Free Amino Acids Are Absorbed Faster and Assimilated More Efficiently than Protein in Postlarval Senegal Sole (Solea senegalensis) 1

ABSTRACT To improve the formulation of diets for the early stages of marine fish, assimilation rates of free amino acids (FAA) and protein in postlarval Senegal sole (Solea senegalensis) were determined. Fish (2.45 ± 0.87 mg dry weight) were tube fed 36 nL of a diet of FAA containing L-[35S] methionine (FAA diet) or bovine serum albumin, containing L-[methylated-14C] bovine serum albumin (Prot-diet), both at a concentration of 4.08 g/L. A time series was performed, and the amounts of label in incubation water, liver, gut and body carcass were quantified. The FAA diet was absorbed with a 3.5-times-higher transfer rate (P < 0.001) from the gut into the larval body tissues compared with the Prot-diet. The FAA diet also was assimilated with greater efficiency than the Prot-diet (80% versus 58%, P = 0.001). If we assume that the label present in the gut represents amino acids incorporated into the intestinal tissue, the assimilation efficiencies for the two diets were 89 and 64%. Therefore, FAA seems to be superior to protein as a dietary source of amino acids in Senegal sole postlarvae. However, because the absorption dynamics of protein and FAA differ, care should be taken when using the sources together to avoid amino acid imbalance. J. Nutr. 130: 2809–2812, 2000.

KEY WORDS: • Solea senegalensis • fish larvae • absorption • protein • free amino acids.

There are direct and indirect literature data that suggest intestinal function, and especially proteolytic ability, is not fully developed at the time when fish larvae switch their feeding mode from endogenous yolk to exogenous live prey. Rust (1995) examined the developmental nutrient assimilation in six fish species by using a method of controlled tube feeding of radiolabeled nutrients. His results indicated that larvae that develop gastric digestion during metamorphosis (most marine fish of commercial interest belong in this group) initially assimilate simple forms of amino acids (AA)3 more efficiently than more complex forms [assimilation order: free AA (FAA) > peptides > protein]. His results (Rust 1995) also indicated that the differences in assimilation decreased as the larvae approached metamorphosis.

Due to their fast growth rate, fish larvae have a high requirement for AA, the building blocks for the protein synthesis. In addition, in marine fish larvae, including Senegal sole (Parra et al. 1999), AA are an important energy source (Conceição et al. 1998, Finn 1994, Finn et al. 1996, Fyhn 1989, Rønnestad and Fyhn 1993, Rønnestad and Naas 1993, Sivaloganathan et al. 1998). Therefore, there is an exceptional demand for AA by fish larvae. The need implies that the delivery of AA from intestinal protein digestion in marine fish larvae may be insufficient to cover the demand.

It has been proposed that freely dissolved AA may prevent nutritional deficiency during the developmental window from first feeding until the intestine is fully differentiated and able to digest ingested proteins (Fyhn 1989, Rønnestad et al. 1999, Walford et al. 1991).

To specifically test the assimilation rates of FAA and protein in the early stages of marine fish, postlarval Senegal sole (Solea senegalensis) were tube fed controlled amounts of diets containing [35S] and [14C]AA. To our knowledge, this is the first report on the kinetics of absorption of AA during the early life stages of marine fish.

MATERIALS AND METHODS

Larvae. The larvae originated from a group of naturally spawned eggs collected from a tank of wild-caught Senegal sole (S. senegalensis) maintained at the University of Algarve. The larvae were reared according to the protocol described by Dinis et al. (1999), in which the larvae were kept in tanks (100 L) at the University of Algarve in a recirculating system (temperature 21°C, salinity 34 g/L). Larvae opened their mouths 2 d after hatching (DAH) and started to feed on rotifers (Brachionus plicatilis, small strain) enriched with Tetraselmis sp. and Isochrysis sp. Artemia nauplii (Bass Entrée; INVE, Dendermonde, Belgium) were introduced at 3 DAH and became the only prey at 6 DAH. From d 10 on, the larvae were fed Artemia metanauplii (RH; INVE) enriched for 24 h with Tetraselmis sp. and Isochrysis sp.

On 20 DAH, larvae were transferred to flat-bottomed tanks at the University of Algarve. On the day of experimentation (23 DAH), groups of larvae (2.45 ± 0.87 mg dry weight, means ± SD, n = 12; weight determined from larvae from the main group) were transferred under temperature control (21°C) to the radioisotope laboratory, where radioactive diets were tube fed. Only larvae with an empty gut at the time of injection were used. All animal procedures and handling were in compliance with the Guide for the Care and Use of Laboratory Animals (NRC 1996).

Experimental set-up. To specifically test the functionality of the digestive system, we used an in vivo method to control tube feeding

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3 Abbreviations used: AA, amino acids; BSA, bovine serum albumin; DAH, days after hatch; FAA, free amino acids.
of fish larvae that was described by Rust et al. (1993) and modified by Rønnestad et al. (2000) with additional modifications as described here.

The in vivo set-up consisted of a stereo dissecting microscope with a micromanipulator. A nanoliter injector (World Precision Instruments, Sarasota, FL) was fastened to the micromanipulator. A plastic capillary (0.19-mm diameter; Sigma Chemical Co., St. Louis, MO) was fastened to the nanoliter injector. Before the injection, the larvae were tranquillized (33 μg/L MS-222; Sigma Chemical Co.) and thereafter gently placed in a droplet of clean seawater. With the fish in position, the capillary was gently passed through the mouth and the esophagus into the stomach. This operation was controlled visually, because all of the larvae used in the experiments had transparent and semitransparent gut/stomach areas. A single injection of the test diet was then deposited into the stomach lumen with the nanoliter injector. After capillary withdrawal, the larvae were gently rinsed for any spillage through three successive transfers to wells (20 mL) that contain clean seawater. The larvae were transferred between the wells with a pipette with as little water as possible and then transferred to single-larva incubation wells with 2 mL of clean seawater. Visual observations of a diet that contains food coloring in a pilot study showed that the ventilation movements of the opercula effectively removed any contamination in the mouth during the first steps of the rinsing series. The total transfer time was approximately 75 s.

**Assimilation study.** To specifically study the assimilation of FAA and proteins, solutions were injected according to the described system for in vivo tube feeding. The series consisted of six groups of six larvae that were incubated for 15 min, 30 min, 1 h, 2 h, 4 h, 8 h and 24 h (FAA only) after being fed a single injection (36 nL; 4.08 g/L) of an FAA-containing or a protein-containing diet (see later). The injected volume was based on a pilot study in which we gently injected increasing volumes while we observed the distention of the stomach wall. The volume (not nutrient concentration) is equivalent to three Artemia nauplii.

FAA assimilation rates were determined by using an aqueous diet that consists of FAA (FAA diet: 32.35 mmol/L that contain L-[35S]methionine (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and the following L-AA (analytical grade, Sigma Chemical Co.): 4.21 mmol/L alanine, 1.74 mmol/L arginine, 2.24 mmol/L aspartic acid, 3.14 mmol/L glutamic acid, 2.61 mmol/L glycine, 0.51 mmol/L histidine, 1.52 mmol/L isoleucine, 2.70 mmol/L leucine, 2.35 mmol/L lysine, 1.31 mmol/L methionine, 1.14 mmol/L phenylalanine, 1.53 mmol/L proline, 2.22 mmol/L serine, 1.57 mmol/L threonine, 0.37 mmol/L tryptophan, 0.69 mmol/L tyrosine and 1.96 mmol/L valine.

For studies on protein assimilation rates, an aqueous diet that contains bovine serum albumin (BSA; Sigma Chemical Co.) added L-[methyl-14C]BSA (DuPont New England Nuclear, Boston, MA) was injected (Protdiet). Both diets were prepared using seawater diluted 1:3 in distilled water. The final activity was 43.7 and 0.87 Bq/mL for the FAA diet and the Protdiet, respectively.

The larvae were tube fed and incubated individually as described earlier. After the set time, the larvae were gently lifted by the tail with a forceps and rapidly transferred to a slide on ice for dissection of the gut compartment. Once the gut and the liver was dissected, each compartment lasted 4–5 min. Any liquid remnant on the slide was included with the gut compartment. The operation was controlled visually, because all of the larvae used in the experiments had transparent and semitransparent gut/stomach areas. A single injection of the test diet was then deposited into the stomach lumen with the nanoliter injector. After capillary withdrawal, the larvae were gently rinsed for any spillage through three successive transfers to wells (20 mL) that contain clean seawater. The larvae were transferred between the wells with a pipette with as little water as possible and then transferred to single-larva incubation wells with 2 mL of clean seawater. Visual observations of a diet that contains food coloring in a pilot study showed that the ventilation movements of the opercula effectively removed any contamination in the mouth during the first steps of the rinsing series. The total transfer time was approximately 75 s.

**Statistical analysis.** Results are given as mean ± SD (n = 6). Mean values were compared with a t test after a check for equality of variances through F-test (Microsoft Excel 97; Microsoft Corporation, Redmond, WA). Differences were considered statistically significant at P < 0.05.

**RESULTS**

Larvae of Senegal sole were very tolerant of the slight stress imposed by handling, anesthetics and the microtube feeding technique used in the present study. No deaths (0 of 360) occurred in any of the experimental or pilot series.

The FAA diet deposited into the digestive tract was absorbed with a 3.5-times-higher transfer rate (P < 0.001) from the gut lumen into the larval body compartment compared with the Protdiet (Fig. 1). This is based on data from 15 min after tube feeding, when only 20.6 ± 4.7% of the injected 35S-labeled FAA diet remained in the gut compartment (Fig. 1C). Of the 80% that was absorbed, 20% was found in the liver, and the remaining 60% were distributed among the remaining body tissues. In comparison, for protein-fed larvae at the same time (i.e., 15 min after tube feeding), 72.0 ± 6.1% of the protein remained in the gut compartment (Fig. 1D).

The processing of the labeled FAA diet in the gut compartment was mainly finished 1 h after deposition (Fig. 1C). For protein, the intestinal processing took much longer; the data indicate that it may take as long as 8 h to complete absorption (Fig. 1D).

Assimilation efficiency was assessed as the sum of liver and body compartments at 8 h after tube feeding was calculated for the two diets. These calculations showed that FAA were absorbed with higher efficiency than AA from the Protdiet (~80% versus ~58%, P = 0.001). If we assume that the label present in the gut at this time represents AA incorporated into the intestinal tissue and this compartment is included in the calculations, the values are 89 and 64%, respectively.

**DISCUSSION**

In the present study, methionine was the only labeled AA in the FAA diet, and it remains to be shown whether it is representative of the absorption rate and assimilation efficiency of other FAA in the early life stages of fish. The studies of Rust (1995) indicated a higher rate of assimilation for methionine than for a mixture of AA in striped bass (Morone saxatilis). Rønnestad et al. (2000) studied the assimilation efficiency of a mixture of 20 L-[14C]AA in Japanese flounder larvae and found comparable values (79.5 ± 7.1%) to those for the assimilation of L-[35S]methionine in the present study (80% or 89%, depending on assumptions for calculation). Further studies are necessary to determine the absorption rates of individual FAA.

The [14C]BSA used in the present study was produced through methylation with the use of formaldehyde and sodium cyanohydrate. This radiolabeling procedure will produce a BSA that has chemical properties similar to unlabeled BSA. The labeling modifies primary amines (mainly lysine) in the protein and has the advantage that the charge properties of the modified residues are preserved (Jentoft and Dearborn 1979). It therefore reasonable to assume that the results obtained for the Protdiet are representative of both the digestion and absorption of dietary BSA.

The tested FAA diet showed that FAA seems to be an amino acid source with 40% better total assimilation efficiency...
than the tested protein diet (BSA) for Senegal sole postlarvae. This is in line with the results from Rust (1995), who found better assimilation efficiencies for FAA than for \textit{Escherichia coli} protein for striped bass, especially in the initial larval stages. The present results are intriguing because the Senegal sole stage that was tested had a well-defined stomach that contained the first gastric glands (Ribeiro et al. 1999a) and a mature intestinal enzyme profile (Ribeiro et al. 1999b). Therefore, even though Senegal sole early postlarvae have an apparently well-developed digestive system, have completed metamorphosis, and have acquired a benthic behavior, their protein digestive capacity seems to still be somewhat limited. This may explain at least in part the historic difficulties in the adaptation of young sole to artificial diets (Dinis et al. 1999, Howell 1997). This limited protein digestive capacity supports the findings that sole postlarvae benefit from having at least part of their dietary AA in a simpler molecular form (Day et al. 1997). This is also supported by the fact that in nature, \textit{Solea} postlarvae, as older stages, feed on polychaete worms and other invertebrates (Dinis et al. 1999), which, like all marine invertebrates, have a high content of FAA (Fyhn 1989).

In addition to the overall effect on AA assimilation efficiency, this study also shows that there are large differences in AA absorption rates between FAA and protein in Senegal sole postlarvae. Based on the data at 15 min after tube feeding, the FAA was absorbed into the larval tissues/body carcass 3.5 times faster. The data for assimilation efficiency show the differences between diets at the time when diet processing is complete, whereas the absorption rate calculated here describes the flow rate of nutrients from the gut into body tissues. The data for absorption rates are more relevant for continuously feeding fish larvae. Their high potential growth rates (30–100%/d; Kamler 1992), together with their dominant use of AA as a fuel (Rønnestad and Naas 1993), imply a large demand for precursor AA. The larval growth rate may therefore depend on the flow of a balanced AA solution from the intestine and into the tissue cells. In the present study, the injected AA constituted only \textasciitilde{}0.01% of the protein in the Senegal sole larva and 0.40% of the FAA pool. Therefore, the composition of the AA mixture that was tube fed to the fish is not likely to significantly influence the composition of the fish FAA pool. However, when fish are fed a larger meal (the real situation), a faster absorption of FAA may lead to transient AA imbalances and consequently to decreased protein utilization if crystalline AA are used to supplement dietary protein. Such effects have been shown in pigs and other animals (Batterham 1974, Rérat 1993 and 1995); faster absorption has been documented when FAA are the source of AA rather than protein. Poor utilization of dietary FAA has also been observed in white sturgeon (Ng et al. 1996). The observations from the present study may explain 1) the poor results verified when crystalline AA are used in microdiets for larval fish with inclusion levels above 10% (López-Alvarado and Kanazawa 1995), 2) the limitations in the use of high levels of protein.

\begin{figure}
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\caption{Assimilation of labeled diets in postlarval Senegal sole tube fed 36 nL (4.08 g/L) of a diet of free amino acids containing \textit{L}-[\textsuperscript{35}S]methionine (FAA-diet) or bovine serum albumin containing \textit{methylated}-\textsuperscript{14C}bovine serum albumin (Protein-diet). Values are means \pm SD (n = 6). Fraction of label retained in the body and the fraction expelled to the incubation water (A and B). Compartmental distribution of label within the body (dissected larvae) in percent of total label fed (C and D). The dissected larvae consisted of (g/100 g dry weight): 5.9 \pm 0.6 gut, 4.7 \pm 0.9 liver and 89.4 \pm 1.0 body/carcass (means \pm SD, n = 6). Due to technical difficulties the series on Prot-diet at 30 min was repeated. These larvae (n = 8) were older and larger (P < 0.001) than the main group: 11.48 \pm 3.92 versus 2.45 \pm 0.87 mg dry weight.}
\end{figure}
hydrolysates in diets for larval and juvenile fish (Cahu et al. 1999, Carvalho et al. 1997, Espe et al. 1999) and 3) the poor results when alternative protein sources are supplemented with crystalline AA in juvenile and adult fish (Davies and Morris 1997, Murai et al. 1986).

The liver serves an important function in screening and processing the nutrients absorbed from the gut. The high initial retention of labeled methionine in the liver in comparison with the low liver retention of labeled AA from protein (Fig. 1D) suggests that the liver has some buffering capacity for high AA absorption rates. However, the liver retention of the absorbed FAA reported here is only about half of the peak value of 42% reported in pigs (Rérat 1993). The high rates of whole body protein synthesis that seem to characterize fish larvae and postlarvae (Conceição et al. 1997a and 1997b, Houlihan et al. 1993) compared with older fish probably also contribute to an high overall retention efficiency of dietary FAA.

Postlarval sole had a higher retention efficiency and absorption rate for FAA than for intact protein, which supports the important role of AA sources that are simpler than protein, such as FAA (López-Alvarado and Kanazawa 1995, Rønnestad et al. 1999) or protein hydrolysates (Cahu et al. 1999, Carvalho et al. 1997, Day et al. 1997) in the nutrition of fish larvae and postlarvae. The challenge is to find proper feed production technologies and the appropriate inclusion levels so the buffering capacity of the larvae for rapidly absorbed AA can be used to the maximum extent without reducing protein utilization, growth or survival rates.

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