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Dietary protein/lipid ratio affects growth and amino acid and fatty acid absorption and metabolism in Senegalese sole (*Solea senegalensis* Kaup 1858) larvae

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Abstract

Studies with fish larvae have reported poor performance associated with quantitative lipid imbalances in the diet and a lower dietary protein/neutral lipid ratio has been shown to result in an increased accumulation of lipid droplets in the enterocytes and in reduced fatty acid (FA) absorption efficiency in larval Senegalese sole. The present study examined the effect of dietary protein/neutral lipid ratios on amino acid (AA) absorption efficiency and metabolism, gut histology and growth in Senegalese sole larvae. Larvae were fed either non-enriched *Artemia* (NEA) or *Artemia* enriched on a soybean oil emulsion (EA) following larval settlement (at 16 days after hatching—DAH). AA absorption efficiency and metabolism were determined at 27 and 33 DAH by feeding the larvae on ^{14}C-AA-labeled *Artemia*. The effect on FA absorption was also verified at 28 DAH by tube feeding a lipid mixture containing the ^{14}C-labeled triacylglycerol triolein (TRI). A significantly lower growth was obtained with the EA diet, which also lead to an increased lipid accumulation in the gut epithelium. Feeding larvae with EA resulted in lower ^{14}C-FA absorption (significantly lower gut and body retention and higher label evacuation) at 28 DAH but no effect was noted in ^{14}C-AA absorption 24 h after feeding, at 27 and 33 DAH. However, larvae fed NEA evacuated a significantly higher amount of ^{14}C-AA at 3 h after feeding and presented a significantly higher AA catabolism. This indicates a faster AA absorption in NEA-fed larvae, which may allow more time for its metabolic use. On the other hand, a higher net AA absorption may be achieved in larvae fed NEA through a more rapid clearance of the lumen and sustained ingestion of the diet. The significantly 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.
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Keywords: Fish larvae; Neutral lipid accumulation; Protein; Amino acids; Food intake

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1. Introduction

Fish larvae are characterized by an extremely rapid growth coupled with high demands for energy and structural components (Conceição, 1997). Lipids, by being an important source of metabolic energy, components of biological membranes and precursors of essential metabolites (Sargent et al., 1989), have been extensively studied in marine larval nutrition. However, most studies are concerned with the requirements for essential fatty acids (EFA) (Watanabe et al., 1983; Sargent et al., 1997, 1999) and relatively few studies have examined the impact of lipid level on larval nutrient digestion and absorption. It has been hypothesized that the attempt to simulate the larva's natural diet and to meet larval requirements using oil sources poor in EFA may result in a high, possibly excessive, supply of neutral lipid (Sargent et al., 1989). Such a quantitative imbalance may have undesirable consequences during the digestive and absorptive processes and thus be detrimental to larval growth and performance. In fact, several authors have reported poor larval performance with a high dietary lipid level, attributing it to a decrease in larval digestion ability or in fatty acid (FA) assimilation efficiency (Kjørsvik et al., 1991; Hoehne, 1999; Olsen et al., 2000). In addition, high dietary neutral lipid content has been associated with the accumulation of large lipid droplets in the enterocytes of carp, gilthead seabream, Artic charr and rainbow trout (Diaz et al., 1997; Fontagné et al., 1998; Salhi et al., 1999; Olsen et al., 1999; Caballero et al., 2002, 2003), possibly reducing the nutrient transport rate across the intestinal epithelium. In a previous study (Morais et al., in press) with Senegalese sole larvae, it was shown that a lower dietary protein/neutral lipid ratio resulted in a tendency for lower growth and in an increased lipid accumulation within the gut epithelium, concurrently with a lower FA absorption efficiency (higher evacuation and lower gut retention of tube fed lipid-bound and free FA ^{14}C-labels). However, growth is mainly the result of protein deposition and it is not known whether the dietary protein/lipid ratio may also affect this process. Therefore, the present study was conducted in order to examine the effect of the previously tested dietary regimes—non-enriched *Artemia* (NEA) or *Artemia* enriched on a soybean oil emulsion (EA)—on the amino acid (AA) absorption capacity and metabolism of Senegalese sole larvae. The effect on growth, histological appearance of the midgut enterocytes and FA absorption was also analysed to confirm previous results.

2. Materials and methods

2.1. Larval rearing and diets

Senegalese sole (*Solea senegalensis* Kaup 1858) eggs were obtained from naturally spawning wild caught broodstock, maintained at the University of Algarve. Larvae were reared in a recirculating system until settlement, in 70 l cylindrical–conical tanks, at a temperature of 21±1 °C and a salinity of 35 g/l. At mouth opening (2 days after hatching—DAH), larvae were fed on *Artemia* AF (INVE Aquaculture NV, Belgium) nauplii until 9 DAH, when *Artemia* EG (INVE Aquaculture NV, Belgium) metanauplii enriched on Algamac-2000 (Aquafauna Bio-Marine Inc., CA, USA) were then fed. At 14 DAH frozen enriched metanauplii were introduced and replaced live *Artemia* (standard protocol, as after settlement sole larvae stay at the bottom of the tanks and do not feed well on actively swimming *Artemia* metanauplii). A transition period of 3 days was used between each prey type. When most larvae had settled at the bottom of the tank (16 DAH) they were transferred to 3 l flat bottom trays (200 larvae tray^{-1}) and were fed solely on frozen *Artemia* EG metanauplii, either enriched on a soybean oil emulsion (EA) or non-enriched (NEA), until the end of the experiment (36 DAH). Each treatment was tested in triplicate trays and larvae were fed in excess three times per day.

A single batch of NEA and of EA was prepared at the start of the experiment and was kept frozen for the duration of the experiment. The EA treatment was prepared by enriching *Artemia* on a soybean oil emulsion that was prepared by mixing well the following ingredients (per 100 g of emulsion): 86 g of commercial soybean oil (OliSoja, Sovena, Portugal), 5 g of soybean lecithin (Sorgal, Portugal), 3 g of Tween 80 (Sigma-Aldrich), 2 g of alginic acid (Sigma-Aldrich), 2 g of a vitamin mixture containing

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35% vitamin C (Sorgal, Portugal) and 2 g of a mixture including 50% vitamin E (Sorgal, Portugal). *Artemia* was hatched according to Van Stappen (1996) and, at instar II, was enriched in 100 l cylindrical–conical tanks, at a density of 150 nauplii per milliliter of seawater (35 g/l), at 28 °C and with strong aeration. The soybean oil emulsion was blended with water in a high-speed blender and enrichment was conducted over a period of 16 h, with two doses of 0.3 g emulsion per liter being added at 0 and 8 h after the start of enrichment. The NEA was prepared in the same manner, only without the addition of the enrichment emulsion.

2.2. Radiolabelled Artemia trials

Two trials were conducted in which sole larvae were fed radiolabelled *Artemia*, either NEA or EA, at 27 DAH (NEA treatment—1.57 mg DW; EA treatment—1.68 mg DW; n=20 per treatment, pooled sample) and at 33 DAH (NEA treatment—4.12 mg DW; EA treatment—3.41 mg DW; n=15 per treatment, pooled sample). *Artemia* was radiolabelled with a [U-^{14}C] protein hydrolysate (1.85 MBq ml^{-1}, Amersham Pharmacia Biotech Ltd, UK). In a previous study it was shown that *Artemia* enriched in this manner accumulate the majority of the label in the protein fraction (and the remaining in the free AA fraction) (Morais et al., 2004a), enabling studies on *Artemia* AA digestion and absorption.

Newly hatched *Artemia* were transferred to two 50 ml vials containing *ca* 8000 nauplii each in 40 ml of seawater (28 °C, 34 g/l). The *Artemia* in one vial was enriched (EA) and radiolabelled for 14 h by adding a single 0.6 g/l dose of soybean oil emulsion and 150 µl of [U-^{14}C] protein hydrolysate. The other vial (NEA) received only 150 µl of [U-^{14}C] protein hydrolysate. Both vials were sealed and connected to a ^{14}C-KOH trap (1000 ml, 0.5M) to avoid release of the radiolabel into the atmosphere (Morais et al., 2004a). At the end of the radiolabeling period, *Artemia* metanauplii were thoroughly washed and, before being fed to the larvae, triplicate samples were collected for 6% trichloroacetic acid (TCA) extraction.

The trials were conducted under temperature and salinity controlled conditions (23±1 °C, 35 g/l) and with continuous illumination. Approximately 14 h prior to the start of the trial and 30 min following their last feeding, larvae were transferred to smaller trays and transported to the radioisotope experimental room, where they were acclimated and unfed. Each tray contained a total of 36 larvae per treatment (12 larvae per replicate). Larvae from the EA and NEA treatments were allowed to feed on radiolabelled EA and NEA, respectively, for a period of 25 min. Of these, 27 larvae (9 larvae for each incubation period of 1, 3 and 24 h) with a full gut were transferred to the incubation setup, after being allowed to swim for 1 min in seawater (for rinsing). The incubation setup is described in Rønnestad et al. (2001). Individual 20 ml scintillation vials (Sigma-Aldrich) containing 8 ml of fresh seawater were tightly sealed with a rubber seal that was punctured by a metal tube inducing a gentle airflow just above the incubation water and a plastic capillary tube which was connected to a metabolic trap (20 ml vial with 5 ml KOH 0.5M). The CO_2 diffused into the air of the incubation vial passes through the KOH of the metabolic trap, where it is retained by conversion to HCO_3^-. At the end of each incubation period—1, 3 and 24 h—each larva was washed in clean seawater and the larval gut (also containing the liver) was separated from the remaining body by dissection. After sampling the larvae, the incubation vial was resealed and 1 ml of hydrochloric acid (HCl 0.1M) was added gradually, diffusing any remaining CO_2 from the water into the metabolic trap (Rønnestad et al., 2001). The incubation water and KOH–CO_2 (metabolic trap) were mixed with 12 ml of scintillation cocktail (Ultima Gold XR, Packard Bioscience). Body and gut were placed in separate 6 ml scintillation vials and, together with the samples of radiolabelled *Artemia*, were submitted to a 24 h 6% TCA extraction (1 ml, with periodical shakings, at 4 °C). After removal of the TCA soluble fraction (containing free AA and small peptides) into a clean 6 ml vial, the TCA precipitate (mostly protein) was solubilised (1 ml of 30% hydrogen peroxide, 60 °C, 24 h). The TCA soluble and precipitate fraction of gut and body were prepared for radioactivity counting by adding 4 ml of Ultima Gold XR. The samples were counted on a Beckman LS 6000IC liquid scintillation counter (Beckman Instruments Inc, Fullerton, USA). Results are presented as a percentage of the counts found in each compartment in relation to the total counts.

2.3. Tube feeding trial

At 28 DAH a tube feeding trial was conducted in order to analyse the effect of the NEA and EA diets on FA absorption efficiency. Two trays containing 12 larvae (4 larvae from each triplicate larval rearing tray) were transported to the experimental room, where they were acclimated 14 h before the trial. Of these, 9 larvae per treatment were tube fed a radio-labelled triolein (TRI; 3.7 MBq/ml; Amersham Pharmacia Biotech UK Limited) mixture after a 25 min period of feeding on either NEA or EA (6,000 defrosted metanauplii per tray), depending on the treatment being tested. The TRI mixture was prepared by adding the labeled TRI to commercial soybean oil, to obtain a specific activity of 222 dpm/nl, as described in Morais et al. (in press). The larvae were tube fed with a plastic capillary (0.19 mm inner diameter, Sigma, St. Louis, MO, USA), which deposited 23 nl of the mixture in the foregut through a nanolitre injector (World Precision Instruments, Sarasota, FL, USA) (Rønnestad et al., 2001). After allowing the larvae to swim in rinsing seawater for about 2 min, they were transferred to the same incubation setup used in the radiolabelled *Artemia* trials. At the end of the 24 h incubation period, the larvae were removed from the incubation vials, washed and dissected into gut (plus liver) and body. These two compartments were placed in separate 6 ml scintillation vials (Sarstedt) and solubilised in 1 ml of 30% w/v hydrogen peroxide (Sigma-Aldrich) at 60 °C for 24 h, after which 4 ml of scintillation cocktail (Ultima Gold XR, Packard Bioscience) were added. The incubation water and KOH–CO_2 (metabolic trap) were prepared and sample counting was done as described above.

2.4. Larval growth and survival

At the end of the experiment (36 DAH), 20 larvae per triplicate tray were removed and their total length (TL) was individually measured. The wet weight (WW) of each larva was simultaneously recorded and the dry weight (DW) was determined individually after freeze-drying during 48 h in a Savant VLP120 ValuPump (Savant Instruments Inc., NY, USA). The condition factor of each larva was calculated using the formula: $WW(g)/TL(cm)^3 \times 100$. Survival was determined by counting the larvae remaining at the end of the experiments and was corrected for the individuals sampled.

2.5. Artemia and larval protein and lipid composition

Samples of NEA and EA were collected, washed, frozen in liquid nitrogen and later freeze-dried. Triplicate samples were analysed for the determination of total protein content, according to a method modified from Lowry (Rutter, 1967), and total lipid content, using a Bligh and Dyer (1959) procedure modified for small samples. The same methods were used for the quantification of total protein and lipid content of larvae fed NEA or EA. After determining the individual DW, the 20 larvae from each replicate were pooled, re-hydrated, homogenised (Ultra Turrax T25, IKA Labortechnik) and freeze-dried. Each sample was then divided in triplicate aliquots on which the analyses were performed. Results are presented as a percentage of sample DW.

2.6. Histological analysis

At 28 DAH, 4 larvae per tray were sampled and immediately fixed in formol-calcium for about 60 h. After dehydration, the larvae were included in historesin (Leica) and 2 μm sections were cut in a Microm HM 340 E (Microm International, Germany) and stained with toluidine blue. Histological sections of two larvae from each replicate tray (6 per treatment) were examined under an optical microscope. Observation of the histology slides focused on

Table 1
Growth and survival of larvae fed non-enriched *Artemia* (NEA) and enriched *Artemia* (EA) at the end of the experiment (36 DAH)

	NEA	EA
Total length (mm)	15.1 ± 1.1^{a}	14.1 ± 1.1^{b}
Wet weight (mg)	35.0 ± 6.7^{a}	28.1 ± 5.9^{b}
Dry weight (mg)	6.2 ± 1.3^{a}	4.8 ± 1.0^{b}
Condition factor	1.01 ± 0.08^{NS}	0.99 ± 0.09^{NS}
Survival (%)	93.8 ± 3.5^{NS}	91.0 ± 4.3^{NS}

Data are means±standard deviation (S.D.) (n=60 per treatment for growth results and n=3 per treatment for survival). Values with different superscript letters are significantly different ($P<0.05$). NS—not significantly different.

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Table 2
Total protein (% DW), total lipid (% DW) and protein/lipid ratio of the *Artemia* diets and of larvae fed NEA and EA, at the end of the experiment (36 DAH)

	Total protein (% DW)	Total lipid (% DW)	Protein/Lipid
Artemia			
NEA	33.6±2.2	11.5±0.2	2.9
EA	25.3±1.3	21.9±0.4	1.2
Larvae			
NEA	52.8±1.5^NS	26.0±5.0^NS	2.0
EA	53.0±2.3^NS	28.2±2.4^NS	1.9

Results are means (n=3)±S.D. NS—not significantly different.

the appearance of the intestinal wall in the midgut region.

2.7. Statistical analysis

The results from the radiolabelled *Artemia* and tube feeding trials (for each compartment and incubation period), larval growth, survival, total protein and lipid content are given as mean values and standard deviations (S.D.) and were analysed by one-way ANOVA. All percentage data were arcsin ($x^{1/2}$) transformed and the assumption of homogeneity of variance was previously checked using the Bartlett's test (Zar, 1996). When significant differences were found at the P<0.05 level the Tukey HSD multiple range test was performed, using the software Statistica 6 (StatSoft Inc., Tulsa, USA).

3. Results

3.1. Larval growth and survival

The NEA diet induced a significantly (P<0.05) higher larval growth (TL, WW and DW) while no marked differences were found in condition factor. Survival was high and not significantly affected by dietary treatment (Table 1).

3.2. Artemia and larval total protein and lipid composition

The tested *Artemia* diets presented different protein/lipid ratios, with NEA having a higher relative total protein and lower total lipid content than EA (Table 2). However, no significant differences were found in the total protein and lipid composition of the larvae fed these diets.

3.3. Histological analysis

The observation of the histology slides of 28 DAH larvae revealed the presence of numerous larger vacuoles within the enterocytes of the midgut region of larvae fed EA (Fig. 1B) in contrast with what was observed in larvae fed NEA (Fig. 1A) where the vacuoles were smaller and the enterocytes showed a denser appearance.

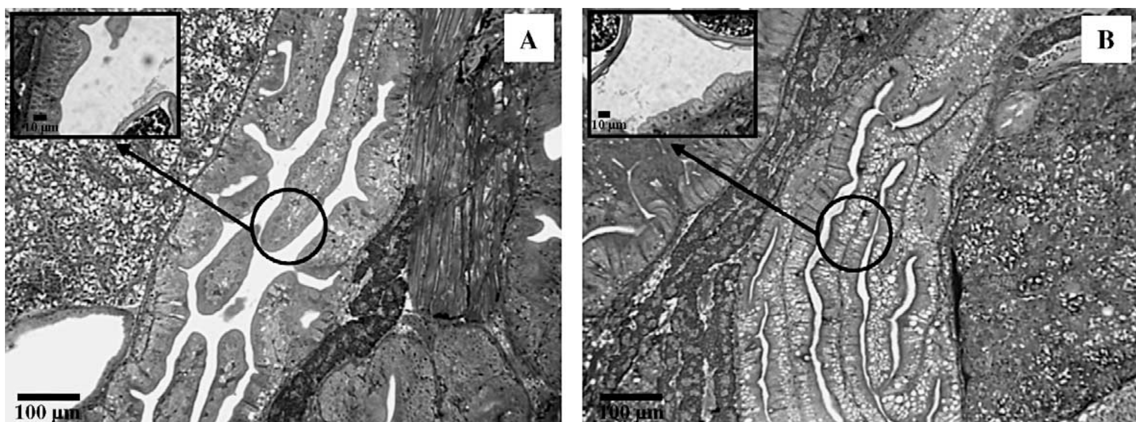

Fig. 1. Histological sections (2 µm historesin sections stained with toluidine blue) of the midgut region of 28 DAH *Senegalese sole* larvae fed NEA (A) or EA (B).

3.4. Radiolabelled Artemia trials

Analysis of the radiolabelled *Artemia* revealed that most of the label absorbed by the *Artemia* was incorporated into the TCA precipitate fraction (mostly protein) (Morais et al., 2004a) (27 DAH trial—NEA: 92.0±0.6%, EA: 93.4±1.1%; 33 DAH trial—NEA: 94.9±0.0%, EA: 94.6±0.1%), with the remaining being found in the TCA soluble fraction (mostly free AA).

Fig. 2 shows the distribution of ^{14}C-label between the different compartments at different incubation

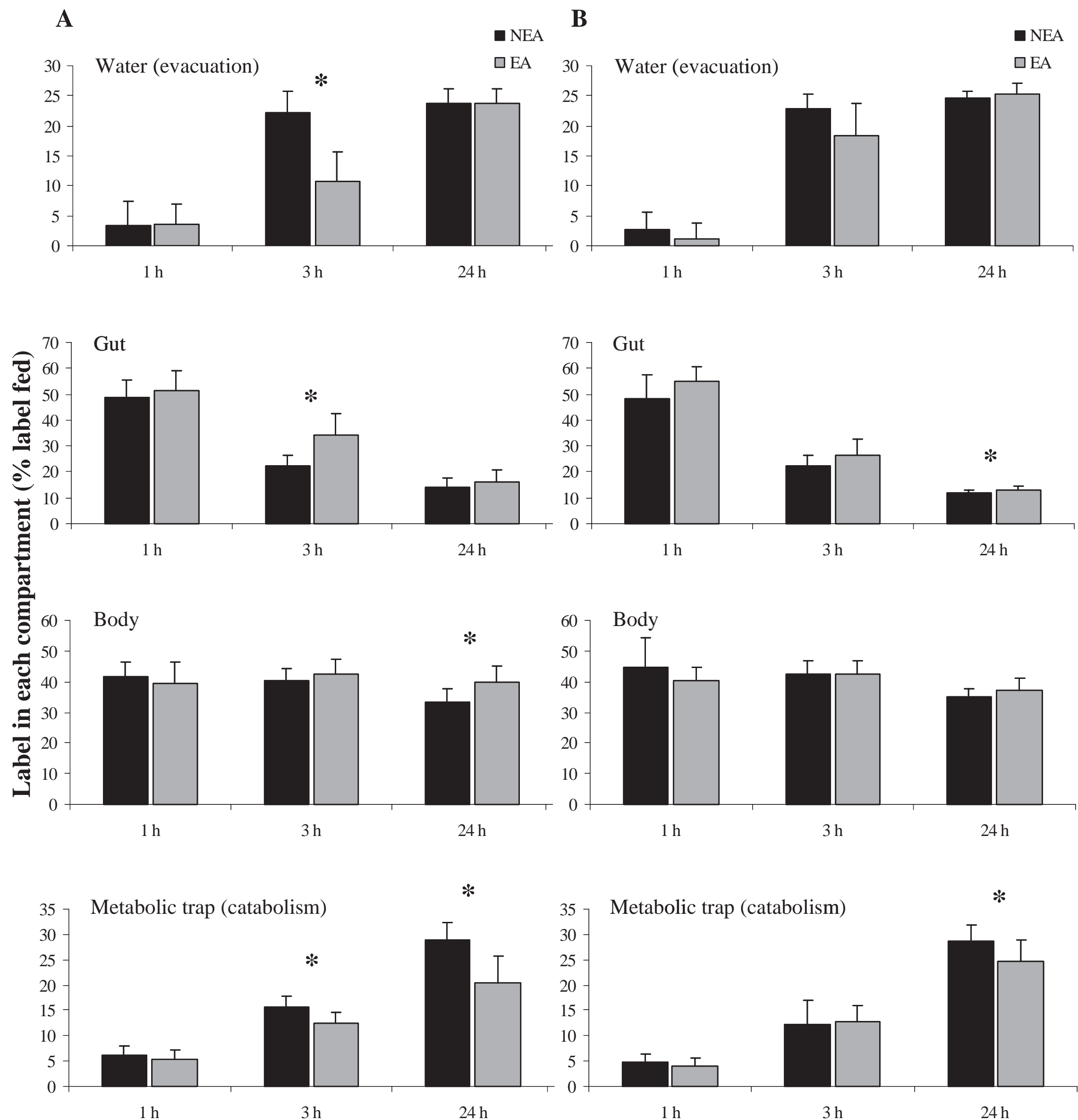

Fig. 2. Label in each compartment (% of total label fed) of 27 DAH (A) and 33 DAH (B) Senegalese sole larvae at 1, 3 and 24 h after feeding on either radiolabelled NEA or EA. Values are means (n=9)±S.D. Asterisks indicate significant differences (P<0.05) between NEA and EA at each incubation time.

periods, when larvae were fed either radiolabelled NEA or EA, at 27 DAH (Fig. 2A) and 33 DAH (Fig. 2B). In terms of evacuation, in spite of a significantly higher amount of the label being found in the incubation water of 27 DAH larvae fed NEA at 3 h, no significant differences were found at the end of the 24 h incubation period. Therefore, total label absorption (body, gut and catabolised, in relation to total label fed) was identical in 27 DAH (76% in both treatments) and 33 DAH (75% in both treatments) larvae fed either NEA or EA. There was a tendency for higher retention of label in the gut and body of larvae fed EA. However, significant differences in the gut were only found at 3 h in 27 DAH larvae and at 24 h in 33 DAH larvae, while in the body this was only observed at 24 h in 27 DAH larvae. In general, a higher catabolism of the AA label was noted in larvae fed NEA, which was statistically significant at 3 and 24 h in 27 DAH larvae and at 24 h in 33 DAH larvae.

The analysis of the label partition between the TCA fractions in the gut and body compartments revealed a rapid incorporation of the AA label into the TCA precipitate (mostly protein) of both of these compartments but particularly in the gut. There was 71–74% and 69–76% of the total radiolabel in the gut TCA precipitate fraction of 27 DAH and 33 DAH larvae, respectively, following 1 h of incubation. This value increased up to 81–86% (27 DAH) and 82–84% (33 DAH) at 24 h. In the body, at 1 h of incubation, label incorporation in the TCA precipitate was 51–52% and 54–55% of the total label of 27 DAH and 33 DAH larvae, respectively, increasing gradually to 89% in both ages after 24 h. No significant differences in label TCA partition were found between treatments.

3.5. Tube feeding trial

Tube feeding TRI at 28 DAH resulted in a significantly higher label retention in the gut and body of larvae fed NEA (Gut: 14.2±12.1%; Body: 11.7±8.4%), in comparison with larvae fed EA (Gut: 3.8±2.1%; Body: 5.2±2.7%), after a 24 h incubation period. No significant differences were found between larvae fed NEA or EA in the water (NEA: 33.6±18.0%; EA: 50.4±20.6%) or metabolic trap (NEA: 40.5±9.5%; EA: 40.5±17.5%) compartments, although label evacuation tended to be higher in larvae fed EA (probably not found statistically significant due to the large S.D. measured in this compartment).

4. Discussion

Imbalances in the lipid composition of the diet, either quantitative (lipid excess—i.e., low protein/lipid ratio) or qualitative (lipid class; e.g. phospholipid deficiency) have been reported to result in poor larval growth and performance (Kanazawa et al., 1983; Kjørsvik et al., 1991; Geurden et al., 1995; Coutteau et al., 1997; Hoehne, 1999; Pousão-Ferreira et al., 1999; Olsen et al., 2000). In addition, feeding larvae with triacylglycerol (TAG) rich diets and/or phospholipid-poor diets commonly results in an accumulation of lipid vacuoles in the basal zone of the enterocytes, which indicates good digestion and absorption of dietary TAG but a reduced transport capacity (Diaz et al., 1997; Fontagné et al., 1998; Olsen et al., 1999; Salhi et al., 1999; Izquierdo et al., 2000; Caballero et al., 2002, 2003). This accumulation is thought to be a natural occurrence in fish, with enterocytes acting as temporary sites of lipid storage resulting from the slow lipid processing occurring between FA assimilation from the digestive tract to secretion of lipoproteins into the serum (Noaillac-Depeyre and Gas, 1974; Sire and Vernier, 1981). However, unbalanced diets may extend this process further. Dietary phospholipid deficiencies may have a marked effect on lipid transport and lead to the accumulation of large amounts of lipid vacuoles in the enterocytes, as the endogenous synthesis of phospholipids may be insufficient to maintain an optimal rate of lipoprotein synthesis (Fontagné et al., 1998; Olsen et al., 1999; Salhi et al., 1999; Izquierdo et al., 2000). In rats, it has been shown that an increased rate of TAG infusion leads to an increased transport time from the endoplasmatic reticulum (ER) to the Golgi apparatus, up to a point were saturation of TAG occurs in the ER, inhibiting further lipid transport (Mansbach and Dowell, 2000). In some cases, these extensive lipid accumulations have been considered pathological, as they may damage enterocyte integrity and prevent normal nutrient transport (Olsen et al., 1999).

In a previous study conducted by the authors using the same dietary treatments used here for rearing Senegalese sole larvae (Morais et al., in press), it was

shown that when sole were fed EA, a diet with a higher neutral lipid content, there was a tendency for lower growth (although not statistically significant) and a higher accumulation of lipid droplets in the enterocytes. This lipid accumulation was shown to occur concurrently with a lower FA assimilation efficiency, with larvae fed EA presenting a significantly higher evacuation and lower gut retention of several lipid-bound and free FA tube fed labels. This lower FA assimilation efficiency may partly explain the tendency for lower growth noted in larvae fed high lipid diets. However, growth is mainly the deposition of protein and, therefore, a considerable effect in growth might be expected if high neutral lipid diets also affect AA absorption and metabolism. Kjørsvik et al. (1991) reported an overload of the digestive capacity and alterations in protein absorption in the hindgut of turbot larvae fed rotifers with a high lipid content. On the other hand, Olsen et al. (1999) described pinocytotic activity related to protein hydrolysis in Artic charr and noted that it was not affected by diets inducing differences in accumulation of lipid droplets in the enterocytes. Therefore, the present study was conducted in order to examine the effect of dietary protein/neutral lipid ratio on AA absorption and metabolism in Senegalese sole larvae.

Given the importance of EFA in larval growth and survival (Watanabe et al., 1983; Sargent et al., 1997, 1999), effects caused by differences in dietary total lipid content may be masked by differences in dietary EFA level. Therefore, in order to compare a higher dietary lipid supply with a diet containing no lipid enrichment, as is the case of NEA, soybean oil was chosen as the neutral lipid for *Artemia* enrichment because of its deficiency in EFA, DHA, eicosapentaenoic acid (EPA) and arachidonic acid (ArA). However, in spite of this concern, the enrichment with soybean oil had likely a small diluting effect on the naturally occurring ArA, EPA and DHA levels in *Artemia*. In addition, the dietary phospholipid level, as well as the phospholipid/neutral lipid ratio, which are known to affect larval fish growth and lipid transport (Kanazawa et al., 1983; Geurden et al., 1995; Coutteau et al., 1997; Fontagné et al., 1998; Olsen et al., 1999; Salhi et al., 1999; Izquierdo et al., 2000), may have differed between dietary treatments as a result of the enrichment with soybean oil in the EA treatment, which increased the neutral lipid level in this diet. Although this difference would have been reduced by the addition of 5% soybean lecithin in the soybean oil emulsion, it likely did not as the EA diet resulted in the accumulation of lipid droplets in the enterocytes, which has been described as being caused by an insufficient dietary intake of phospholipids resulting in an impaired lipoprotein synthesis (Fontagné et al., 1998; Olsen et al., 1999; Salhi et al., 1999; Izquierdo et al., 2000). Unfortunately, the FA and lipid class composition of the experimental *Artemia* used in the present study was not assessed. Consequently, the possibility exists that a decrease in phospholipid content, as well as a dilution of the relative amounts of highly unsaturated FA in the EA treatment, may have contributed to a growth reduction.

Despite the EFA deficiency of both NEA and EA, Senegalese sole appeared to perform well, in terms of growth and survival, on these diets. Similar results have been previously reported with this species (Morais et al., 2004b, in press) and with *Solea solea* larvae (Howell and Tzoumas, 1991).

The results from the present experiment confirmed the tendency for a lower growth in larvae fed EA observed by Morais et al. (in press) and also that feeding sole larvae with EA affects the histological appearance of the midgut mucosal epithelial cells, with larger lipid inclusions being noted in larvae fed EA. The tube feeding trial also confirmed previous results (Morais et al., in press) showing that feeding larvae with a lower dietary protein/lipid ratio, as is the case of EA, results in a significantly lower FA (product of TRI digestion) retention in the gut and body, together with a higher label evacuation. However, when larvae were fed *Artemia* radiolabelled with a [U-^{14}C] protein hydrolysate, in which most of the label is incorporated into the *Artemia* protein fraction (Morais et al., 2004a), the absorption efficiency of the AA label after a 24 h incubation period (75–76%) was independent of dietary treatment. These results suggest that the intracellular lipid inclusions in the enterocytes do not affect AA absorption, supporting the observations made by Olsen et al. (1999) in Artic charr.

In spite of no differences being found in AA absorption efficiency after 24 h, the absorbed AA label was significantly more catabolised and therefore less retained in larvae fed NEA, which appears to

contradict the significantly higher growth that was obtained in this experiment using the NEA diet. The percentage of label in the incubation water at 3 h, particularly in 27 DAH fish, appears to indicate a faster evacuation in larvae fed NEA, which points to a faster processing of the *Artemia* meal by these larvae. Therefore, AA absorption probably occurred earlier in these larvae, with more time being allowed for its metabolic use. In the present study the absorption rate did not affect total (net) absorption since the larvae were fed a single *Artemia* meal in a 24 h period. However, in a continuous feeding situation, a higher net AA absorption may be achieved in larvae fed NEA through a more rapid clearance of the gut lumen leading to sustained appetite and ingestion of the diet. A higher growth with NEA could thus be explained by a higher food intake in larvae fed with this diet. An increased intake of lower energy formulated diets was reported to result in significantly faster growth in juvenile seabream (Marais and Kissil, 1979). In the same study, however, it was not clear whether the reduction in feed intake with increasing soybean oil content was due to an excess of energy or if the nature of this oil has a somewhat deterrent effect. This is less likely in the present study where the soybean oil is incorporated into the *Artemia* FA composition, thus minimizing taste effects.

It has been suggested that fish eat primarily to satisfy their energy needs and studies have shown that food intake may be regulated according to the energy content of the diet, with a higher voluntary food intake being measured for low energy diets (Lee and Putnam, 1973; Boujard and Médale, 1994; Santinha et al., 1999; Sæther and Jobling, 2001). In this study food intake was not directly quantified but total dpm measured in larvae fed NEA was much higher than in those fed EA and the difference was larger than could be expected from differences in *Artemia* radiolabel incorporation efficiency (Morais et al., 2004a), which indicates that larvae fed NEA might have ingested a higher number of prey. A higher food intake in larvae fed NEA would also explain the faster evacuation observed in these larvae as meal size has been positively related with evacuation rate (Werner and Blaxter, 1980; Boehlert and Yoklavich, 1984). In addition, it might also justify the higher catabolism of absorbed AA label. Helland and Grisdale-Helland (1998) suggested that in adult Atlantic halibut higher protein consumption may result in increased deamination of dietary AA for energy purposes.

In conclusion, the long term feeding of a higher neutral lipid diet (EA) affected the capacity of larvae to absorb dietary FA and this was most probably caused by the higher accumulation of lipid droplets observed within the gastrointestinal mucosa, which may function as a physical barrier to efficient lipid absorption leading to an increased evacuation. On the other hand, AA absorption appeared to be independent of dietary lipid level and not affected by the presence of lipid droplets in the enterocytes. This suggests that the mechanisms associated with lipid and amino acid absorption are fundamentally different. Additionally, larvae fed the low lipid diet (NEA) demonstrated higher AA catabolism coupled with a slightly reduced AA retention but a faster AA absorption rate. The significantly 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 (i.e. a lower energy diet).

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