstrated that increasing prey density increased ingestion rates and stimulated digestive enzyme synthesis. However, the researchers did not observe an increase in larval growth associated with the increasing ingestion rates. Additionally, they did not detect any positive effects on development associated with the lipid content of rotifers during the first stages of development. In contrast, Zambonino-Infante and Cahu (1999) showed that a diet with high lipid content improved larval development in European sea bass *Dicentrarchus labrax*. Morais et al. (2004) demonstrated for the same species that the use of different neutral lipid sources in the diet did not affect lipase at transcriptional level, in contrast to that observed by Zambonino-Infante et al. (1996) and Cahu et al. (2003). Péres et al. (1996) suggested that diet composition can affect expression of digestive enzymes at the transcriptional and translational levels. In this sense, more experiments using formulated microdiets with different nutritional composition need to be performed to increase the existent knowledge of the digestive physiology of fish larvae.

1.5.3 Trypsinogen

In fish, as in other vertebrate species, trypsinogen is the inactive form of trypsin, an important proteolytic digestive enzyme present early in larval development, when the gastric glands have not yet developed and there is no pepsin activity for acid digestion. The expression of trypsinogen is specifically localized in the exocrine pancreas (Murray et al. 2004; Darias et al. 2007a; Figure 1.6c,d). Douglas and Gallant (1998) described three different trypsinogens in *Pleuronectes americanus* with apparently diverse functions throughout the larval period. Trypsinogen 2 seems to be most important during digestive system development since it is the first detected and the most highly expressed (Murray et al. 2004).

There are few studies concerning trypsinogen during the larval development (Srivastava et al. 2002; Murray et al. 2004; Darias et al. 2007a). In *Paralichthys olivaceus*, trypsinogen 1 expression occurred at 1 dph (Srivastava et al. 2002), while in *Pleuronectes americanus*, the expression of trypsinogen 2 was observed 5 dph and displayed a maximum peak of expression during metamorphosis (Murray et al. 2004). In *Pagrus pagrus*, trypsinogen gene expression was detected from hatching and the maximum levels of expression occurred after first feeding and subsequently remained constant during the first month of development (Darias et al. 2007a). These authors observed a decrease in trypsinogen expression from 50 dph, suggesting a reduction of the importance of trypsin in the digestive process after metamorphosis to the juvenile stage.

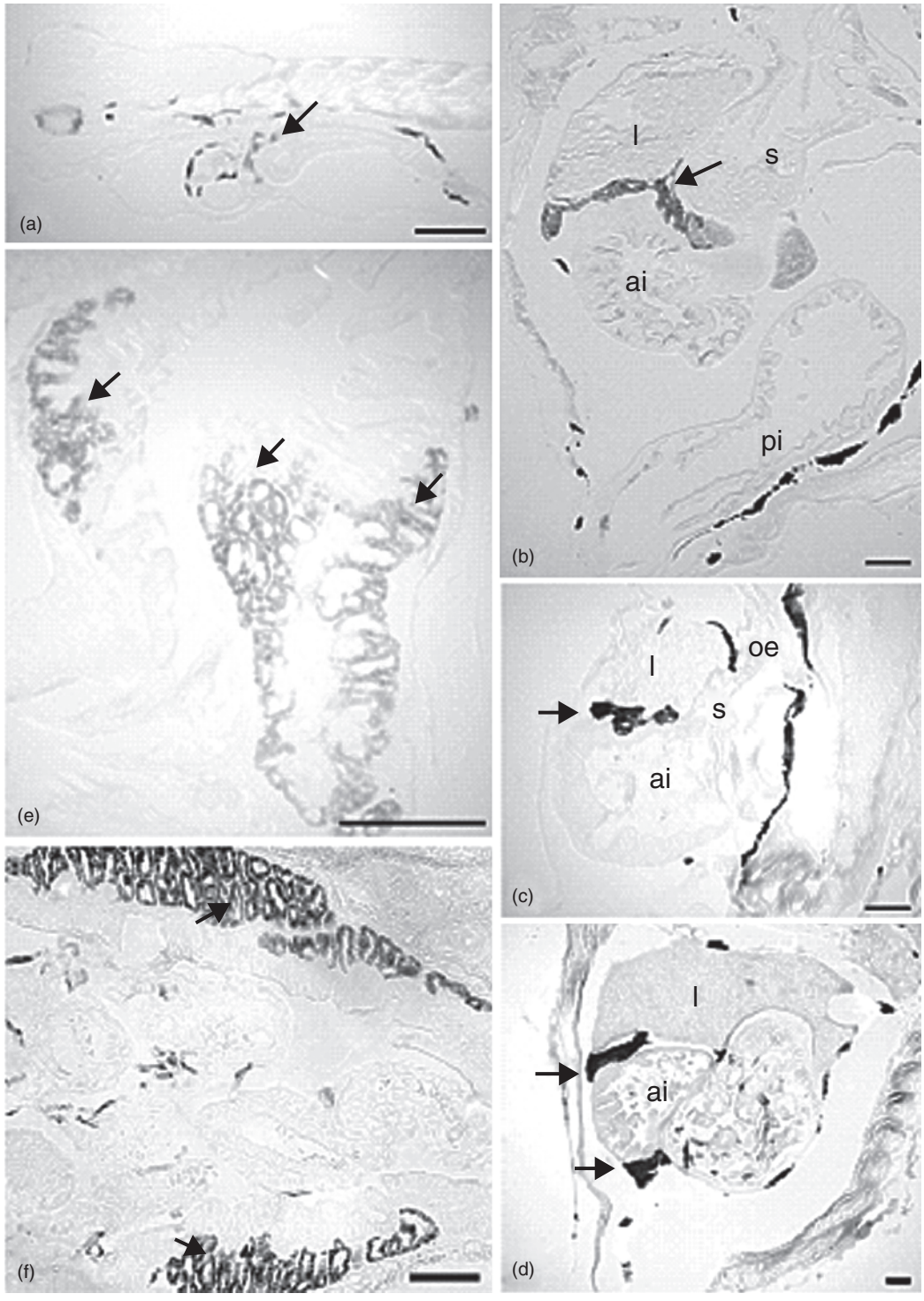

Figure 1.6 Localization of the gene expression of different digestive enzymes in developing red porgy (*Pagrus pagrus*) larvae by *in situ* hybridization. (a) Amylase gene expression at 2 dph in the exocrine pancreas. (b) Bile salt–activated lipase gene expression at 30 dph in the exocrine pancreas. (c, d) Trypsinogen expression at 9 and 35 dph, respectively, in the exocrine pancreas. (e) Proton pump expression at 40 dph in the gastric glands of the stomach. (f) Pepsinogen expression at 50 dph in the gastric glands of the stomach. ai = anterior intestine, oe = esophagus, l = liver, pi = posterior intestine, s = stomach. Scale bar = 100 µm.

1.5.4 Pepsinogen and proton pumps

In general, gastric gland development (Figure 1.4) is thought to indicate the transition from the larval to the juvenile stage (Kapoor et al. 1975; Govoni et al. 1986; Segner et al. 1994). Gastric glands produce pepsinogen and HCl secretion to the lumen of the stomach; an acidic environment is necessary to convert pepsinogen into pepsin. The α subunit of the proton pump (H^+/K^+-ATPase) is responsible for the maintenance of HCl production.

Histological detection of gastric glands does not imply that the glands are fully functional. In fact, the first signs of pepsinogen expression were detected 30 dph in *Pagrus pagrus*, 4 days after the complete formation of the gastric glands (Darias et al. 2007b). Huang et al. (1998) obtained similar results in *Paralichthys dentatus*, where they detected the expression of pepsinogen around 1 week after gastric gland formation. However, the first signs of gastric gland formation and pepsinogen expression occurred simultaneously in *Pleuronectes americanus* and initial pepsinogen expression occurred concurrently with the expression of the α-subunit of the proton pump (H^+/K^+-ATPase) (Douglas et al. 1999). Both pepsinogen and proton pump genes are expressed in the gastric glands and their expression progressively increases during maturation of the gastric glands and stabilizes at the juvenile stage (Douglas et al. 1999; Gawlicka et al. 2001; Darias et al. 2007b; Figure 1.6e,f).

The replacement of live prey by formulated microdiets for larval feeding is of fundamental interest for the marine fish larvae rearing industry. The development of a functional stomach is necessary to reach complete digestive capacity. However, it is important to mention that *Sparus aurata* and *Sciaenops ocellatus* are completely weaned before gastric glands are developed and become functional and are typically reared using a standard protocol (Lazo et al. 2000b; Elbal et al. 2004; Yúfera et al. 2004). These results imply that a functionally developed stomach is not required to adequately wean marine fish larvae with formulated microdiets. The study of the expression and function of genes associated with different proteolytic activities will help in understanding the real digestive capacities of developing larvae. Douglas et al. (1999) showed the existence of different types of pepsinogen in *Pleuronectes americanus*, which were expressed consecutively during larval development. It is interesting to note that pepsinogen IIa was expressed as early as 13 dph, before the gastric glands were formed. Several authors have found acid protease activity before stomach development even in species that never develop a true and functional stomach, such as the puffer fish *Takifugu rubripes* (Kurokawa et al. 2005). Therefore, a better understanding of the enzymes implicit in digestion and their functionality, in combination with knowledge of the natural diet of the larvae, will aid in optimizing the nutritional composition of formulated microdiets to be compatible with their digestive physiology. The timing and quantities of gene expression for the digestive enzyme precursors provide an insight into the larval digestive strategy. For instance, in *Pagrus pagrus*, the expression of trypsinogen was five times higher than all the other enzyme genes studied (such as amylase or BAL) and gives an idea of the importance of protein digestion in early developing larvae even though pepsin activity is not yet present (Darias et al. 2005).

In fish, as well as in amphibians, reptiles, and birds, pepsinogen and HCl are synthesized by one type of gastric cell called oxynticopeptic cells (Helander 1981) that possess characteristics of the oxyntic cell (HCl secretor cells) and zymogen cells (enzyme secretor cells) of mammals (Murray et al. 1994). Some authors suggest the existence of a unique cell type that has different morphologies depending on whether they produce HCl (light cells) or pepsinogen (dark cells) under a transmis-

sion electron microscope (Elbal and Agulleiro 1986; Arellano et al. 1999). Gawlicka et al. (2001) confirmed that only one type of cell, the oxynticopeptic cell, is responsible for the synthesis and expression of the different types of pepsinogens and proton pump. They also showed that the mucous neck cells of the stomach epithelium of *Pleuronectes americanus* had HCl, but not pepsinogen, secretory function. This may be due to the need of fish, in an aqueous medium where they continuously drink water, to increase the HCl concentration to counteract the dilution and neutralization of gastric juices (Kapoor et al. 1975). However, in *Pagrus pagrus*, the gene expression of the proton pump was exclusively localized in the gastric glands (Darias et al. 2007b), in contrast to that found in *Pleuronectes americanus*. Since the latter species ingests bigger prey, it might need a higher concentration of HCl and pepsin activity to adequately digest its food. Nevertheless, more studies of the neck cells of the mucosa are needed to test whether there are structural and functional differences among species. Gawlicka et al. (2001) and Darias et al. (2007b) reported that the expression of pepsinogen and proton pump occurred simultaneously in *Pleuronectes americanus* and *Pagrus pagrus*, respectively. The simultaneous secretion of pepsinogen and HCl could be a physiological strategy for promoting fast conversion of pepsinogen into active pepsin (Bal and Ghoshal 1992). In *Pagrus pagrus*, the expression of both genes begins 30 dph and have similar copies of mRNA that increase with larval development. However, in *Pleuronectes americanus*, the expression of pepsinogen is constant from 20 dph onward (Douglas et al. 1999).

Ongoing studies of the molecular mechanisms underlying the gastrointestinal functions of fish larvae reared under different nutritional and rearing conditions will help improve our understanding of the digestive physiology of commercially important fish species.

1.6 Assessing the nutritional condition of fish larvae: histological biomarkers and digestive enzymes

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 nonoptimal 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 and 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 of reliability, sensitivity, time response, size and age specificity, field versus laboratory estimates, processing time, costs, and requirements. These 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, digestive and metabolic enzyme activities), histological criteria, or various combinations of the abovementioned methods (Ferron and Leggett 1994; Catalan 2003; Gisbert et al. 2008; see also the discussion in Chapter 14). Although there are a wide variety of nutritional condition indices, this section will only cover those related to digestive system organization (histological biomarkers) and function (pancreatic and intestinal enzyme activities).

1.6.1 Histological biomarkers

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 1.1).

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

Table 1.1 Cellular criteria used to grade tissues and assess the nutritional condition in teleost larvae.

	Grade (condition)		
Tissue	**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 25–50% 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

Data rewritten from Margulies (1993), Catalan (2003), and Gisbert et al. (2004b).

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 suboptimal feeding or starvation in several fish species (Ferron and Leggett 1994; Catalan 2003; Gisbert et al. 2008). However, Catalan and Olivar (2002) reported that cell heights of the posterior intestine in 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 vary 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 very low-density lipoproteins (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. 1997b). 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.

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 1.1). 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 among 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 gallbladder) and a decrease in glycogen and lipid deposits stored in the hepatocytes. The liver is also a good biomarker for the 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 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 with 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 1.1).

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

1.6.2 Digestive enzymes

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 and Clemmesen 1992; Lamarre et al. 2004; Cara et al. 2007) to intestinal brush border and cytosolic enzymes (Zambonino-Infante and Cahu 2007; Zambonino-Infante et al. 2008).

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 3 or 4 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, like free amino acids (Zambonino-Infante and Cahu 1994a, 1994b) or some nonbiodegradable particles (Pedersen and Andersen 1992), can enhance pancreatic secretion, 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, for example, 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 and Morais 2007); 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 et al. 2001; 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 the 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 leucine-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; consequently, disparity between diet composition and larvae digestive features may delay or prevent the genetically programmed sequence of intestinal development (Zambonino-Infante and 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 and Cahu 1994a). 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, independent 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.

1.6.3 Gene expression

Results of digestive enzyme 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 constitutes 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 been 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 enzyme 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 the 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 enzyme 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 transcription and translation in European sea bass larvae (Péres et al. 1996). 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 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 mykiss*) fed a hyperglucidic diet compared with 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 larval nutritional condition.

1.6.4 Indirect methods for assessing nutritional condition

The nutritional condition of a fish larva can 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 and Lewis-McCrea 2007). Recent studies have demonstrated that the degree of fish larval ossification is influenced by diet (see Chapter 7) and is an adequate indicator of larval quality. The ossification status has been shown to be correlated with osteocalcin gene expression (Mazurais et al. 2008; Darias et al. 2010a). This gene is specifically localized in bone and constitutes the most specific marker for bone mineralization (Lian and Stein 1995). Moreover, its expression level can be correlated with dietary levels of several nutrients, thus providing a suitable 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, transient receptor potential cation channel, subfamily V, member 6 (TRPV6) expression, which codes for the most important intestinal Ca^{2+} transporter, can be modulated by dietary vitamin D_3 levels, consequently affecting intestinal maturation and therefore larval development (Darias et al. 2010b). Low levels of vitamin mix have been shown to induce skeletal malformations correlated with the modulation of genes involved in osteoblast determination and differentiation such as bone morphogenetic protein 4 (BMP4), insulin growth factor 1 (IGF1), and peroxisome proliferator–activated receptor γ (PPARγ) (Mazurais et al. 2008). Similarly, inadequate dietary retinol levels alter morphogenesis through the modulation of homeobox protein Hox-D9 (Hoxd9) and retinoic acid receptor γ (RARγ) gene expression, provoking a variety of skeletal deformities (Villeneuve et al. 2006; Mazurais et al. 2009).

Genomic research technologies such as microarrays appear to be useful tools not only for studying mechanisms to explain phenotypes but also for exploratory interest, which is useful in the search for markers. With the application of the recent advances

in genomics research, studies of larval fish nutrition will advance rapidly, improving our capabilities to assess the nutritional status of fish larvae under different nutritional and rearing conditions. Such resources will contribute to the ultimate goal of understanding digestive capabilities during ontogeny in fish larvae that can lead to successful weaning to microdiets.

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