Marine fish larvae are generally reared on live preys, whose lipid content needs to be enhanced to meet the larval requirements for essential fatty acids (EFA). However, 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. In fact, a number of authors have reported poor larval growth associated with a high dietary lipid content and, although different explanations have been proposed, 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 hypothesized: 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 ultimate goal of this work is thus to contribute to a better understanding of the factors which may limit the optimum utilization of high dietary neutral lipid levels by marine fish larvae. A more detailed understanding of the larval digestive physiology and how it may be influenced by dietary lipid components is indispensable to enable better inert diet formulations in a near future.

In **chapter 1**, a general introductory overview of the present status of knowledge of different aspects of larval nutrition research, particularly concerning aspects of lipid digestion and absorption capacity and how these may be influenced by dietary composition, as well as of the effect of dietary lipid level and composition on food intake, is given.

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 fatty (FA) composition of the prey. *Artemia* were radiolabelled by adding a ^{14}C-protein hydrolysate into their hatching or enrichment media and the results showed that a large proportion of the ^{14}C-label was rapidly incorporated into the TCA precipitate (mostly protein) fraction, which means that the method has also potential applications in the study of *Artemia* digestibility, protein and amino acid (AA) metabolism in marine fish larvae. However, this method resulted in a high variability in the incorporation of labelled free AA by the *Artemia*, which made difficult an accurate estimation of food intake. Measurements of *Artemia* digestibility by Atlantic herring (*Clupea harengus*) larvae were made and revealed a fast and high capacity to digest *Artemia* and a considerable catabolism of the absorbed AA for energy production.

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 and metabolism of a triacylglycerol (triolein; TRI) was compared with that of oleic acid (OA), which is the FA that composes TRI in its free form (i.e., not requiring digestion). A better absorption was measured for the free fatty acid (FFA), but reducing the volume of tube fed TRI improved lipid absorption to a greater degree than OA, indicating a problem of lipid absorption at high doses. In addition, the process of lipid digestion was described through video image analysis, which revealed a considerable gut contractile activity that appeared effective in emulsifying and mixing the tube fed lipid, while signs of chemical degradation during lipid digestion were also noticed.

Chapter 4 examined 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, lipase specific activity and mRNA level in European seabass (*Dicentrarchus labrax*) larvae. Lipase activity was significantly affected by the source of dietary lipid but not by its quantity. Differences in the FA composition of the diet, related to the specificity of lipase towards FA differing in chain length and degree of saturation, may explain these results. The physiological digestive response to the fish oil-based diets was, however, age-dependent (i.e., different in 24 and 52 day-old fish), which strengthens the need for further studies at different stages of development, as not only nutritional requirements but also digestive function changes between early larvae and later larval/juvenile stages. Nonetheless, growth was not related with lipase specific activity, suggesting that lipase synthesis is not a limiting factor for growth, possibly as a result of a production in excess to dietary needs. Therefore, even if the results indicate a dietary effect on lipase specific activity, the physiological meaning of such a regulation still needs to be clarified.

The aim of the work reported in **chapters 5-7** was to study the effect of dietary neutral lipid level on growth, gut histology, nutrient digestion and absorption efficiency in 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**). 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). In both experiments, feeding sole larvae on a higher neutral lipid diet (EA), compared to NEA, resulted in a lower growth and in an increased accumulation of lipid droplets within the gut epithelium. In **chapter 5**, by tube feeding several ^{14}C-lipids (glycerol tri[1-^{14}C]oleate; TRI or L-3-phosphatidylcholine 1,2, di-[1-^{14}C]oleoyl; PC) or ^{14}C-FFA (OA, stearic acid; SA or docosahexaenoic acid; DHA), it was seen that feeding a diet richer in neutral lipid (EA) generally resulted in a lower FA absorption efficiency (higher label evacuation and lower retention of dietary FA). It thus looks as if the accumulation of lipid

droplets within the gastrointestinal mucosa may present a physical barrier to efficient lipid absorption, which in turn may explain the lower growth observed in larvae fed the higher neutral lipid diet. In addition, tube feeding of OA in different forms (as a FFA or esterified to either TRI or PC) and of different FFA allowed examining how the chemical form in which a FA is supplied in the diet may affect its bioavailability and whether differences may be expected in FA absorption and metabolism according to its chain length and degree of saturation. The results confirmed that, in fish, FA absorption efficiency increases with unsaturation, with the order of decreasing absorption efficiency being DHA>OA>SA. DHA was almost completely absorbed and mostly retained in the tissues, independently of the previous feeding regime, indicating that the accumulation of lipid droplets in the enterocytes may not be an obstacle for the efficient dietary utilization of EFA. Sole larvae had a significantly higher capacity to digested and absorb OA esterified to PC, compared to TRI, indicating that, in order to increase the bioavailability of dietary FA, it might be preferable to supply them as phospholipid (PL) rather than as triacylglycerol (TAG). This might be due to a more deficient emulsification and lower efficiency of neutral lipases, compared to phospholipases. Finally, sole larvae appeared to discriminate between the source of OA for its energetic utilization, as this FA in the free form or esterified to PC was significantly less catabolised than TRI. In **chapter 6**, feeding Senegalese sole larvae on ^{14}C-*Artemia* did not result in a significantly different total AA absorption efficiency, suggesting that the lipid inclusions in the enterocytes may not interfere with AA absorption. Nonetheless, the AA absorption rate appeared to be slower in larvae fed EA which, in continuously feeding larvae, may result in a slower clearance of the gut lumen with potential effects in the reduction of food intake. These two experiments had, however, the inconvenient of testing the dietary effect of two *Artemia* diets which differed in their relative FA profile. Therefore, further work was conducted (described in **chapter 7**) using *Artemia* enriched with higher and lower levels of the same lipid emulsion (i.e., with an equivalent relative FA profile) and comparing two

different lipid sources differing in FA composition – fish oil and soybean oil. In this experiment, differences in lipid accumulation within the gut enterocytes were also induced by the experimental diets, with a higher amount of lipid droplets being noticeable in the midgut epithelia of larvae fed *Artemia* enriched with higher fish oil (HF) and soybean oil (HS) doses, compared to the lower lipid treatments (LF and LS). The observed differences appeared to be directly correlated to the dietary quantitative supply of neutral lipid and an effect of dietary lipid source was not clearly noticeable. In addition, the results also confirmed earlier suggestions linking an increase in the total neutral lipid content of the diet and the accumulation of lipid droplets within the gut enterocytes with a decrease in the absorption efficiency of tube fed TRI (but not of OA). Nevertheless, the use of live preys enriched with different doses of lipid emulsion did not enable achieving important differences in total lipid level between the higher and lower lipid diets, which somehow limited the conclusions that could be drawn from this experimental approach.

In **chapter 8** it was analyzed 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). Two experiments were performed in which 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 (experiment 1) or using a MD containing soybean oil and labelled with either ^{14}C-TRI or ^{14}C-OA (experiment 2). In the first experiment, no significant differences were found in larval growth between larvae fed *Artemia* enriched on higher and lower doses of fish oil emulsion (HF and LF). However, larvae fed the HF diet showed a significantly higher food intake and a significantly lower nutrient absorption, supporting the notion of an inverse

relationship between food intake and nutrient absorption efficiency. Therefore, in this case, the total lipid level of the diet did not negatively affect food intake, as observed in older fish. On the other hand, in the second experiment, when larvae were co-fed a MD and *Artemia* enriched on two levels of soybean oil emulsion (HS and LS), a significantly higher dry weight was achieved by larvae fed on the LS diet, which was also significantly more ingested and absorbed. Thus, dietary lipid level significantly affected larval food intake and absorption efficiency but the effect was dependent on lipid source, suggesting that dietary FA composition might be a more determinant factor than total lipid level in the regulation of larval food intake. In addition, food intake did not appear to have been regulated to meet a requirement for EFA. One of the hypothesis that is suggested to explain the results is that the biochemical composition of the food, by influencing the digestive and absorptive processes, can indirectly affect the rate of gut clearance and, consequently, feeding rates. Other potential effects may be at the level of palatability and stimulation of neuroendocrine pathways.

To conclude, the final chapter (**chapter 9**) summarizes and discusses the results obtained during the course of this thesis. Aspects related to the digestion, absorption, transport and metabolism of dietary FA have only started to be investigated in fish larvae but the current perception is that the transport of lipid from the enterocytes into the body may be more of a problem in early larval stages than lipid digestion by itself. Although both factors are likely to intervene, the work presented here gives support to this idea, as the results seem to collectively indicate that the enzymatic capacity is not a limiting factor for fish larvae to deal with high lipid diets, while FA absorption might be reduced at higher dietary neutral lipid levels, particularly when it results in an enhanced accumulation of lipid droplets in the enterocytes. In spite of this, tube feeding free OA always resulted in higher label absorption than TRI, indicating that neutral lipases were not entirely efficient. In addition, the results obtained with Senegalese sole larvae appear to suggest that PL digestion may be more efficient, possibly as a result of a higher activity of phospholipases, compared to neutral

lipases. In terms of FA absorption, it was seen that not all FA are equally affected by the lipid accumulation in the enterocytes and mechanisms of specific absorption and preferential incorporation into structural tissues may exist to minimize EFA deficiency. Nevertheless, lipids are also an important source of metabolic energy and the supply of other FA in sufficient amounts is critical, particularly in fast growing larvae with high developmental energetic demands. Finally, it was also noted that, contrary to what has been observed in juvenile and adult fish, food intake does not appear to be strictly regulated by total energy content of the diet in seabream larvae and that the source of dietary lipid (and its FA composition) may have a more important role in controlling ingestion. Therefore, lipid level in diets for marine fish larvae may have an important impact in several factors influencing growth and development but clearly it cannot be dissociated of its FA composition, which appears to play a central role on the nutritional and physiological impacts of dietary lipid inclusion levels, in terms of ingestion, digestion and absorption.