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Metabolism of tryptophan, methionine and arginine in *Diplodus sargus* larvae fed rotifers: effect of amino acid supplementation

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Summary. Dietary amino acids imbalances have been described when fish larvae are fed rotifers, what may lead to a reduction in growth rate. The tube-feeding technique can be used to assess the effect of free amino acid short term supplementation. In this study supplementation of tryptophan, methionine and arginine were tested in *Diplodus sargus*. Single crystalline ^{14}C amino acids as well as a mix of ^{14}C amino acids were used as tracers to compare results of individual amino acids metabolism with the average of all amino acids. The results show low absorption efficiencies for tryptophan (70%) and arginine (80%) and similar absorption for methionine (90%) when compared with the average of all amino acids. Supplementation of these amino acids seems to be viable but it did not result in higher retention compared to the amino acid mix. This means that tryptophan, methionine and arginine are probably not the limiting amino acid when *Diplodus sargus* larvae are fed rotifers. However, supplementation in these IAA may be required for their roles as precursors of important molecules other than proteins, in order to improve larval quality and/or performance.

Keywords: *Diplodus sargus* – Arginine – Methionine – Tryptophan – Tube-feeding

Introduction

During fish larval stages growth rate is usually very high and, as growth is mainly related to muscle protein deposition (Conceição et al., 1998), a high amino acids (AA) flow is required from food to growing biomass (Rønnestad et al., 2003). The lack of certain indispensable amino acids (IAA) has been shown to affect negatively growth and food conversion efficiencies in juvenile fish (Fauconneau et al., 1992), and increase nitrogen losses (Aragão et al., 2004, 2007) and mortality in fish larvae. The AA profiles of the whole larvae body has been commonly used as a preliminary approach to determine fish larvae AA requirements (Wilson and Poe, 1985; Watanabe and Kiron, 1994). Diets supplemented with IAA in order to simulate fish AA profiles have improved growth rates and feeding efficiencies in several salmonids species (Ogata et al., 1983). Although the whole body AA composition alone is not sufficient to determine the fish larvae requirements, it can provide important information concerning possible AA deficiencies (Conceição et al., 2003). Saavedra et al. (2006a) compared *Diplodus sargus* and rotifers AA profiles and reported potentially severe amino acid imbalances in the live feed and suggested arginine as one of the IAA in deficiency in rotifers. This IAA was also reported by Aragao et al. (2004) as a possible deficient AA in rotifers when fed to gilthead sea bream (*Sparus aurata*). In giant gouramy, *Osphronemus gouramy* fed a defatted soybean meal diet (Suprayudi et al., 2000) and in penaeid shrimp fed plant-protein based diets (Millamena et al., 1998) arginine was found as well to be one of the limiting essential amino acids. Arginine is involved in metabolic pathways such as protein synthesis, urea production, metabolism of glutamic acid and proline, and synthesis of creatine and polyamines (Kaushik et al., 1988; Alam et al., 2002). Arginine deficiency has been shown to cause a reduction on growth and protein retention in European sea bass (Tibaldi et al., 1994) and coho salmon (Luzzana et al., 1998).

Methionine is an IAA required for normal fish growth and metabolic functions (Luo et al., 2005). Methionine is precursor of choline, a vitamin required for fish homeostasis and growth (Kasper et al., 2000). This IAA is commonly reported as the first limiting AA in many fish diets (Tacon and Jackson, 1985; Dabrowski et al., 1989),

especially in those where fish meal has been replaced by large amounts of plant protein. Methionine deficiencies result in reduced growth and survival rates, lower feed efficiency (Goff and Gatlin, 2004) and in the development of bilateral cataracts in rainbow trout (Poston et al., 1977; Walton et al., 1982) and hybrid striped bass (Keembiyehetty and Gatlin, 1993). Supplementation of methionine in diets with deficiency in this AA must be performed in order not to compromise growth (Jackson and Capper, 1982; Takagi et al., 2001). Although this AA was not considered to be in deficiency by Saavedra et al. (2006a) the low relative bioavailability for methionine observed by Saavedra et al. (2006b) in *Diplodus puntazzo*, a species closely related to *Diplodus sargus*, suggests that it might be.

One of the major concerns in the rearing of *D. sargus* is the high incidence of skeleton deformities, especially at the vertebral column (Dores et al., 2006; Saavedra et al., 2007a, b). The level of AA such as tryptophan is reported to be relevant to prevent such problems (Akiyama et al., 1986; Hseu et al., 2003). In salmonids tryptophan deficiency has been reported to induce scoliosis (Akiyama et al., 1986). Tryptophan is also a precursor of the neurotransmitter serotonin, which affects food intake and aggression in fish (Hseu et al., 2003).

Studies of AA metabolism in marine fish larvae are limited by the small larval size and by the difficulty to manipulate the AA composition of the live preys (Rønnestad et al., 2001a). A better knowledge about the larval capacity to digest, absorb and retain several nutrients is crucial for the development of suitable artificial diets for early larval stages of fish (Rønnestad et al., 2001a). The tube-feeding technique developed by Rust et al. (1993) and modified by Rønnestad et al. (2001a) enables to distinguish between the unabsorbed labelled nutrients from the labelled molecules produced either from the catabolism or retention of the absorbed nutrients (Rønnestad et al., 2001b). This technique consists of the use of a ^{14}C tracer which is deposited in the larval stomach by a capillary tube (Rønnestad et al., 2001a). Once the digestion period is over it is possible to determine the fate of the tracer and quantify the retention and catabolism rates. Tube-feeding can be used to determine if an AA is deficient in the diet and therefore limiting growth in fish larvae (Aragão et al., 2004).

The purpose of this study was to verify if arginine, tryptophan or methionine are limiting protein synthesis (and growth) in *Diplodus sargus* larvae and to determine to what extent dietary supplementation with these AA may be efficient. Assessment of supplementation efficiency is important for further studies on the role of these AA on *Diplodus sargus* larvae metabolism in the long term.

Materials and methods

Larvae

Diplodus sargus larvae obtained from natural spawning were reared at IPIMAR Aquaculture Research Station (EPPO, Olhão, Portugal) under standard conditions (Pousão-Ferreira and Dores, 2000). At 25 days after hatching (DAH) *Diplodus sargus* larvae (7.8 ± 0.9 mm, 0.46 ± 0.1 mg, mean $\pm$ S.D., $n = 20$) were transferred to CCMAR (University of Algarve, Faro, Portugal) facilities to perform the tube-feeding trials.

Larvae were kept in small white trays with rotifers at a room temperature of 25 °C. After a 30 min period of acclimation and being fed a rotifers meal, larvae were ready to be tube-fed. Therefore the AA supplements were adding on the rotifer AA contents present in the larval gut.

This study was performed with 25 day after harching (DAH) larvae due to the difficulty of manipulating smaller sizes.

Treatments

For each AA there was a separate trial. Four treatments were carried out for each trial using ^{14}C tryptophan (ARC, USA), ^{14}C arginine (ARC; USA) or ^{14}C methionine (ARC, USA) as target AA, each one using twelve larvae: 1) tracer AA (^{14}C) + free AA supplement; 2) tracer AA (^{14}C) + saline solution; 3) tracer mixture of several AA (^{14}C) + free AA supplement; 4) tracer mixture of several AA (^{14}C) + saline solution. The first two treatments give information about the efficiency of the AA supplementation. Comparing the four treatments it is possible to know if the AA is limiting protein synthesis (Aragão et al., 2004). The free AA supplementation (non-radioactive) was performed dissolving tryptophan (3.6 mg/ml), arginine (50 mg/ml) or methionine (25 mg/ml) in a saline solution (seawater/distilled water 1:3). After dissolving the AA, 5 µl were frozen in liquid nitrogen and then freeze-dried (RVT 400, Savant, NY). The amount of free amino acids given to the larvae was always considerably lower than the calculated amount larvae would get from a full stomach of rotifers. For the treatments without supplementation, the same procedure was followed using just 5 µl of the saline solution instead of the free AA solution. Tube-feeding experiments for each AA were carried out twice and in different days and using different larval batches.

Tube-feeding set-up

These experiments followed the methodology described by Rust et al. (1993) and modified by Rønnestad et al. (2001a, b). Larvae were tube-fed using 0.19 mm diameter plastic capillary tube (Sigma-Aldrich) inserted in a nannoliter injector (World Precision Instruments, Sarasota, USA). Before being tube-fed, larvae were lightly anesthetised in a 40 µl/l of Phenoxyethanol (Sigma-Aldrich) solution for 2 min. Two consecutive injections of 9.2 nanoliters were performed in the larval gut. After tube-fed, larvae were rinsed twice in sea water and transferred to a sealed incubation well (8 ml seawater), connected to a metabolic trap well (5 ml KOH, 0.1 M) by an air tube. For further details on method see Rønnestad et al. (2001a).

Radioactivity counting

Once digestion was completed (6 h, observed using a binocular microscope), larval body was removed from the incubation well and placed in a 6 ml scintillation plastic vial. 0.5 ml of Solvable (Packard Instruments) was added in order to dissolve the larvae during a period of 24 h at 30 °C. In order to obtain the results from AA catabolism the protocol from Rønnestad et al. (2001a) for collecting CO_2 from incubation water was

followed and once it was over the incubation and metabolic trap vials were collected. Ultima Gold scintillation liquid (Packard Instruments) was added to all larval body, metabolic trap and incubation vials before being counted (DPM) in a Beckman LS 6000IC liquid scintillation counter (Fullerton, CA).

Data analysis

Differences between treatments were assessed using one-way parametric ANOVA for all tryptophan and methionine treatments. For arginine absorption data a non-parametric ANOVA, Kruskal-Wallis, was performed. Schéffe test was used to perform the post-hoc comparisons. All data were calculated subtracting the blanks and was expressed as a percentage of the tracer fed, i.e., the sum of the DPM counted in three vials (incubation water, metabolic trap and larval body). When the incubation water presented differences between treatments, retention and catabolism proportions were also calculated using the metabolic trap and larval body sum as 100%.

Results

Larval survival rate after 6 h incubation period was $94.1 \pm 6.5\%$. In the tryptophan experiment, significant differences were found for larval body ($F_{3,38} = 6.1$, $p = 0.002$) between the ^{14}C Trp treatments and the ^{14}C Mix without supplementation treatment. When radioactive tryptophan was tube-fed, a lower radioactive content was counted in the larval body (Fig. 1). In the incubation chamber (water) significant differences were found ($F_{3,38} = 27.8$, $p < 0.001$) between ^{14}C Trp treatments and ^{14}C Mix treatments. An almost three times higher percentage was observed for the tryptophan tube-fed larvae compared with the mix treatments (Fig. 1). The mix treatment with tryptophan supplementation presented significantly higher percentages ($F_{3,38} = 3.41$, $p = 0.03$) in the metabolic trap whereas all other treatments presented similar values (Fig. 1).

The absorption efficiency (retention $\pm$ catabolism) was significantly higher ($F_{3,38} = 27.8$, $p < 0.001$) for the ^{14}C Mix treatments (approximately 90%) compared to the 68% observed in the TRP ^{14}C treatments (Fig. 1). However, retention and catabolism presented the same proportion of the absorbed AA in all treatments (Fig. 2). The retention percentage was around 75% whereas catab-

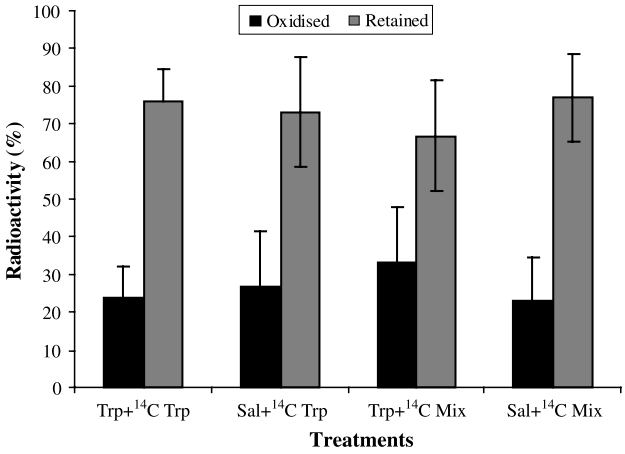

Fig. 2. Proportion of catabolised (*black columns*) and retained in body (*grey columns*) ^{14}C AA of tube-fed *Diplodus sargus* larvae. Trp + ^{14}C Trp: Trp ^{14}C supplemented with crystalline tryptophan; Sal + ^{14}C Trp: Trp ^{14}C without supplementation; Trp + ^{14}C Mix: ^{14}C Mix supplemented with crystalline tryptophan; Sal + ^{14}C Mix: ^{14}C Mix without supplementation. Values are mean and standard deviation ($n = 10$)

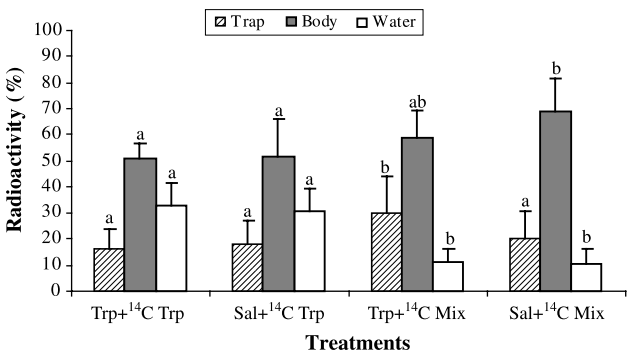

Fig. 1. Proportion of the tube-fed ^{14}C amino acid mix (Mix) or ^{14}C tryptophan (Trp) catabolised (*black stripes columns*), retained in body (*grey columns*) and evacuated into water (*white columns*) of *Diplodus sargus* larvae. Trp + ^{14}C Trp: Trp ^{14}C supplemented with crystalline tryptophan; Sal + ^{14}C Trp: Trp ^{14}C without supplementation; Trp + ^{14}C Mix: ^{14}C Mix supplemented with crystalline tryptophan; Sal + ^{14}C Mix: ^{14}C Mix without supplementation. Values are mean and standard deviation ($n = 10$). Different letters in the same compartment indicate significant differences ($p < 0.05$)

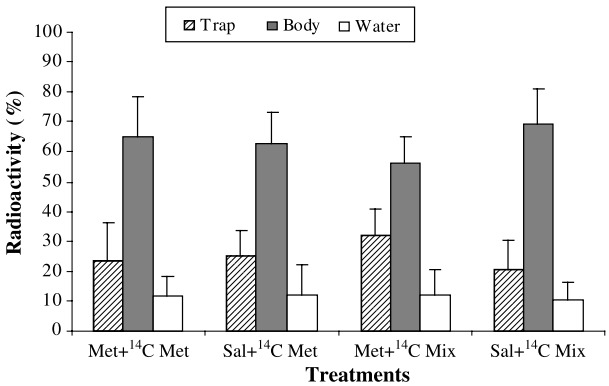

Fig. 3. Proportion of tube-fed ^{14}C amino acid Mix and Methionine catabolised (*black stripes columns*), retained in body (*grey columns*) and evacuated into water (*white columns*) of *Diplodus sargus* larvae. Met + ^{14}C Met: Met ^{14}C supplemented with crystalline methionine; Sal + ^{14}C Met: Met ^{14}C without supplementation; Met + ^{14}C Mix: ^{14}C Mix supplemented with crystalline methionine; Sal + ^{14}C Mix: ^{14}C Mix without supplementation. Values are mean and standard deviation ($n = 10$)

olism proportion was 25% of the absorbed tube-fed ^{14}C AA.

Methionine tube-feeding showed no significant differences (Fig. 3). The larval body retained around 60% of the total tube-fed ^{14}C AA. The catabolised proportion was twice the non absorbed tube-fed ^{14}C AA (Fig. 3).

In the arginine experiment, significant differences were found for the incubation water ($F_{3,46} = 2.6$, $p = 0.02$), which presented higher values for the ^{14}C arginine treatments (Fig. 4). ^{14}C AA mix treatments showed significantly higher absorption efficiencies (around 89%) than ^{14}C Arginine treatments (around 84%) (Fig. 4). No significant differences were found among treatments for retention and catabolism levels (Fig. 5).

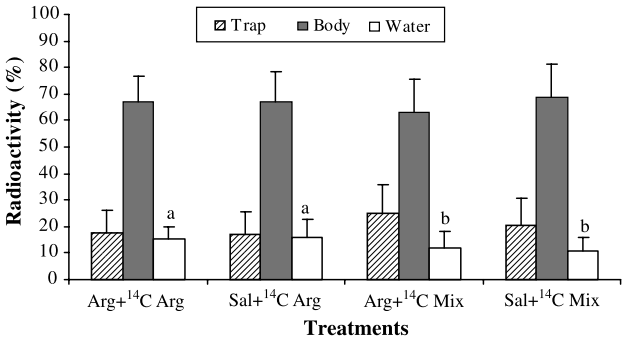

Fig. 4. Proportion of the tube-fed ^{14}C Mix and Arg counted in metabolic trap (*black stripes columns*), larval body (*grey columns*) and incubation water (*white columns*) of *Diplodus sargus* larvae. Arg + ^{14}C Arg: Arg ^{14}C supplemented with crystalline arginine; Sal + ^{14}C Arg: Arg ^{14}C without supplementation; Arg + ^{14}C Mix: ^{14}C Mix supplemented with crystalline arginine; Sal + ^{14}C Mix: ^{14}C Mix without supplementation. Values are mean and standard deviation ($n = 10$)

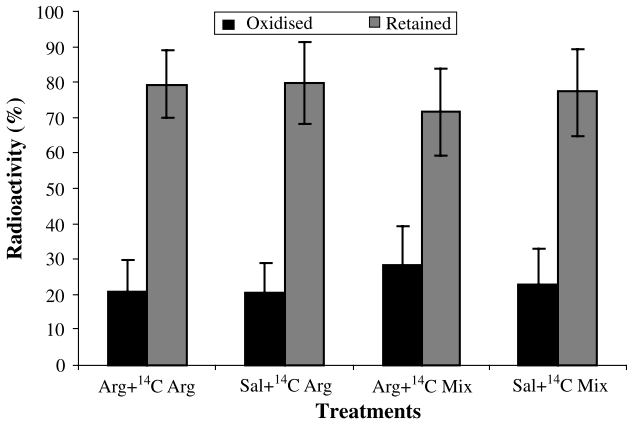

Fig. 5. Proportion of catabolised (*black columns*) and retained (*grey columns*) ^{14}C of tube-fed *Diplodus sargus* larvae. Arg + ^{14}C Arg: Arg ^{14}C supplemented with crystalline arginine; Sal + ^{14}C Arg: Arg ^{14}C without supplementation; Arg + ^{14}C Mix: ^{14}C Mix supplemented with crystalline tryptophan ; Sal + ^{14}C Mix: ^{14}C Mix without supplementation. Values are mean and standard deviation ($n = 10$)

Discussion

This was the first tube-feeding study with *Diplodus sargus.* Larvae seemed to resist well to handling and survival rate was high. However, as previously reported for *Solea senegalensis* (Rønnestad et al., 2001a, b; Aragão et al., 2004), standard deviations were high, probably due to biological variation and/or to different tube-fed doses in the larval gut. In *Diplodus sargus* some part of the radioactivity dose is lost by regurgitation. This was observed in preliminary trials using a blue food-grade dye. To avoid higher variation, fish were tube-fed with a fairly full stomach, after a rotifer meal, as suggested by Rønnestad et al. (2001a).

Tryptophan and arginine evacuation was significantly higher than for the average of all AA, suggesting that the absorption of these two AA is less efficient compared to other AA. This low absorption was especially severe for tryptophan (approximately 70%). There is not much published information about AA absorption efficiencies in fish but, in humans, published reports (Wahbeh and Christie, 2006) indicate tryptophan absorption efficiency is six times lower than methionine and four times lower than arginine. For the three AA studied, at least two-carrier mediated transport systems specific to basic (arginine) and neutral free AA (FAA) are involved in the transport across the brush-border membrane (Aragão et al., 2004). If no competitive interactions exist between FAA then transport rates are supposed to be similar between basic and neutral FAA (Applebaum and Rønnestad, 2004). However, interactions between AA seem to have some influence in the AA uptake and often transporters have overlapping specificities (Aragão et al., 2004).

For both tryptophan and arginine no significant differences were found between AA catabolism and AA retention among treatments. This means that although these AA presented lower absorption efficiency, they were not more retained when supplemented, suggesting these AA are not limiting growth in *Diplodus sargus* larvae fed rotifers. Arginine retention efficiencies reported for *Solea senegalensis* (Rønnestad et al., 2001a) are higher than the ones obtained in this study for *Diplodus sargus* (80 vs. 65%, respectively). This may be explained by varying efficiencies in different fish species, or due to the different age/developmental stage of larvae tested. Fish larvae digestive tract is maturing during the first 30 days of life in both species (Ribeiro et al., 1999; Ortiz-Delgado et al.,

2003), and the study with *S. senegalensis* was performed in older fish.

No significant differences between treatments were observed in the methionine trial. This AA presented an absorption efficiency of approximately 90%, similar to the average of all AA. However, from the 90% absorbed methionine, 20% was used for catabolism and therefore this AA retention was just 70%. Other studies presented slightly higher retention rates for methionine and free AA mixture such as Rønnestad et al. (2000a, b, respectively) who reported AA retention efficiencies of 80% for Senegal sole and Japanese flounder. On the other hand, the results seem to indicate that this AA is more efficiently retained compared to arginine or tryptophan. In addition, it seems methionine is not limiting *D. sargus* growth as a higher retention would be expected as well as lower percentage of this AA being canalised to energy production when supplemented. These results confirm the observations by Saavedra et al. (2006a), that suggested methionine was not deficient in *D. sargus* diet when larvae were fed rotifers.

When rotifers and *Diplodus sargus* AA profiles were compared low correlations were found, suggesting rotifers had strong AA imbalances for this species (Saavedra et al., 2006a). However, the present study shows that for at least arginine, tryptophan and methionine, rotifers seem to fulfil *Diplodus sargus* amino acid nutritional needs. This apparent contradiction, in particular for the case of arginine, probably means that none of these three AA is the first limiting AA for *Diplodus sargus* larvae fed rotifers. An alternative explanation is that this species may have high obligatory AA demand for energy production rendering the dietary AA imbalance of little significance (Conceição et al., 2003).

In conclusion, the three AA studied do not seem to be limiting growth in *Diplodus sargus* larvae fed rotifers. Furthermore, this study shows that the supplementation of arginine, tryptophan and methionine in *Diplodus sargus* larval diets seems to be viable. However, both arginine and tryptophan presented lower absorptions efficiencies than the average of all AA. This lower absorption could be a limitation factor for diet supplementation as more AA quantities need to be added in order to obtain the desired AA absorption. Supplementation of these IAA may be required even if they are not limiting AA, as all three have roles as precursors of important molecules other than proteins. Protein synthesis is likely to have precedence over synthesis of such molecules. Therefore, dietary shortage of methionine, arginine or tryptophan, may have important consequences in terms of larval quality and performance.

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