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, in some cases, poor larval growth has been obtained concurrently with high lipid content and an imbalanced lipid class composition (neutral lipid excess) of the diet. The exact mechanisms behind these observations have not been completely clarified, but several effects of high dietary neutral lipid level, which can potentially have a negative influence on larval growth, may be speculated. In this thesis, a series of experiments were conducted with the objective of elucidating the mechanisms underlying and limiting nutrient (essentially lipid) digestion and absorption in marine fish larvae, as well as potential effects of dietary lipid level and fatty acid (FA) composition on food intake. The present chapter will discuss and summarize the main findings of this research, with the aim of better understanding the factors which may limit the utilization of high dietary neutral lipid levels by marine fish larvae. The ultimate goal of this work is thus to contribute to a better knowledge of larval nutritional physiology and therefore enable better inert diet formulations in the near future.

1. Methodological aspects

The tube feeding methodology had been employed previously to study the absorption and metabolism of water soluble amino acids (AA), peptides and proteins in fish larvae (e.g., Rønnestad et al., 2000, 2001; Rojas-Garcia and Rønnestad, 2003; Aragão et al., 2004) but had never been used to deliver lipids. Given the differences in chemical and physical characteristics of lipid and aqueous solutions, preliminary work was conducted (**chapter 3**) in order to test the tube feeding setup using pure lipid mixtures. For instance, the higher viscosity and density of lipids, in combination with the small capillary diameter, might have eventually posed a problem for tube feeding. In addition, some data was collected regarding

lipid digestion and absorption by Atlantic herring (*Clupea harengus*) larvae. It was observed that the tube fed lipid was highly and very rapidly evacuated and, in order to reduce this problem in the subsequent tube feeding studies, larvae were tube fed the lipid and FA labels after being allowed to feed on *Artemia* for a period of time (30 min.). In this case, the tube fed label represented a "supplement" to what would normally be a standard meal. This corresponds to a more natural situation, where the *Artemia* might stimulate the larval digestive system in several ways, as reviewed by Kolkovski et al. (1997) and Kolkovski (2001): 1) by stimulating larval digestive enzyme secretion or contributing with their own enzymes (although this is controversial, as some studies have measured a negligible contribution); 2) by supplying gastric hormones or stimulating the release of hormonal factors that can improve the activation of digestive processes; or, finally, 3) by mechanically stimulating the intestinal motor activity (e.g., peristaltic movements), which may in turn trigger other steps of the digestive process. In addition, prior feeding with *Artemia* will result in the presence of other nutrients in the larval gastrointestinal tract, such as phospholipids (PL), peptides and carbohydrates, which may aid emulsification of the neutral lipid.

The study described in **chapter 2** was carried out with the objective of developing a method that could later be used to quantify the larval intake of *Artemia*. However, it was found that this method, using a uniformly labeled ^{14}C-protein hydrolysate, resulted in a high variability in the incorporation of labelled free AA by the *Artemia*, which made difficult an accurate estimation of food intake. This problem was solved afterwards (**chapter 8**) by radiolabeling the *Artemia* using liposomes with ^{14}C-oleic acid (OA), which was found to result in a more uniform labelling of the *Artemia*, thus allowing more precise estimates of food intake to be made with seabream larvae. This uniform labelling might have been caused by the fact that a very large proportion of the ^{14}C-OA was incorporated into the structural polar lipid fraction of the *Artemia*, which was surprising, as a larger amount of label was

expected to accumulate in the neutral lipid. It is not known whether supplying the label within the polar membrane of liposomes may affect its metabolic use by *Artemia*.

Although it is not directly relevant to the objectives of the work presented in this thesis and was therefore not mentioned elsewhere, an interesting observation was made over the course of the *Artemia* radiolabeling experiments which is worth pointing out, for future reference. Independently of the method that was used to radiolabel *Artemia* (either through the inclusion of a ^{14}C-protein hydrolysate or ^{14}C-FA-liposomes in the enrichment media), the *Artemia* enriched with higher lipid levels systematically accumulated a lower amount of radioactivity (dpm), even though the same amount of radiolabel was added to the enrichment media. It thus appears that non-enriched *Artemia* or *Artemia* enriched with a lower dose of emulsion were either filter-feeding more actively or were able to filter a larger proportion of labeled liposomes or ^{14}C-protein hydrolysate and therefore incorporated the label more efficiently. These observations strengthen the importance of an accurate determination of the radioactivity in the labeled *Artemia* in the different treatments used in a given study, in order be able to estimate with precision differences in larval food intake in relation to the total lipid level of the prey. In addition, in the liposome radiolabeling trials, a larger amount of the ^{14}C-FA was incorporated into the lipid fraction of the radiolabelled *Artemia* in the higher lipid treatments, while in the lower lipid treatments a higher percentage of the label was found in the non-lipid fraction. Therefore, when *Artemia* was enriched with a lower amount of lipid, a higher proportion of this lipid may have been used as an energy substrate and was thus less accumulated in the lipid fraction.

Initially it was attempted to quantify food intake based on a single meal, i.e., by allowing the larvae to feed on *Artemia* during a period of time shorter than the evacuation time (estimated to be around 2 h for seabream) and then measuring the radioactivity in the gut. Nonetheless, this methodology was soon changed to a cold-chase approach (**chapter 8**), for several reasons. Quantifying food intake based on a single meal, particularly the first morning

meal, may not give reliable results, given that at this time the appetite is at its maximum. As the larvae have an empty gut at the start of the trial, they might respond to gut fullness, without the biochemical composition of the diet having a regulatory effect on feeding. Therefore, the cold chase approach has some advantages, as it allows extending the period of time during which food intake is being measured and at the same time permits better measurements of label absorption. When feeding continuously on a labeled diet, it is not possible to distinguish between label which is in the gut lumen from that accumulating in the gut tissues, after absorption. However, by allowing larvae to feed on radioactive diet during an amount of time shorter than the evacuation time and by examining the quantity of label retained in the gut after the radioactive meal has been evacuated, it becomes possible to quantify label absorption into the gut tissues.

2. Effect of dietary lipid on lipid digestion and lipase activity

The study conducted in **chapter 3** with Atlantic herring larvae also aimed at better understanding the mechanisms underlying and limiting lipid digestion and absorption in this species. A visual description of the actions taking place in the gut during the passage of a pure neutral lipid (triolein, TRI) was made through the analysis of video-tape recordings and showed that Atlantic herring was able to process the tube fed lipid, both through mechanical and chemical action. Contractions of the midgut wall caused the mechanical breakdown of large lipid droplets, an active dispersion and emulsification throughout the midgut, and they most probably insured considerable mixing between the lipid droplets and the bile salts and pancreatic secretions being released close to the anterior midgut sphincter (Pedersen, 1984). As the mass of the lipid droplets moved to the posterior midgut, strong retrograde contractions of the midgut and hindgut likely increased the retention time in the gut and also

allowed a better emulsification and mixing with bile and pancreatic secretions. Signs of chemical action during lipid digestion were also noted. For instance, the fact that the smaller lipid droplets do not fuse back together indicates the presence of bile salts in the gut lumen. In addition, the gradual change in the appearance of the lipid droplets, from a translucent and clear to an opaque and whitish look, was most probably a result of the chemical mechanisms occurring during digestion. Additionally, the digestive and absorptive efficiency of tube fed ^{14}C-TRI and of the FFA that it is comprised of, ^{14}C-OA, was also examined in Atlantic herring. Different volumes of ^{14}C-TRI were also tube fed to the larvae, in order to study the effect of lipid dose on digestive capacity. The results showed that increases in the volume of tube fed TRI enhanced only marginally label absorption, while leading to a steep rise in evacuation. At a comparable volume (50.6 nl of tube fed lipid), OA, which does not require digestion, was significantly less evacuated and better absorbed than TRI. However, a somewhat higher absorption and lower evacuation of TRI compared to OA was measured by reducing the volume of tube fed TRI from 50.6 nl to 9.2 nl. The metabolic studies, together with the video image analysis, suggested that the high dose of TRI may be too large for the enzymes to hydrolyze efficiently (i.e., was possibly beyond the plateau of enzymatic capacity of the Atlantic herring larvae), which explains the increase in absorption at lower doses. Nevertheless, an inadequate digestive capacity could not fully explain the results, suggesting that there is also likely an absorption efficiency component.

The results obtained in the studies presented in **chapters 4, 7 and 8** with European seabass (*Dicentrarchus* labrax), Senegalese sole (*Solea senegalensis*) and gilthead seabream (*Sparus aurata*) suggest that lipase enzymatic activity may not be a growth limiting factor when feeding larvae on high lipid diets. Even if the lipase specific activity has been shown to be affected by dietary lipid (in seabass larvae, in relation to lipid source but not lipid level), these effects were not sufficient to affect seabass growth (**chapter 4**). In this case, not even the size of the larvae appeared to influence the results, as the segmental activity at 52 DAH closely

followed the specific activity (data not shown in **chapter 4**). On the other hand, in **chapter 7**, Senegalese sole that were fed on *Artemia* enriched with high and low levels of fish oil, compared to soybean oil-enriched *Artemia*, had a slightly higher segmental activity (i.e., in the dissected digestive tract), as a result of their larger size, but when lipase activity was expressed as specific activity (U/mg protein) to standardize for differences in size, no significant differences were found between treatments. These results are not that surprising, having in mind that fish larvae are characterized by extremely high growth rates and thus elevated nutritional demands (Conceição, 1997), which imply a high digestive capacity, i.e., a digestive system that is highly efficient and able to deal with their natural food. A large excess of pancreatic lipase secretion in relation to dietary needs has been reported in humans (Brannon, 1990) and, if this is also the case in young fish, a diminished lipase specific activity might not necessarily affect growth. Very little has been studied in fish but evidence so far points in that direction. In larval red drum fed a microparticulate diet, live prey or both, digestive enzyme activity was not the limiting factor for growth (Lazo et al., 2000). A high enzyme-substrate ratio has also been measured for proteolytic enzymes in larval herring, and the possibility for a very fast proteolysis may explain the fast evacuation rates observed in these larvae (Pedersen, 1984; Pedersen et al., 1987). Lipase may thus be produced in sufficient amounts for the digestion of all ingested prey, as had already been suggested by Hoehne-Reitan et al. (2001a), and therefore may not be limiting for growth in normal hatchery conditions, at least when feeding on live prey. On the other hand, as will be discussed below, a lower digestive capacity in larvae fed higher neutral lipid diets could not entirely explain the results obtained in the experiments that are presented in this thesis and the results give some support to the notion that lipid transport may be more of a problem than lipid digestion *per se* when rearing larvae on high lipid diets (Izquierdo et al., 2000). An example of this was seen in **chapter 8**, where the regulation of food intake and nutrient absorption according to lipid level and source was studied in seabream larvae. In the

experiment conducted with soybean oil, the same trend was noted in terms of food intake and absorption when the microdiet (MD) was labelled with either free ^{14}C-OA or ^{14}C-TRI (which needs to be digested prior to absorption), indicating that the differences observed between larvae fed the high and low soybean oil diets were not due to differences in the capacity to digest the diets.

In spite of these considerations, all the radiotracer studies in which the absorption efficiency of OA in its free form was compared with that of TRI (**chapters 3, 5, 7 and 8**) revealed that the free FA (FFA) was more absorbed than when it still has to be digested by neutral lipase. This indicates that the digestive capacity was not 100% efficient to deal with the tube fed neutral lipid. Even knowing that the typical lipid composition of the larva's natural diet is around 20-30% of dry weight in spring (Tocher and Sargent, 1984; Sargent et al., 1989), the lack of reliable data on larval feeding rates does not enable to determine whether the amount of tube fed lipid corresponds to a realistic load for a single meal. Therefore, it can be questioned whether methodological problems may partly explain some of the results obtained. However, even if the larval digestive capacity is sufficient to deal with their diet, it is not that surprising that a FA is faster and better absorbed in its free form than when esterified to TAG. Still, these results are not in agreement with previous observations by Izquierdo et al. (2000, 2001) in gilthead seabream larvae, as will be discussed below.

Based on the results presented in **chapter 5**, where OA esterified to PC was much less evacuated and more absorbed into the gut tissue than when it was supplied esterified to TRI (independently of the previous feeding regime), it was suggested that, in Senegalese sole larvae, neutral lipase activity may be less efficient than that of phospholipase A_2 (PLA_2) or that TRI may have had a more deficient emulsification. In addition, PC and free OA were absorbed and metabolised by the larvae in a comparable way, which again indicates a very high efficiency of PLA_2 digestion and absorption of the products of PL digestion. However, even though the quantities that were tube fed were very small (23 nl) and probably did not

represent an extreme physiological challenge, it should be noted that the PC mixture that was tube fed had a lower specific activity than the other mixtures. Therefore, even though it is unlikely that the difference in the number of PC and TRI molecules that were tube fed (34% less PC molecules, having in mind that there are 2 [1-^{14}C]-OA in each PC molecule, compared to 3 in TRI) may have affected the results, it should still be kept in mind and future studies should attempt keeping a constant load.

Contrary to what has been shown by some authors working with fish (Borlongan, 1990; Zambonino Infante and Cahu, 1999), lipase activity in the experiments conducted with seabass (**chapter 4**) and Senegalese sole (**chapter 7**) larvae was not affected by total lipid quantity. However, as discussed in **chapter 4**, the differences in the dietary lipid level (17 vs. 24% total lipid) might have not been sufficiently marked to have a measurable effect on lipase activity. In particular, the low lipid diet should have included a lower amount of total lipid, as the 17% lipid level might have been too close to the level of 20% when, according to Zambonino Infante and Cahu (1999), a plateau is reached and no further stimulation occurs in seabass lipase activity by dietary lipid. In **chapter 7**, the differences in total lipid content between the *Artemia* enriched with high and low doses of oil emulsion were even less marked (20 vs. 23 % for soybean oil-enriched *Artemia* and 23 vs. 25% in the fish oil treatments). Additionally, 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. In accordance with this theory, Hoehne-Reitan et al. (2001b) showed 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.

The work conducted with seabass larvae (**chapter 4**) showed, however, that when studying the effect of dietary lipid on lipase activity, its quantity cannot be dissociated of its source

(i.e., FA composition). The FA content of the diet might act directly on the regulation of enzymatic activity, either through increased enzyme-substrate stability and/or enhanced pancreatic secretion (Singer, 1987; Brannon, 1990). The FA specificity of lipolytic enzymes has been relatively well studied and the effects of FA on the activity of pancreatic lipase are related to both the acyl chain length and degree of saturation. Fish lipases have a preference for polyunsaturated FA (PUFA) as substrates, followed by monounsaturated FA (MUFA), with saturated FA (SFA) being more resistant to lipolysis (see references in general introduction). It had been suggested earlier that the neutral lipase activity may be influenced by the dietary FA composition in seabream larvae (Izquierdo et al., 2000). In seabass larvae, at 24 days after hatching (DAH), the same tendency as that reported by Izquierdo et al. (2000) was observed, that is, a higher lipase activity was measured in larvae that were fed a PUFA-rich fish oil diet, compared to the MUFA-rich triolein diet. However, this effect was age dependent, as at 52 DAH the diet containing fish oil caused a significant reduction in lipase specific activity. In the case of the diet formulated with coconut oil the effect on lipase activity was more constant, with a high lipase activity being induced by this diet both at 24 and 52 DAH. The age-dependence of the physiological digestive response to the fish oil-based diets remains unresolved and should be the focus of further studies at different stages of development, as not only nutritional requirements but also digestive function may show important changes between early larvae and later larval/juvenile stages. It can be speculated that the long term feeding of seabass larvae with more digestible diets containing long chain FA, such as those using fish oil, may cause an adaptative response leading to a lower secretion of pancreatic lipolytic enzymes. On the contrary, in a dietary regime containing less digestible oils, an elevated secretion or lipolytic activity would be maintained. The experiment described in **chapter 7** was not as conclusive but a non-significant trend for a lower lipase specific activity in 32 DAH Senegalese sole fed *Artemia* enriched with a high dose of fish oil was observed, giving some support to the results discussed in **chapter 4**. The

results obtained with seabass thus suggest the existence of a regulatory mechanism of neutral lipolytic enzyme secretion and activity according to the dietary FA composition in marine fish larvae. Nevertheless, the causes explaining the differences in lipase activity according to the dietary lipid source were not completely clarified and further studies should be conducted. Studies of this nature are particularly difficult given the multiplicity of factors (some of which are still not very well studied in fish) which may intervene and interact – the effect of FA nature on lipid digestibility, stimulation of neuroendocrine pathways and other post-absorptive mechanisms. For instance, in mammals, unsaturated lipids induce lipase activity more than saturated lipids when dietary lipid intake is moderate and the lipase content is not maximal but it is generally no longer effective when dietary lipid intake is high and lipase content is maximal (Brannon, 1990).

On the other hand, as mentioned above, growth was not related to lipase enzymatic activity, suggesting that these two factors might be independent. Therefore, even if the results indicate a dietary effect on lipase specific activity, the physiological consequences of such a regulation remains unclear.

3. Effect of dietary lipid level on lipid droplet accumulation and nutrient absorption

The study conducted with Atlantic herring (**chapter 3**) showed that reducing the amount of TRI that was tube fed from 50.6 nl to 9.2 nl improved lipid absorption as much as tube feeding OA, which does not require digestion previous to absorption into the enterocytes. As mentioned above, these results indicated that the limiting step for the utilization of high dietary lipid levels in Atlantic herring may be more a question of lipid absorption into the enterocyte and posterior transport into the body than of lipid digestion, although the two factors are probably involved. This had been suggested, although not clearly shown by other

authors (Izquierdo et al., 2000) and was also confirmed by some of the studies conducted on Senegalese sole and seabream (**chapters 5, 7 and 8**).

Two experiments were performed with Senegalese sole larvae where, by using diets containing a higher and lower level of neutral lipid - *Artemia* enriched on a soybean oil emulsion (EA) and non-enriched *Artemia* (NEA), respectively - an accumulation of lipid droplets within the enterocytes was induced. This enabled studying to what extent these lipid accumulations may influence the capacity of sole larvae to digest and absorb FA (**chapter 5**), as well as proteins and AA (**chapter 6**). In both experiments the larvae fed on the higher neutral lipid diet (EA) presented a higher amount of larger lipid vacuoles in the midgut enterocytes (mostly in the basal zone), concurrently with a lower growth. In the experiments described in **chapter 5**, when Senegalese sole larvae that were fed either EA or NEA from 16 to 32 DAH were tube fed lipid mixtures containing ^{14}C-OA either in the free form or esterified to a TAG (glycerol tri[1-^{14}C]oleate or triolein; TRI) or to a PL (L-3-phosphatidylcholine 1,2, di-[1-^{14}C]oleoyl; PC), a significant effect of the *Artemia* diet (two-way ANOVA) was noted on the absorption of the labels. This effect was particularly marked when TRI was tube fed, being a non significant trend for the free OA and PC labels. The larvae which had been fed EA showed a significantly increased evacuation of the labelled FA, suggesting that the long term feeding of a diet higher in neutral lipid may affect the capacity of larvae to efficiently absorb dietary FA. Therefore, it appears that the accumulation of lipid droplets observed 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 these experiments. In early studies performed with rats, it was thought that the rate limiting step for the esterification in the intestinal mucosa was the FA absorption from the lumen but it was later discovered that an increase in the esterification rate resulted in an increased OA flux from the lumen (Borgström, 1977). Therefore, the intracellular FA pool may regulate the uptake into the enterocytes. This may also explain

why, in fish larvae, a higher accumulation of lipid in the enterocytes might eventually lead to an impaired FA absorption and increased evacuation. However, the results presented in **chapter 5** suggest that this effect depends also on the FA nature, as different FA may show a specific pattern of digestion, absorption and utilization, as will be discussed below. On the other hand, the results obtained in **chapter 8** with seabream larvae revealed that diets differing on their total lipid level are absorbed with a different efficiency but, in this case, the histological analysis of the gastrointestinal tract did not reveal any obvious difference in lipid accumulation between larvae fed the higher and lower lipid diets, independently of the lipid source (soybean or fish oil). This may be due to species-specific differences or to the fact that in these experiments there was a higher dietary supply of PL, included in the MD or in the form of liposomes added to the *Artemia* enrichment. Therefore, contrary to what was observed with Senegalese sole, differences in nutrient absorption could not be attributed to the accumulation of lipid droplets in the enterocytes in these experiments. The causes and consequences of these accumulations and the mechanisms involved in the changes in enterocyte absorptive efficiency are still not completely understood and further work is thus necessary to fully understand the mechanisms of FA absorption in the enterocytes and how these may be affected by dietary lipid level and composition.

The mechanisms associated with lipid and protein absorption are fundamentally different. Nonetheless, considering that growth is mainly the result of protein deposition, a study was conducted to examine whether a higher neutral lipid regime inducing the accumulation of lipid droplets in the gut epithelium may also affect AA absorption and metabolism (**chapter 6**). Few studies have examined this interaction and the existing results are contradictory. Kjørsvik et al. (1991) reported alterations in protein absorption in the hindgut of turbot larvae fed rotifers with a high lipid content while Olsen et al. (1999), when describing pinocytotic activity related to protein hydrolysis in Artic charr, noted that it was not affected by diets inducing differences in the accumulation of lipid droplets in the enterocytes. Senegalese sole

larvae that were fed either NEA or EA from 16 DAH onwards were allowed to feed on *Artemia* radiolabelled with a [U-^{14}C] protein hydrolysate, in which most of the label is incorporated into the *Artemia* protein fraction, as described in **chapter 2**. Both dietary treatments had very similar high absorption (75-76%) of the AA label after a 24 h incubation period, which suggests that the intracellular lipid inclusions in the enterocytes did not affect AA absorption, in accordance with the observations made by Olsen et al. (1999). Nevertheless, the significantly higher amount of label found in the incubation water of larvae fed the NEA treatment, 3 h after feeding, appears to indicate a faster evacuation and thus a more rapid processing of the *Artemia* meal by these larvae. AA label catabolism was also significantly higher at 3 and 24 h in the NEA treatment, which might be explained by the faster AA absorption resulting in more time for its metabolic use. Therefore, in a continuous feeding situation, a higher net AA absorption may be achieved in larvae fed NEA through a more rapid clearance of the lumen leading to sustained appetite and ingestion of the diet, as will be discussed further below. The higher growth of larvae fed NEA may thus be explained by a higher FA absorption efficiency and/or by an increased ingestion rate of a diet containing a lower lipid level.

However, the two studies presented in **chapters 5 and 6** have the inconvenience of testing the dietary effect of two *Artemia* diets which differed in their relative FA profile, even though the higher lipid treatment was achieved through enrichment with soybean oil, which avoided increasing the dietary supply of EFA that would have greatly affected growth. In addition, in carnivorous juvenile and adult fish, it has been shown that the inclusion of plant-derived oils may result in a reduction of the transport rate across the gut epithelia and thus in the accumulation of lipid droplets in the enterocytes, possibly leading to tissue damage and compromised gut integrity (Olsen et al., 1999, 2000, 2003; Caballero et al., 2002, 2003). Indeed, it has been hypothesized that the dietary FA composition may influence not only the composition, morphology and fluidity of the intestinal cells but also the physiological

mechanisms involved in intestinal lipid metabolism and transport (Sire and Vernier, 1981; Caballero et al., 2002, 2003). One of the suggested potential metabolic effects would be in the intracellular pathways of TAG and PL reacylation, which appear to be affected by the nature of dietary lipids (Sire and Vernier, 1981; Pérez et al., 1999; Izquierdo et al., 2000; Olsen et al., 2000), even though Oxley et al. (2005) did not find evidence of this, following replacement of fish oil by vegetable oil in the diet of Atlantic salmon. Additionally, an increase in the dietary lipid level causing a change in the FA composition of the brush border membrane (more specifically, a decrease in the level of PUFA and a concomitant increase in MUFA) of seabass juveniles has been reported by Cahu et al. (2000) and was linked to a significant reduction in the brush border enzyme activities, possibly as a result of changes in membrane fluidity. Having this in mind, a follow-up study was conducted with Senegalese sole using *Artemia* diets enriched with higher and lower levels of the same lipid emulsion (i.e., with an equivalent FA profile) and comparing two different lipid sources – fish oil and soybean oil (**chapter 7**). In this experiment, differences in lipid accumulation within the basal zone of the gut enterocytes were also induced by the experimental diets, with a higher amount of lipid droplets being noticeable in the anterior intestine 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 free OA). Nevertheless, the experimental approach that was followed, namely the use live preys enriched with different doses of lipid emulsion, did not enable achieving important differences in total lipid level between the high and low treatments, which may also explain the lack of substantial differences in growth between these diets. As in many other areas of

fish larval nutrition, the development of inert diets enabling a better control of the dietary composition is highly needed, in order to proceed with the investigation of the effects of total lipid level and lipid source on larval performance and digestive physiology.

The accumulation of lipid droplets within the enterocytes appeared to significantly affect the absorption of OA esterified to TRI (**chapters 5 and 7**). However, the same effect was not as marked for OA in its free form or esterified to PC, in the tube feeding experiment described in **chapter 5**, while in the work presented in **chapter 7** no effect of lipid droplet accumulation on the absorption of free OA was noticeable. It could be speculated that the rise in dietary neutral lipid level may depress the neutral lipase activity which is responsible for TRI lipolysis but the lipase determinations performed in **chapter 7** do not support this hypothesis, indicating that the differences may be more at the absorption level. Therefore, no convincing hypothesis can be proposed at this time to explain the differences observed in absorption efficiency between TRI and free OA when larvae are fed diets differing in neutral lipid level but it is possible that effects at the digestion level interact with effects at the absorption level to produce the observed results.

4. Effect of dietary lipid source on lipid absorption and metabolism

The work carried out with Senegalese sole larvae by tube feeding FFA differing on chain length and degree of saturation (**chapter 5**) showed quite clearly that different FA are absorbed differently, as it is well established in existing literature for adult fish and mammals. The results confirm that, in fish, FA absorption increases with unsaturation (see references cited in the general introduction), with the order of decreasing absorption being DHA (docosahexaenoic acid; 22:6n-3) > OA > SA (stearic acid; 18:0). Therefore, just as noted for the dietary effect of lipid on lipase activity, when analysing the effect of neutral lipid on

absorption efficiency, its level cannot be dissociated of its biochemical composition, as different FA will be affected differently by high lipid inclusion levels, both at the digestion and absorption levels. In particular, DHA was seen to be almost completely absorbed and mostly retained in the tissues, independently of the previous feeding regime. Therefore, it may be that, as a result of the high specificity of digestive enzymes towards (n-3) PUFA (see references in the general introduction) and of the presumed higher affinity of cytosolic FA-binding proteins and higher rate of esterification of long-chain PUFA, as well as their preferential incorporation into polar lipids (Sire and Vernier, 1981; Poirier et al., 1997; Pérez et al., 1999; Izquierdo et al., 2001), the accumulation of lipid droplets in the enterocytes may not be an obstacle for the efficient dietary utilization of EFA. This being the case, effects on growth would then be explained by energy deficiencies caused by a lower absorption of SFA and MUFA which are normally directed into energy-producing catabolic pathways, eventually sparing protein for growth.

Besides analysing how FA differing on chain length and degree of saturation are absorbed and metabolised by the larvae, it was attempted to verify the effect of dietary inclusion of OA in different chemical forms – as a FFA, esterified to TRI or to PC (**chapter 5**). OA is a good subject for nutritional studies and has been widely used in mammalian work, as it was found to be one of the FA that most efficiently stimulates both TAG and PL synthesis and secretion in IPEC-1 cells (Wang et al., 1997). This question is of considerable nutritional interest and should be considered when formulating diets, as the chemical form in which a FA is supplied in the diet affects its utilisation (digestion, absorption and metabolism) and therefore its bioavailability (see Favé et al., 2004 for a comprehensive review on this subject). This is particularly the case in organisms such as marine fish larvae, which are presumed to have immature digestive and absorptive systems (see general introduction). Due to their different chemical structures, TAG and PL have different metabolic pathways, mainly in what concerns the processes of enzymatic hydrolysis, absorption and incorporation into lipoproteins (Sala-

Vila et al., 2004). Potential beneficial effects of using PL as the FA vector may be a higher digestion efficiency by PLA_2 compared to neutral lipases, a higher oxidative stability of the FA esterified to PL (particularly important for the essential long-chain PUFA) and the presence of higher amounts of lysophospholipids that will improve FA absorption by a more efficient transport of the product of lipolysis to the enterocyte (Favé et al., 2004). The results presented in **chapter 5** with OA reveal that in order to increase the bioavailability of dietary FA, it might be preferable to supply them in the form of PL rather than TAG. The advantages of using PL as fatty acid carriers instead of TAG in the nutrition of marine fish larvae have been previously discussed, particularly concerning highly unsaturated FA (HUFA) of the (n-3) series (Geurden et al., 1998; Salhi et al., 1999; Izquierdo et al., 2000, 2001; Gisbert et al., 2005). However, having in mind the high specificity of fish lipases towards PUFA, as well as their preferential absorption and reacylation into PL (references in general introduction and Sire and Vernier, 1981; Poirier et al., 1997; Pérez et al., 1999; Izquierdo et al., 2001), one might consider that the dietary chemical form would be less important for (n-3) HUFA. Still, Gisbert et al. (2005) showed that the best growth and survival performance in seabass larvae was achieved with a MD supplying moderate levels of DHA and eicosapentaenoic acid (EPA) in the PL fraction rather than in TAG, and this was explained by the ability of young larvae to better modulate PLA_2 expression than that of neutral lipase. Indeed, as mentioned above, the results obtained in **chapter 5** suggest that PLA_2 activity may be more efficient than neutral lipase. On the other hand, Geurden et al. (1998), observed a higher incorporation of HUFA in postlarval turbot fed a diet containing HUFA-PL than when providing these FA in the diet as ethyl esters supplemented with PC. This was hypothesized to result from the fact that a part of the PL is not hydrolyzed, so that the HUFA are absorbed esterified to the PC molecule, which might be more efficient than the uptake of HUFA in the FFA form. Given the essentiality of these nutrients for larval growth and proper development, this subject deserves further attention.

Nonetheless, the tube feeding results do not support the idea that the incorporation of dietary FFA into larval lipids is less efficient than that of TAG or PL, as was shown for seabream by Izquierdo et al. (2000, 2001). These authors suggest that a better utilization of TAG, compared to FFA, could be explained by a lower capacity of reacylation or transport of FFA or to its preferential utilization as an energy source in the enterocyte. In Senegalese sole larvae, free OA was incorporated into the gut and body tissues at least with the same efficiency as PL (or even slightly higher) and considerably more than TAG (**chapters 5 and 7**). The results obtained in **chapter 8** for gilthead seabream larvae, concerning the absorption of labeled FA from a MD containing either ^{14}C-OA or ^{14}C-TRI, also show a higher absorption of the FFA compared to the TAG but, in this case, the results were obtained in different trials and therefore can not be easily compared. Furthermore, there were differences in MD size which may have affected total intake. Therefore, further studies on the digestive processes of both species are necessary to determine if these differences are related to the species or the type of feed used in those studies, as discussed in **chapter 7**.

The tube feeding trials presented in **chapters 5 and 7** showed that OA esterified to TRI was highly catabolised, while OA in the free form or esterified to PC were significantly less catabolised. Initially it was suggested that Senegalese sole larvae may be able to discriminate between the molecular forms of OA in the diet for its subsequent catabolism, although at the time no compelling hypothesis was proposed to explain these results (**chapter 5**). The higher retention and lower catabolism of the OA esterified to PL was not a very surprising result and might have a simple physiological explanation. The intestinal digestion of PL in fish has not been very well studied but it is generally agreed that the mechanisms are probably similar to the mammalian system in which PLA_2 catalyses the hydrolysis of the FA ester bond at the *sn*-2 position, producing a FFA and 1-acyl lysoglycerophospholipid (Iijima et al., 1997; Izquierdo and Henderson, 1998). Therefore, given that after lipid digestion one half of the radiolabelled OA will probably stay esterified to the PC backbone in the form of

lysophosphatidylcholine, it is conceivable that this lipid digestion product, once absorbed by the enterocytes, will be directed via the PL biosynthetic pathway, thus leading to the accumulation of the labeled OA in cellular membranes. On the other hand, the high catabolism of OA when it is tube fed as a TAG (TRI) might also be expected. Given the specific and non-specific nature of intestinal lipases in adult and juvenile fish, the intraluminal hydrolysis of TAG is complete or almost complete, leading to the release of FFA, glycerol and 2-monoacylglycerols (2-MAG) (see references in general introduction). Therefore, even if not much is known regarding the mechanisms of lipid digestion in larval fish and some dissimilarities may exist, the luminal digestion of TRI should result in the release of either all radiolabelled FA moieties or at least of 2 free OA molecules and another esterified to 2-MAG, which will then be absorbed. Caballero et al. (2003) noted that OA appears to be reesterified mostly into TAG and a large accumulation of OA in TAG of trout enterocytes has been shown (Pérez et al., 1999). Thus, it is quite likely that the OA reesterified into TAG is highly utilized for energetic purposes by fish larvae, as has been postulated by Rodríguez et al. (1994) and Izquierdo et al. (2001). However, the results obtained for the absorption and metabolism of the tube fed free OA were more surprising and, for the reasons discussed above, free OA was expected to be absorbed and metabolized in a similar way to that of TRI rather than to that of PC. Nonetheless, it was later suggested (**chapter 7**) that this is probably simply a consequence of the differences in total amount of OA label that was absorbed when it was supplied as TRI or in the free form (and a similar reasoning can also be applied for OA-PL). Hence, the results obtained in **chapter 5** and later confirmed in **chapter 7** substantiate the existing idea that OA is preferentially utilized for energetic purposes (Rodríguez et al., 1994; Izquierdo et al. 2001), being largely directed via a catabolic route and only when absorbed in larger amounts (e.g., when supplied in a free form) it is more highly directed into anabolic pathways, thus accumulating in the body and gut tissues. In support of this, a direct relationship was observed in **chapter 7** between the absorption efficiency of TRI in different

dietary treatments and the proportion of label retained in the tissues, while an inverse correlation was noted with the percentage of label found in the metabolic trap.

5. Effect of dietary lipid on food intake

The control of appetite in fish, as in higher vertebrates, is highly complex and appears to involve numerous external and internal factors, which makes it an extremely challenging subject to study. The involvement of neuropeptides and neurotransmitters in the neural control of food intake in fish, as in mammals, has been recently suggested (Lin et al., 2000; Jensen, 2001), and a multitude of hormones may intervene (Le Bail and Boeuf, 1997). In addition, a multiplicity of environmental factors and many genes are involved (Silverstein, 2002). Regarding nutritional aspects, dietary digestible energy content is a well documented factor that has been shown to affect food intake (see general introduction). However, even this may be complex to establish as, in extreme cases, when fish are fed diets of very high or low energy density, stomach fullness and the control of gastric emptying may be responsible for the regulation of food intake (Lee and Putnam, 1973; Ogata and Shearer, 2000; Gélineau et al., 2001). On the other hand, in the case of very high lipid diets, food intake may also be increased to meet the requirements for essential nutrients (Yamamoto et al., 2002). Therefore, it is not surprising how little is known regarding the regulation of food intake in fish and the lack of work in larval fish, whose study is even further complicated by their tiny size and by their specific and little studied metabolic and physiological characteristics.

Two experiments were carried out to study the regulation of food intake and nutrient absorption in seabream larvae by dietary lipid level and FA composition (**chapter 8**). In one experiment, larvae were fed *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 MD containing soybean oil, at a corresponding lipid level. After a period of feeding on the experimental diets, trials were conducted using ^{14}C-markers in order to quantify food ingestion and absorption. When seabream larvae were fed diets containing higher and lower amounts of soybean oil (HS and LS, respectively), they fed significantly more on the LS diet. Therefore, in this case, the total lipid level of the diet was inversely correlated with food intake, as observed with older fish. In addition, possibly as a result of the significantly lower food intake of the HS diet, larvae on this treatment reached a significantly lower dry weight at the end of the experimental period, similarly to what had been noted with Senegalese sole larvae fed EA (**chapters 5 and 6**). However, when *Artemia* were enriched with higher or lower doses of fish oil instead (HF and LF), the opposite effect was noted on ingestion, i.e., larvae on the HF diet had a significantly higher food intake. In this experiment, however, no significant differences were found in dry weight between the HF and LF treatments, which may indicate that the LF diet met the minimum EFA requirement for growth in seabream larvae. Therefore, the experiments presented in **chapter 8** showed that in seabream larvae, 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 and that the source of the dietary lipid may have a more important role in controlling ingestion. Some of the reasons which may explain these results will be discussed below. In addition, food intake did not appear to have been regulated to meet a requirement for EFA as both diets which were more ingested (HF and LS) presented higher or similar levels of EFA than their counterpart treatment.

The work conducted with Atlantic herring (**chapter 3**) suggested that an increment of lipid level above the digestive and absorptive capacity of the larvae may potentially lead to a faster evacuation of the diet, indicating that dietary lipid input may affect ingestion and total food intake by influencing evacuation rates. However, in this study, an artificial situation of feeding pure lipid was induced and it is uncertain whether the same would occur in larvae

feeding a complete diet in which emulsifying agents such as PL, proteins and carbohydrates, are also present. Being this the case, it is not surprising that an increase in dietary lipid level may affect differently food intake depending on its FA composition, as was observed with seabream larvae (**chapter 8**). Having in mind the results obtained with Atlantic herring, it could be hypothesised that increasing the levels of lipids which are more easily digested and absorbed by the larvae may not have the same effect on evacuation and subsequent re-feeding rates as increasing the dietary input of a poorly digested and absorbed lipid which may more rapidly "exhaust" the larval digestive and absorptive capacity. However, if this was the case, a diet containing higher levels of soybean oil, which is most probably a less digestible oil compared to fish oil, should be evacuated more rapidly, thus leading to a higher ingestion rate. However, this hypothesis does not agree with the results presented in **chapter 8**, where the HS diet was in fact significantly less ingested than the LS regime. Nevertheless, one other hypothesis is that, when feeding a complete diet, the digestive system of the larvae will function in a way that it will digest a meal as completely as possible before evacuation. If this is the case, then the inverse situation would be observed, that is, the higher the lipid digestibility the faster the digestive system is emptied, meaning that sustained appetite can be maintained and food intake may be higher. Thus, even if in the Atlantic herring study it is suggested that evacuation rate may be accelerated when the larval digestive capacity is exhausted, if the larval digestive capacity is sufficient to handle high dietary lipid loads, as appeared in the subsequent studies, the digestion and absorption rates may be a more determining factor affecting gut transit time and therefore ingestion rates. In sum, one of the suggested hypothesis that is discussed in **chapter 8** 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, the feeding rates. In fact, food intake and absorption efficiency are commonly interrelated (Werner and Blaxter, 1980; Ryer and Boehlert, 1983; Boehlert and Yoklavich, 1984). In support of this, Hadas et al. (2003) showed that when

gilthead seabream larvae were fed a PC-supplemented MD, an enhanced ^{14}C-OA transport from the gut enterocytes into the larval body was noted, along with an increased ingestion of this diet. Therefore, FA digestibility may have an indirect effect on food intake. Given the high digestibility of long chain (n-3) PUFA, *Artemia* enriched with fish oil may be more digestible for marine fish larvae than with soybean oil. If the HF *Artemia* is digested and absorbed faster than the LF diet, the gut of the larvae feeding on this diet may be cleared sooner, thus leading to a higher food intake. On the other hand, *Artemia* enriched on higher levels of soybean oil (HS) may have detrimental effects on lipid digestion and absorption, which might delay gastric emptying and thus result in a lower ingestion rate. However, the absorption efficiency of the radioactive labels was analyzed and does not support this hypothesis, given that the LF diet was more absorbed from the gut into the body tissues than the HF *Artemia*, while the soybean oil diets appear to have been both equally efficiently absorbed. The results obtained with the fish oil treatments support the hypothesis that a higher food intake may cause a decrease in nutrient absorption efficiency, possibly as a result of more rapid passage through the gut (Werner and Blaxter, 1980; Ryer and Boehlert, 1983; Boehlert and Yoklavich, 1984), while in the soybean oil experiment total absorption appeared to be correlated with food intake.

In the study that was conducted with Senegalese sole larvae (**chapter 6**), even though food intake was not directly measured, the data appears to suggest that larvae fed NEA may have a faster AA absorption which may result in a more rapid clearance of the lumen and sustained ingestion of the diet, again giving some support to the idea that the total lipid and FA composition of the diet, by affecting absorption rates, may potentially have an effect on total food intake, which in turn will determine growth.

Another parameter which may potentially exert some effect on food intake and that has not been investigated in fish larvae is palatability. When feeding *Artemia*, even if a direct orosensory or taste effect of the oils is unlikely, as they are absorbed and converted into the

Artemia's body nutrients, *Artemia* washing after enrichment might be insufficient to remove all residues of the lipid emulsions from the exosqueleton. In the case of formulated MD, as the one used in the soybean oil experiment, taste may have a more important effect. In addition, the possibility exists that the dietary FA composition may exert an influence at the hormonal level, such as through effects on the release of the gastrointestinal hormone cholecystokinin (CCK), which is involved in the control of gastric emptying and food intake, and whose secretion can be affected by the chemical nature of the FA (see references in general introduction).

However, none of the discussed hypothesis appears to clearly and fully explain the results, and further studies are necessary to confirm and clarify the mechanisms behind these results. Given the enormous importance that food intake may have on fish growth and thus on aquaculture productivity, a substantial research effort should be directed into this subject in the near future.

6. Conclusions and future perspectives

One of the central objectives in marine larviculture for at least the last two decades has been the replacement of live feeds by inert formulated diets, which will have significant implications for the future economic viability of aquaculture. However, in order to achieve this, a detailed understanding of the larval digestive physiology and how it is modulated by the diet is essential. The current perception, based on the few results obtained so far by studying lipid digestion and transport in fish larvae is that the transport of lipid from the enterocytes into the body may be more of a problem in small larvae than lipid digestion (Izquierdo et al., 2000). Although both factors are likely to intervene, the work presented here gives support to this idea. Atlantic herring larvae were able to deal quite effectively with pure

lipids administered directly into the gut, showing considerable mechanical and chemical action that was effective in emulsifying the lipid in the absence of exogenous dietary emulsifiers. In tracer experiments conducted with various species (Atlantic herring, Senegalese sole and gilthead seabream), free OA was always more efficiently absorbed than TRI, indicating that neutral lipases were not entirely efficient. The results obtained with Senegalese sole larvae suggest that PL digestion may be more efficient, possibly as a result of a higher activity of phospholipases, compared to neutral lipases. Nonetheless, in European seabass, even though the dietary FA composition significantly affected lipase activity, no correlation was found between enhanced lipolytic activity and weight. It has been suggested that digestive enzymes are produced in excess to standard dietary needs and this might be particularly relevant in larvae with relatively short guts, where a high digestive activity may compensate for the short gut transit time. Therefore, existing data seems to collectively indicate that the enzymatic capacity is not a limiting factor for fish larvae to deal with high lipid diets.

The main obstacle to high lipid inclusion levels in diets for marine fish larvae may thus be at the absorption level. Feeding larvae on high neutral lipid diets, particularly when PL supply is deficient, results in the accumulation of lipid droplets in the enterocytes, which may in turn reduce the absorption of the FA products of luminal digestion. Even so, not all FA are equally affected by the lipid accumulation in the enterocytes and mechanisms of specific PUFA absorption and preferential incorporation into structural tissues probably exist to minimize EFA deficiency. Nevertheless, lipids are also an important source of metabolic energy and the supply of SFA and MUFA in sufficient amounts is critical, particularly in fast growing larvae with high developmental energetic demands. Very little is known regarding the cytological and biochemical aspects of lipid absorption in fish and virtually no studies exist for fish larvae. The events occurring between luminal lipolysis and assembly of lipid digestion products into the lipoproteins that will transport the lipid into the body tissues is still a

mystery. If so little is known concerning the natural mechanisms of lipid absorption, studying the impact of dietary composition and how it may affect these processes is a major challenge.

The results presented in this thesis suggest that gilthead seabream larvae, contrary to juvenile and adult fish, do not simply regulate their food intake according to total lipid level. A stringent control of food intake according to dietary energy level or a lipostatic regulation of food intake may not be so likely in fish larvae which have a much higher demand for energy and structural components that are required for growth, early organ development and physiologically demanding processes, such as metamorphosis. This study has also shown that dietary FA composition seems to play a major role on nutrient absorption and ingestion rate but the exact mechanisms behind these observations still need to be clarified.

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, at the ingestion, digestion and absorption levels.

In the future, studies should be conducted to describe the normal pathways and dynamics of lipid absorption at the cellular level and how these may be affected by quantitative and qualitative changes in the dietary lipid supply in fish larvae. This may be accomplished through histological methodologies, mostly through electronic microscopy (EM) and EM together with radiotracer studies (autoradiography). In addition, it is hoped that future studies based on molecular biology and functional genomics approaches will allow more insight into the physiological mechanisms occurring inside the larval enterocyte and their regulation by the diet. For instance, work should be conducted to identify genes which are implicated in the enterocyte lipid metabolism and that are regulated by the quantity and quality of lipid in the diet, and whose study may provide important insights into the molecular events involved in the regulation of TAG reesterification, intracellular trafficking and lipoprotein synthesis and its relationship with the accumulation of lipid droplets in the fish enterocytes. A few examples

of enzymes and proteins which are expected to be affected by the total lipid level in the diet and have different affinities towards different FA are: the MAG acyltransferase (mediates the acylation of 2-MAG in the neutral lipid reesterification pathway) (Oxley et al., 2005), the intestinal FA binding protein (involved in the intracellular transport of FA) (Sire and Vernier, 1981), the microsomal triglyceride transfer protein (MTP; catalyses the lipid transport between membranes and may be essential for the assembly and secretion of lipoproteins) (Jamil et al., 1995; Marza et al., 2005) and some apolipoproteins which are present in the lipoproteins responsible for lipid transport from the enterocytes into the body circulation (Wang et al., 2001).

On the other hand, knowledge in this field is expected to advance greatly in the near future with the improvement of formulated diets, which will boost nutritional studies and lead to considerable progress which can then be directly re-invested in the development of increasingly better formulations. In particular, even though quite a lot has been done to study EFA requirements, more attention needs to be given to the balance between EFA and other FA which function as metabolic fuel, to determine optimal ratios of neutral to polar lipid that enable an optimized utilization (absorption and retention) of the dietary total lipid fraction, as well as to the global macronutrient composition of the diet, namely the optimal concentration of protein relative to lipid levels (i.e., protein-energy balance), to verify whether a protein-sparing effect may be achieved in marine fish larvae.

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