Dietary neutral lipid level and source in Senegalese sole (*Solea senegalensis*) larvae: effect on growth, lipid metabolism and digestive capacity

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Running title: Dietary lipid level and source in S. sole larvae

Abstract

Contrary to larval essential fatty acid (EFA) requirements, the effect of dietary neutral lipid supply has been little investigated in marine fish larvae. The present work investigates the effect of feeding Senegalese sole larvae on *Artemia* enriched with higher or lower doses of lipid emulsion. Two lipid sources – soybean oil and fish oil – were compared. From 16 days after hatching (DAH) onwards, larvae were fed one of four experimental treatments: *Artemia* enriched on a high or low dose of soybean oil emulsion (HS and LS) or *Artemia* enriched on a

high or low dose of fish oil emulsion (HF and LF). In terms of growth, the dietary lipid level did not have a significant effect while the soybean oil treatments induced a lower growth than the fish oil-enriched *Artemia*. The fatty acid (FA) composition of the larvae closely reflected the dietary quantitative and qualitative FA profile. Only slight dietary effects were noted in the activity of trypsin, lipase and alkaline phosphatase. A higher amount of lipid droplets was noticeable in the posterior intestine epithelia and in the hepatocytes of larvae fed *Artemia* enriched with higher lipid doses, while LS-*Artemia* induced the lower lipid accumulation on the basal zone of the enterocytes, in accordance with the lowest total lipid level measured in this treatment. These results suggest an important effect of dietary total lipid level on lipid accumulation in the enterocytes and on FA absorption. At 33 DAH a tube feeding trial was conducted with ^{14}C-labelled oleic acid (OA) or triolein (TRI), showing that the lower accumulation of lipid droplets in the larvae fed LS was associated with a significantly higher absorption and retention in the gut and body tissues of the TRI label. For OA no significant differences between treatments were found. TRI label was considerably more evacuated than free OA, indicating that sole larvae may have a lower capacity to incorporate a triacylglycerol, which needs to be digested. Finally, OA appears to be preferentially utilized for energy production, accumulating more in larval tissues when absorbed in higher amounts.

Keywords: Triacylglycerol level; *Artemia* enrichment; Lipid absorption; Lipid vacuoles; Enterocytes; Digestive enzymes; Oleic acid; Triolein

Abbreviations: ARA, arachidonic acid (20:4n-6); DAH, days after hatching; DHA, docosahexaenoic acid (22:6n-3); EFA, essential fatty acids; EPA, eicosapentaenoic acid (20:5n-3); FA, fatty acids; FAME, fatty acid methyl esters; HUFA, highly unsaturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

1. Introduction

Lipids are important components in diets for larval fish species, given their central role as a source of energy and essential fatty acids (EFA) (Rainuzzo et al., 1997; Sargent et al., 1997, 1999). Therefore, in the last decades, a large amount of research effort has been directed towards the development of lipid enrichment products and strategies, in order to raise the EFA content, particularly of the n-3 highly unsaturated fatty acids (HUFA), of the nutritionally deficient live preys used in marine larviculture (Rodríguez et al., 1996; Rainuzzo et al., 1997; Han et al., 2000). However, excessive dietary lipid contents or unbalances in lipid class composition found occasionally in enriched live preys have been suggested to affect fatty acid (FA) digestion and absorption (Salhi et al., 1995, 1997, 1999; Diaz et al., 1997) and have been related to poor larval growth and performance in several species (Kjørsvik et al., 1991; Hoehne, 1999; Pousão-Ferreira et al., 1999; Izquierdo et al., 2000; Olsen et al., 2000a). In larvae of Senegalese sole (*Solea senegalensis* Kaup 1858), the long term feeding of a higher neutral lipid *Artemia* resulted in reduced growth, higher lipid droplet accumulation within the gut enterocytes (mostly in the basal zone) and lower capacity of larvae to absorb dietary FA (Morais et al., 2005ab). Since in these previous studies the *Artemia* neutral lipid content was increased by enrichment with a soybean oil emulsion, rich in linoleic acid (18:2n-6), while the low lipid *Artemia* was non-enriched, these live preys not only differed in their lipid content but also in their FA composition. Moreover, despite both types of *Artemia* were deficient in HUFA, the enrichment with soybean oil may have caused a dilution of the EFA naturally present in *Artemia* (mostly eicosapentaenoic acid – EPA, 20:5n-3; and arachidonic acid – ARA, 20:4n-6), possibly contributing to the observed changes in growth. In addition, lipid absorption could also have been affected by the change in *Artemia* FA profile, since it has been shown in carnivorous juvenile and adult fish 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, 2000b, 2003; Caballero et al., 2002, 2003). Besides, dietary FA profile may influence not only the composition and morphology 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).

Having this in mind, the present study was conducted in order to clarify the importance of total neutral lipid and FA contents in *Artemia*, on dietary lipid absorption and growth performance of fish larvae. Hence, this study aimed to determine the effect of feeding Senegalese sole larvae with *Artemia* enriched with two different types of oil (soybean and fish oil), at two inclusion levels, on larval growth, on the morphology of the gut mucosa and on digestive and absorptive processes such as enzyme activity, FA absorption and metabolism.

2. Materials and methods

2.1. Larval rearing and experimental diets

Larvae were obtained from IPIMAR-CRIPSul (Olhão, Portugal) at 14 days after hatching (DAH), with an average wet weight of 3.73 ± 0.17 mg (n=5, 20 pooled larvae). Until this age they had been fed *Artemia* enriched in a mixture of microalgae – *Nannochloropsis* sp. and *Isochrysis* sp. (50:50, on a volume basis). At this time the larvae had already settled and were transferred to a recirculation system of 12 three L flat bottom trays, which were stocked with 200 larvae each. During two acclimation days the larvae in all the trays were fed *Artemia* enriched for 12 h, at 250 nauplii mL^{-1}, with a mixture of Easy DHA Selco (INVE Aquaculture NV, Dendermonde, Belgium; two doses, at 0 h and 9 h, according to manufacturer's instructions) and Microfeed (EWOS, Bathgate, Scotland; 0.4 g L^{-1} in the same two doses).

From 16 DAH onwards the larvae were fed one of four experimental treatments, in triplicate trays: *Artemia* enriched on a high dose of soybean oil emulsion (HS), *Artemia* enriched on a low dose of soybean oil emulsion (LS), *Artemia* enriched on a high dose of fish oil emulsion (HF) and *Artemia* enriched on a low dose of fish oil emulsion (LF). The enrichment oil emulsions were prepared by mixing (per 100 g of emulsion): 86 g of commercial soybean oil (OliSoja, Sovena, Portugal) or fish oil (mostly sardine oil; Sorgal, Ovar, Portugal), 5 g of soybean lecithin (Sorgal), 3 g of Tween 80 (Sigma-Aldrich, St. Louis, MO, USA), 2 g of alginic acid (Sigma-Aldrich), 2 g of a vitamin mixture containing 35% vitamin C (Sorgal) and 2 g of another mixture including 50% vitamin E (Sorgal). The enrichments were then prepared by blending the emulsions with water (1g emulsion : 100-200 g water) in a high speed blender and were conducted during 16 h at a density of 150 instar II nauplii mL^{-1} of seawater (35 g L^{-1}), at 28-30 ºC and with strong aeration. In the high lipid enrichments (HS and HF), 0.6 g L^{-1} were added in two doses (0.4 g L^{-1} at 0 h and 0.2 g L^{-1} at 8 h), while a single dose of 0.04 g L^{-1} was added at the start of the enrichment in the low lipid treatments (LS and LF). A single batch of enriched *Artemia* from each treatment was produced and was kept frozen for the duration of the experiment. Samples of *Artemia* from each treatment were collected in three eppendorfs and into liquid nitrogen, for the determination of total lipid and FA composition. Larvae were fed frozen enriched *Artemia* (after thawing in seawater), in excess to satiation, three times daily. Photoperiod was 12 L: 12 D, salinity was kept at 33 g L^{-1}, temperature varied between 20.5 and 22 ºC and oxygen level was maintained at 93-97 %.

2.2. Sampling

Larvae were collected at the start of the experiment (n=5, 20 pooled larvae each) and were stored in liquid nitrogen, for later FA analysis. At 23 DAH, after 7 days of feeding on the experimental diets, 20 larvae were collected from each tray, washed in distilled water, frozen

and later freeze-dried in a Savant VLP120 ValuPump (Savant Instruments Inc., Holbrook, NY, USA), for the determination of individual dry weight (DW). Close to the end of the experiment, at 32 DAH, larvae were sampled again for the determination of DW and protein content (20 larvae $tray^{-1}$), optical histological analysis (20 larvae $tray^{-1}$, fixed in 10% buffered formalin-calcium) and total lipid and FA analysis (20 larvae $tray^{-1}$, stored in liquid nitrogen until analysis). Sampling for enzyme analysis was conducted at 34 DAH (20 larvae $tray^{-1}$, washed in distilled water and stored in liquid nitrogen).

2.3. Total protein, total lipid and fatty acid analysis

The larvae used in the measurement of individual DW at 32 DAH were later pooled, re-hydrated and homogenised (Ultra Turrax T25, IKA Labortechnik, Staufen, Germany), followed by freeze-drying. Each freeze-dried sample (one per tray) was then divided in triplicate aliquots on which total protein content was determined according to a method modified from Lowry (Rutter, 1967). The same method was used to determine the total protein of the experimental diets. Lipids were extracted with a chloroform:methanol (2:1, v:v) mixture, as described by Folch et al. (1957). The fatty acid methyl esters (FAME) were obtained by transesterification with 1% sulphuric acid in methanol and were purified by adsorption chromatography on NH2 sep-pack cartridges (Waters, S.A., Milford, Massachussets), as decribed by Fox (1990), and separated and quantified by gas-liquid chromatography following the conditions described by Izquierdo et al. (1990). FAME were identified by comparison to external standards.

2.4. Enzyme analysis

The larvae (20 per replicate tray) that were kept in liquid nitrogen were defrosted on ice and their digestive tracts were dissected and homogenized (Ultra Turrax T25) in 1.5 mL of ice cold distilled water. The homogenate was used immediately for the determination of trypsin

activity and was kept frozen (-20 ºC) until the remaining enzymatic determinations that were performed for alkaline phosphatase and lipase activities. Trypsin activity was assayed using BAPNA (Na-benzoyl-DL-arginine-pnitroanilide) (Sigma-Aldrich) as substrate (Tseng et al., 1982), while the activity of alkaline phosphatase was measured following the method of Bessey et al. (1946), using as subtrate p-nitrophenylphosphate (pNPP) (Sigma-Aldrich). Lipase activity was assayed according to a spectrophotometric method slightly modified from Iijima et al. (1998), using as substrate p-nitrophenyl myristate (Sigma-Aldrich) (Morais et al., 2004a). Enzyme specific activities were expressed as μmoles of substrate hydrolyzed per minute, per mg of protein (i.e. U mg protein^{-1}) at 25 °C for trypsin, 37 °C for alkaline phosphatase and 30 °C for lipase. Enzyme activities were also expressed as segmental activities, i.e., total activity per larval segment (digestive tract). Protein was determined by the Bradford procedure (Bradford, 1976).

2.5. Histological analysis

To examine the histological appearance of the gut and liver of 32 DAH larvae, 10 larvae per tray fixed in 10% buffered formalin-calcium were dehydrated in a graded ethanol series and embedded in paraffin. Serial 4 μm sections were stained with haematoxylin and eosin (H&E) (Martoja and Martoja-Pierson, 1970) and examined under light microscopy. Evaluation of histological changes (amount of lipid vacuoles) in gut and liver was carried out using a four-graded examination scheme: 0 = not observed; 1 = scarce; 2 = moderate; 3 = numerous.

2.6. Tube feeding trial

A tube feeding trial was conducted at 33 DAH, using the methodology and experimental setup described by Rønnestad et al. (2001) and Morais et al. (2005a). This method allows following the metabolic fate of a tracer nutrient into the following compartments: retention in

body and gut, catabolism (CO_2 trap) and evacuation. Larvae from each dietary treatment were tube fed a mixture of either ^{14}C-radiolabelled glycerol tri[1-^{14}C]oleate (or triolein, TRI; Amersham Pharmacia Biotech UK Limited, 3.7 MBq mL^{-1}) or [1-^{14}C]oleic acid (OA, 18:1n-9; Amersham Pharmacia Biotech UK Limited, Little Chalfont, Bucks, UK; 3.7 MBq mL^{-1}) and the absorption and metabolism of these labels were examined after an incubation period of 24 h. The tube feeding mixtures were prepared by adding the labels to "cold" purified triolein (VWR Prolabo, Fontenay-sous-Bois, France), to obtain a specific activity of 222 dpm nL^{-1} mixture and, after flushing with nitrogen (N_2) to evaporate all traces of solvents, the solutions were stored at -20 ºC until use. On the day before the trial, 30 min after the last meal, 10 larvae were removed from each triplicate tray and pooled in smaller trays (two trays per treatment, one for tube feeding TRI and another for OA, containing a total of 15 larvae each). These trays were transported to the radioisotope experimental room, where the larvae were acclimated and kept unfed overnight. Temperature and salinity were maintained at 22 ºC and 35 g L^{-1}, respectively, and the trial was conducted under continuous illumination. Thirty minutes before tube feeding *ca*. 15,000 *Artemia* metanauplii of the enrichment being tested were added to each tray and the larvae were allowed to feed for 30 min. Six larvae from each treatment were tube fed 46 nL of the radiolabel mixture (in two microinjections of 23 nL). After 24 h, each larva was removed from the incubation setup, rinsed in clean saltwater and the gut was separated from the remaining body by dissection. Both compartments were transferred separately to 6 mL scintillation vials (Sarstedt, Rio de Mouro, Portugal), and were solubilised in 200 µL of 30% w/v hydrogen peroxide (Sigma-Aldrich) at 60 ºC for 24 h. After cooling, 4 mL of scintillation cocktail (Ultima Gold XR, Packard Bioscience, Monza, Italy) were added to each 6 mL vial. The 20 mL scintillation vials (Sigma-Aldrich) with 8 mL of incubation seawater (water compartment, containing the evacuated label) and the 20 mL vials containing 5 mL of KOH 0.5M that had been gradually acidified by the addition of 1 mL of hydrochloric acid (HCl 0.1M) (metabolic trap, including the $^{14}CO_2$ resulting from catabolism)

were prepared for scintillation counting by adding 12 mL or 10 mL, respectively, of Ultima Gold XR (Rønnestad et al. 2001; Morais et al., 2005a). The samples were counted on a Beckman LS 6000IC (Beckman Instruments Inc, Fullerton, USA) liquid scintillation counter and the results are presented as a percentage of dpm in each compartment, in relation to the total counts.

2.7. Statistical analysis

The data obtained for each treatment in terms of larval growth, total protein and lipid content, FA composition (only of total FA classes and of the quantitatively and qualitatively most important FA), enzymatic activity, as well as the distribution of label in each compartment after tube feeding, were compared through one-way and two-way (to examine the combined effect of the factors "lipid level" and "lipid source") analysis of variance (ANOVA), using the software Statistica 6 (StatSoft Inc., Tulsa, USA). The assumption of homogeneity of variance was checked using the Bartlett's test and a significance level of 0.05 was employed (Zar, 1996). Data from the tube feeding trial (percentage of counts found in each compartment) and all other percentage data were $\arcsin(x^{1/2})$ transformed. When significant differences were found, the Tukey HSD multiple range test was performed. All data is given as mean values with standard deviations (S.D.).

3. Results

3.1. Biochemical composition of the experimental diets

The total protein content of the enriched *Artemia* varied between 28.7 and 34.9% while, for the total lipid content, the higher lipid diets (HS and HF) contained a higher lipid level than

the corresponding low enrichment treatments (LS and LF), as expected, even if the difference was not too pronounced (particularly in the case of the fish oil diets) (Table 1).

In terms of FA composition, the differently enriched *Artemia* showed a different FA profile, reflecting the quantity and especially the composition of the oil used in the enrichment emulsion (Table 2). Thus, soybean oil-enriched *Artemia* (HS and LS), were characterized by a higher level of polyunsaturated FA (PUFA) of the n-6 series, mainly linoleic acid (18:2n-6), although the ARA content was lower than in *Artemia* enriched with fish oil. In terms of their n-3 HUFA content, particularly docosahexaenoic acid (22:6n-3; DHA) and EPA, zero and very low amounts, respectively, were found in both soybean oil-enriched *Artemia*. Between the two soybean oil-enriched *Artemia* the main difference was the higher content of saturated FA (SFA), particularly palmitic (16:0) and stearic (18:0) acids, in LS *Artemia* which also showed the lowest content of total PUFA. Fish oil-enriched *Artemia* (HF and LF) were characterized by a higher level of ARA and n-3 PUFA, particularly of the HUFA DHA and EPA. These two later FA were present in lower amounts in LF *Artemia*, which had a higher linolenic acid (18:3n-3) content.

3.2. Larval growth and biochemical composition

At 23 DAH no statistically significant differences in growth were noted between larvae fed the different experimental regimes ($P > 0.05$; one-way ANOVA), while at 32 DAH the soybean oil treatments appeared to induce a lower growth than the fish oil diets, although only significantly for LS (Fig. 1). When a two-way ANOVA was performed, the factor "lipid level" was not found to significantly affect larval growth, while "lipid source" had a significant effect both at 23 DHA ($P < 0.05$) and at 32 DAH ($P < 0.001$).

In terms of total protein and lipid content (Table 1), no significant differences were noted between the larvae fed different experimental regimes, although larvae fed on HF *Artemia* tended to have slightly higher total lipid content and a correspondingly lower total protein

composition, while the inverse appeared to occur for larvae fed the LF *Artemia*. Analyzing the results through a two-way ANOVA revealed a significant effect of "lipid level" on the larval protein content, with larvae fed the low lipid treatments showing a higher protein level, but no significant differences were found in total lipid content.

The FA composition of the larvae and the changes noted from 14 to 32 DAH generally reflected the dietary FA profile (Table 3). Larvae of 32 DAH showed a reduction in SFA, particularly in those fed HS *Artemia*, in comparison with those at the beginning of the experiment (14 DAH). Larvae fed soybean oil-enriched *Artemia* (HS and LS) had a significantly lower content of EPA, DHA and n-3 HUFA and a higher level of n-6 PUFA. Larvae fed HS *Artemia* presented the significantly highest total n-6 PUFA content, particularly of linoleic acid, and the significantly lowest ARA level, together with significantly lower contents of n-3 PUFA and HUFA than LS *Artemia*. On the other hand, larvae fed fish oil-enriched *Artemia* presented significantly lower n-6 and higher n-3 PUFA contents, as well as higher HUFA levels (mainly EPA and DHA), which were significantly highest in HF larvae. The two-way ANOVA has shown a significant effect of both "lipid level" and "lipid source" on all the analyzed FA and total FA classes, except for SFA (only "lipid level" was significant), HUFA and n-3 PUFA (only "lipid source" was significant). However, in all cases there was also a significant interaction between factors.

3.3. Larval enzymatic activity

At 34 DAH there were no significant differences in trypsin specific activity in terms of protein content, but HS-fed larvae showed a significantly lower trypsin segmental activity (Fig. 2). Similarly, for lipase no significant differences were found in terms of specific activity. However, the LS treatment was responsible for a significantly lower lipase segmental activity than the LF treatment. The activity of alkaline phosphatase, an enzyme involved in nutrient absorption, was also assayed. In this case, the LF treatment was responsible for a

higher specific activity (although only significantly different from HS) and segmental activity. When a two-way ANOVA was performed to analyze enzymatic specific activity, significant differences were only found for alkaline phosphatase for the factor "lipid level", being the low lipid *Artemia* responsible for a significantly higher alkaline phosphatase specific activity in larvae fed these diets. In terms of segmental activity, significant differences were found in all assayed enzymes for the factor "lipid source", with the soybean oil-enriched *Artemia* inducing lower enzymatic activities. The factor "lipid level" was significantly different for trypsin segmental activity (but in this case there was also a significant interaction between factors) and for alkaline phosphatase segmental activity (same differences as with specific activity).

3.4. Histological analysis

The examination of the histological sections showed that, in general, lipid accumulation in the enterocyte was higher in the posterior intestine than in the anterior intestine. In the anterior intestine, a scarce accumulation of lipid was observed in all diets while in the posterior intestine very large lipid accumulations were detected in the basal zone of the enterocytes (Table 4 and Fig. 3). This was particularly noticeable in larvae fed *Artemia* enriched with higher lipid doses (HS and HF). The LS diet induced the lowest lipid accumulation in the posterior intestine enterocytes. With respect to the liver, larvae fed diets containing the highest lipid levels showed an intense accumulation of lipid vacuoles in the hepatocytes, being this moderate for larvae fed the LS and LF diets (Table 4 and Fig. 4).

3.5. Tube feeding trial

When the larvae submitted to the different dietary regimes were tube fed either TRI or OA (the free FA that composes the triacylglycerol TRI), considerable differences were seen in the absorption and metabolism of these labels (Fig. 5). Firstly, TRI was much more evacuated by the larvae, independently of their feeding regime. Therefore, TRI was little retained in the gut

and body tissues, compared to OA. In addition, the absorbed TRI was mostly catabolised. If the amount of catabolised label is expressed in terms of percentage of total absorbed label, a catabolism of 88%, 41%, 75% and 72% of total absorbed TRI was measured for the HS, LS, HF and LF treatments, respectively, while the catabolism was significantly lower for the tube fed free OA – 17%, 23%, 19% and 19% for larvae fed HS, LS, HF and LF, respectively.

Tube feeding TRI resulted in significant differences between larvae submitted to different experimental treatments, in the amount of label that was found in the analyzed compartments. Larvae fed the LS treatment showed a significantly lower TRI evacuation than larvae fed the HS *Artemia*, while larvae fed fish oil-enriched *Artemia* (HF and LF) showed an intermediate and non-significantly different evacuation. Consequently, larvae fed the LS *Artemia* presented a higher absorption of TRI and the significantly highest label retention in the gut and body tissues. No significant differences were measured between treatments in the metabolic trap compartment but when the catabolism results were expressed in terms of percentage of total label absorbed, the LS treatment showed a significantly lower TRI catabolism, compared to the remaining treatments, which were not significantly different from each other. The LS treatment induced such large differences that in most compartments (except in the metabolic trap) the two-way ANOVA detected a significant effect of the factor "lipid level" (and also of "lipid source" in the gut compartment), but always accompanied by a significant interaction between both factors. On the other hand, when OA was tube fed, no significant differences were found (both through one-way and two-way ANOVA) in the way the larvae submitted to different dietary treatments absorbed and metabolized this label.

4. Discussion

Enriching *Artemia* with different doses of lipid emulsion did not enable achieving large differences in total lipid level between the high and low lipid *Artemia*. The effect of total neutral lipid level on Senegalese sole larval growth and nutrient absorption efficiency has been addressed before but using non-enriched *Artemia* as the lower lipid diet (Morais et al., 2005ab). However, even if larger differences in total lipid level were achieved in such way, the experimental treatments most probably differed on their relative FA profile and it can be argued that the observed effects might be linked to the FA composition of the diet and are not only due to dietary lipid level. The present and previous studies have evidenced the problems faced with approaches using live feed to study the relative importance of dietary lipid levels and FA compositions in growth performance, digestive physiology and lipid metabolism, as it is very difficult to accurately manipulate both simultaneously in a living organism. The need for effective formulated diets, allowing better control of the dietary composition and limiting the effect of factors acting simultaneously, is clear and vital, in order to advance in this field. Fortunately, considerable progress has been achieved in the development of inert diets in the last few years (Koven et al., 2001; Cahu et al., 2003; Langdon, 2003; Yúfera et al., 2003) and some prototype diets are now becoming available for experimental work.

In the present study, feeding Senegalese sole on *Artemia* enriched with a high or low dose of lipid emulsion did not affect significantly larval growth and the type of lipid source appeared to have a more important effect. Thus, fish oil-enriched *Artemia* induced a better growth than the soybean oil-enriched one, in agreement with the higher n-3 HUFA content of the former. However, growth reduction was slightly and non-significantly more pronounced in larvae fed soybean oil-enriched *Artemia* at lower lipid levels, denoting a situation of both marginal EFA, energy and/or protein deficiencies. Accordingly, larval FA profile closely

reflected the quantitative and qualitative FA composition of their diet, as is commonly found in fish larvae and has been described before for Senegalese sole larvae (Morais at al., 2004b).

Slight dietary effects could be noted in the activity of enzymes involved in the digestive and absorptive processes, mostly in terms of segmental activity. However, the results indicate a relationship between larval size and the enzymatic activity in the dissected digestive tracts, as the two-way ANOVA revealed significant differences in segmental activity for all assayed enzymes for the factor "lipid source". The soybean oil-enriched *Artemia* treatments, which induced a lower larval growth and a lower digestive tract weight (data not shown), were also responsible for lower enzymatic activities. Nonetheless, the soybean oil in higher doses may have had a particularly depressing effect on trypsin activity, as a significantly lower segmental activity (and a non significant trend in specific activity; one-way ANOVA) was measured in larvae fed the HS diet, whose size was not significantly different from the remaining treatments. At 34 DAH the LS treatment was responsible for a significantly lower lipase segmental activity than the LF *Artemia* (one-way ANOVA). In a previous study performed with seabass larvae, the dietary lipid level was not found to affect lipase activity but the source of dietary lipid had a significant affect (Morais et al. 2004a). The differential lipase response was explained as being possibly caused by differences in the FA composition of the diet, related to the specificity of lipase towards FA differing in chain length and degree of saturation. The method utilized in the present experiment is most likely determining the activity of the non-specific bile salt-activated lipase which has been shown to have a higher affinity for PUFA (Iijima et al., 1998; Izquierdo et al., 2000). Nevertheless, as mentioned above, the smaller size of LS larvae must be at least partly responsible for the reduction in segmental lipase activity. In terms of specific activity (i.e., after standardizing for larval size using protein content), no significant differences were found, even if there was a trend for a lower specific activity in larvae fed the HF *Artemia*. A reduction in lipase specific activity caused by formulated diets containing fish oil has been noted in 52 DAH seabass by Morais et

al. (2004a), where it was suggested that the higher digestibility of the diets containing PUFA and HUFA could eventually cause an adaptative response leading to lower lipase secretion. Regarding the alkaline phosphatase activity, a trend for higher activity was noted when larvae were fed lower lipid diets (particularly LF), and this "lipid level" effect was significant when a two-way ANOVA was performed, both in terms of segmental and specific activity. The alkaline phosphatase is involved in nutrient absorption and transport across the intestinal epithelia and its activity has been related to food intake (Fraisse et al., 1981). Ribeiro et al. (1999) has found a trend for higher alkaline phosphatase activity in Senegalese sole that were seen to feed more actively. In a previous study conducted with Senegalese sole larvae it was shown that feeding the larvae with *Artemia* enriched on a soybean oil emulsion, when compared to non-enriched *Artemia*, appeared to result in a slower amino acid absorption and it was suggested that this may result in a slower clearance of the gut lumen with potential effects in the reduction of food intake (Morais et al., 2005b). Nevertheless, in the present study the food intake was not measured and even if the possibility exists that a higher dietary lipid level might potentially lead to a lower food intake in sole larvae, this can only be speculated at the moment and further studies are necessary.

Increased lipid content in *Artemia* markedly increased the amount of lipid droplets found in the posterior intestine epithelia, denoting the higher lipid absorption in these larvae. On the contrary, the lowest enterocyte lipid accumulation found in larvae fed *Artemia* enriched with a low dose of soybean oil is in agreement with the lowest lipid level found in this dietary treatment. It has been suggested that the dietary FA composition may alter the intestinal membrane composition, leading to changes in its morphological structure and fluidity, and may affect intestinal lipid metabolism and transport (Caballero et al., 2002, 2003). One of the suggested potential metabolic effects would be in the intracellular pathways of triacylglycerol (TAG) and phospholipid (PL) reacylation, which appear to be affected by the nature of dietary lipids (Sire and Vernier, 1981; Izquierdo et al., 2000; Olsen et al., 2000b). In this respect, van

Greevenbroek et al. (1995), working with Caco-2 cells derived from a human colorectal carcinoma, noted that the intracellular lipid resynthesis differed according to the nature of the FA, with palmitic acid (16:0) increasing PL synthesis, while the presence of linoleic acid (18:2n-6) in the culture media led to higher amounts of TAG being synthesized. In fish, relatively little is known regarding the pathways of lipid reesterification within the enterocytes and how these may be affected by dietary FA nature. Olsen et al. (2000b) found that replacing a part of dietary linseed oil by 16:0 reduced significantly lipid droplet accumulation in the enterocytes. In addition, Caballero et al. (2004) found that in seabream the intestinal TAG and PL biosynthesis is affected by the type and FA composition of the vegetable oil included in the diet, with soybean oil diets resulting in a high production of phosphatidylcholine. Moreover, 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 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 involved in the intestinal reacylation of digested lipid into TAG or PL. In the present study, the differences in lipid accumulation within the enterocytes appeared to be directly correlated to the dietary quantitative supply of neutral lipid and no dietary influence in terms of FA composition was noted.

The lipid vacuoles were localized mostly in the basal zone of the enterocyte in all treatments, implying a reacylation of the dietary FA. In addition, no lipid accumulation was observed in the enterocyte intercellular spaces, indicating that there were no problems of lipid transport into the body circulation. Salhi et al. (1999), when feeding larval seabream (*Sparus aurata*) with high dietary neutral lipid content, also observed increased lipid vacuoles in the basal zone of the enterocytes, suggesting a reduction in the lipid transport rate from the intestinal mucosa to the blood. In rats, it was noted that the rate of FA esterification may

regulate FA absorption from the lumen (Borgström, 1977) and, in adult fish, lipid accumulation within the enterocytes has been reported and is believed to result from a slower lipid processing, compared to mammals, 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 (Sire and Vernier, 1981). The results from the present study give support to this idea and the intracellular location of the lipid droplets denote a problem at the level of lipoprotein synthesis and/or secretion rather than in FA reacylation.

Similarly to what was observed in the enterocytes of sole larvae, the accumulation of lipid droplets in the hepatocytes was higher in the higher lipid diets (HS and HF), independently of their lipid source, suggesting an important effect of dietary total lipid level.

The histology results also showed that the posterior intestine section of the digestive tube of sole was more active in absorbing dietary lipid, than the anterior intestine. The capacity for lipid absorption by the intestinal epithelium has been noted from the onset of exogenous feeding in many fish species, although the specific localization of the predominant absorption site in the digestive tract segment varies with species (Izquierdo et al., 2000). In carp, rainbow trout, cod and Artic char, lipid absorption was demonstrated to occur mainly in the proximal region of the intestine and in the pyloric caeca (Noaillac-Depeyre and Gas, 1974; Sire et al., 1981; Lie et al., 1987; Olsen et al., 1999), whereas in turbot the posterior area of the gastrointestinal tract, mainly the posterior intestine and rectum regions, was described as being more active (Koven et al., 1994).

When sole larvae that had been fed on different experimental diets were tube fed ^{14}C-labeled TRI, the LS treatment induced a significantly lower label evacuation than the HS diet, together with a significantly higher absorption into the gut and body tissues. However, no

significant differences in TRI absorption were noted between larvae fed HF or LF *Artemia*. The total dietary lipid level might explain these results, as the difference between the total lipid of the *Artemia* enriched with higher or lower doses of fish oil (HF and LF) was lower than that obtained when enriching the *Artemia* with a soybean oil emulsion (HS and LS). This difference in total lipid level of the diets was probably of nutritional significance, as the histological appearance of the larval gut mucosa appears to confirm. Therefore, these results substantiate previous suggestions that a high dietary neutral lipid supply may result in an overload of the enterocyte with lipid droplets, which in turn may decrease FA absorption efficiency (Morais et al., 2005a).

In addition, the tube feeding results show that, independently of the feeding regime and in comparison to OA, TRI was much more evacuated into the incubation water and was little retained in the gut and body, corroborating previous results that sole larvae have a lower capacity to absorb a TAG, which still needs to be digested, compared to a free FA (Morais et al., 2005a). Nonetheless, these results are not in agreement with previous observations by Izquierdo et al. (2000, 2001) in larvae of seabream, where OA was better incorporated into the body lipids when it was provided in a microdiet esterified to TAG rather than as free FA. The authors suggested a limited capacity of reacylation or transport of free FA, or its preferential utilization as energy source in the enterocyte, as possible explanations. This discrepancy between both studies could be related to differences in the activity of the enzymes involved in lipid digestion in each fish species, since a lower total bile salt-activated lipase activity has been found in another flat fish, *Psetta maxima*, in comparison with several sparids, including gilthead seabream (Izquierdo et al., 2000). More studies on the digestive processes of both species are necessary to determine if this difference is species-specific or related to methodological differences, such as the type of feed used in these studies.

On the other hand, in contrast with what was observed with TRI, when OA was tube fed no significant differences were found in the way the larvae absorbed and metabolized this label,

which might indicate that the factors which were affecting the absorption of the triacylglycerol label (TRI) did not affect the absorption of a free FA. Differences in lipase activity and thus in the ability of larvae submitted to different dietary regimes to digest TRI can not explain these results which must then be related to absorption aspects. The reason why the lipid droplet accumulation within the basal region of the enterocytes had an influence on TRI absorption but did not affect free OA can not be explained and contradict the previous study where this accumulation affected both TRI and OA absorption (Morais et al., 2005a). Differences in the dietary quantitative supply of neutral lipid and in the subsequent accumulation of lipid in the enterocytes might partly explain the differences between the present and the latter study.

The tube feeding trial also showed that a considerably higher proportion of OA is used for the production of energy (i.e. catabolised) when supplied as TRI rather than in the free form. This has been noted previously in Senegalese sole larvae (Morais et al., 2005a) and it was suggested that the metabolic fate of OA depends on its dietary molecular form, being significantly more catabolised when supplied esterified to a TAG (TRI), the usual provider of FA for beta-oxidation, than when esterified to a PL (L-3-phosphatidylcholine-1,2-di-oleoyl) or in the free form. It seems, however, puzzling that free OA is absorbed and subsequently metabolized differently from when it is tube fed esterified to TRI which, in adult fish, is at least partly digested to free OA and 2-monoacylglycerol or even entirely to OA and glycerol before absorption (Sire et al., 1981; Koven et al., 1994), although the digestive process is not completely known in larvae and differences may exist. Nevertheless, these results may be explained simply by differences in total absorption of OA when supplied as TRI or in the free form. If OA is highly utilized for energetic purposes by fish larvae, as has been postulated by Rodríguez et al. (1994) and Izquierdo et al. (2001), it could be possible that when it is absorbed in very large amounts, as appears to be the case of free OA, a more substantial fraction would be directed into anabolic pathways, enhancing its accumulation in the gut and

other body tissues. Thus, when larvae were fed LS *Artemia*, a significantly higher amount of label was absorbed, meaning that a larger proportion of FA will remain after catabolism, explaining the higher label retention in the gut and body tissues of these larvae. On the contrary, in the HS treatment, a significantly lower amount of TRI was absorbed compared to larvae fed LS *Artemia*, resulting in a much higher proportion of label being catabolised (and thus less retained) when expressed as a percentage of total label absorbed.

The results described in the present paper confirm earlier suggestions linking an increase in total neutral lipid content of the diet with the accumulation of lipid droplets within the basal zone of the gut enterocytes and a decrease in the absorption of labeled FA, i.e. in the rate of lipid export from the enterocyte into the body circulation. However, the differences between the total lipid content of the high and low enriched *Artemia* did not allow finding an effect on larval growth, which was only affected by the type of lipids. Thus, the enrichment of *Artemia* with fish oil, rich in HUFA, slightly enhanced growth, both at high and low doses. In this case, the enhanced growth of larvae fed these *Artemia* is likely due to the higher dietary supply of EFA, for which there are specific mechanisms of absorption and reacylation into structural lipids, independently of the presence of lipid droplet accumulation in the enterocytes. Finally, OA appeared to be preferentially utilized for energy production, accumulating more in larval tissues when absorbed in higher amounts (e.g. when supplied as free OA or as TRI in larvae fed LS-*Artemia*).

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Table 1. Total protein and lipid content (% of dry weight, DW) of the experimental diets and of the larvae, at 32 DAH. Values for the *Artemia* correspond to a single enrichment batch, while values for 32 DAH larvae are means (n=3) ± S.D.

Treatment	Total protein (% DW)	Total lipid (% DW)
Enriched *Artemia*		
HS	32.8	23.2
LS	28.7	20.0
HF	34.9	25.2
LF	33.6	23.0
Larvae (32 DAH)		
HS	55.2 ± 2.1	27.0 ± 1.3
LS	55.8 ± 0.2	27.8 ± 1.6
HF	53.8 ± 0.7	28.8 ± 0.9
LF	57.3 ± 0.4	25.4 ± 1.3

Table 2. Fatty acid composition (g/100g fatty acids) of the enriched *Artemia*.

	HS	LS	HF	LF
14:0	0.56	0.71	1.60	1.16
14:1n-5	0.55	0.66	0.63	0.75
14:1n-7	0.17	0.26	0.29	0.25
15:0	0.14	0.15	0.16	0.18
15:1n-5	0.45	0.55	0.50	0.60
16:0iso	0.05	0.08	0.07	0.08
16:0	13.85	18.10	14.02	12.49
16:1n-9	0.65	0.43	0.27	0.37
16:1n-7	1.99	2.27	4.46	3.71
Me 16:0	0.04	0.05	0.08	0.07
16:1n-5	0.49	0.73	0.56	0.62
16:2n-6	1.23	1.27	1.01	1.23
17:0	0.06	0.11	0.58	0.40
16:2n-4	0.71	1.10	0.74	0.72
16:3n-4	0.54	0.62	0.73	0.77
16:3n-3	0.36	0.38	0.37	0.44
16:4n-3	0.16	0.18	0.19	0.22
16:4n-1	0.00	0.04	0.08	0.02
18:0	8.37	11.18	6.01	6.02
18:1n-9	19.88	17.62	18.45	18.85
18:1n-7	6.27	7.21	7.19	7.79
18:1n-5	0.08	0.09	0.13	0.12
18:2n-9	0.34	0.41	0.37	0.45
18:2n-6	19.12	9.40	4.67	5.40
18:2n-4	0.10	0.06	0.00	0.09
18:3n-6	0.36	0.44	0.43	0.48
18:3n-4	0.00	0.00	0.00	0.09
18:3n-3	17.15	18.35	17.35	21.54
18:4n-3	2.01	2.30	2.32	2.67
18:4n-1	0.00	0.00	0.06	0.00
20:0	0.26	0.33	0.20	0.20
20:1n-9	0.45	0.46	0.20	0.02
20:1n-7	0.08	0.10	1.58	1.04
20:2n-9	0.03	0.00	0.05	0.05
20:2n-6	0.27	0.26	0.23	0.24
20:3	0.00	0.09	0.10	0.00
20:4n-6	0.56	0.73	0.97	0.98
20:3n-3	0.44	0.50	0.48	0.58
20:4n-3	0.36	0.41	0.64	0.60
20:5n-3	1.48	1.81	6.82	5.47
22:1n-11	0.00	0.00	1.53	1.08
22:1n-9	0.29	0.45	0.18	0.15
22:4n-6	0.00	0.00	0.00	0.05
22:5n-3	0.00	0.15	0.73	0.40
22:6n-3	0.00	0.00	2.98	1.59
Totals				
SFA	23.34	30.71	22.71	20.60
MUFA	31.35	30.81	35.96	35.34
PUFA	45.22	38.48	41.32	44.06
HUFA	2.40	3.10	12.14	9.09
n-3 PUFA	21.97	24.07	31.87	33.51
n-6 PUFA	21.54	12.09	7.32	8.37
n-3/n-6	1.02	1.99	4.35	4.01

Table 3. Fatty acid composition (g/100g fatty acids) of the larvae at the start (14 DAH) and close to the end (32 DAH) of the experimental period. Values are means (n=2-3) ± S.D. Treatments at 32 DAH with different superscript letters induced significant differences in FA content (one-way ANOVA, $P<0.05$).

	Start	**End of the experiment (32 DAH)**			
	(14 DAH)	**HS**	**LS**	**HF**	**LF**
14:0	1.74 ± 0.15	0.61 ± 0.00	0.79 ± 0.01	1.32 ± 0.05	1.08 ± 0.01
14:1n-5	0.53 ± 0.03	0.39 ± 0.01	0.51 ± 0.01	0.41 ± 0.02	0.49 ± 0.01
14:1n-7	0.40 ± 0.04	0.16 ± 0.01	0.22 ± 0.00	0.27± 0.01	0.26 ± 0.00
15:0	0.10 ± 0.00	0.08 ± 0.00	0.08 ± 0.00	0.06 ± 0.03	0.09 ± 0.01
15:1n-5	0.51 ± 0.04	0.44 ± 0.00	0.58 ± 0.00	0.41 ± 0.02	0.51 ± 0.01
16:0iso	0.18 ± 0.13	0.05 ± 0.01	0.09 ± 0.01	0.06 ± 0.00	0.07 ± 0.00
16:0	17.76 ±1.44	11.45 ± 0.86	13.52 ± 0.09	13.60 ± 1.20	14.20 ± 0.78
16:1n-9	1.00 ± 0.31	1.31 ± 0.04	1.28 ± 0.03	0.67 ± 0.36	0.57 ± 0.01
16:1n-7	3.19 ± 0.10	1.84 ± 0.01	2.18 ± 0.03	4.17 ± 0.12	3.20 ± 0.13
Me 16:0	0.13 ± 0.01	0.04 ± 0.00	0.05 ± 0.00	0.09 ± 0.00	0.07 ± 0.00
16:1n-5	0.67 ± 0.05	0.58 ± 0.01	0.85 ± 0.00	0.57 ± 0.01	0.68 ± 0.00
16:2n-6	0.97 ± 0.06	1.28 ± 0.02	1.26 ± 0.00	0.85 ± 0.02	1.01 ± 0.02
17:0	0.43 ± 0.02	0.12 ± 0.01	0.17 ± 0.01	0.75 ± 0.04	0.52 ± 0.08
16:2n-4	0.66 ± 0.05	0.47 ± 0.00	0.70 ± 0.02	0.64 ± 0.04	0.70 ± 0.02
16:3n-4	0.59 ± 0.04	0.57 ± 0.01	0.72 ± 0.00	0.74 ± 0.02	0.68 ± 0.06
16:3n-3	0.19 ± 0.09	0.28 ± 0.01	0.20 ± 0.01	0.30 ± 0.00	0.27 ± 0.06
16:4n-3	0.49 ± 0.02	0.30 ± 0.11	0.36 ± 0.15	0.33 ± 0.07	0.33 ± 0.05
16:4n-1	0.27 ± 0.02	0.26 ± 0.00	0.30 ± 0.05	0.30 ± 0.03	0.24 ± 0.01
18:0	8.35 ± 0.58	6.78 ± 0.53	8.64 ± 0.14	6.81 ± 0.47	7.89 ± 0.55
18:1n-9	20.14 ± 0.72	21.73 ± 0.13	21.00 ± 0.04	19.11 ± 0.48	19.03 ± 0.44
18:1n-7	6.55 ± 0.24	7.03 ± 0.10	8.64 ± 0.07	7.71 ± 0.24	8.20 ± 0.27
18:1n-5	0.15 ± 0.01	0.08 ± 0.00	0.04 ± 0.06	0.11 ± 0.00	0.11 ± 0.00
18:2n-9	0.27 ± 0.01	0.30 ± 0.01	0.38 ± 0.00	0.35 ± 0.01	0.39 ± 0.01
18:2n-6	10.11 ± 0.44	23.87 ± 0.49^{a}	14.32 ± 0.03^{b}	5.28 ± 0.31^{c}	5.72 ± 0.06^{c}
18:2n-4	0.00 ± 0.00	0.02 ± 0.03	0.02 ± 0.03	0.16 ± 0.00	0.06 ± 0.08
18:3n-6	0.32 ± 0.01	0.31 ± 0.00	0.39 ± 0.00	0.38 ± 0.00	0.40 ± 0.00
18:3n-4	0.00 ± 0.00	0.01 ± 0.02	0.02 ± 0.03	0.06 ± 0.06	0.03 ± 0.05
18:3n-3	10.25 ± 1.17	11.32 ± 0.64^{a}	11.87 ± 0.08^{ab}	12.80 ± 0.20^{b}	14.49 ± 0.57^{c}
18:4n-3	1.16 ± 0.25	0.79 ± 0.07	0.84 ± 0.00	1.38 ± 0.03	1.28 ± 0.06
18:4n-1	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.03	0.03 ± 0.00
20:0	0.39 ± 0.03	0.28 ± 0.02	0.36 ± 0.01	0.23 ± 0.02	0.28 ± 0.03
20:1n-9	0.18 ± 0.10	0.75 ± 0.00	0.79 ± 0.01	1.16 ± 0.03	1.08 ± 0.02
20:1n-7	1.90 ± 0.11	0.27 ± 0.02	0.34 ± 0.01	0.29 ± 0.01	0.30 ± 0.01
20:2n-9	0.12 ± 0.11	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.06	0.04 ± 0.01
20:2n-6	0.36 ± 0.01	0.81 ± 0.02	0.60 ± 0.00	0.30 ± 0.01	0.32 ± 0.00
20:3	0.09 ± 0.01	0.12 ± 0.00	0.18 ± 0.00	0.16 ± 0.01	0.16 ± 0.00
20:4n-6	0.82 ± 0.10	0.60 ± 0.31^{a}	1.32 ± 0.04^{b}	1.60 ± 0.04^{b}	1.56 ± 0.03^{b}
20:3n-3	0.74 ± 0.05	1.13 ± 0.01	1.37 ± 0.02	0.87 ± 0.06	1.08 ± 0.02
20:4n-3	0.39 ± 0.07	0.33 ± 0.02	0.42 ± 0.01	0.63 ± 0.02	0.59 ± 0.00
20:5n-3	1.98 ± 0.45	0.67 ± 0.06^{a}	1.06 ± 0.01^{a}	4.22 ± 0.09^{b}	3.25 ± 0.01^{c}
22:1n-11	1.30 ± 0.09	0.00 ± 0.00	0.05 ± 0.07	0.65 ± 0.07	0.68 ± 0.00
22:1n-9	0.22 ± 0.04	0.10 ± 0.00	0.09 ± 0.00	0.16 ± 0.01	0.16 ± 0.00
22:4n-6	0.08 ± 0.01	0.03 ± 0.04	0.04 ± 0.06	0.16 ± 0.00	0.14 ± 0.00
22:5n-6	0.00 ± 0.00	0.03 ± 0.05	0.06 ± 0.03	0.06 ± 0.05	0.08 ± 0.00
22:5n-3	1.29 ± 0.33	0.62 ± 0.00	0.90 ± 0.01	3.09 ± 0.21	2.23 ± 0.07
22:6n-3	2.98 ± 0.45	1.72 ± 0.06^{a}	2.37 ± 0.05^{a}	6.69 ± 0.20^{b}	5.43 ± 0.18^{c}
Totals					
SFA	29.08 ± 2.27	19.42 ± 1.40^{a}	23.70 ± 0.22^{ab}	22.91 ± 1.67^{ab}	24.21 ± 1.28^{b}
MUFA	36.75 ± 1.07	34.67 ± 0.17^{a}	36.56 ± 0.17^{a}	35.69 ± 0.96^{a}	35.27 ± 0.90^{a}
PUFA	34.14 ± 3.19	45.88 ± 1.29	39.74 ± 0.06	41.40 ± 1.21	40.52 ± 0.38
HUFA	7.53 ± 1.38	4.01 ± 0.23^{a}	6.18 ± 0.00^{b}	16.44 ± 0.48^{c}	13.29 ± 0.05^{d}
n-3 PUFA	19.49 ± 2.81	17.15 ± 0.63^{a}	19.39 ± 0.08^{b}	30.31 ± 0.74^{c}	28.94 ± 0.44^{c}
n-6 PUFA	12.65 ± 0.47	26.94 ± 0.71^{a}	18.00 ± 0.03^{b}	8.62 ± 0.41^{c}	9.24 ± 0.13^{c}
n-3/n-6	1.54 ± 0.19	0.64 ± 0.01	1.08 ± 0.01	3.52 ± 0.09	3.13 ± 0.00

Table 4. Evaluation of the histological appearance (accumulation of lipid droplets) in the gut and liver of 32 DAH Senegalese sole larvae fed different experimental diets (n=10). 0 = not observed; 1 = scarce; 2 = moderate; 3 = numerous.

Diet	Gut		Liver
	Anterior intestine	Posterior intestine	
HS	1	3	3
LS	1	1	2
HF	1	3	3
LF	1	2	2

Captions to figures

Fig. 1. Larval dry weight (mg), at 23 and 32 DAH. Values are means (n=60) ± S.D. Columns with different letters are significantly different (one-way ANOVA, $P<0.05$). NS – non significantly different.

Fig. 2. Enzymatic specific (U or mU / mg protein) or segmental (U or mU / segment) activity of the enzymes trypsin, lipase and alkaline phosphatase in Senegalese sole larvae, at 32 DAH. Values are means (n=3) ± S.D. Columns with different letters are significantly different (one-way ANOVA, $P<0.05$). NS – non significantly different.

Fig. 3. Histological appearance of the anterior intestine of 32 DAH Senegalese sole larvae (H&E, x400). Large lipid accumulations were detected in the basal zone of the enterocytes in larvae fed HS (a) and HF (c) *Artemia*, while scarce lipid accumulation was observed in the enterocytes of larvae on the LS treatment (b), and intermediate on the LF treatment (d). Bar = 25μm.

Fig. 4. Histological appearance of the liver of 32 DAH Senegalese sole larvae (H&E, x400). An intense accumulation of lipid vacuoles was observed in the hepatocytes of larvae fed HS (a) and HF (c) *Artemia* and less so in larvae fed the LS (b) and LF (d) treatments. Bar = 25μm.

Fig. 5. Label found in each compartment (% of total label tube fed), 24 h after tube feeding *S. senegalensis* larvae that were previously fed different experimental diets (HS, LS, HF or LF) with either ^{14}C-TRI (triolein) or ^{14}C-OA (oleic acid). Values are means (n=6) ± S.D. Different letters in different dietary treatments which were tube fed the same radiolabel indicate significant differences (one-way ANOVA, $P<0.05$). NS – non significantly different.

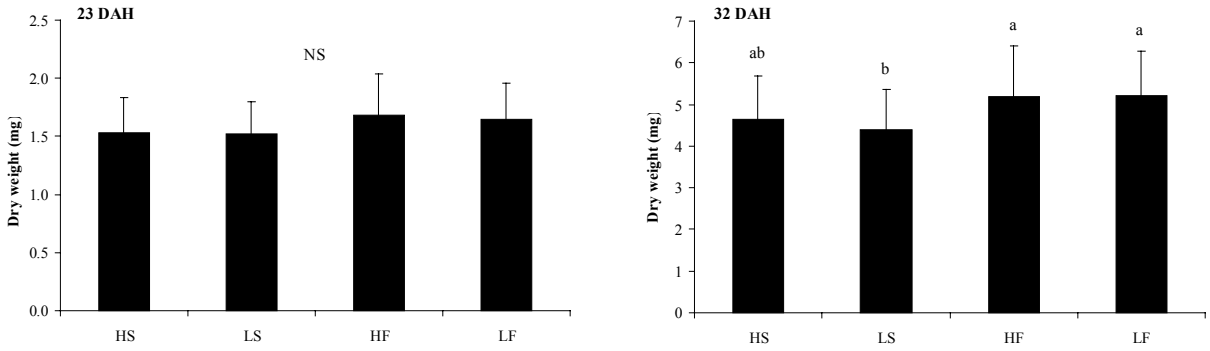
23 DAH
NS
Dry weight (mg)
2.5
2.0
1.5
1.0
0.5
0.0
HS
LS
HF
LF
32 DAH
ab
b
a
a
Dry weight (mg)
7
6
5
4
3
2
1
0
HS
LS
HF
LF

Fig. 1

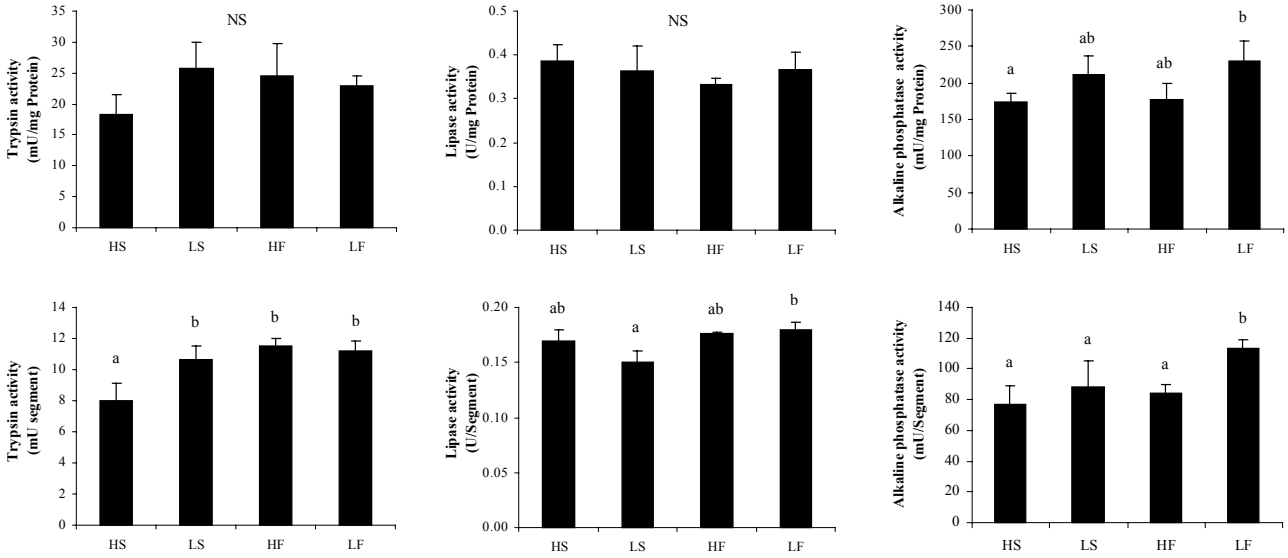
Trypsin activity (mU/mg Protein)
NS
Lipase activity (U/mg Protein)
NS
Alkaline phosphatase activity (mU/mg Protein)
a
ab
ab
b
Trypsin activity (mU/ segment)
a
b
b
b
Lipase activity (U/Segment)
ab
a
ab
b
Alkaline phosphatase activity (mU/Segment)
a
a
a
b
HS
LS
HF
LF

Fig. 2.

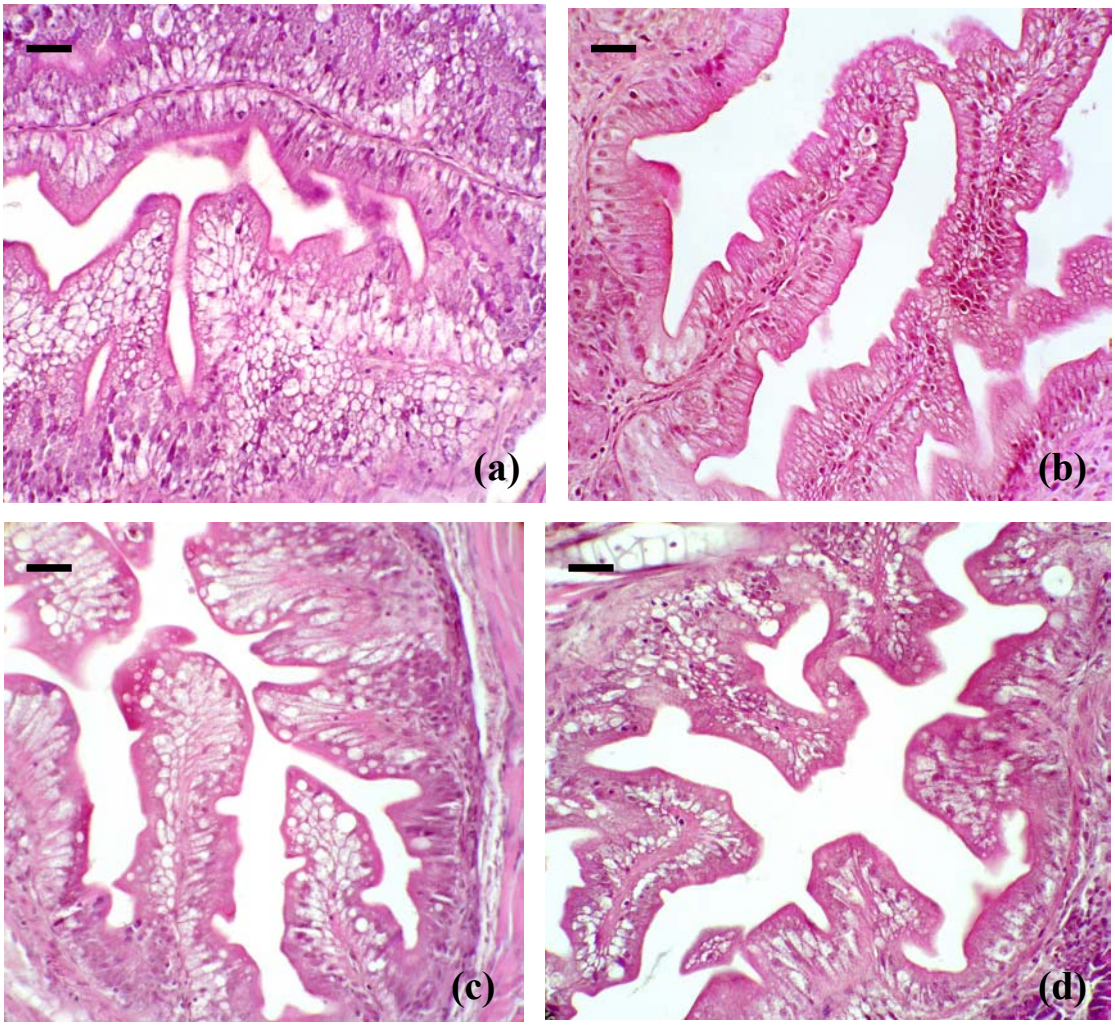

Fig. 3

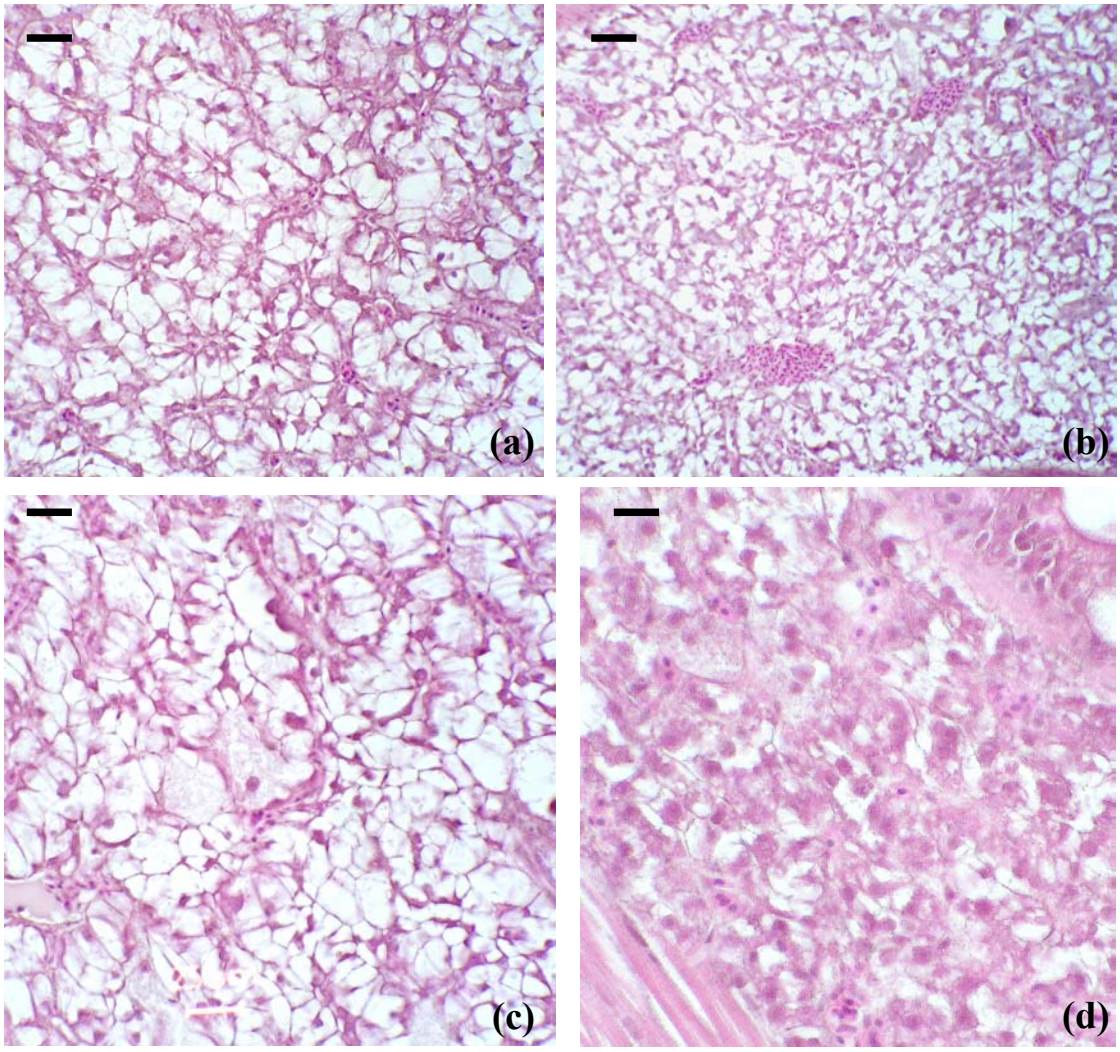

Fig. 4

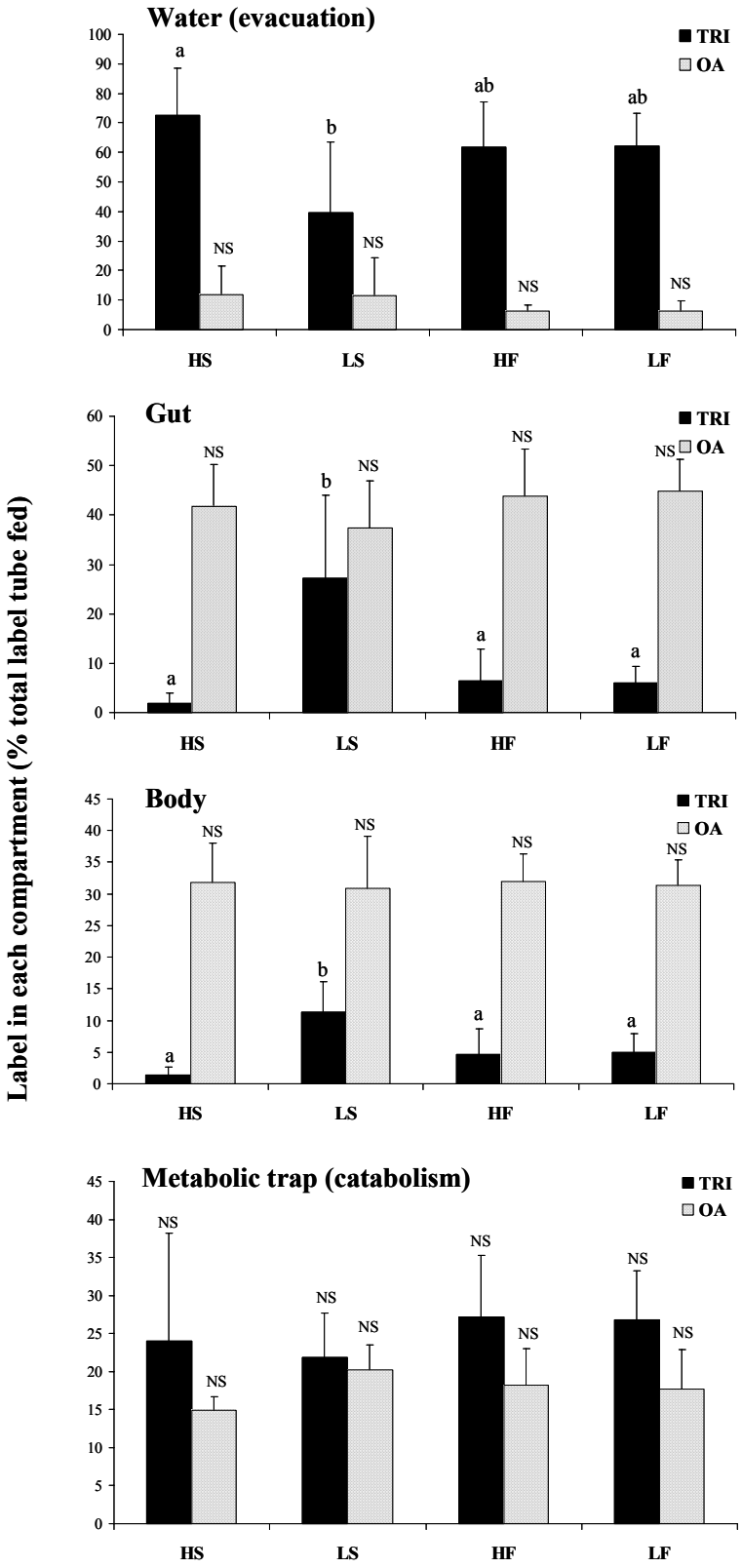
Water (evacuation)
Gut
Body
Metabolic trap (catabolism)
TRI
OA
Label in each compartment (% total label tube fed)
HS
LS
HF
LF
a
b
ab
NS

Fig. 5