Tyrosine and phenylalanine supplementation on *Diplodus sargus* larvae: effect on growth and quality

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Abstract

Dietary amino acids (AA) supplementation may improve growth rate and quality in marine fish larvae. Phenylalanine is the sole precursor of tyrosine, and this AA is the precursor of several molecules with key roles in regulation of metabolism and growth, stress response, and pigmentation. In the present study, three experimental diets were tested: a balanced diet supplemented with phenylalanine, a balanced diet supplemented with phenylalanine and tyrosine and, as control, a non-supplemented balanced diet. Trials started with newly hatched larvae and lasted 25 days. Rotifers were fed with FAA enriched liposomes in order to obtain a convenient AA profile until 20 DAH. The effect of the supplemented diets was assessed in terms of survival, growth rate, skeletal deformities, enzyme activities of AA catabolism, ammonia excretion and resistance to a temperature stress test. Results presented similar survival, growth, enzyme activities of AA catabolism and nitrogen excretion in all treatments. A high level of skeletal deformities was registered and significant differences were found between control and phenylalanine treatment for the percentage of vertebral compression in the trunk region of the vertebral column (30% in the control and 5% in the phenylalanine group). A significantly higher survival to a temperature stress test was obtained for the larvae fed a diet supplemented with phenylalanine and tyrosine. The results obtain in this study

suggest that supplementation of phenylalanine / tyrosine in fish diets might be useful, in order to reduce skeletal deformities, and especially when using high larval densities or other rearing conditions prone to stress larvae. The present study confirms the idea that there are AA requirements for other metabolic processes in addition to the ones for growth.

Key words: *Diplodus sargus*, amino acids, tyrosine, phenylalanine, stress, skeletal deformities

1. Introduction

Diplodus sargus is considered to be a promising new species for aquaculture in Southern Europe due to its high market price and demand (Pousão-Ferreira et al., 2001, Ozorio et al., 2006, Santos et al., 2006) and for its adaptability to the sea bream (*Sparus aurata*) rearing techniques (Sá, 2007). *D. sargus* farming is restricted to Greece although in Portugal there is already some production at a small scale (Sá, 2007). *D. sargus* has also been involved in restocking programmes in the southern coast of Portugal where the landings decreased from 200.3 t to 75.2 t between 1987 and 2004 (Santos et al., 2006). Constraints to white sea bream production are a decrease in the growth rate when reaching the juvenile stages (Cejas et al., 2003) and the presence of several skeletal deformities, especially in the vertebral column (Dores et al., 2006; Saavedra et al., 2007a,b).

Ten amino acids (AA) are considered indispensable for normal fish growth (Wilson, 1989). AA are also a major energy source during the larval stage (Rønnestad et al., 1999; Rønnestad and Conceição, 2005). During this period a high amino acid flow is required from food to growing biomass (Rønnestad et al., 2003). Growth and food conversion efficiencies can be affected by the composition of the dietary amino acids (Conceição et al., 2003) and in order to maximize performance the AA profile of the diet should be as close as possible to the larval AA requirements (Watanabe and Kiron, 1994; Conceição et al., 2003).

Phenylalanine is an aromatic indispensable AA, precursor of tyrosine which is the precursor of thyroxine, required for normal growth and metabolic processes (Khan and Abidi, 2007). Tyrosine is an aromatic semi-indispensable AA, precursor of dopamines and the adrenocortical hormones norepinephrine and adrenaline. Dopamines regulate central and peripheric nervous system activity and therefore can be related to the control of stress in the fish (Lehnert and Wurtman, 1993). Tyrosine has been shown to reduce acute stress such as cold exposure in rodents when supplemented in the diet (Brady et al., 1980; Lehnert et al., 1984). Tyrosine is also the precursor of melanin which plays an important role in fish pigmentation.

Manipulation of rotifers and *Artemia* AA profiles have been difficult in the past (Aragão et al., 2004) but recently Barr and Helland (2007) developed a technique to enrich live feed with free amino acids (FAA) using liposomes. This technique enables the study of FAA supplementation in early larvae stages using live feed which can be later switched to inert diets. Inert diets are still more efficient in terms of supplementation because only the FAA profile of live feed can be changed (Conceição et al. 2003). However, early marine fish larval weaning is still difficult to achieve with success (Kolkovski et al., 1997) and so live feed FAA supplementation is an important advance in fish larvae nutrition studies. Yúfera et al. (1999, 2002) have developed cross-linked casein-walled capsules and showed this diet was able to substitute with some success live food in *Sparus aurata* early larval stages. Saavedra et al. (2007a,b) presented interesting results introducing this microencapsulated diet in *D. sargus* early larvae feeding protocol. Larvae fed on the microencapsulated diet presented still a lower survival rate when compared to the live feed protocol but growth rate was similar and when larvae were fed an AA balanced diet they presented higher larval quality and lower nitrogen excretion (Saavedra et al., 2007a).

The present study was conducted to investigate the possible effects of a diet supplemented with tyrosine and phenylalanine on *Diplodus sargus* larvae quality. Quality was referred in terms of survival, growth, skeletal malformations and stress resistance.

2. Materials and methods

2.1 Husbandry and experimental set-up

This study was carried out at the Aquaculture Research Station of INIAP/IPIMAR, in Olhão, South of Portugal during 2006.

Diplodus sargus eggs were obtained from a mix of wild and farmed broodstock consisting of 38 fish with an average weight of $869.2 \text{ g} \pm 319.8 \text{ g}$, a biomass and tank density of 33 kg and 4 kg/m^3 , respectively.

After collected from the incubators, first feeding larvae were transferred to 200l conical cylindrical fibreglass tanks at a density of 80 larvae/ l. Water passed through a biological filter and then through a mechanical and sterilized by a UV filter before entering the tank. The system worked in a semi-closed circuit and water temperature was maintained constant at $20.3 \pm 0.3 \text{ }^\circ\text{C}$, oxygen at $6.3 \pm 0.4 \text{ mg/l}$ and salinity at $37 \pm 1 \text{ ppt}$. Water flow started at 0.4 l/min and then was slowly increased with larvae age until a maximum of 1 l/min , at 20 DAH. Photoperiod was 24 hours Light.

This experiment lasted 25 days and three treatments were tested: a control diet presented a balanced AA profile, balanced diet supplemented with phenylalanine and a balanced diet supplemented with tyrosine.

2.2. Cross-linked casein-walled capsules formulation

Cross-linked casein-walled capsules were used in this trial due to its lower free amino acid leaching properties when compared to other kind of inert diets (Yúfera et al., 2002). To formulate the control diet, the indispensable amino acid profiles from larval carcass were used (Saavedra et al., 2006) as they are commonly used as a good indicator of fish amino acids requirements (Wilson and Poe, 1985; Watanabe and Kiron, 1994; Conceição et al., 2003). The microencapsulated diet consisted on 70% protein, of which 31.1% was casein (minimum required to obtain this type of capsules) (table 1.) The percentages of the protein sources of the diet were manipulated in order to obtain an AA

diet profile as close as possible to the larval carcass profile. AA in deficiency were supplemented in the form of free crystalline indispensable AA to balance the diet. Once the balanced diet was formulated phenylalanine and tyrosine was supplemented to the phenylalanine and tyrosine diets, respectively, in the form of free crystalline AA. Diets were formulated to be isonitrogenous at intake, taking into account the amino acid leaching estimates provided by Yúfera et al. (2002) meaning that to all free AA in the diet a leaching loss allowance was added (*e.g.*, if the estimated leaching percentage of a given free AA is 50% its content in the diet was increased in the same proportion). Microencapsulated diets were prepared according to Yúfera et al. (1999).

2.3 Feeding protocol

Feeding protocol consisted of *Brachionus plicatilis* enriched with Protein Selco (INVE Aquaculture, Belgium). One hour before given to the larvae, rotifers were boosted with liposomes filled with FAA. From 3 to 14 DAH 5 Br/ml were given to the larvae in the morning and afternoon. From 15 to 20 DAH, rotifers were decreased to half in both meals. Dry feed was introduced at day 15. From 15 to 18 DAH 1.5 g of micro diet was given to each tank and from 19 DAH until the end of the experiment it was increased to 2 g.

Tetraselmis sp. ($12 \times 10^3 \pm 2 \times 10^3$ cells/ml) and *Isochrysis galbana* ($73 \times 10^3 \pm 14 \times 10^3$ cells/ml) were added to the tank twice a day (morning and afternoon).

Liposomes were filled with free amino acids according to Barr and Helland (2007), using the free amino acid quantities detailed in table 1. Due to the low tyrosine solubility, the liposomes from this treatment were enriched with tyrosine and phenylalanine as well in order that the supplemented amount would be the same in both treatments.

2.4 Ammonia excretion

Ammonia excretion trials were carried out using 10 fish larvae per tank enclosed in 45 ml spherical glass vials for two hours. Trials were performed both in fasted larvae (about 12 hours after last meal) and in fed larvae (about 4 hours after feed addition to tanks). The vials were filled with oxygen saturated sea water and sealed without any air bubbles. Five replicates from each treatment were used as well as three blanks *per* treatment. Trials were carried out at a temperature of 20 °C and 38 g/l salinity. When trials were finished, larvae were rinsed in distilled water and frozen for dry weight determination. The sea water was filtered, 20 µl of sulphuric acid 25% was added and then frozen in 20ml plastic flasks for ammonia quantification. Ammonia concentration was determined according to Berthelot (Grasshoff, 1983). Samples were treated with alkaline citrate, sodium hypochloride and fenol in the presence of sodium nitroprussiate which catalyzes the reaction. The blue colour formed by indofenol plus ammonia reaction was measured at 630 nm. Ammonia excretion (expressed as $\mu\text{mol g DW}^{-1} \text{ h}^{-1}$) was calculated as follows:

$\text{MNH}_4^+ = \Delta[\text{NH}_4^+] V_{\text{H}_2\text{O}} \text{DW}^{-1} \Delta T^{-1}$, where $\Delta[\text{NH}_4^+]$ is the difference between the sample and the blank water, $V_{\text{H}_2\text{O}}$ is the volume of water in the spherical glass vial, DW is the larvae dry weight, and ΔT is the time duration of the trial.

2.5 Deformity analysis

At 25 DAH, 30 larvae were taken from each tank to examine the occurrence of deformities in the vertebral column. Cartilage was stained with alcian blue (40 minutes) whereas ossified bone was stained with alizarin red (2 hours), according to Gavaia et al. (2000). To determine deformities, Koumoundouros et al. (2001) development descriptors were used as a standard. In order to identify the regions of the vertebral column most affected by deformities, the vertebral column was divided into three regions: trunk (1 to 11 vertebrae), caudal (12 to 20) and preurostyle (remaining three and urostyle).

2.6 Enzymatic analysis

At 25 DAH, 50 larvae per tank were collected and immediately frozen at -196 °C to determine the metabolic activity of the following enzymes: alanine amino-transferase (ALAT), aspartate aminotransferase (ASAT) and glutamate dehydrogenase (GDH) analysis.

Samples were first homogenized in ice-cold buffer (30 mM HEPES, 0.25 mM sucrose, 0.5 mM EDTA, 5 mM K₂HPO₄, 1mM DTT, pH 7.4)(maximum of 10 volumes of buffer per sample weight), using an UltraTurrax and then centrifuged at 1000 g for 10 min at 4 °C. The supernatant was collected and centrifuged again at 15000 g for 20 min at 4 °C. Enzyme activities were assayed on the supernatant at 37 °C using spectrophotometric procedures, alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) using bioMerieux Enzyline kit, and glutamate dehydrogenase (GDH) using the following assay conditions: 175 mM Tris, 100 mM semi-carbazine, 1.1mM NAD, 1mM ADP, 5 mM leucine, started by 100 mM glutamic acid, and the reaction was monitored by following the synthesis of NADH at 340 nm (Aragão et al., 2003).

Enzyme activity units (IU), defined as micromoles of substrate converted to product per minute at assay temperature, are expressed per gram of soluble protein (specific activity). The soluble protein content of fish was determined on the homogenates by the method of Bradford (1976) using bovine serum albumin (BSA) as the standard.

2.7 Stress Test

At 25 DAH a temperature stress test was performed. This test was chosen because it presented good preliminary results in previous trials with *D. sargus* larvae. The test consisted on taking 10 larvae from each tank, reducing their water volume to 20 ml (water temperature approximately 21°C). The larvae were then transferred into a 2 l bucket containing 400 ml of sea water at a temperature of 10 °C. Five minutes later, water was added until a final volume of 1.8 l was reached. After waiting five more minutes the number of dead larvae is counted therefore obtaining the survival rate. This trial was repeated three times for each tank (nine replicates *per* treatment).

2.8 Sampling and biochemical analysis

Larvae samples were taken from every tank at 0, 15 and 25 DAH for dry weight determination. Each sample consisted of 40 and 20 larvae at 25 DAH. These samples were gently rinsed with distilled water and immediately frozen in liquid nitrogen at -196 °C and then freeze-dried (RVT 400, Savant, NY) and weighed.

2.8.1 Amino acids analysis

Protein-bound amino acids samples (microencapsulated diet) were hydrolysed in 6M HCL at 108°C over 24h in nitrogen-flushed glass vials. A reversed-phase high pressure liquid chromatography (HPLC) in a Waters Pico-Tag amino acid analysis system, using norleucine as an internal standard, was used. The resulting chromatograms were analysed with Breeze software (Waters, USA).

Rotifers free amino acids levels were analysed by High Pressure Liquid Chromatography (HPLC) in a Pico-Tag Amino Acid Analysis System (Waters, USA), using norleucine as internal standard and according to the procedures described by (Cohen et al., 1989). Resulting peaks were analysed with the Breeze software (Waters, USA).

2.9 Data analysis

The relative growth rate (RGR, % DW day⁻¹) was calculated using the following formula: $RGR = (e^{(\ln DW_t - \ln DW_0) / t} - 1) * 100$, being DW_t and DW₀ the final and initial dry weights respectively and t the trial duration.

All amino acids data are expressed in weight percentage of the Indispensable amino acids (IAA) pool ((weight of one AA) x (weight of all AA)⁻¹ x 100), to enable comparisons between live food and larval amino acids profiles.

All data were analyzed by a one-way parametric Analysis of Variance (ANOVA) at a minimum significance of p<0.05 with Statistica© (v.5.5). Data from the stress test were transformed (arcsin) to satisfy ANOVA parametric assumptions. Post-Hoc analysis was performed using Scheffé test.

3. Results

3.1 Free Amino acids profile of rotifers and microencapsulated diet

FAA profiles from rotifers showed higher percentages of lysine, arginine, threonine and histidine in the free AA enriched rotifers (Table 3). Rotifers from phenylalanine and tyrosine/phenylalanine treatments presented higher percentages of phenylalanine compared to control and tyrosine/phenylalanine treatment had the highest tyrosine content (Table 3).

3.2 Growth and larval survival

The relative growth rate (RGR) presented no significant differences between treatments at the ages of 15 DAH and 25 DAH (Table 5). No differences were found either in larval dry weight from the different treatments (Table 5).

At the end of the experiment, survival rate was 5.98 ± 1.42 % for the control treatment, 4.78 ± 1.35 % for the diet supplemented with tyrosine and 4.77 ± 0.85 % for the phenylalanine supplemented diet (Table 5). No significant differences were found for survival.

3.3. Enzymatic activity

Alanine aminotransferase (ALAT) presented similar values between treatments as well as aspartate aminotransferase (ASAT) and glutamate dehydrogenase (GDH) (Table 8). No significant differences were found in the activity of any enzymes studied.

3.4. Ammonia excretion

The ammonia excretion presented no significant differences between treatments or between fasted and fed larvae (Fig. 1).

3.5 Temperature stress test

The stress test showed significant differences between treatments ($F_{2,24}=3.5$, $p=0.04$) (Fig. 2). When submitted to a sudden drop of temperature at 25 DAH, larvae fed a diet supplemented with tyrosine presented significantly higher survival rate when compared with the other treatments (Fig. 2).

ab

b

3.6 Larvae deformities

The incidence of deformities in the vertebral column was $85 \pm 22\%$ in the control, $72 \pm 7\%$ in phenylalanine treatment and $78 \pm 6\%$ in the tyrosine treatment (Fig.3). However, scoliosis was only observed in a maximum of $8 \pm 14\%$ in the control, kyphosis maximum registered percentage was $3 \pm 7\%$ in the phenylalanine treatment and no lordosis cases were observed in this study (Fig. 3). Most deformities were located in the preurostyle region, mainly total or partial vertebrae fusions. In the trunk region the most common deformity was vertebrae compression, which was significantly higher ($F_{2,6}=5.3$, $p=0.048$) in the control group when compared to the phenylalanine group. No other significant differences were found (Fig.3). Caudal region presented the lowest deformity incidence. In this region mainly abnormal shape vertebrae were found, which were usually between the 16 and the 20 vertebrae. The most observed deformity in all vertebral column regions was the presence of several hypertrophic vertebrae. These vertebrae, which in the trunk region were observed mainly after the fifth vertebrae, occurred in some treatments (control) in 50% of the larvae (Fig.3).

4. Discussion

The aim of this study was to find the effects of diets supplemented either with phenylalanine or tyrosine on *D. sargus* larval quality. Supplementation was performed using enriched rotifers on early larval stages and a microencapsulated diet in later larval stages. FAA supplementation of rotifers was carried out successfully using the Barr and Helland (2007) technique. Although this technique has been a major advance, survival rate did not increased compared to other *D. sargus* studies (Saavedra et al., 2007a) where a casein microencapsulated diet replaced 90% of the live feed at 15 DAH. Survival rate was approximately the same to previous work with *D. sargus* weaning at 20 DAH. *D. sargus* weaning is usually between 35 and 40 DAH because only after 30 DAH the digestive tract seems to be fully developed (Ortiz-Delgado et al, 2003) and the

stomach functional (Cara et al., 2003). However, it would be useful to know the effect of AA supplementation in earlier phases in larval quality since in terms of vertebral column deformities a high incidence already occurs after 25 DAH. The adaptation phase to the microencapsulated diet was probably a critical period, especially to the smaller larvae, and conducted to an increase in the mortality rate which does not occur in a later weaning. Such a moderate success in weaning marine fish larvae in early larval phases have been previously reported (Kolkovski et al., 1997, Cahu and Infante, 2001).

Growth rate and dry weight were approximately the same to the ones reported by Saavedra et al. (2006, 2007a). One of the faced limitations in this work was the low solubility of tyrosine in water, which did not permit a higher tyrosine enrichment of rotifers. To overcome this, a supplementation of phenylalanine in the tyrosine enriched rotifers was given, since this AA can be converted into tyrosine (Khan and Abidi, 2007).

Both enzymes activities of key enzymes of AA catabolism (ALAT, ASAT and GDH) and ammonia excretion were approximately the same in all groups studied, and so were survival and growth rates, what confirms that the control diet used was well balanced in AA, and phenylalanine and tyrosine were not limiting growth. A diet with unbalanced AA profile has been shown to cause higher mortality, and a tendency for lower growth, in gilthead seabream (*Sparus aurata*) larvae (Aragão et al. 2007). The values obtained for the activity of the three main enzymes involved in AA catabolism were slightly higher than the ones reported for juvenile gilthead seabream (Gómez-Requeni et al., 2003). Fish larval stages are usually characterized by a very high growth rate (Conceição et al., 1998). During this phase, AA seem to be the major energy source (Rønnestad et al., 1999) and so it is not surprising that the enzymes involved in the AA metabolism have higher activities in larvae than in juveniles. Saavedra et al. (2007) using a casein microencapsulated AA balanced diet obtained similar values of nitrogen excretion and no differences between fed and fasted larvae.

A high level of skeletal deformities was observed in larvae at 25 DAH. At this stage control group presented 85.0 ± 21.8 % larvae with deformities and phenylalanine and tyrosine groups showed percentages of 71.7 ± 7.1 % and 78.3 ± 5.8 %, respectively. However, only a small proportion of larvae presented serious skeletal malformations

such as kyphosis and scoliosis which had values lower than 10%. No lordosis cases were observed in this study probably due to the preventive measures such as oil surface removal and close-control of water current. The rearing protocol used allowed for a normal swim bladder inflation and a non-intensive swimming activity which are usually pointed out to be the major causes of lordosis (Chatain, 1994). Preurostyle was the vertebral column region most affected by deformities in all treatments. In *Solea senegalensis*, Gavaia et al. (2002) observed the same, more than half of the deformities occurred in the caudal region. Although a high standard deviation was observed, the most common skeletal abnormality were vertebral fusions, approximately 25% in the control and tyrosine treatments and 15% in the phenylalanine treatment, followed by the presence of hypertrophic vertebrae (around 20%). However, it was in the trunk region that the later type of deformity was more frequent, reaching more than 50% of the observed larvae in the control group. The only significant difference found for deformities was in the number of compressions in the trunk region of the vertebral column. A percentage of 30% was obtained for control, which was significantly higher than the observed in the larvae from the phenylalanine group (less than 5%). In most cases the phenylalanine group presented lower frequency of skeletal deformities but, probably due to the high standard deviations observed for the other two groups, no other significant differences were found. There is no literature support for a special role of phenylalanine in preventing skeletal deformities. However, it is possible that this AA was deficient or less available in microencapsulated diet and its supplementation may increase the synthesis of molecules involved in the skeleton ossification. The fact that no lower frequency of skeletal deformities were verified in the tyrosine supplemented group, that would parallel the phenylalanine effect, is somewhat surprising, once tyrosine is the direct precursor for the hormones that may affect skeletogenesis. However, this may result from the low absorption efficiency for tyrosine already shown in *D. sargus* larvae (Saavedra et al., unpublished results). Phenylalanine supplementation may be more efficient, what could explain the results of the present study.

Hypertrophic vertebrae were the most common deformity observed in this study, and this does not bring many concerns. These hypertrophic vertebrae are described to be a result of accelerated organogenesis not followed by an increase of the body size (Gould, 1977). As development proceeds, the smaller vertebrae tend to grow faster and/or the

larger vertebrae to grow slower, and finally homogeneous vertebrae size and shape is obtained (Lewis et al., 2004). Lewis and Lall (2006) also reported high frequency of hypertrophic vertebrae when studying Atlantic halibut larvae.

One of the most interesting results in this study was the higher survival rate to a temperature stress test observed in the larvae supplemented with tyrosine. Tyrosine is precursor of norepinephrine (NE) and dopamine and it seems to enhance NE synthesis (Lehnert and Wurtman, 1993) and therefore may prevent stress-induced NE depletion in the animal brain (Deijen et al., 1999). Physical stress is known to increase the release of norepinephrine (NE) (Dimsdale and Moss, 1980) and in animal studies, stress-induced depletion of brain NE was followed by a reduction in the explorative and motor behaviour or behaviour depression (Lehnert et al., 1984). In rats given a tyrosine-enriched diet it was not observed either a NE depletion or behaviour impairments after stress induction (Brady et al., 1980). In humans, young men exposed to cold and hypoxia showed lower stress symptoms such as tension or fatigue after being given a tyrosine supplement (Banderet and Lieberman, 1989). Although there is a lack of information on this matter related to fish, laboratory studies strongly suggest that tyrosine supplementation may serve to reduce cognitive and behavioural effects of exposure to stress (Deijen et al., 1999). The fact that no stress mitigation effects were verified in the phenylalanine supplemented group, may suggest that fish larvae capacity to convert phenylalanine in tyrosine may be insufficient.

In conclusion, tyrosine and phenylalanine supplementation in *D. sargus* diet did not seem to cause any effect on growth or survival. However, phenylalanine seemed to have an impact on decrease of the percentage of vertebral compressions in the trunk region of the vertebral column and tyrosine supplementation increased larval resistance when *D. sargus* larvae were submitted to a temperature stress test. The present study confirms the idea that there are AA requirements for other metabolic processes in addition to the ones for growth.

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References

- Aragão, C., Conceição, L.E.C., Dias, J., Marques, A.C., Gomes, E. and Dinis, M.T. 2003. Soy protein concentrate as a protein source for Senegalese sole (*Solea senegalensis* Kaup 1858) diets: effects on growth and amino acid metabolism of post-larvae. *Aquaculture Research* 34, 1443-1452.
- Aragão, C., Conceição, L.E.C., Dinis, M.T. and Fyhn, H.J. 2004. Amino acid pools of rotifers and *Artemia* under different conditions: nutritional implications for fish larvae. *Aquaculture*. 234, 429-445.
- Aragão, C., Conceição, L.E.C., Lacuisse, M., Yúfera, M., Dinis, M.T. 2007. Do dietary amino acid profiles affect performance of larval gilthead seabream? *Aquatic Living Resources* 20, 155-161.
- Banderet, L.E. and Lieberman, H.R. 1989. Treatment with tyrosine, a neurotransmitter precursor, reduces environmental stress in human. *Brain Res. Bull.* 22, 759-762.
- Barr, Y. and Helland, S. 2007. A simple method for mass production of liposome, in particular large liposomes, suitable for delivery of free amino acids to filter feeding zooplankton. *Journal of liposome research* 17, 79-88.

- Brady, K., Brown, J.W. and Thurmond, J.B. 1980. Behavioral and neurochemical effects of dietary tyrosine in young and aged mice following cold-swim stress. *Pharmacol. Biochem. Behav.* 12, 667-674.
- Cahu, C. and Zambonino Infante. 2001. Substitution of live food by formulated diets in marine fish larvae. *Aquaculture* 200, 161-180.
- Cara, J.B., Moyano, F.J., Cárdenas, S., Fernández-Díaz, C. And Yúfera, M. 2003. Assessment of digestive enzyme activities during larval development of white sea bream. *J. Fish Biol.* 63, 48-58.
- Cejas, J.R., Almansa, E., Villamandos, J.E, Badía, P., Bolanos, A. And Lorenzo, A. 2003. Lipid and fatty acid composition of ovaries from wild fish and ovaries and eggs from captive fish of white sea bream (*Diplodus sargus*). *Aquaculture* 216, 299-313.
- Chatain, B. 1994. Abnormal swimbladder development and lordosis in sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*). *Aquaculture*, 119, 371-379.
- Cohen, S.A., Meys, M., Tarvin, T.L. 1989. The Pico-Tag Method - A Manual of Advanced Techniques for Amino Acid Analysis. Waters, Bedford, USA.
- Conceição, L.E.C., Ozório, R.O.A., Suurd, E.A. and Verreth, J.A.J. 1998. Amino acid profiles and amino acid utilization in larval African catfish (*Clarias gariepinus*): effects of ontogeny and temperature. *Fish Physiol. Biochem.* 19, 43-57.
- Conceição, L.E.C., Grasdalen, H. and Rønnestad, I. 2003. Amino acid requirements of fish larvae and post-larvae: new tools and recent findings. *Aquaculture* 227, 221-232.
- Deijen, J.B., Wientjes, C.J.E., Vullingsh, H.F.M., Cloin, P.A. and Langefeld, J.J. 1999. Tyrosine improves cognitive performance and reduces blood pressure in cadets after one week of combat training course. *Brain Res. Bull.* 48, 203-209.
- Dimsdale, J.E. and Moss, J. 1980. Plasma catecholamines in stress and exercise. *JAMA* 243, 340-342.

Dores, E.; Ferreira, I.; Mendes, A.I. & Pousão-Ferreira, P. 2006. Growth and skeleton anomalies incidence in intensive production of *Diplodus sargus*, *Diplodus cervinus* and *Diplodus vulgaris*. *Aqua 2006*, 9-13 Maio 2006, Florença, Itália, Books of Abstracts, pp. 262.

Gavaia, P.J., Sarasquete, C. and Cancela, M.L. 2000. Detection of mineralized structures in very early stages of development of marine Teleostei using a modified Alacian blue-Alizarin red double staining technique for bone and cartilage. *Biotech. Histochem.* 75, 79-84.

Gavaia, P.J., Dinis, M.T. Cancela, M.L. 2002. Osteological development and abnormalities of the vertebral column and caudal skeleton in larval and juvenile stages of hatchery-reared Senegal sole (*Solea senegalensis*). *Aquaculture* 234, 429-445.

Gómez-Requeni, P., Mingarro, M., Kirchner, S., Calduch-Giner, J.A., Médale, F., Corraze, G., Panserat, S., Martin, S.A.M., Houlihan, D.F, Kaushik, S.J. and Pérez-Sánchez, J. 2003. Effects of dietary amino acid profile on growth performance, key metabolic enzymes and somatotrophic axis responsiveness of gilthead sea bream (*Sparus aurata*). *Aquaculture* 220, 749-767.

Grasshoff, K. 1983. Methods of seawater analysis-*Verlag chemie-NY*, pp 317.

Khan, L and Bamji, M.S. 2007 Tissue carnitine deficiency due to dietary lysine deficiency: triglyceride accumulation and concomitant impairment in fatty acid oxidation. *J. Nutr.*, 109, 24-31.

Kolkovski, S., Arieli, A. and Tandler, A. 1997. Visual and chemical cues stimulate microdiet ingestion in sea bream larvae. *Aquacult. Int.* 5, 527-536.

Koumoundouros, G, Sfakianakis, D.G., Maingot, E., Divanach, P. and Kentouri, M. 2001. Osteological development of the vertebral column and of the fins in *Diplodus sargus* (Teleostei: Perciformes: Sparidae). *Mar. Biol.* 139, 853-862.

- Lehnert, H., Reinstein, D.K., Stowbridge, B.W. and Wurtman, R.J. 1984. Neurochemical and behavioral consequences of acute and uncontrollable stress: effects of dietary tyrosine. *Brain Res.* 303, 215-223.
- Lehnert, H. and Wurtman, R.J. 1993. Amino acid control of neurotransmitter synthesis and release: Physiological and clinical implications. *Psychother.Psychosom.* 60, 18-32.
- Lewis, L.M., Lall, S.P. and Witten, P.E. 2004. Morphological description of the early stages of spine and vertebral development in hatchery-reared larval and juvenile Atlantic halibut (*Hippoglossus hippoglossus*). *Aquaculture*, 241, 47-59.
- Lewis, L.M. and Lall, S.P. 2006 Development of the axial skeleton and skeletal abnormalities of Atlantic Halibut (*Hippoglossus hippoglossus*) from first feeding through metamorphosis. *Aquaculture* 257, 124-135.
- Ozório, R.O.A, Valente, L:M:P, Pousão-Ferreira, P. and Aires Oliveira-Teles. 2006. Growth performance and body composition of white seabream (*Diplodus sargus*) juveniles fed diets with different protein and lipid levels. *Aquacult. Res.*, 37, 255-263.
- Ortiz-Delgado, J.B., Darias, M.J., Cañavate, J.P., Yúfera, M., Sarasquete, C., 2003. Organogenesis of the digestive tract in the white seabream, *Diplodus sargus*. Histological and histochemical approaches. *Histol. Histopathol.* 18, 1141-1154.
- Pousão-Ferreira, P., Dore, E., Morais, S. and Narciso, L. 2001. Lipid requirements of the white seabream (*Diplodus sargus*) larvae. In: Larvi 2001- Fish and shellfish larviculture symposium. Special Publication, N° 30, European aquaculture Society. Oostende, Belgium, 187.
- Rønnestad, I., Thorsen, A. and Finn, R.N. 1999. Fish larval nutrition: a review of recent advances in the roles of amino acids. *Aquaculture* 177, 201-216.
- Rønnestad, I., Tonheim, S.K., Fyhn, H.J., Rojas-García, C.R., Kamisaka, Y., Koven, W., Finn, R.N., Terjesen, B.F. and Conceição, L.E.C. 2003. The supply of amino acids during early feeding stages of marine fish larvae: A review of recent findings. *Aquaculture* 227, 147-164.

Rønnestad, I. and Conceição, L.E.C. 2005. Aspects of protein and amino acids digestion and utilization by marine fish larvae. In *Physiological and Ecological Adaptations to Feeding in Vertebrates* (J. Matthias Starck and Tobias Wang, eds.), Science Publishers: Enfield, New Hampshire, USA, pp. 389-416.

Sá, R. Estudos de nutrição em juvenis de sargo-legítimo (*Diplodus sargus*). 2007. PhD Thesis, Science Faculty of Porto University.

Saavedra, M., Conceição, L.E.C., Pousão-Ferreira, P. and Dinis, M.T. 2006. Amino acid profiles of *Diplodus sargus* (L., 1758) larvae: implications for feed formulation. *Aquaculture*, 261, 587- 593.

Saavedra, M., Yúfera, M., Conceição, L.E.C., Pousão-Ferreira, P. and Dinis, M.T. 2007a. Effect of an amino acid balanced and unbalanced diet on *Diplodus sargus* metabolism and larval quality. World Aquaculture Society, San Antonio, Texas.

Saavedra, M., Barr, Y., Conceição, L.E.C., Pousão-Ferreira, P. and Dinis, M.T. 2007b. Effect of tryptophan and lysine supplementation on *Diplodus sargus* larvae quality. World Aquaculture Society, San Antonio, Texas.

Santos, M.N., Lino, P., Pousão-Ferreira, P. and Monteiro, C.C. 2006. Preliminary results of hatchery-reared seabreams released at artificial reefs of the Algarve coast (Southern Portugal): A Pilot Study. *Bull. Mar. Sci.* 78 (1): 177-186.

Watanabe, T. and Kiron, V. 1994. Prospects in larval fish dietetics. *Aquaculture* 124, 223-251.

Wilson, R. P. and Poe, W.E. 1985. Relationship of whole body and egg essential amino acid patterns to amino acid requirement patterns in channel catfish *Ictalurus punctatus*. *Comp. Biochem. Physiol.* Vol 80B, No2, 385-388.

Wilson, R. 1989. Amino acids and proteins. In: Halver, J. (Ed.), *Fish Nutrition*. Academic Press, San Diego, 11-151.

Yúfera, M., Pascual, E. and Fernández-Díaz. 1999. A highly efficient microencapsulated food for rearing early larvae of marine fish. *Aquaculture* 177, 249-256.

Yúfera, M. Kolkovski, S., Fernández-Díaz and Dabrowski, K. 2002. Free amino acid leaching from a protein-walled microencapsulated diet for fish larvae. *Aquaculture* 214, 273-287.

Table 1. Amino acid quantities added to the liposomes used to enriched control, phenylalanine (Phe) and tyrosine (Tyr) rotifers for one meal. Data are expressed as g of AA *per* million of enriched rotifers.

g/million rotifers	His	Lys	Arg	Thr	Met	Phe	Tyr
Control	0.047	0.183	0.100	0.050	0.023	0.000	0.000
Phe	0.047	0.183	0.100	0.050	0.023	0.055	0.000
Tyr	0.047	0.183	0.100	0.050	0.023	0.052	0.003

Table 2. Ingredient composition of balanced, phenylalanine and phenylalanine/tyrosine diets. ¹AgloNorse, Norway ²Rieber & Son, Norway ³CPSP 90, Sopropêche (Boulogne sur Mer, France), ⁴Acid hydrolysate, Sigma- Aldrich (Germany),⁵ Maialab (Portugal), ⁶ PREMIX (Portugal) ⁷Acofarma (Spain), ⁸DL- alpha-Tocopherol Acetate Powder, ICN (USA), ⁹ Rovimix Stay C-35, Roche ^{*}Sigma-Aldrich, purity $\geq 98\%$, ⁺ Fluka (Germany) $\geq 98\%$.

Ingredients (%)	Control	Phe	Tyr
Fish Meal ¹	15.9	15.9	15.9
Squid Meal ²	7.3	7.3	7.3
Hydrolised Protein Concentrate ³	7.3	7.3	7.3
Casein ⁴	31.1	31.1	31.1
Fish Oil ⁵	15.9	15.9	15.9
Vitamin+Mineral Complex ⁶	3.5	3.5	3.5
Agar ⁷	0.5	0.5	0.5
Vitamin E ⁸	0.7	0.7	0.7
Vitamin C ⁹	1.4	1.4	1.4
Threonine [*]	5.5	5.5	5.5
Arginine [*]	5.5	5.5	5.5
Phenylalanine [*]	1	2	1
Cystein [*]	1.4	1.4	1.4
Lysine [*]	4.2	4.2	4.2
Tyrosine [*]	-	-	4.7

Table 3. Free amino acid profile of *Brachionus plicatilis* (g/100g IAA). Non enriched rotifers (Blank), rotifers enriched with balanced amino acid liposomes (control), rotifers enriched balanced amino acid liposomes plus phenylalanine supplementation (Phe) and rotifers enriched with balanced amino acid liposomes plus tyrosine/phenylalanine supplementation (Tyr). AA are expressed in percentage (g indispensable AA/ 100g IAA). Values are mean and standard deviation.

	Blank	Control	Phe	Tyr
Leu	8.36±0.04	3.13±0.19	2.65±0.00	2.87±0.02
Lys	10.26±0.22	20.98±0.72	17.33±0.04	16.95±0.14
Arg	12.05±0.24	18.35±0.07	16.65±0.82	15.12±0.20
Val	14.08±0.15	5.62±1.22	4.05±0.01	4.87±0.07
Thr	7.05±0.10	14.39±0.34	11.50±0.25	11.22±0.10
Ile	11.89±0.17	4.18±0.02	3.59±0.00	4.21±0.14
His	10.20±0.36	18.80±0.65	15.63±0.29	15.84±0.09
Met	4.07±0.12	5.28±0.14	4.38±0.07	4.29±0.23
Phe	8.24±0.15	3.87±0.39	18.20±0.44	17.29±0.06
Tyr	13.80±0.19	5.40±0.14	6.00±0.18	7.34±0.04

Table 4. Total amino acids (Protein AA+ FAA) profiles of the microencapsulate casein diet of Control, Tyrosine (Tyr) and Phenylalanine (Phe) treatments. AA are expressed in percentage (g IAA/100g IAA). Values are mean and standard deviations.

	CONTROL	TYR	PHE
IAA			
Leu	13.8 ± 0.3	12.4 ± 0.4	14.4 ± 0.4
Lys	14.3 ± 0.3	11.3 ± 0.6	12.3 ± 0.8
Arg	13.2 ± 0.4	11.0 ± 0.9	11.3 ± 0.3
Val	8.9 ± 0.1	8.0 ± 0.3	8.8 ± 0.3
Thr	10.5 ± 0.1	8.4 ± 0.7	8.9 ± 0.3
Ile	7.3 ± 0.2	6.6 ± 0.1	7.2 ± 0.4
His	4.2 ± 0.1	3.8 ± 0.2	4.3 ± 0.0
Met	3.6 ± 0.7	3.3 ± 0.9	4.8 ± 0.4
Cys	7.8 ± 0.9	7.4 ± 2.6	9.9 ± 2.8
Phe	8.9 ± 0.2	7.9 ± 0.3	10.2 ± 0.4
Tyr	7.5 ± 0.2	20.0 ± 1.3	7.8 ± 0.3
DAA			
Glu+Gln	16.2 ± 0.2	15.4 ± 0.5	16.6 ± 0.2
Ala	4.0 ± 0.0	3.6 ± 0.1	4.1 ± 0.2
Gly	4.5 ± 0.1	3.8 ± 0.1	4.3 ± 0.0
Asp+Asn	7.7 ± 0.1	7.3 ± 0.2	8.0 ± 0.0
Ser	5.2 ± 0.2	4.9 ± 0.1	5.4 ± 0.0
Pro	8.2 ± 0.2	7.6 ± 0.3	8.2 ± 0.2

Table 4. Growth and survival of *Diplodus sargus* fed a balanced diet (control), a balanced diet supplemented with tyrosine and a balanced diet supplemented with phenylalanine. Values are mean and standard deviations (for growth n=20 larvae).

	Control	Tyr	Phe
Dry weight (mg)			
0 DAH	0.03± 0.00	0.03 ± 0.00	0.03 ± 0.00
15 DAH	0.23 ± 0.03	0.22 ± 0.02	0.22 ± 0.03
25 DAH	0.59 ± 0.11	0.68 ± 0.05	0.59 ± 0.12
RGR (%/day)			
0 – 15 DAH	15.04 ± 1.26	14.67 ± 0.37	14.73 ± 0.45
15 -25 DAH	10.03 ± 1.05	12.18 ± 0.96	10.51 ± 1.34
Survival (%)	5.98 ± 1.42	4.78 ± 1.35	4.77 ± 0.85

Table 5. Specific activity (IU/ g protein) of amino acid metabolic enzymes in 25 DAH *Diplodus sargus* larvae from control, tyrosine and phenylalanine diets (n=50 larvae *per* tank, three tanks *per* treatment).

	CONTROL	TYR	PHE
ALANINE AMINOTRANSFERASE (ALAT)	1219.0 $\pm$ 253.3	910.6 $\pm$ 229.7	1059.4 $\pm$ 114.7
ASPARTATE AMINOTRANSFERASE (ASAT)	2396.2 $\pm$ 384.0	2404.5 $\pm$ 509.7	2242.1 $\pm$ 195.3
GLUTAMATE DEHYDROGENASE (GDH)	324.2 $\pm$ 46.0	328.0 $\pm$ 49.5	300.4 $\pm$ 26.5

Fig. 1. Mass-specific ammonia excretion of fasted and fed from control, tyrosine and lysine treatments at 25 DAH. Values are mean and standard deviations (five replicates *per treatment*).

Fig 2. *Diplodus sargus* larval survival rate after being submitted to a temperature stress test. Values are mean and standard deviations (n=9). Different letters represent significant differences.

Fig 3. Percentage of deformities in the vertebral column of 25 DAH *D. sargus* larvae from control, phenylalanine and tyrosine treatments. Values are mean and standard deviations (n=60 larvae per treatment). Different letters represent significant differences. HV- hypertrophic vertebrae, PF- partial fusions, TF- total fusions, COMP- compressions, AV- abnormal shape vertebrae, DEF- total of deformed larvae.