Lipids are an important source of metabolic energy and of essential fatty acids (EFA), structural components of biological membranes and precursors of essential metabolites (e.g. eicosanoids) (Sargent et al., 1989). These functions are particularly important during the fast growing and developing larval stages, which have high demands for energy and structural components. Therefore, given the importance of dietary lipid utilization for the success of larval rearing, a great deal of attention has been paid during the last years to larval lipid nutrition. Qualitative aspects, especially the requirements for EFA and their effects on survival, growth, development and pigmentation have been extensively studied in marine fish larvae (Rainuzzo et al., 1997; Sargent et al. 1997, 1999). Dietary requirements for phospholipids (PL) have also been relatively well studied (Coutteau et al., 1997). However, other aspects of lipid nutrition have been more neglected – in particular, relatively few studies have examined quantitative lipid needs and the effect of total lipid levels and source in larval diets, while aspects such as digestion, absorption, transport and metabolism of dietary fatty acids (FA) have only started to be investigated in fish larvae (Izquierdo et al., 2000).

1. Marine larviculture: constraints and perspectives

Aquaculture represents one of the fastest growing food producing sector, with a yearly growth of ten percent from the late 1980s to the late 1990s, while livestock meat production has only been growing at around three percent over the same period. In contrast, a 3.4% reduction in global fisheries catches occurred in the decade from 1989 to 1998. During the past fifty years, from 1950 to 2001, the aquaculture contribution to total fish production has increased from 3.2% to over 34%. In terms of global aquaculture production, the production of marine fishes is still small compared to other aquatic products, but has reached 1.2 million tons (1.8% of total fish production, including marine fisheries capture), corresponding to USD

5.3 billion, in 2003 (statistics from FAO, Fisheries Global Information System). With the current trends of global population growth and with the world's natural fisheries resources being fully exploited (many are already overexploited), aquaculture faces a great challenge in the next few decades to meet the expected increasing deficit in fish supplies.

One of the major bottlenecks for the expansion of marine fish culture has been the production of suitable amounts of good quality larval and juvenile fish. A main constraint for the development of larviculture has been an insufficient information on the nutritional needs of the larval stages, which are characterized by extremely high growth rates (10-100% per day; Conceição, 1997) and developmental demands, that imply very high nutritional and metabolic requirements. Larvae follow a pattern of trophic ontogeny, changing diet with increasing size, resulting from changes in digestive requirements (Govoni et al., 1986). It is clear that the quantitative and qualitative dietary requirements of marine fish larvae are greatly different from those of adult fish but, except for EFA, very little is known about the specific requirements of larvae (Person Le Ruyet et al., 1993). In addition, there are important differences in what concerns the morphology and physiology of the digestive tract, as well as in larval metabolism. Therefore, the particular morphological and physiological characteristics of the larval digestive system, as well as the changes occurring during the larval ontogeny and in response to dietary regime, must be considered as they will clearly affect dietary nutrient utilization and limit the type of diet that should be offered. However, few studies have examined the dietary influence on the regulation of gastrointestinal tract development, enzymatic and hormonal response.

In nature, larval nutritional requirements are satisfied by an enormous diversity of natural prey, whereas in culture conditions larvae are fed on a limited number of prey organisms - mostly rotifers and *Artemia* and more rarely copepods. The widespread use of rotifers and *Artemia* in marine hatcheries is mostly explained by their easiness of culture in large densities and maintenance in artificial conditions, as well as good acceptability by the larvae.

Nevertheless, important disadvantages are that their nutritional composition is deficient, variable and difficult to manipulate in a controlled manner. In addition, the production of live preys represents a significant expenditure in terms of manpower, infrastructure, time and energy. Furthermore, eventual problems of shortage in the supply of *Artemia* cysts may become critically limiting to the expansion and intensification of aquaculture production (Sorgeloos et al., 2001). Therefore, in the last decades, the problems inherent to the production of live feeds and to the manipulation of their nutritional profile has stimulated intense research in the development of inert diets for marine fish larvae. This would not only lead to a substantial reduction in infrastructure, labour and production costs but also permit the supply of a more convenient (off-the-shelf) and nutritionally consistent diet and allow more detailed and controlled studies on the nutritional requirements of fast growing larvae (Person Le Ruyet et al., 1993). In the last few years considerable progress has been achieved in the development of inert diets (Koven et al. 2001; Cahu et al. 2003; Yúfera et al. 2003, 2005) and some prototype diets are now becoming more widely available. Nevertheless, in an industrial scale, and for the majority of the cultivated species, inert diets still lead to worse survival and growth than live preys, particularly when they are used in complete replacement of rotifers and *Artemia*. An inert formulated diet, in order to produce good larval survival and growth, has not only to be ingested (i.e., need for chemical and visual stimuli) but also meet the nutritional requirements of the larva. In addition, it must contain ingredients that may be easily digested and absorbed by the larva's digestive tract and promote an endocrine response (release of gastrointestinal hormones) that will stimulate enzyme secretion or activate zymogens in the larval gut (see Cahu and Zambonino Infante, 2001 and Kolkovski et al., 2001 for a complete review). Therefore, in order to successfully achieve the objective of complete replacement of live food by inert diets, a detailed understanding of the larval digestive physiology and how it may be influenced by the dietary components is indispensable.

This introduction will focus on aspects related to the lipid nutrition of marine fish larvae and therefore an overview of the present knowledge of the digestive and absorptive mechanisms in fish will be given.

2. Digestion and absorption of dietary lipids

Lipid digestion, absorption and transport have been insufficiently studied in fish, particularly in fish larvae. However, parallels have been found between the digestive and absorptive processes of fish and mammals and it is presumed that the mechanisms are generally similar (Sire et al., 1981; Honkanen et al., 1985). The process of lipid digestion can be divided in two distinct parts: (1) lipid digestion, which is the hydrolytic cleavage of dietary FA esters in the gastrointestinal tract, and (2) the absorption of the resulting FA into the enterocytes (Olsen et al., 1998). Within the gastrointestinal lipid digestion phase, three steps can be distinguished: 1) the dispersion of bulk lipid droplets into finely divided emulsion particles, 2) the enzymatic hydrolysis of fatty acids at the emulsion-water interface, and 3) the desorption and dispersion of insoluble lipid products into an absorbable form (Carey et al., 1983). There is a large volume of literature dealing in great depth with all aspects of mammalian and human lipid digestion and absorption (e.g., Borgström, 1977; Carey et al., 1983; Tso and Fujimoto, 1991; Nordskog et al., 2001). This section will briefly describe the main mechanisms based on the mammalian model, comparing it with what is known for fish in general, as no specific data exists for larval fish.

2.1. Digestion

In humans and most vertebrates the digestion of dietary lipids begins in the stomach, through the chemical action of gastric (acid) lipase, which is secreted by the gastric mucosa.

Gastric lipase digests triacylglycerols (TAG) but not PL or cholesteryl esters. Its main hydrolytic products are diacylglycerols (DAG) and FA. Given that dietary lipids may be retained in the stomach for some time, up to 30% of the total dietary TAG may be digested (Borgström, 1977; Carey et al., 1983; Nordskog et al., 2001). The stomach also plays a very important role in the mechanical emulsification of dietary fat, which is an essential prerequisite for the efficient hydrolysis by pancreatic lipase in the intestinal lumen. The mechanical emulsification of lipids is aided by the DAG and FA resulting from acid lipolysis, as well as by dietary PL, peptides from protein digestion, complex polysaccharides and membrane-derived PL, which function as emulsifiers in the acid milieu of the stomach (Borgström, 1977; Carey et al., 1983; Nordskog et al., 2001). However, in this aspect, substantial differences exist between fish and humans, as studies looking at the *in vivo* lipid digestion in cod and turbot have shown that gastric digestion appears to be insignificant (Lie et al., 1987; Koven et al., 1994ab; Izquierdo and Henderson, 1998). This is even more the case for fish larvae, which lack a functional stomach.

The bulk of TAG digestion occurs in the duodenum (Tso and Fujimoto, 1991). The lipid is in the form of crudely emulsified droplets when entering the small intestine, which are then mixed with fluids arising from the pancreas, gallbladder and small intestine. As a result of the abrupt rise in pH, the FA in the emulsion become partially ionized and migrate to the interface of the emulsion (Carey et al., 1983; Nordskog et al., 2001). At the same time, biliary lipids released from the gallbladder in the form of lipid micelles come into contact with the emulsion particles and are transferred to the surface of the emulsions. These two events markedly increase the surface of the emulsion and, together with the mechanical emulsification, create smaller stabilized particles. As the enzyme rich pancreatic secretions are released, pancreatic lipase-colipase, phospholipase A_2 and non-specific esterases hydrolyze both core and surface components. The digestion products create an increased

pressure in the emulsion surface, which causes the "budding off" of bilayered fragments, probably as unilamellar liposomes (Carey et al., 1983).

Honkanen et al. (1985), working with killifish, a stomachless saltwater fish species, pointed out several similarities in the digestive processes of fish and mammals, from the location of the major site of lipid digestion and absorption (proximal one third of the intestine), to the concentration of bile salts in the gallbladder, as well as the concentrations of bile salts and lipolytic products in the intestine lumen during lipid digestion. In some fish species, such as cod and Artic char, lipolysis and lipid absorption has been seen to occur mainly in the pyloric caeca and anterior ileum regions, where pancreatic enzymes are secreted and gastrointestinal contents are temporarily retained (Lied and Lambertsen, 1982; Lie et al., 1987; Olsen et al., 1998, 1999). The organization of the pyloric caeca may enhance lipid digestion by promoting a prolonged exposure of lipids to digestive enzymes (Gjellesvik et al., 1992). However, in other species, such as turbot, lipid digestion and absorption appears to take place mainly in the hindgut and rectum regions (Koven et al., 1994ab; Izquierdo and Henderson, 1998). The continuation of lipid digestion in the posterior gastrointestinal tract may be an advantage for certain fish species, particularly in strict carnivores with short digestive tracts (Koven et al., 1994a and references cited therein).

Two different neutral lipase activities have been identified in the pancreatic juice of higher animals – the classic pancreatic lipase or the pancreatic lipase-colipase system (bile salt inhibited and substrate specific) and the bile salt-dependent lipase or carboxyl ester lipase (non-specific and bile salt stimulated) (Borgström, 1977). In mammals, digestion of TAG is mostly due to pancreatic lipase, which is more active at the *sn*-1 and *sn*-3 positions of the TAG molecule, releasing 1,2-DAG, 2-monoacylglycerols (2-MAG) and free FA (FFA). This requires the presence of a cofactor, called colipase, that binds at the lipid-aqueous interface and provides a high affinity anchor site for the pancreatic lipase molecule which would otherwise be inhibited by the bile salts that induce its desorption from the lipid-water interface

(Borgström, 1977; Carey et al., 1983; Tso and Fujimoto, 1991; Nordskog et al., 2001). Although 1-MAG may be formed through isomerization of 2-MAG in an aqueous medium, this transformation is slower than the uptake of MAG by the small intestine and therefore the predominant form in which MAG is absorbed is as 2-MAG (Nordskog et al., 2001). Pancreatic bile salt-dependent lipase is also found, but in minor amounts in the mammalian pancreatic juice. It is a glycoprotein with broad substrate specificity that requires the presence of bile salts to hydrolyze insoluble FA esters and presents a relatively high affinity for DAG and particularly for esters of long-chain polyunsaturated FA (PUFA), which are more resistant to hydrolysis by pancreatic lipase (Borgström, 1977; Carey et al., 1983).

The exact nature of the neutral lipases found in fish is still not completely established as different studies of digestive lipolysis have yielded results that differ as to which types of lipases can be found. In addition, the existence of a diffuse pancreas in most fish species and the higher difficulty in obtaining purified enzyme extracts renders the investigation of digestive lipases in fish more challenging than in mammals (Tocher and Sargent, 1984). Brockerhoff (1966), when analyzing the isomeric forms of the glycerides found in the intestinal tract of cod during lipid digestion, initially assumed that fish neutral lipases were similar to the mammalian pancreatic lipase, with a specificity for 1,3 ester bonds. Léger et al. (1977) also found evidence of a mammalian type pancreatic lipase in rainbow trout, which appeared to be activated by a cofactor, but its optimum activity required bile salts. Léger and Bauchart (1972) had earlier suggested that trout lipase has a mechanism of action that differs from that of mammals, combining a 1,3 specificity with a specificity for the nature of the FA, which may superimpose each other. On the other hand, Patton et al. (1975), found significant complete hydrolysis (including 2-MAG) as well as a faster cleavage of C20 esters (which are normally resistant to pancreatic lipase) than of C18 esters in the intestinal extracts of several fish species. Tocher and Sargent (1984) reported that, during lipid digestion in rainbow trout, TAG was rapidly hydrolysed to FFA and 2-MAG, which may be further hydrolyzed but only

at a very slow rate, indicating a classical mammalian specificity for primary ester bonds. Even so, the enzymatic activity was dependent on the presence of bile salts and the presence of a cofactor similar to the mammalian colipase could not be shown. Other studies have also found the activity of neutral lipase to be bile salt-stimulated (Lie and Lambertsen, 1985; Ozkizilcik et al., 1996). In addition, the only lipolytic enzyme found in the pancreas and pyloric caeca of cod was a pancreatic bile salt-dependent or non-specific lipase that has been found to be homologous to the mammalian one (Gjellesvik, 1991; Gjellesvik et al., 1992). Therefore, several authors have described lipolytic enzymes in fish with characteristics differing from that of mammalian pancreatic lipase. This might be expected, given that a considerable proportion of the energy intake of marine fish is in the form of lipids rich in PUFA which are frequently located in the position 2 of the TAG molecule and that are not readily hydrolyzed, independently of position, by mammalian 1,3 specific pancreatic lipase (Gjellesvik et al., 1992; Iijima et al., 1998). Therefore, even if the presence of a 1,3 specific pancreatic lipase-colipase or even other types of lipase cannot be excluded, it is now commonly accepted that the major digestive lipase in teleosts appears to be bile-salt dependent and non-specific, producing mainly FFA and glycerol (Lied and Lambertsen, 1982; Tocher and Sargent, 1984; Lie and Lambertsen, 1985; Lie et al., 1987; Gjellesvik, 1991; Gjellesvik et al., 1992; Koven et al., 1994ab; Iijima et al., 1998; Olsen et al., 1998; Hoehne-Reitan et al., 2001ab; Murray et al., 2003; Perez-Casanova et al., 2004).

The FA specificity of lipolytic enzymes has been substantially studied and the effects of FA on the activity of pancreatic lipase are related to both the acyl chain length and degree of saturation (Brannon, 1990; Linscheer and Vergroesen, 1994). Gjellesvik (1991) has performed a detailed comparative study between human and cod bile salt-dependent lipase and determined that although the enzymes are homologous, there are minor but significant differences in FA specificity – while the human enzyme was found to be more effective in hydrolyzing shorter chain saturated FA esters (even though its major physiological substrates

are long chain FA), in cod it was more active towards esters of long chain PUFA (up to 22 carbon atoms). Since then, many other authors have reported a FA specificity with a preference for PUFA as substrate (regardless of position), followed by monounsaturated FA (MUFA) and finally saturated FA (SFA). Within MUFA and SFA, digestibility appears to decrease with increasing chain lengths (Austreng et al., 1979; Lie and Lambertsen, 1985, 1991; Gjellesvik, 1991; Koven et al., 1994b; Lie et al., 1987; Iijima et al., 1998; Olsen et al., 1998; Johnsen et al., 2000).

The digestion of dietary PL occurs entirely in the small intestine by pancreatic phospholipase A_2 (PLA_2), which acts at the *sn*-2 position to yield a lysophospholipid and a FA. PLA_2 is secreted as an anionic zymogen and is activated by a tryptic cleavage which has an absolute requirement for calcium ions. Its activity is also dependent on the presence of bile salts, at a 2:1 bile salt to phosphatidylcholine (PC) molar ratio for optimal activity (Carey et al., 1983; Nordskog et al., 2001). PLA_2 activity has also been found in several fish species, with similar activities and dependence characteristics as the mammalian form (Iijima et al., 1990; 1997; Ozkizilcik et al., 1996; Izquierdo and Henderson, 1998).

2.2. Absorption and intracellular metabolism

Until recently it was thought that the uptake of lipid digestion products by the enterocytes was through a passive diffusion process. The dispersed lipids, being bilayers themselves, are very soluble in membranes. Given their high intraluminal concentration, it is possible that lipids can move passively down their concentration gradient from the gut lumen via apical membranes of the enterocytes to the cytosol (Carey et al., 1983; Nordskog et al., 2001). However, findings by several investigators (reviewed by Nordskog et al., 2001) have raised the possibility that some lipids may be taken up via energy-dependent carrier mediated processes and have indicated the existence of a FA binding protein associated with the brush border membrane which plays a role in the absorption of FA (particularly of long chain FA)

by the enterocytes. Sire and Vernier (1981) suggested the presence of a transport protein with characteristics similar to the mammalian FA binding protein, which was used to explain the early absorption peak that was observed when trout were fed on a PUFA rich diet. The transport of lysophosphatidylcholine is also controversial – questions still exist as to whether it is passive or carrier-mediated (Tso and Fujimoto, 1991).

Once absorbed by the enterocyte, the products of lipid digestion migrate from the site of absorption to the smooth endoplasmatic reticulum (ER), where complex lipids are resynthesized, being deposited in large lipid droplets of the mucosal epithelial cells. In the enterocyte, 2-MAG and FA enter the monoacylglycerol pathway, in which 2-MAG are reacylated into TAG through the consecutive action of MAG acyl transferase and DAG acyl transferase. The enzymes involved in this pathway are known as the TAG synthetase complex and are located on the cytoplasmic surface of the smooth ER (Tso and Fujimoto, 1991; Nordskog et al., 2001). There is a second pathway in the intestinal mucosa that forms TAG, which is the α-glycerophosphate pathway. In this case, there is a stepwise acylation of glycerol-3-phosphate to form phosphatidic acid that is hydrolyzed to form DAG and then converted to TAG. PL, as well as cholesterol esters, may also be synthesized through this route. This pathway may occur in both the rough and smooth ER. The relative importance of the MAG pathway and the α -glycerophosphate pathway depends on the supply of 2-MAG and FA but the MAG pathway is normally the most important for the resynthesis of TAG. During normal lipid absorption, when 2-MAG is present in sufficient amounts, the MAG pathway is preponderant and inhibits the alternative pathway. Only when the supply of 2-MAG is lacking or is insufficient, the α-glycerophosphate pathway becomes the major route for the formation of TAG (Tso and Fujimoto, 1991; Nordskog et al., 2001). As for the absorbed lysophosphatidylcholine, a fraction is reacylated into PC which may rapidly appear in lipoprotein membranes on the enterocyte and be used for the transport of the lipids out of the cell as chylomicrons. The remaining is further hydrolyzed to glycero-3-phosphorylcholine

(which is then transported via the portal blood for use in the liver), glycerophosphate (partly reutilized to form TAG), glycerol and phosphate (Borgström, 1977; Tso and Fujimoto, 1991; Nordskog et al., 2001).

After the products of lipid digestion have been reconverted to TAG, they are packed with PL, dietary cholesterol and specific proteins (apoproteins) into large lipoprotein complexes, mainly chylomicron particles and very low density lipoproteins (VLDL), which consist of a hydrophobic core containing neutral lipids (TAG and cholesterol ester) and a hydrophilic coat with the more polar components (PL and protein). These are then collected in the lymphatic system, enter the blood vascular system through the thoracic duct and are transported to the liver and other tissues, for storage or energy production (Rogie and Skinner, 1985).

Studies performed on several fish species have shown that the digested FA are absorbed and reesterified into TAG in the intestinal cells, resulting in the production of a lipoprotein aggregate (chylomicron or VLDL-type) (Sire et al., 1981; Honkanen et al., 1985; Iijima et al., 1990; Lie and Lambertsen, 1991). Nevertheless, in fish, as a result of the non-specificity of pancreatic lipase, the hydrolysis of dietary TAG is mostly complete, resulting in the production of FFA and glycerol. For this reason, in teleosts, the α-glycerophosphate (or glycerol-3-phosphate) pathway appears to be the major pathway responsible for the biosynthesis of both TAG and PL. In carp (Noaillac-Depeyre and Gas, 1974) and trout (Bauermeister et al., 1979; Rogie and Skinner, 1981, 1985), chylomicrons are produced in the intestine in a manner that is basically similar to that of mammals, with TAG being synthesized in the smooth ER, processed in the Golgi system and finally discharged into the lateral and basal intercellular spaces and drained into the lamina propria. However, the precise role of the Golgi system during the absorption of dietary lipids in fish is still controversial, as movement into the intercellular spaces has also been reported to occur directly from the ER. Thus, the traffic of lipoproteins may follow two alternative pathways: (1) direct exocytosis

from the ER or (2) via the Golgi complex, as occurs in mammals (Noaillac-Depeyre and Gas, 1974; Sire et al., 1981; Vernier and Sire, 1986).

Fish are characterized by producing smaller lipoproteins with more surface constituents than their human counterparts. Due to the predominance of the α-glycerophosphate pathway, PL synthesis in fish is higher than that in mammals, for the same dietary intake of lipids. On the other hand, fish diets contain high concentrations of protein and the anterior intestine is capable of absorbing extensively the amino acids (AA) resulting from protein digestion. These facts, taken together, give a likely explanation as to why fish epithelial cells synthesize mainly particles closer to the size of VLDL than mammalian chylomicrons given that, in comparison with mammals, the rate of production of surface material for a given amount of dietary lipid is higher and thus the resulting lipoproteins are smaller (Sire and Vernier, 1981; Sire et al., 1981; Babin and Vernier, 1989). In addition, there is evidence that the size of the lipoprotein varies according to the nature of the ingested meal. Just as in mammals, the degree of saturation of the micellar FA affects the nature of the lipoprotein particles and increasing the unsaturation of dietary lipids increases the lipoprotein size. Thus, after feeding SFA, the particle size is that of VLDL, while feeding unsaturated FA results in the production of chylomicron-type particles (Sire et al., 1981; Olsen et al., 1999). Furthermore, the intracellular pathways of TAG and PL reacylation may be affected by the nature of dietary lipids (Sire and Vernier, 1981; Izquierdo et al., 2000; Olsen et al., 2000a) which, in turn, will determine the amount of lipoprotein surface material being produced (Sire and Vernier, 1981). For instance, van Greevenbroek et al. (1995), working with Caco-2 cells derived from a human colorectal carcinoma, noted that palmitic acid (16:0) increased PL synthesis, while the presence of linoleic acid (18:2n-6) in the culture media led to higher amounts of TAG being synthesized. Olsen et al. (2000a) found that replacing a part of dietary linseed oil by 16:0 reduced significantly lipid droplet accumulation in the enterocytes of Artic char, while Pérez et al. (1999) noted that different radiolabelled FA incubated *in vitro* with trout

enterocytes showed a different post-absorptive reesterification fate, particularly concerning PL synthesis. Nevertheless, in a recent experiment conducted by Oxley et al. (2005), the replacement of fish oil by vegetable oil in the diet of Atlantic salmon did not affect significantly the lipid class composition of the gut mucosa or the activity of the enzymes MAG acyltransferase, DAG acyltransferase or DAG cholinephosphotransferase, which are involved in the intestinal reacylation of digested lipid into TAG or PL.

3. Dietary lipid imbalances

The intensive larval rearing of the majority of marine fish species continues to be based on the provision of cultured live food during the early larval stages, which commonly have a deficient nutritional composition, particularly in terms of EFA. To overcome this nutritional deficiency of *Artemia* and rotifers, enrichment products and protocols have been developed over the last decades to supply these zooplankton with sufficient levels of EFA (e.g., Rainuzzo et al., 1994, 1997; Rodríguez et al., 1996; Han et al., 2000). Therefore, in order to meet the EFA requirements of the larvae and thus achieve good growth, survival and larval quality, the general approach is to simulate the biochemical composition of the larva's natural diet (marine plankton), which is particularly rich in (n-3) PUFA. However, the natural marine phytoplankton and zooplankton is composed mostly of (n-3) PUFA-rich PL, while many of the enrichment methodologies use lipid sources rich in TAG, which have a lower (n-3) PUFA content compared to polar lipids (Sargent et al., 1989). Consequently, in order to supply high levels of EFA, live prey fed to larvae may in some cases have an excessive total lipid content, with a high neutral lipid composition. In fact, a few studies have reported poor larval growth, even when the EFA composition of the diets appeared to meet larval requirements (Pousão-Ferreira et al., 1999). In order to explain these results, the authors have hypothesized that

quantitative and qualitative imbalances in the macronutrient composition of the diet, particularly neutral lipid excess, may adversely affect larval digestion and absorption ability (Kjørsvik et al., 1991a; Pousão-Ferreira et al., 1999; Izquierdo et al., 2000; Olsen et al., 2000b; Hoehne-Reitan et al., 2001a; Gisbert et al., 2005) resulting in poor growth and larval performance. Additionally, Gara et al. (1998) suggested that the overall level of lipid intake may have a significant influence on flatfish metamorphosis success.

On the other hand, larvae fed TAG rich diets commonly show an accumulation of lipid vacuoles in the basal zone of the enterocytes, which indicates a good digestion and absorption of dietary TAG but a reduced transport capacity (Diaz et al., 1997; Fontagné et al., 1998, 2000; Salhi et al. 1999; Izquierdo et al., 2000). Loewe and Eckman (1988) reported a lipid accumulation in the epithelium of the intestine of coregonid (*Coregonus fera*) larvae, which was particularly intense between 7 and 13 days after start feeding, but diminished after two weeks. The authors claimed that this was due to a lower basal transport rate across the membrane of the enterocyte during the earlier period of larval development, which becomes more efficient later on. It was suggested that even if the morphological appearance of the intestinal cells remains unchanged, the "biochemical machinery" or the intracellular compartments responsible for the assembly of the lipoproteins might be less developed in the early larval stages (Loewe and Eckman, 1988). Deplano et al. (1991) and Sarasquete et al. (1995) also observed that in first feeding European seabass and gilthead seabream there was an accumulation of lipid droplets in the enterocytes that decreased in a later larval stage and explained this by a low capacity for lipoprotein synthesis in early stages as well. Nevertheless, similar lipid accumulations have been reported in adults of many fish species and they are probably a natural occurrence resulting from slow lipid processing, where there is a temporal separation between FA absorption and secretion by the intestinal tissue (Noaillac-Depeyre and Gas, 1974; Sire and Vernier, 1981). In poikilothermic fish there are differences between the rate of luminal lipid digestion and diffusion of FA into the gut epithelia, which are not

affected by water temperature, and the rate of reacylation into TAG and lipoprotein synthesis, which are slowed down by low water temperatures (Austreng et al., 1979; Sire and Vernier, 1981). Noaillac-Depeyre and Gas (1974) observed two types of lipid inclusions in the enterocytes of the carp: small lipid particles or lipoproteins (<100nm in diameter) that normally accumulate in the intercellular spaces and were implicated in the direct transport of absorbed FA into the blood circulation, and lipid droplets (that may reach several microns in diameter), which seem to act as temporary sites of lipid storage. However, unbalanced diets may lead to even greater lipid accumulation in the form of lipid droplets by enhancing the discrepancy between the rates of absorption and lipoprotein synthesis.

In terms of dietary qualitative lipid imbalances, the effect of PL deficiency has been quite extensively studied (Coutteau et al., 1997), while changes in lipid assimilation and transport (more specifically an abnormal lipid accumulation in the gut enterocytes) due to imbalances in the EFA in the diet, and in particular to an insufficient level of docosahexaenoic acid (DHA), has only been recently reported in larval Striped trumpeter (Bransden et al., 2005). A beneficial effect of dietary PL supplementation has been observed on the larval growth and survival of several fish species (Kanazawa et al., 1983; Kanazawa, 1993; Geurden et al., 1995). One observed effect of dietary PL supplementation in gilthead seabream has been an increase in diet ingestion rate, suggesting that PC may act as an age-dependent attractant (Koven et al., 1998; Izquierdo et al., 2001; Hadas et al. 2003). A role of PL in enhancing lipid emulsification, in the absence of sufficient levels of larval bile salts, had also been suggested by Koven et al. (1993) but could not be substantiated in subsequent studies (Geurden et al., 1995; Fontagné et al., 1998; Hadas et al., 2003). The more important effect of dietary PL appears to be related to its role in lipid transport from the enterocytes into the body tissues (Hadas et al., 2003; Fontagné et al., 1998; Olsen et al., 1999; Salhi et al., 1999). Numerous studies have shown that larval diets deficient in PL lead to the accumulation of large amounts of lipid vacuoles in the enterocytes, probably due to insufficient lipoprotein synthesis (Diaz et

al., 1997; Fontagné et al., 1998, 2000; Olsen et al., 1999; Salhi et al., 1999; Izquierdo et al., 2000). Lipoprotein production by the enterocyte involves a complex sequence of biosynthetic events that bring together specific apoproteins, PL (mostly PC), cholesterol, cholesterol ester, carbohydrate and reesterified TAG (Vernier and Sire, 1986). Iritani et al. (1984) reported that the α-glycerophosphate acyltransferase activity of fish is extremely low compared to other animals and it is now well established that marine fish, particularly in the larval stages, have a limited capacity for endogenous "de novo" PL biosynthesis, which may be insufficient to maintain an optimal rate of lipoprotein synthesis. Therefore, a specific dietary requirement for dietary polar lipid for the transport of lipids through the intestinal membranes (by enhancing lipoprotein synthesis) has been suggested (e.g., Geurden et al., 1995; Fontagné et al., 1998; Olsen et al., 1999; Salhi et al., 1999; Hadas et al., 2003). In addition, a higher dietary requirement for PL is also probable in the earlier larval stages, to sustain the fast growth and organogenesis which likely require a high rate of membrane synthesis and turnover. However, in what concerns its role in lipoprotein assembly and lipid export, there is probably not an absolute dietary requirement for PL, as it should depend on the total dietary lipid level (including neutral lipid), as well as on the dietary FA composition (if different FA are used preferentially for TAG or PL biosynthesis). For instance, in carp larvae, Fontagné et al. (2000) observed that 2% PL supplementation in a high lipid diet (12% total lipid) was not sufficient for the optimal absorption of neutral lipid, while the same PL level was efficient in the lower lipid diets (6-8%) tested by Geurden et al. (1995). So far, no studies have examined the balance between total neutral and polar lipid levels, in order to establish optimal ratios for FA digestion and absorption, enabling an optimized energetic and structural utilization of the dietary total lipid fraction.

4. Larval digestive and absorptive capacity

Fish larvae are characterized by dietary requirements and digestive systems that differ from that of adults (Govoni et al., 1986). For instance, not only the enzymatic capacity but also the mechanisms of regulation of the pancreas, seem to differ between larvae and adults (Kurokawa and Suzuki, 1996). It was originally believed that marine fish larvae have a poorly developed digestive system and immature digestive mechanisms, which was used to explain the nutritional problems in rearing fish larvae, particularly on artificial diets (Lauff and Hofer, 1984; Walford and Lam, 1993). The larval gastrointestinal tract, although functional, in that it can process prey encountered in the wild at first feeding, lacks a stomach and remains structurally and functionally less complex than in adults and this lower complexity was often associated to a lower digestive enzyme production (Govoni et al., 1986; Rust, 2002). This apparent lack of complexity is a puzzling issue that has fascinated many researchers, considering that fish larvae must be remarkably efficient in utilizing their diet, in order to meet their extremely fast growth and high developmental needs.

Several studies have shown significant levels of pancreatic and intestinal digestive enzymes at the onset of exogenous feeding (Cousin et al., 1987; Izquierdo et al., 2000; Hoehne-Reitan et al., 2001b) and have found nutrient absorption capacities even before complete absorption of yolk reserves (Pedersen et al., 1987; Loewe and Eckman, 1988; Kjørsvik et al., 1991b; Segner et al., 1994; Diaz et al., 1997; Calzada et al., 1998; Ribeiro et al., 1999a,b). In addition, the liver, pancreas and gall bladder are differentiated at hatching and functional before first feeding in many fish species (Govoni et al., 1986; Boulhic and Gabaudan, 1992; Segner et al., 1994; Bisbal and Bengtson, 1995; Kurokawa and Suzuki, 1996; Ribeiro et al., 1999a). Morphological observations of the digestive tract just after the start of exogenous feeding revealed the presence of lipid vacuoles in the larval intestine of several species (e.g., Dover sole, Summer flounder, seabream, Senegalese sole), showing

evidence of efficient absorption of dietary FA (Boulhic and Gabaudan, 1992; Bisbal and Bengtson, 1995; Sarasquete et al., 1995; Ribeiro et al., 1999a).

The ultrastructural characteristics of the enterocytes of pre-feeding coregonid, turbot, European seabass and gilthead seabream larvae were described as being essentially the same as in adult fish (Loewe and Eckman, 1988; Segner et al., 1994; Diaz et al., 1997; Calzada et al., 1998; Elbal et al., 2004) and the existence of functional lipid absorption structures, such as well developed ER and Golgi apparatus at the time of first feeding has been noted (Deplano et al., 1991; Segner et al., 1994; Diaz et al., 1997). Therefore, Segner et al. (1994) considers that the enterocytes are not only cytologically differentiated but also physiologically functional at first feeding and suggests that in the subsequent stages of ontogenetic development the intestine experiences mainly quantitative changes, such as an increase in length and mucosal surface area, as well as an intensification of the activities of brush border enzymes. Nevertheless, Deplano et al. (1991) noted a lower development of the ER and Golgi system, compared to later stages, and considered that the enterocytes are still not completely developed in European seabass at the start of exogenous feeding. In addition, as mentioned above, there are a few suggestions that, despite the relatively normal morphological appearance of the enterocytes, the earlier larval stages may have a low capacity for lipoprotein synthesis, which becomes increasingly effective in more advanced stages of larval development (Loewe and Eckman, 1988; Deplano et al., 1991; Sarasquete et al., 1995; Elbal et al., 2004). This might be due to a higher availability of surface material for lipoprotein synthesis as a result of an increase in PL synthesis capacity and/or an improved protein digestion in later stages of development (Elbal et al., 2004). Deplano et al. (1991) hypothesized that in the more advanced stages of larval development the capacity for cellular intestinal absorption may be even greater than in the adult, which would explain their rapid growth in spite of the apparently lower differentiation of their digestive tract.

Few studies have been performed regarding the development of lipase activity during larval ontogeny. Lipase activity was detected as early as 2 days after hatching (DAH) - corresponding to first feeding - in the brush border and cytoplasm of enterocytes of Senegalese sole (Ribeiro et al. 1999a). In red drum, lipase specific activity was detectable at hatching, peaked on day 3, just prior to first feeding, and subsequently decreased (Lazo et al., 2000). Similarly, Hoehne-Reitan et al. (2001b) noted that bile salt-dependent lipase (BSDL) is present in turbot immediately after hatching and increased at the time of first feeding, while in haddock BSDL transcripts were noted at hatching and increased during the first 4 days (Perez-Casanova et al., 2004). In gilthead seabream (Izquierdo et al., 2000) and winter flounder (Murray et al., 2003) larvae, BSDL activity was detected at first feeding and increased significantly during the first days of seabream larval development. Changes in lipolytic activity during the ontogeny of Striped bass were studied by Ozkizilcik et al. (1996), who found low activities of TAG hydrolase, PLA_2 and wax ester (WE) hydrolase until 5-6 DAH. At the time of hatching, PLA_2 was the dominant lipolytic enzyme and with the onset of feeding all enzymes increased significantly, although less drastically for PLA_2. TAG hydrolase became the lipolytic enzyme with the highest activity after feeding was initiated. In addition, feeding stimulated the enzymatic activity of TAG hydrolase and PLA_2, whose activity was significantly higher in fed, compared to unfed larvae. The authors have estimated that first feeding Striped bass larvae are able to digest 47% of their daily lipid ingestion – more specifically, 17, 71 and 94% of dietary TAG, WE and PL, respectively.

There are thus strong indications that fish larvae are capable of efficiently digesting and absorbing lipids from the start of exogenous feeding. However, there is very little information concerning the maximal digestive and absorptive capacity for lipids and FA in marine fish larvae and how it may be affected by compositional differences of lipids in the diet (Planas and Cunha, 1999).

5. Dietary stimulation of lipase activity

In the larvae of fish species studied so far, the exocrine pancreas is functional and pancreatic and intestinal enzyme activity is detected before first feeding, suggesting that enzymatic capacity is not induced by the diet (Ribeiro et al., 1999ab; Hoehne-Reitan et al., 2001b; Zambonino Infante and Cahu, 2001). Therefore, as in mammals (Henning, 1987), the temporal pattern of development is genetically determined, at least in the early larval stages, although this may change latter in development. In turbot larvae, for instance, BSDL activity appears to follow an ontogenetically programmed pattern of development during the initial larval period, up to 5 DAH, while a dietary effect started to be noticeable at 7 DAH, when the BSDL content increased exponentially in fed larvae and decreased in starved larvae (Hoehne-Reitan et al., 2001b). In Pacific threadfin larvae a similar stimulation of lipase activity by feeding and a decrease in unfed larvae was also observed at the time of first feeding (Kim et al., 2001).

Nevertheless, relatively few studies have examined the influence of dietary composition on the regulation of digestive enzymatic activities, gastrointestinal tract function and development in fish. Still, diet quality appears to have a direct effect on the onset of the maturation processes of the digestive tract (Cahu et al., 1995; and see also Cahu and Zambonino Infante, 2001; and Zambonino Infante and Cahu, 2001; for a thorough review). In particular, dietary lipids have been observed to induce an earlier maturation of enterocytes in European sea bass (Zambonino Infante and Cahu, 1999) and red drum larvae (Buchet et al., 2000). In addition, total dietary lipid content has been reported to stimulate lipolytic enzymes both in mammals (Wicker and Puigserver, 1989; Brannon, 1990; Spannagel et al., 1996) and in fish (Borlongan, 1990; Zambonino Infante and Cahu, 1999). Studies looking at the quantitative lipid supply in marine fish larvae have described a stimulation of the lipolytic activities of pancreatic lipase and PLA_2 by an increase in their substrate level in the diet, until

a plateau is attained (Zambonino Infante and Cahu, 1999; Buchet et al., 2000). The existence of these plateaus of lipolytic enzyme expression thus suggests that there is a maximal capacity for enzyme synthesis (Buchet et al., 2000; Zambonino Infante and Cahu, 1999, 2001). In white sturgeon, an increased activity of the brush border lipase was shown histochemically in larvae fed higher lipid diets (Gawlicka et al., 2002). Nonetheless, in turbot larvae no stimulation of lipase synthesis was obtained by an increase in dietary lipid level (Hoehne-Reitan et al., 2001a) and it was hypothesized that the effect might depend on the larval stage of development or that there may be a lower or higher threshold of dietary lipid content in order to induce a change, as claimed by Brannon (1990). Moreover, it has been suggested that the intestinal receptors for intraluminal stimulants require only a very low concentration for activation, meaning that the length of the intestine exposed to these products is the main determinant of pancreatic secretory response (Singer, 1987). If this is the case in marine fish larvae, the ingestion rate of the diet may also play a major role in determining lipase activity. The results of Hoehne-Reitan et al. (2001a), showing that the BSDL content of turbot larvae was not significantly affected by the lipid level of the prey, but appeared to be a function of the ingestion rate, are in agreement with this theory. In addition, Perez-Casanova et al. (2004) also suggest that BSDL synthesis in haddock larvae might be stimulated by the amount of ingested prey.

The quality of the dietary lipid source is also an important parameter that may affect gastrointestinal tract development and digestive enzyme activity, although few studies have focused on this. TAG are generally the quantitatively most important lipid class in fish diets and their constituent fatty acid composition, in terms of carbon chain length and the degree of saturation, has been shown to affect digestion and absorption (Austreng et al., 1979; Linscheer and Vergroesen, 1994; Koven et al., 1994b; Olsen et al., 1998; Johnsen et al., 2000). Izquierdo et al. (2000) reported that neutral lipase activity is influenced by the FA

composition of dietary lipids, as gilthead seabream larvae showed an increased lipase activity when they were fed rotifers containing TAG rich in 20:5n-3 instead of MUFA.

Additionally, endocrine factors may also be quite important in mediating a lipase activity response to dietary FA level and composition but, so far, have not been examined in fish. Dietary lipid has been shown to influence the release of cholecystokinin (CCK) and secretin, which are gastrointestinal hormones involved in the stimulation of pancreatic enzyme secretion (Singer, 1987; Brannon, 1990; Spannagel et al., 1996). The chemical nature of the FA, particularly their carbon chain length, is known to affect also CCK secretion in humans and other mammals although the results obtained by various authors with diverse species reveal that different animals may show a discriminatory sensitivity to different FA (Hopman et al., 1984; Douglas et al., 1990; Matzinger et al., 2000). For instance, in humans, medium chain TAG (MCT) are a weaker stimulus to CCK secretion than long chain TAG (Hopman et al., 1984; Matzinger et al., 2000) and it is hypothesized that this is the result of the rapid absorption of MCFA, with only a small part of the upper intestine being exposed to these FA (Hopman et al., 1984). On the other hand, in rats, MCT were found to be the most potent stimulators of CCK secretion (Douglas et al., 1990) and it has been suggested that a relatively accelerated rate of digestion and absorption of MCFA may allow these metabolites to interact more rapidly with receptors in the gut or in the circulatory system (Denbow et al., 1992). A stimulatory role on the relative synthesis of lipase has also been described for secretin, whose plasma level increases in response to the products of lipid digestion (Brannon, 1990). In fish, evidence is starting to accumulate that CCK has the same functions as in mammals, namely in the stimulation of pancreatic digestive enzymes secretion and bile release (gallbladder contraction) (Aldman et al., 1992; Holmgren, 1993; Aldman and Holmgren, 1995; Einarsson et al., 1997), in slowing down gastric emptying (Holmgren, 1993; Olsson et al., 1999) and in the control of food intake (Himick and Peter, 1994; Gélineau and Boujard, 2001). CCK producing cells have been identified in larval stages of several fish (Garcia-Hernandez et al.,

1994; Reinecke et al., 1997; Kurokawa et al., 2000; Kamisaka et al., 2001, 2002ab, 2003). In Japanese eel (*Anguilla japonica*) larvae, CCK mRNA was expressed in the larval intestine at the onset of exogenous feeding and it is presumed that CCK controls pancreatic enzyme secretion from first feeding (Kurokawa et al., 2004). There are thus indications that CCK participates in the regulation of the digestion of larval stages of Japanese eel, Atlantic halibut, Atlantic herring and European seabass (Koven et al. 2001; Rojas-García and Rønnestad, 2002; Cahu et al., 2004; Kurokawa et al., 2004). However, except for preliminary work on the CCK-mediated stimulation of pancreatic secretion by proteins and AA (Koven et al., 2002; Rojas-García and Rønnestad, 2002; Cahu et al., 2004), the adaptation of pancreatic secretion to other dietary components in fish remains completely unknown.

6. Dietary lipid and food intake

The most commonly proposed explanation for the lower larval growth which is sometimes obtained with high lipid diets has been related to an overload of the digestive and absorptive capacity caused by excess lipid, as discussed above. However, another alternative or additional possibility is that the higher dietary lipid level, resulting in more energy-dense diets, may induce a lower food intake and consequently an insufficient intake of protein and essential nutrients (e.g. highly unsaturated FA; HUFA) and micronutrients. This possibility has been suggested (Conceição et al., 1998; Pousão-Ferreira et al., 1999; Gawlicka et al., 2002) but has not been directly tested in marine fish larvae. In addition, the qualitative dietary lipid composition might affect food intake through influences on digestion and absorption processes and gut clearance rate. Influences of the chemical nature of the dietary FA at the hormonal level, such as effects on the release of the gastrointestinal hormone CCK, which is involved in the control of gastric emptying (Holmgren, 1993; Olsson et al., 1999) and food

intake (Himick and Peter, 1994; Gélineau and Boujard, 2001), as discussed above, may potentially be involved.

In spite of the lack of studies at the larval stage, the effect of dietary total lipid level on food intake has been one of the most frequently addressed questions in juvenile and adult fish nutrition studies, where it is normally aimed to partly replace protein, which is the most expensive component of fish diets, by a cheaper energy source, but without inducing negative effects on growth and flesh quality (i.e., adiposity). A regulation of food intake based on dietary digestible energy content has now been well established in a variety of fish species, meaning that fish are able to adjust their ingestion to meet a certain set energy level (Lee and Putnam, 1973; Marais and Kissil, 1979; Boujard and Médale, 1994; Santinha et al., 1999; Ogata and Shearer, 2000; Yamamoto et al., 2000; Sæther and Jobling, 2001; Gélineau et al., 2001, 2002; Boujard et al., 2004; Skalli et al., 2004). A few exceptions have been found (e.g., Alanärä, 1994), being suggested that a minimum difference in energy content between the diets is required in order to induce a regulation of feeding activity. In addition, a regulation of food intake to meet a set energy requirement may not occur when the diet has such a low energy density that stomach volume becomes limiting or if very high energy-density diets are ingested in larger amounts to avoid an under filled stomach (Lee and Putnam, 1973; Ogata and Shearer, 2000). In these cases, as discussed in Gélineau et al. (2001), stomach fullness may be responsible for the short-term food intake regulation in fish. On the other hand, if a reduced feed intake of a high lipid diet results in an insufficient supply of other important nutrients, such as essential AA, vitamins and minerals, fish may subsequently increase their food intake to meet their requirements for other essential nutrients (Yamamoto et al., 2002).

A lower ingestion of high lipid (i.e. high energy) diets may therefore result in a lower intake of protein, which is in fact the building block for growth. However, in most cases, growth is similar to that obtained with a lower lipid diet, as a result of a protein-sparing effect, meaning that the energy from lipid spares protein for deposition (rather than being used for

energy production), leading to better growth performance and food utilization efficiency (Lee and Putnam, 1973; Marais and Kissil, 1979; Boujard and Médale, 1994; Santinha et al., 1999; Ogata and Shearer, 2000; Yamamoto et al., 2000; Sæther and Jobling, 2001; Gélineau et al., 2001, 2002; Boujard et al., 2004; Skalli et al., 2004). In fish larvae nothing has been documented concerning the existence of a potential protein-sparing effect. Results from the adult can not be extrapolated given that fish larvae, as already discussed, have much higher requirements for energy and structural components, while dietary lipid (particularly EFA) is also extremely important for early organ development and for physiologically demanding processes such as metamorphosis. These differences in requirements, coupled with changes in the structure of the gastrointestinal tract, as discussed above, are likely to result in a dissimilar metabolism. Therefore, given the potential impact of these factors in larval growth, studies in this area are essential, as they are likely to contribute to major improvements in the productivity of marine hatcheries.

7. Aims and outline of this thesis

The attempt to meet larval requirements using poor sources of EFA (predominantly neutral lipids) may result in excessive lipid content and in an imbalanced lipid class composition of the diet, and a number of authors have reported poor larval growth associated with a high lipid content of the diet (Kjørsvik et al., 1991a; Hoehne-Reitan et al., 2001a; Pousão-Ferreira et al., 1999; Izquierdo et al., 2000; Olsen et al., 2000b; Gawlicka et al., 2002). Different explanations have been proposed but the exact mechanisms behind these observations have not been completely clarified. Several effects of high dietary neutral lipid levels which may have a potential negative influence on larval growth can speculated: at the digestion and absorption level, assuming that a high dietary lipid content may eventually result in a lower

digestive effectiveness or decreased activity of digestive enzymes and in a reduced absorption efficiency, and at the ingestion level, if a lipid-rich diet results in a lower food and thus protein intake (i.e., if a mechanism of regulation of food intake according to dietary energy level exists in larvae, as in juvenile and adult fish). In this thesis, work was carried out on commercially valuable species (Atlantic herring, Senegalese sole, European seabass and gilthead seabream), with the objective of investigating the effects of neutral lipid level and lipid source on some of these key factors influencing larval growth.

The work described in **chapter 2** was conducted in order to establish a radiolabeling methodology for *Artemia*, to allow the measurement of live food intake in larval fish and enable studies on the relationship between larval ingestion rate and the neutral lipid level and FA composition of the prey. In addition, given that a large proportion of the ^{14}C-label was incorporated into the protein fraction of the *Artemia*, it was found that the developed methodology has also potential applications in the study of larval digestive capacity towards dietary protein. In **chapter 3**, the tube feeding of a pure lipid was tested, while the capacity of Atlantic herring (*Clupea harengus*) larvae to digest, absorb and metabolize lipids was analyzed after tube feeding a ^{14}C-lipid mixture and incubating the larvae in metabolic chambers. The absorption efficiency of a TAG (triolein; TRI) was compared with that of oleic acid (OA), which is the FA that composes the TAG in its free form (i.e., not requiring digestion). In addition, the process of lipid digestion was observed and described through the analysis of video images. **Chapter 4** examines the effect of formulated diets differing in neutral lipid level (7.5 or 15% of oil in microdiet) and lipid source (fish oil, triolein and coconut oil) on growth and survival, body biochemical composition and lipase specific activity and mRNA level of European seabass (*Dicentrarchus labrax*) larvae. The main objective of these experiments was to verify whether the total amount and FA composition of dietary lipid may influence lipase activity and synthesis and if this effect may in turn have a significant impact on growth. The aim of the work reported in **chapters 5-7** was to study the

effect of high neutral lipid diets in growth, gut histology, nutrient digestion and absorption efficiency, digestive enzymatic activity (only in chapter 7) and larval FA composition (only in chapter 7) of Senegalese sole (*Solea senegalensis*) larvae. In these experiments, it was attempted to induce an accumulation of lipid droplets in the enterocytes by feeding the larvae on high neutral lipid diets, to investigate whether these lipid vacuoles may affect the absorption and metabolism of dietary FA (**chapters 5 and 7**) and AA (**chapter 6**). This was tested by tube feeding several ^{14}C-lipids (TAG or PL) or ^{14}C-FFA (stearic acid, OA or DHA) or by feeding the larvae on ^{14}C-*Artemia*, radiolabelled as described in **chapter 2**, followed by incubation in metabolic chambers, up to 24 h after feeding. In **chapter 5**, the tube feeding of OA in different forms (as a FFA or esterified to either TAG or PL) and of different FFA allowed examining how the form in which a FA is supplied in the diet may affect its utilization and therefore its bioavailability and whether differences may be expected in FA absorption and metabolism according to its chain length and degree of saturation. In **chapters 5 and 6** Senegalese sole larvae were fed either *Artemia* enriched on a soybean oil emulsion (EA) or on non enriched *Artemia* (NEA), which were both deficient in EFA. However, the FA profile of these diets was very likely different and therefore the work described in **chapter 7** used *Artemia* enriched with higher and lower levels of the same lipid emulsion (i.e., with an equivalent relative FA profile) and compared two different lipid sources differing in FA composition – fish oil and soybean oil. Finally, **chapter 8** analyzes whether, in gilthead seabream (*Sparus aurata*) larvae, food intake may be regulated by total dietary neutral lipid level, as reported in juvenile and adult fish, and if this may be influenced by the lipid source (soybean oil or fish oil). In a first experiment, seabream larvae were reared on *Artemia* enriched on one of two levels of a fish oil emulsion, while in a second experiment larvae were co-fed *Artemia* enriched on one of two levels of soybean oil emulsion, together with a microdiet (MD) containing soybean oil, at a corresponding lipid level. Food intake and nutrient absorption were tested using *Artemia* enriched with fish oil emulsion and labelled

with ^{14}C-OA-liposomes (1st experiment) or using a MD containing soybean oil and labelled with either ^{14}C-TRI or ^{14}C-OA (2nd experiment). The dietary effects on growth and biochemical composition of the larvae were also analyzed. To conclude, the final chapter (**chapter 9**) reviews and discusses the results obtained in all the experiments, in view of the global aim of this thesis which was to clarify some of the effects of dietary neutral lipid level and source on lipid digestion, absorption and food intake and how these factors may affect larval growth, hoping to contribute to a better understanding of larval digestive physiology which will aid inert diet formulation in the future.

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