UNIVERSIDADE DO ALGARVE

AMINO ACID REQUIREMENTS OF WHITE SEABREAM (*Diplodus sargus*) LARVAE: EFFECTS ON GROWTH AND PERFORMANCE

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To my Mum

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Abstract

Diplodus sargus is a potential species for aquaculture. Constraints to its production are a high incidence of skeletal deformities and stagnation of the growth rate in juvenile stage, possibly caused by dietary imbalances. This thesis is focused on the amino acid (AA) requirements of *Diplodus sargus* larvae, on the formulation of diets with AA supplementation and also identifies larvae skeletal deformities patterns (Chapter 2). *D. sargus* AA requirements were estimated using the AA profiles from larval carcass. It was observed that the AA profiles were relatively constant during larval ontogeny, and dietary imbalances were identified in rotifers and *Artemia* (Chapter 3). Tyrosine, lysine, tryptophan and arginine supplementation was tested by tube feeding and lower gut absorptions efficiencies were observed for these AA. Also, these AA do not seem to be limiting *D. sargus* growth (Chapter 4 and 5). Based on the results from Chapter 3, a microencapsulated casein diet with a balanced AA profile was formulated and was compared to an unbalanced AA diet and to a live feed diet (control) (Chapter 6). Results showed lower ammonia excretion and less skeletal deformities in larvae fed a balanced diet. Tryptophan supplementation in the diet was tested because of its involvement in skeleton ossification (Chapter 7). However, a decrease on larval growth and no effect on skeletal deformities were observed. The effect of tyrosine and phenylalanine supplementation on *D. sargus* larvae was tested and a higher survival to a temperature stress test was observed in larvae fed a tyrosine supplementation (Chapter 8). Larvae given a phenylalanine supplementation had a decreased incidence on vertebral compressions. In conclusion, an AA balanced diet reduces skeletal deformities and improves nitrogen utilization. Skeletal deformities do not seem related to tryptophan deficiency. Supplementation of phenylalanine and tyrosine lead to a decrease on skeletal deformities and better resistance to a stress test, respectively. From the AA studied; tryptophan, lysine, phenylalanine, arginine and tyrosine; none seems to be limiting *D. sargus* larval growth.

Key-words: Diplodus sargus, Amino acids, deformities, growth, microencapsulated diets

Resumo

A aquacultura tem vindo a ganhar cada vez mais importância e é responsável, neste momento, por cerca de 40% do pescado fornecido a nível mundial. O desenvolvimento desta actividade tem possibilitado ultrapassar problemas como a estagnação das capturas de peixes da pesca tradicional e o aumento da procura de pescado gerado pelo aumento da população mundial. Em Portugal, a aquacultura tem tido um desenvolvimento pouco acelerado e, baseia-se essencialmente, no cultivo de robalo e pregado, no Norte e dourada e robalo, no Sul. No entanto, a comercialização destas espécies tem estado em crise e, portanto, é necessário apostar no cultivo de novas espécies para promover o crescimento desta indústria na região do Mediterrâneo.

O sargo é uma espécie com bastante interesse comercial e que tem apresentado um decréscimo nas capturas de pesca. O *Diplodus sargus* tem sido cultivado experimentalmente em Portugal, estando ligado a programas de repovoamento, sendo já cultivado em pequena escala em países como a Grécia. Para que a cultura do sargo passe de extensiva a intensiva é necessário formular rações específicas para esta espécie e daí ser fundamental determinar os seus requisitos nutritivos. Até agora os protocolos de cultivo do sargo têm tido como base os protocolos de cultivo da dourada (*Sparus aurata*). Os principais problemas relacionados com a aquacultura desta espécie são uma elevada incidência de deformações esqueléticas e uma desaceleração da taxa de crescimento nos juvenis, problemas, esses, que podem estar relacionados com facto de não se conhecer os requisitos nutricionais desta espécie.

Existem dez aminoácidos (AA) considerados indispensáveis para o crescimento normal dos peixes. A optimização do crescimento está intimamente relacionada com o conhecimento do metabolismo das proteínas, de forma a ser fornecida proteínas de qualidade na quantidade apropriada. Para além disso, mais de 50% da composição larvar, em peso seco, é proteína e, por isso, não é de estranhar que o crescimento esteja relacionado com a qualidade da proteína fornecida às larvas. A juntar a este facto há, também, que considerar que a taxa de crescimento destas larvas é bastante elevada, podendo exceder os 50% peso do corpo/ dia e que o crescimento se deve, essencialmente, à deposição de proteína nos músculos. Estudos que examinaram a importância do catabolismo dos AA na alimentação exógena das larvas de peixe são escassos, mas os dados existentes parecem apontar para que os AA sejam o substrato

energético mais importante na fase larvar. Assim, as altas necessidades energéticas, juntamente com uma elevada taxa de crescimento das larvas, levam a considerar que deve existir um elevado requisito de AA nesta fase. Este requisito de AA durante as primeiras fases de desenvolvimento larvar dos peixes é normalmente satisfeito, em situações de cultivo, pelo alimento vivo (rotíferos e *Artemia*), uma vez que o aparelho digestivo não está totalmente funcional e algumas enzimas estão ausentes. Mais tarde, os requisitos passam a ser satisfeitos por dietas inertes, formuladas tendo em vista os requisitos nutricionais da espécie em causa.

Este trabalho centra-se fundamentalmente no estudo dos requisitos de AA de larvas de *Diplodus sargus* e na formulação de várias dietas, algumas suplementadas com AA específicos, tendo em vista uma maior sobrevivência, maior crescimento e qualidade larvar. Assim os objectivos deste trabalho são estimar os requisitos em AA das larvas de sargo, formular dietas onde o perfil de AA é corrigido, e testar o seu efeito na sobrevivência, crescimento e qualidade larvar. Uma vez obtida uma dieta com perfil de AA equilibrado, testou-se ainda o efeito da suplementação de certos AA: uns por serem considerados como potencialmente limitantes (lisina), outros por poderem, eventualmente, minimizar as deformações esqueléticas (triptofano) e outros por poderem ter efeitos que não no crescimento (fenilalanina e tirosina). Estes objectivos foram alcançados através de ensaios zootécnicos, com cerca de 25 dias de duração, onde se testou o efeito da suplementação a médio prazo, e de ensaios de alimentação por capilar, onde se testou o efeito imediato da suplementação no metabolismo das larvas de sargo.

Iniciou-se este estudo com a caracterização do tipo de deformações que ocorrem na fase larvar do *Diplodus sargus* (Capítulo 2). Embora este trabalho se centre essencialmente no estudo dos requisitos em AA desta espécie, a identificação das deformações esqueléticas, assim como a sua incidência, é importante uma vez que este é, talvez, o problema mais grave que o cultivo desta espécie enfrenta. A frequência de deformações do sargo em cativeiro pode atingir valores superiores a 50%, conduzindo à eliminação de grande parte destes indivíduos por não apresentarem um aspecto comercial aceitável. Por esta razão e porque a frequência de deformações é um dos parâmetros estudados em quase todos os ensaios zootécnicos deste trabalho, foi determinado o padrão de deformações de larvas de sargo dos 0 aos 30 dias de idade. Este padrão foi, mais tarde, utilizado como modelo nos ensaios de nutrição. Uma vez determinado o perfil de deformações esqueléticas, verificou-se que as malformações

começam a desenvolver-se a partir dos 13 dias de idade e que a incidência de deformações pode atingir frequências na ordem dos 80% logo aos 15 dias de idade. A região da coluna vertebral mais afectada, é a zona do do preurostilo, entre a 21 e a 23 vértebra. Para além disso, as deformações mais comuns parecem ser as compressões vertebrais (com valores acima de 50% em certas idades larvares). Outro tipo comum de malformações é a presença de vértebras hipertróficas, vértebras que apresentam um tamanho superior à média das outras vértebras. Este tipo de deformação não é muito grave pois a homogeneidade do tamanho acaba por ser restabelecida mais tarde com o crescimento da larva. As malformações mais graves, que afectam o aspecto exterior do peixe, como a escoliose, lordose e cifose, foram observadas em percentagens inferiores a 15%. Confirma-se portanto que as deformações são, realmente, um problema preocupante no cultivo larvar do sargo e que, embora as deformações mais graves estejam presentes em frequências mais baixas, as outras malformações podem afectar a *performance* do peixe e diminuir a sua taxa de crescimento.

O estudo dos requisitos em AA das larvas de sargo foi realizado utilizando o perfil de AA de carcaças de larvas. Existem vários estudos que demonstram uma correspondência elevada entre este perfil e os requisitos em aminoácidos em larvas de peixes. O perfil de AA das larvas de sargo foi estudado dos zero aos 45 dias de idade e verificou-se que este não se alterava significativamente (Capítulo 3). Para além disso, comparando o perfil de AA das larvas e as suas dietas (rotíferos e *Artemia)*, foi possível identificar-se a lisina, fenilalanina, arginina, treonina e cisteína como AA em possível deficiência na dieta. Estes AA em aparente deficiência não pareceram alterar-se muito com a idade larvar. No entanto, estes desequilíbrios nutricionais parecem ser mais acentuados nos rotíferos. Estes desequilíbrios ao nível dos AA podem ser corrigidos através da suplementação. A suplementação pode ser realizada directamente no alimento vivo através, por exemplo, da utilização de lipossomas com AA livres incorporados, ou pode ser efectuada através da utilização de dietas inertes. Os primeiros ensaios de suplementação foram realizados utilizando a técnica de alimentação por capilar. Esta técnica permite estudar o destino de nutrientes marcados com carbono radioactivo, isto é, determinar a percentagem que foi absorvida, oxidada ou retida. No caso do triptofano e da lisina, verificou-se que estes AA eram mais absorvidos quando suplementados mas que a sua eficiência de absorção ao nível do intestino era bastante mais baixa do que a média dos outros aminoácidos (Capítulo 4 e 5). Para além disso, estes AA não parecem estar a limitar o crescimento das larvas de sargo. Foi testada

também a alimentação por capilar da arginina, um AA que foi considerado como potencialmente limitante, e verificou-se, igualmente, uma eficiência de absorção inferior à média dos outros AA, quando suplementado, e que este AA também não parece ser limitante do crescimento larvar. A suplementação da lisina e tirosina utilizando rotíferos enriquecidos com lipossomas com AA livres incorporados (Capítulo 5). Verificou-se que também estes AA não estão a limitar o crescimento das larvas de sargo. Neste último ensaio a suplementação da tirosina não foi eficiente. Os restantes ensaios de suplementação foram realizados através da utilização de dietas inertes. Embora a adaptação ao alimento inerte de larvas de peixes não seja muito fácil, dietas mircroencapsuladas de caseína têm apresentado bons resultados ao nível da sobrevivência de larvas de dourada, uma espécie semelhante ao sargo. No entanto, esta dieta deve ser administrada em regime de co-alimentação com o alimento vivo para facilitar a digestão. Uma vez determinados os AA potencialmente limitantes do crescimento do sargo, formulou-se uma dieta microencapsulada de caseína cujo perfil era aproximado do perfil ideal de AA, e os AA em deficiência foram suplementados na forma de AA livres. A dieta equilibrada é considerada como tendo vantagens como, por exemplo, a maximização do crescimento e taxas de conversão alimentares. A dieta equilibrada foi comparada com uma dieta microencapsulada, equivalente mas suplementada com apenas AA cristalinos dispensáveis, e com o alimento vivo (controlo) (Capítulo 6). As dietas microencapsuladas foram administradas em co-alimentação com 10% de alimento vivo, uma vez que o alimento vivo parece ser estimulante da digestão larvar. Este estudo iniciou-se com larvas de 1 dia de idade e durou 25 dias. Os resultados demonstraram que a excreção de amónia era menor em larvas que ingeriam a dieta com perfil de AA mais próximo do das larvas (dieta equilibrada) e que havia uma menor incidência de deformações ao nível da coluna vertebral (cerca de metade do alimento vivo) e um menor número de fusões vertebrais aos 25 dias de idade larvar. Esta diminuição na excreção de azoto não foi, no entanto, reflectida num aumento do crescimento larvar. Isto pode ser devido a uma maior consumo de alimento pelas larvas alimentadas com dietas desequilibradas, ou aos AA não utilizados no catabolismo terem sido canalizados para outras funções que não o crescimento. Neste ensaio, as larvas alimentadas com alimento vivo apresentaram o dobro da sobrevivência confirmando, assim, ainda uma dependência elevada das larvas neste tipo de alimento e sugere que, provavelmente, o alimento vivo devia ter sido fornecido numa percentagem maior na co-alimentação.

Considerando que as deformações esqueléticas são um dos principais problemas no cultivo larvar testou-se o efeito da suplementação de triptofano e lisina na dieta (Capítulo 7). A deficiência de triptofano tem sido apontada, noutras espécies de peixes, como nos salmonídeos, como indutora de um aumento na percentagem de lordoses. A suplementação foi realizada, numa primeira fase (1 aos 15 dias de idade) através do enriquecimento dos rotíferos, uma hora antes de serem fornecidos às larvas, com lipossomas com AA cristalinos livres incorporados. Numa fase posterior (15 aos 25 dias de idade), a suplementação passou a ser realizada nas dietas microencapsuladas de caseína. Os resultados indicaram que suplementação da dieta com triptofano não só não teve influência nas deformações, como teve um impacto negativo ao nível do crescimento larvar, uma vez que as larvas alimentadas com esta dieta apresentaram um menor peso seco e um crescimento mais baixo. O mesmo efeito negativo no crescimento foi já descrito para juvenis de truta e robalo alimentados com suplemento de triptofano. Isto pode ter sido devido a um efeito negativo deste AA no paladar da ração, o que já foi descrito para outros peixes como a carpa. Esta diminuição no crescimento também pode estar relacionada com uma diminuição no apetite das larvas, uma vez que o triptofano é precursor da serotonina, neurotransmissor envolvido no controlo do apetite; Um aumento de triptofano pode ter conduzido a um aumento de serotonina que levou a uma diminuição da ingestão de alimento. A suplementação da lisina não teve qualquer efeito na sobrevivência, crescimento ou qualidade larvar do sargo. No último ensaio zootécnico foi testado o suplemento de dois AA, tirosina e fenilalanina, que estão ligados à biosíntese de várias hormonas e outras moléculas (Capítulo 8). Este ensaio foi realizado durante 25 dias e a suplementação foi realizada através do enriquecimento dos rotíferos com AA livres, incorporados em lipossomas, até aos 15 dias e na dieta microencapsulada de caseína a partir dessa idade. Os resultados sugerem um efeito da tirosina na resposta ao stress, uma vez que larvas alimentadas com a dieta suplementada com este AA tiveram uma maior sobrevivência quando submetidas a um teste de stress (diminuição brusca da temperatura). A tirosina é precursora das catecolaminas, hormonas directamente ligadas ao stress. A fenilalanina teve um efeito positivo na incidência de compressões de vértebras. A prevenção da incidência deste tipo de deformação é muito importante já que esta é a deformação mais comum e ocorre durante todas as fases larvares.

Em conclusão, o perfil de AA das larvas de sargo mantém-se relativamente constante ao longo da ontogenia larvar. Isto pode dever-se ao crescimento acelerado

desta espécie na fase larvar e/ou a uma metamorfose pouco acentuada que não leva à existência de diferenças ao nível do perfil de AA. Através da comparação dos perfis de AA das larvas e suas dietas, foram identificados vários possíveis desequilíbrios nutricionais, especialmente nos rotíferos. No entanto, dos AA estudados; lisina, triptofano, metionina tirosina e arginina; nenhum parece estar a limitar o crescimento de larvas de sargo, tal como foi demonstrado nos ensaios de alimentação por capilar. Uma dieta com perfil de AA aproximado do perfil de AA de larvas de sargo (dieta equilibrada) parece apresentar vantagens ao nível da excreção de azoto e prevenção de deformações mas não na taxa de crescimento. No entanto, o alimento vivo parece ser ainda fundamental para não haver redução na sobrevivência larvar. A utilização das dietas de caseína poderá ter vantagens ao nível da qualidade larvar, uma vez que as larvas apresentaram melhores perfis de ácidos gordos. A elevada percentagem de deformações na coluna vertebral das larvas de sargo não parece ser reduzida com suplemento de triptofano nem lisina. Por outro lado, quando a dieta é suplementada com tirosina e fenilalanina a resistência ao stress parece aumentar e o número de compressões de vértebras da coluna vertebral diminuir, respectivamente. Esta tese demonstra que os AA têm outras funções importantes para além do crescimento como na prevenção de deformações esqueléticas (fenilalanina) e resistência ao stress (tirosina).

Este trabalho é, apenas, o primeiro passo no conhecimento dos requisitos nutricionais das larvas de sargo, é necessário aprofundar o estudo de outros nutrientes, como ácidos gordos, vitaminas e minerais, para que esta espécie possa ter uma dieta inerte formulada de acordo com todos os seus requisitos nutricionais. Para além disso, embora tenha sido observada a influência de certos AA na incidência de deformações esqueléticas, a percentagem observada neste trabalho continua a ser demasiado elevada para se considerar este problema resolvido.

Palavras-chave: *Diplodus sargus,* aminoácidos, deformações, crescimento, microencapsulados

Chapter 1

General Introduction

1.1. Aquaculture and the supply of fish

World population is rising and food supply must increase in order to meet global food demands. The contribution of aquaculture to global supply of fish, molluscs and crustaceans is rising at an average of 8.8 % per year since 1970 (FAO, 2006a). In 2006, capture fisheries and aquaculture provided 161 million tonnes of food fish (FAO, 2006b), 42% of each were delivered by aquaculture. This industry has been managing to overcome problems such as stationary capture fisheries (FAO, 2006a) and is having an important role in the increase of family livelihoods in developing countries. Non-governmental agencies have been implementing aquaculture programmes in third world countries in order to teach poor farmers how to obtain an extra income with fish production without much extra costs.

Most fish supplied by aquaculture are delivered from countries in Asia and Pacific region, which represent more than 90% of global fish production. The top five countries producers of fish are located in this region, being China the most important one. There is a wide diversity of farmed species in the world, which varies according to the region. Asia and the Pacific region account for a large fraction of the global production of aquatic plants, cyprinids, penaeids and oysters. Northwest Europe produces more than half of the farmed salmonids and Central and Eastern Europe are dominant in carp farming (FAO, 2006a). In the Mediterranean region, fish production is mainly seabream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) and Greece is the top producer. Due to market saturation the price of these fish decayed and aquaculture in the Mediterranean region is now facing a crisis (Abéllan and Basurco, 1999). Diversification of the farmed species is crucial to boost once again aquaculture in this region (Ortiz-Delgado et al., 2003).

1.2 Aquaculture in Portugal

Portugal is a country with great roots to the sea and fisheries are an important activity employing a considerable percentage of the active population. Aquaculture represents an important alternative to traditional fisheries but the Portuguese aquaculture contribution to fish demand is still very limited. In 2005, fish farming produced 6 669 tons with a value of 34 450 euros, representing 5.1% and 16%, respectively, of the capture fisheries (129 691 tons) (DGPA, 2005).

Marine aquaculture in Portugal is exploited mainly in extensive or semi-intensive systems and ninety species are farmed (DGPA, 2005). From these, nine are bivalves being the main produced group. Finfish culture is essentially gilthead seabream (Sparus aurata) in the South and sea bass (*Dicentrarchus labrax*) and turbot (*Psetta maxima*) in the North. There is a rising interest on senegalese sole (*Solea senegalensis*), which is produced at a small scale in extensive systems.

1.3. Diplodus sargus and its potential to aquaculture

Diplodus sargus (Linnaeus, 1758) is a sub-tropical species distributed along the Mediterranean, although rare in the Black Sea, and in the Atlantic coast, from Bay of Biscay to Cape Verde and the Canaries Islands (Whitehead et al., 1986). It can be found in littoral waters on rocky bottoms and sand close to rocks. *D. sargus* is a eurihaline species, entering in brackish waters and lagoons in spring and returning to sea at the end of autumn (Whitehead et al., 1986). Juveniles are omnivorous, feeding on worms and algae and adults are carnivorous, feeding on worms, molluscs and crustaceans. It reproduces from January to March in the eastern Mediterranean and from March to June in the western Mediterranean (Whitehead et al., 1986).

D. sargus is a potential candidate for aquaculture in the Mediterranean region (Quemener et al., 2002) due to its high price, demand and flesh quality (Abellan and Garcia- Alcazar, 1995; Pousão-Ferreira et al., 2001; Ozorio et al., 2006; Santos et al., 2006). First experimental studies on *D. sargus* farming started in 1980´s and were focused on the reproductive cycle, embryonic and larval development in intensive system (Divanach et al., 1982; Micale et al., 1987). However, it was only recently due to the need of market diversification in the Mediterranean area that research on this species was boosted (Abellan et al., 1994; Abellan and Garcia-Alcazar, 1995; Cejas et al., 2003; Cejas et al., 2004). *D. sargus* potential for aquaculture is increased by its similarity to *Sparus aurata* rearing technique, the easiness to obtain broodfish (Abellán and Basurco, 1999) and broodstock usually spawns naturally in captivity, producing approximately 2000 eggs/g.

Another factor stimulating *D. sargus* culture is the decrease in the capture fisheries which, just in the southern coast of Portugal, has decreased from 200.3 t to 75.2 t between 1987 and 2004 (DGPA). For this reason *D. sargus* has been produced in Portugal at pilot scale for restocking purposes (Santos et al., 2006).

The main constraints to *D. sargus* farming are a decrease on the growth rate when approaching the juvenile stage (Cejas et al., 2003) and a high incidence of skeletal deformities. *D. sargus* also seems to present some agonist behaviour and hierarchic relationships triggered by high densities, food distribution or light incidence (Caballero and Castro, 1999, 2003; Castro and Caballero, 1998, 2004). This behaviour usually leads to heterogeneous sizes and stimulates cannibalism in the tanks.

D. sargus is produced at small scale in Greece, Spain (FAO) and in Portugal at pilot scale.

1.4. Importance of Amino acids

Optimization of growth is closely related to the knowledge of protein metabolism aiming for a supply of better protein quality delivered in the right quantity (Conceição *et al.*, 2003). Larval composition is over 50% protein, in dry weight, and larval growth rate may exceed 50% body weight/ day (Conceição, 1997). Considering growth is mainly muscle protein deposition (Conceição et al, 1998) and amino acids (AA) are the major energy source in larval stages (Ronnestad et al., 1999) there is a high AA requirement in this development stage of fishes. AA requirements in fish vary between species (Conceição, 1997). Within a species it depends on fish size and age; water temperature; protein/ energy balance; dietary AA profiles; digestibility of the diet and diet composition (Wilson, 1989).

There are ten AA in fish considered indispensable: lysine, arginine, histidine, methionine, leucine, isoleucine, phenylalanine, valine, tryptophan and threonine (Wilson, 1989). Cysteine and tyrosine are considered a dispensable AA although they can only be synthesised from methionine and phenylalanine, respectively. Manipulations of AA composition to obtain dietary AA profiles as similar as possible to larval AA requirements can be very advantageous as it can increase both growth rates and food conversion efficiencies (Akiyama et al., 1995; Conceição et al., 2003). The ideal dietary AA profile depends on the efficiency of absorption individual AA, on the profile of proteins being synthesised and on the use of AA for energy production (Conceição, 1997). The absorption of individual AA in the larval gut is associated to different transport systems (Jürss and Bastrop, 1995) and may occur at different rates (Dabrowski, 1983). This variation in the absorption rates of the different AA affects the availability of the different AA and may increase catabolism (Cowey and Walton, 1989; Jürss and Bastrop, 1995).

Indispensable AA profile of fish carcass has been used as a good indicator of larval AA requirements (Wilson and Poe, 1985; Watanabe and Kiron, 1994). Comparing AA profiles from fish carcass and larval diets is a first approach to determine dietary imbalances (Conceição et al., 2003). This comparison can only be seen as a first step but it provides important information on the possible dietary amino acid deficiencies (Conceição et al., 2003). Once larval AA profiles have been determined is possible to formulate a better balanced diet. AA balanced diets present several advantages as they usually increase protein synthesis and decrease nitrogen excretion (Aragão et al., 2004a). In salmonids, diets supplemented with indispensable AA in order to simulate fish AA profiles improved growth rates and feeding efficiencies (Ogata et al., 1983). Also, some fish such as juvenile rainbow trout, in presence of several AA diets, select preferentially balanced diets (Yamamoto et al., 2000).

In rearing conditions, fish larvae AA requirements are normally supplied by live feed such as rotifers and *Artemia* and weaning is done some weeks after hatching (Cahu and Zambonino Infante, 2001). An early weaning, for example of Sparidae larvae, might compromise larval growth and survival (Yúfera et al., 1996, Fernández-Díaz and Yúfera, 1997). This is due to the lack of some enzymes in the beginning of fish ontogeny and an immature digestive tube which incapacitates the full digestion of inert diets (Rønnestad *et al.*, 2003). High levels of citosolic peptidases seem to exist on the first days after hatching, that later on start decreasing with the rising concentration of other enzymes (Cahu and Zambonino Infante, 2001). These physiological changes suggest AA requirements might change throughout fish larval ontogeny (Conceição *et al.*, 2003), especially when fish metamorphosis is pronounced (Aragão et al., 2004a). Although there is still a high dependence on live feed for larval rearing, several studies have reported AA imbalances in rotifers and *Artemia* (Conceição et al., 1998;

Conceição et al., 2003; Aragão et al., 2004a). One way to overcome such problems is enriching live feed, which has been successfully achieved for fatty acids but not so much for AA. Until recently, manipulations of AA profiles of live feed have been difficult (Aragão et al., 2004b). Barr and Helland (2007) developed a technique which made an important advance in this study area. They developed a technique to enrich live feed with free AA using liposomes, enabling AA supplementation in early larval stages. It remains to be demonstrated whether this technique can be upscaled from the research to the commercial level.

It is possible to introduce an inert diet in co-feeding with live feed some days after hatching. Yúfera et al. (1999, 2002) have developed cross-linked casein-walled capsules and were able to replace with some success live feed in *Sparus aurata* early larval stages. However, during the first feeding days the microencapsulated diet had to be used in co-feeding with rotifers, probably to increase diet acceptability and improve the enzymatic capacity to digest the inert diet (Yúfera et al., 1999, 2002). Free AA in the tissues of live feed seem to be important to stimulate the release of digestive enzymes and enhance the ingestion rates as they affect the attractability of the diets (Cahu and Zambonino Infante, 1995). Inert diets are more efficient in terms of supplementation because all dietary AA content can be changed whereas in live feed it is only possible to change partially the free AA profile (Rønnestad et al., 2003). However, inert diet present some problems such as nutrient leaching (Yúfera *et al.*, 2002), which can constraint results interpretation and suggest misleading conclusions (Rønnestad *et al.*, 2003).

The knowledge on AA metabolism and the larval capacity to digest, absorb and retain dietary protein is extremely important to 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 trace the fate of labelled molecules either catabolised or retained, after being absorbed (Rønnestad et al., 2001b). This technique uses a ^{14}C tracer which is injected in the larval gut through a capillary tube (Rønnestad *et al*., 2001a). Once the digestion period is over it is possible to distinguish between absorbed and unabsorbed nutrients 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., 2004a).

Besides an important role on protein synthesis, some AA are also crucial to the synthesis of other molecules and in the development of certain organs or structures. Some AA are precursors of hormones, such as tyrosine in the synthesis of cathecolamines and therefore might have an effect on stress control (Lehnert and Wurtman, 1993). Others such as tryptophan, are involved in the normal development of structures, as skeletal ossification. These AA roles are as important as protein synthesis as they will influence larval quality.

1.5. Larval deformities

Vertebral deformities are the most important skeletal malformation and, unfortunately, a frequent and important problem for aquaculture (Divanach et al., 1996; Koumoundouros et al., 1997a,b; Lewis et al., 2004). Skeletal deformities are often associated with reduced overall hatchery performance, which means, lower survival rates, poor growth rates and feed conversion efficiencies and increased susceptibility to disease (Boglione et al., 2001). While adult fish deformities are well documented (Daoulas et al., 1991, Andrades et al., 1996), few studies report the development of malformations during larval ontogeny (Fraser et al., 2004). Deformities have been reported in the opercules and caudal fin of seabream, *Sparus aurata*, (Koumoundouros

et al., 1997a,b), in the jaw of Atlantic halibut, *Hippoglossus hippoglossus* (Morrison and McDonald, 1995) and stripped trumpeter, *Latris lineate* (Cobcroft et al., 2001) in sea bass spine, *Dicentrarchus labrax* (Koumoundouros et al., 2001). Besides these, there are also abnormalities on pigmentation, scales and swimbladder (Chatain et al., 1994) in a variety of species (Divanach et al., 1997).

Skeletal malformations can have an economical impact because they require manual sorting (Koumoundouros et al., 1997a,b) and they can affect both size and shape of fish (Favaloro and Mazzola, 2000; Boglione et al., 2001). Marketing image, commercial value and production benefits of reared fish also get an impact from skeletal abnormalities (Barahona-Fernandes, 1982; Boglione et al., 1994) because consumers tend to choose fish with standard shape (Koumoundourous et al., 1997a, Boglione et al., 2001).

Identification of potential causes of fish deformities is difficult but is often related to insufficient knowledge of the optimum fish rearing techniques (Koumoundouros et al, 1997; Sfakianakis et al., 2005), including the use of inadequate diets (Cahu et al., 2003). In order to determine the cause of skeletal deformities it is first necessary to identify the structures involved in larval deformities and their time of apparition (Fraser et al., 2004).

1.6. Objectives

This thesis intends to evaluate the AA requirements of *Diplodus sargus* and verify the effect of dietary AA in larval quality. *Diplodus sargus* is a candidate species to aquaculture and there is almost no information about its larval nutritional requirements. Feeding protocols and rearing conditions used for *D. sargus* are adapted from seabream (*Sparus aurata*) and there are no specific inert diets. Probably due to the

implementation of seabream feeding and rearing protocols, there are still serious problems when farming this species farming such as stagnation in juvenile growth and a high incidence of skeletal deformities. Although this study is centered on nutritional requirements, a chapter is dedicated to the knowledge of *D. sargus* skeletal deformities as this is one of the major constraints for farming this species. Once the skeletal deformity pattern is determined, it can be used as a model in following experimental trials. *D. sargus* has its own particularities compared to *Sparus aurata* such as different mouth size and the development of agonistic behaviour as well as hierarchic relationships triggered by some rearing conditions. In order to improve *D. sargus* aquaculture potential it is important to find solutions to these problems, including a better knowledge of larval nutritional requirements and formulation of diets specific for this species.

Considering this, the main objectives of this work are:

a) Identify skeletal deformities affecting *D. sargus* larvae and its incidence in order to establish a pattern which could be used as a reference for later studies.

b) Estimate the AA requirements from *D. sargus* larvae in several ontogeny stages.

c) Test the supplementation efficiency of candidate AA, and verify which AA may be limiting growth of *D. sargus* larvae.

d) Determine if the supplementation of the diets with AA in apparent deficiency or expected requirements other than growth, conducts to a higher survival, growth or quality of *D. sargus* larvae.

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Chapter 2

Development of deformities at the vertebral column in *Diplodus sargus* (L., 1758) early larval stages

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Abstract

One of the bottleneck problems of *Diplodus sargus* farming is a high incidence of deformities at the vertebral column. This study followed three different larval batches from 2 to 30 DAH. During this period, 60 larvae *per* spawn were observed on 2, 8, 13, 15, 18, 21, 23, 25, 27 and 30 DAH and the different types and frequency of vertebral column malformations were recorded. Deformities seemed to appear on 13 DAH, when the percentage of deformed larvae rose from approximately 5 to 40%. On 15 DAH this percentage rose, in some cases, up to 80% of the observed larvae. Serious malformations such as kyphosis, scoliosis and lordosis started appearing towards the 18 DAH but seldom in percentages higher than 15%. Lordosis was usually lower than 10% and more common in later larval stages (27 and 30 DAH). Vertebral fusions and compressions affected especially the preurostyle region, where 20% and 70% of the larvae presented these deformities, respectively. Abnormal shape vertebrae were more incident between vertebrae 15 to 21, affecting therefore both caudal and preurostyle regions. Other observed malformations were hypertrophic vertebrae, more common in the trunk and caudal regions, reaching percentages higher than 50% in the former. This study presents useful information concerning skeletal malformations at the vertebral column of *Diplodus sargus* larvae as it identifies the main observed deformities and ages of highest incidence.

Keywords: *Diplodus sargus*, skeletal deformities, larva, lordosis, kyphosis, scoliosis

2.1. INTRODUCTION

White seabream (*Diplodus sargus*) is a sub-tropical species distributed along the Mediterranean Sea, European and African coasts (Fischer et al., 1987). This species is considered to be a promising new species for aquaculture due to its high market price and demand (Pousão-Ferreira et al., 2001, Ozorio et al., 2006, Santos et al., 2006) and to its adaptability to the sea bream (*Sparus aurata*) rearing techniques (Sá, 2007).

Diplodus sargus farming is restricted to Greece although in Portugal there is some production at a small scale (Sá, 2007). White sea bream production presents several constraints such as a decrease in the growth rate when reaching the juvenile stages (Cejas et al., 2003) and the presence of several skeletal deformities, especially at the vertebral column (Saavedra et al a,b; Dores et al., 2006).

Vertebral deformities are the most important skeletal malformations as a high incidence has been reported in several species reared in wild or in captivity conditions (Divanach et al., 1996). Serious vertebral abnormalities such as scoliosis, lordosis and kyphosis are usually associated with reduced hatchery performance including lower survival rates, poor growth and increased susceptibility to stress and disease (Boglione et al., 2001). In general, vertebral deformities are developed due to an insufficient knowledge of the optimum rearing techniques of the fish at different life stages (Sfakianakis et al., 2005), inadequate nutrition protocols (Cahu et al., 2003) among others.

Skeletal malformations can have an economical impact as they required manual sorting (Koumoundouros et al., 1997a,b) as they can affect both size and shape of the fish therefore decreasing their economical value (Favaloro and Mazzola, 2000; Boglione et al., 2001). Finally, they can make commercialization difficult as the consumers tend to choose fish with normal shape (Koumoundourous et al., 1997a, Boglione et al., 2001). Elimination of skeletal deformities depends on the detection of

their induction factors (Koumoundouros et al, 1997a) which is not easy because fish are subjected to different impact factors. A first step could be the identification of the phase at which malformation incidence starts and, from there, make trials changing the different impact factors such as nutrition, water current or water temperature.

The purpose of this study was to identify the different types and frequency of vertebral column deformities during *Diplodus sargus* early larval stages and determine the larval ages with higher incidence.

2.2. MATERIAL AND METHODS

2.2.1. Husbandry and experimental set-up

This study was performed at the Aquaculture Research Station of IPIMAR, in Olhão, Portugal during 2006. *Diplodus sargus* eggs were obtained from two broodstock tanks consisting of a mix of wild and farmed fish. Tank B1 consisted of 42 individuals with an average weight of 809.9 g ± 208.0 g, kept at a density of 3.2 kg/m^3 and tank B2 had 27 fish with an average weight of 1034.3 g ± 276.3 g, kept at a density of 3.3 kg/m^3 .Sex ratio was approximately 1/1 in both tanks. Three different batches (P1, P2 and P3) from three different spawns were collected from the broodstock tanks, two from B1 (P1 and P3) and one from B2 (P2), and viable eggs incubated at 18ºC with moderated aeration. Newly hatched larvae from each batch was transferred and divided into three 200 L cylindro-conical fibreglass tanks at a density of 80 larvae L^{-1}. The system worked in a semi-closed circuit and water temperature was maintained at 21.5 ± 1.1 º C, oxygen at 5.3 ± 0.6 mg L^{-1} and salinity at 37 ± 1 ppt. Before entering the tank water passed through a biological filter, a mechanical filter (100µm) and a UV light. Water flow

started at 0.6 L min^{-1} and then was slowly increased with larvae age until a maximum of 1 L min^{-1}, from 15 DAH. Photoperiod was 24 hours Light.

Larvae were fed rotifers *Brachionus plicatilis* (5 rotifers mL^{-1}) enriched with Protein Selco® (INVE Aquaculture, Belgium) from 3 to 20 DAH. On 17 DAH, larvae were fed *Artemia* metanauplii (0.25 Artemia mL^{-1}), enriched with Super Selco (INVE, Aquaculture, Belgium). Dry feed (Nippai®, Japan) (increasing from 0.3 g $tank^{-1}$ to 2 g) was given from day 20 until the end of the experiment in cofeeding with *Artemia*. *Tetraselmis* sp and *Isochrysis galbana* were added to the tanks in the morning and in the afternoon, together with the feed, to obtain a final concentration of approximately 150x 10^3 cells mL^{-1}.

2.2.2. Sampling and staining of the larvae

Three larvae samples were taken from each tank on 2, 5, 10, 18, 21, 25 and 30 DAH for dry weight determination. These samples were frozen in liquid nitrogen at -196 ºC, then freeze-dried (RVT 400, Savant, NY) and weighed. Total larval length was measured in 60 larvae *per* treatment on 2, 5, 9, 13, 18, 21, 25 and 30 DAH. Samples of 30 larvae were taken from all tanks on 2 (from the incubator, before being transferred to the larval rearing tanks), 3, 8, 13, 15, 18, 21, 23, 25, 27 and 30 DAH to determine the vertebral column deformities. In the larval samples for deformities, cartilage was stained with alcian blue (40 minutes in average) and ossified bone was stained with alizarin red (2 hours), according to Gavaia et al. (2002). Deformities were identified using Koumoundouros et al. (2001) development descriptors as a standard. In order to simplify the deformities and identified the areas of higher incidence, vertebral column was divided into three regions: trunk vertebrae (1 to 10), caudal vertebrae (11 to 20) and preurostyle vertebrae (21 to 23).

2.2.3. Data analysis

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

Significant differences between larval ages were determined using one-way Analysis of Variance (ANOVA) at a minimum significance of p‹0.05 with Statistisca© (v.5.5) for larval total length. For survival, growth, dry weight and each deformity Kruskal-Wallis non-parametric ANOVA was performed. Scheffé test was performed as post-Hoc test for data with parametric distribution and Nemenyi test for data with non-parametric distribution.

2.3. RESULTS

2.3.1. Larval survival and growth

During the experimental period there were no significant differences in the larval survival from the different spawns. P1 presented a survival rate of 10.2 ± 1.5 %, P2 9.0 ± 0.1 % and P3 had 8.6 ± 0.0 %.

Significant differences between spawns were found for larval total length on 2, 10, 13, 18, 21, 25 and 30 DAH, where P1 presented lower larval length when compared to P2 and P3 (Table 1). On 5 DAH, all spawns presented significantly different larval lengths. The same happened with the larval dry weight, where on 5, 10 and 30 DAH, P1 showed lower larval dry weight (Fig. 1). On 18, 21 and 25 DAH, P1 was only significantly different from P3 (Fig. 1). The relative growth rate (RGR) was significantly different on 5 DAH, where P3 presented higher RGR than P1 and P2 and on 30 DAH, where P1 was significantly different from P2 (Table 2).

Table 1. *Diplodus sargus* total length from 2 to 30 DAH from spawns P1, P2 and P3. Values are mean and standard deviation (n=60 larvae per treatment). Different letters correspond to significant differences (p<0.05).

	Larval age (DAH)							
Larval length (mm)	**2**	**5**	**9**	**13**	**18**	**21**	**25**	**30**
P1	3.63±0.09a	3.79±0.16a	4.61±0.44[a]	5.63±0.48[a]	7.27±0.79	7.51±0.94a	9.35±1.03a	11.31±1.50a
P2	3.79±0.09b	4.00±0.19b	4.92±0.30b	6.09±0.56b	7.06±0.99	8.53±1.18b	10.40±1.49b	13.82±1.10b
P3	3.79±0.09b	3.89±0.16c	4.88±0.34b	6.06±0.54b	7.11±1.08	8.34±1.21b	10.24±1.40b	13.53±1.26b

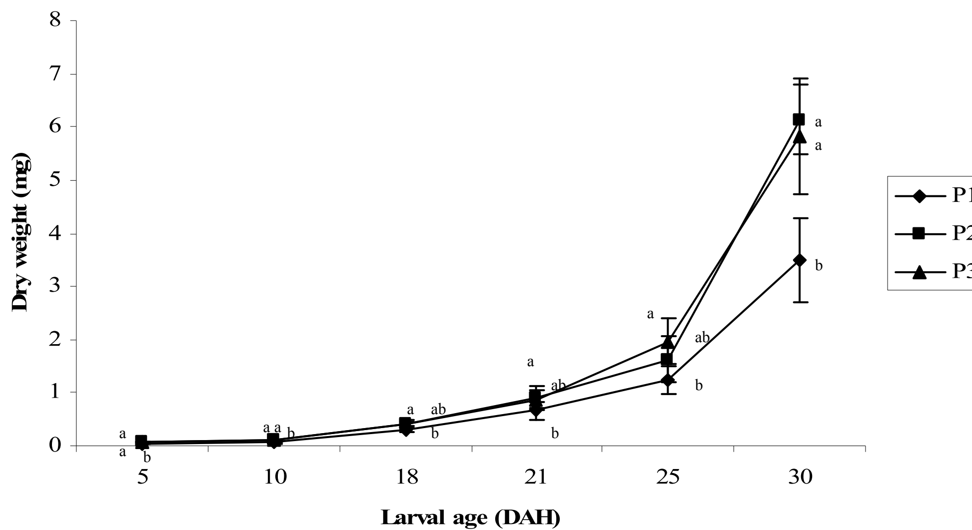

Fig 1. *Diplodus sargus* growth in dry weight (mean and standard deviation) during the 30 days experimental period. Different letters represent significant differences between spawns.

Table 2. *Diplodus sargus* Relative Growth Rate (RGR) from 2 to 30 DAH from spawns P1, P2 and P3. Values are mean and standard deviation (n=12 per treatment). Different letters correspond to significant differences (p<0.05).

	Larval age (DAH)					
RGR (% DW/day)	**2-5**	**5-10**	**10-18**	**18-21**	**21-25**	**25-30**
P1	8.2 ± 6.1a	6.4 ± 5.7	21.3 ± 1.8	30.1 ± 6.2	15.8 ± 6.5	23.0 ± 4.2a
P2	12.6 ± 4.9a	5.7 ± 3.0	19.1 ± 2.1	31.2 ± 11.2	15.9 ± 12.4	28.2 ± 3.3b
P3	22.5 ± 7.6b	4.6 ± 2.6	20.5 ± 2.8	27.4 ± 3.9	22.2 ± 7.9	24.7 ± 7.9ab

2.3.2. Larval deformities

The percentage of deformed *Diplodus sargus* larvae started significantly lower on 3 and 5 DAH ($H_{(9,30)}$=24.6, p=0.003 for P1, $H_{(9,30)}$=19.0, p=0.03 for P2 and $H_{(9,30)}$=20.4, p=0.02 for P3) (Fig. 2). At these early ages the percentage of deformed larvae is approximately 5%, rising to 40% or more on 13 DAH and to 80% on 15 DAH (Fig. 2). From this point on it does not seem to exist a defined pattern but a fluctuation in the number of deformed larvae from 15 to 30 DAH (Fig. 2). Serious vertebral malformations such as kyphosis and scoliosis were first observed on 13 DAH whereas lordosis was only observed on 15 DAH (Fig.3). Significant differences were found only for lordosis where on 30 DAH there were a higher number of larvae presenting this type of deformity ($H_{(7,72)}$=18.7, p=0.01) than in other ages. On 23 DAH larvae presented a significant higher percentage of scoliosis compared with larvae on 13 or 15 DAH ($H_{(7,72)}$=14.3, p=0.046) (Fig.3).

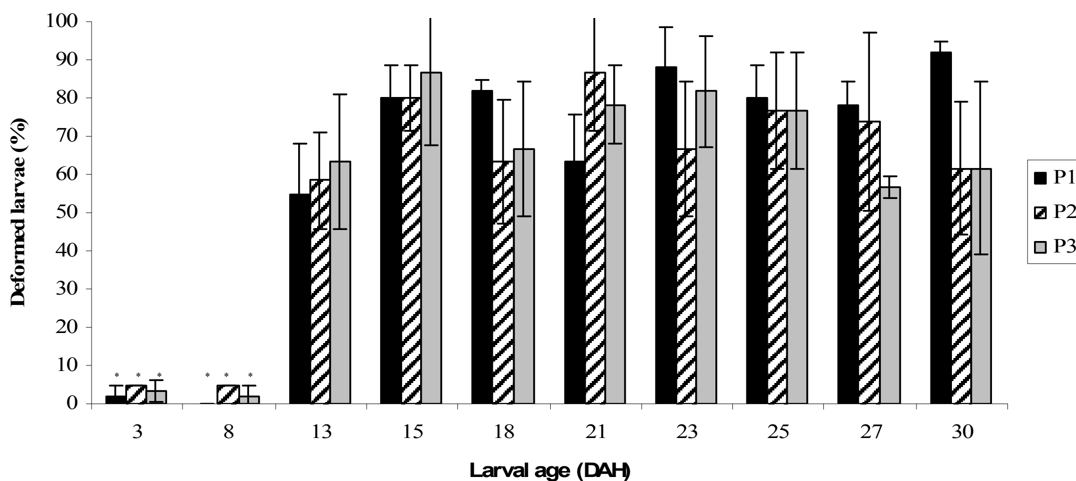

Fig. 2. Percentage of deformed *Diplodus sargus* larvae from 3 to 30 DAH. Values are mean and standard deviation (n=60 per treatment). (* -Correspond to significant differences between larval ages).

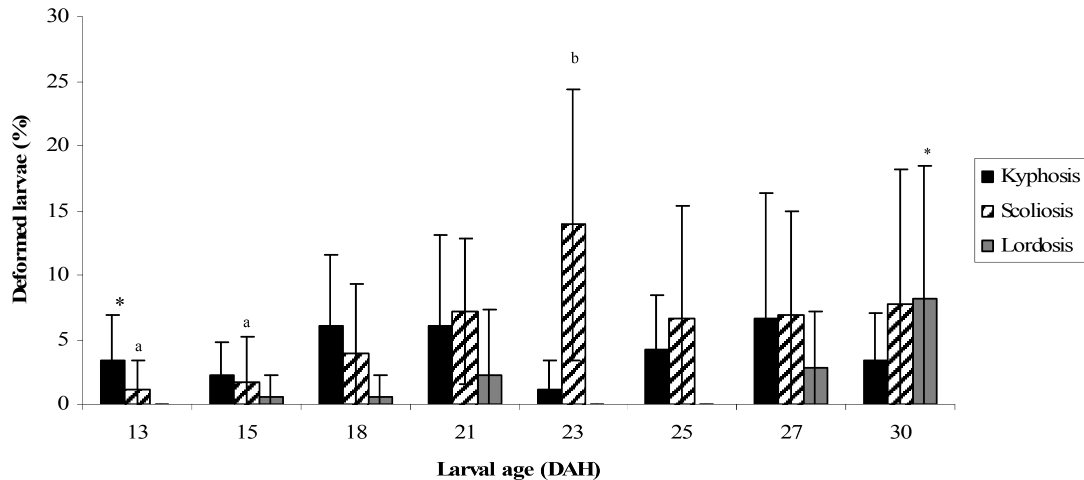

Fig. 3. Frequency of *Diplodus sargus* larvae with Kyphosis, Scoliosis and Lordosis from 13 to 30 DAH. Values are mean and standard deviation (n=180). (* represents one age significant difference from all others and letters correspond to significant differences between several larval ages).

Vertebral fusions start showing on 15 DAH in the trunk region, on 23 DAH in the caudal region and as early as on 13 DAH in the preurostyle region, which seems to be the region most affected by this malformation. However, results show a lower frequency of this type of skeletal deformity with values generally lower than 10%. Significant differences were found for the frequency of larvae with total and partial fusions in the caudal region on different ages ($H_{(7,72)}$=15.5, p=0.03 and $H_{(7,72)}$=15.4, p=0.03, respectively) as well as for the preurostyle ($H_{(7,72)}$=15.3, p=0.03 and $H_{(7,72)}$=20.3, p=0.005 for total and partial fusions, respectively) (Fig. 4).

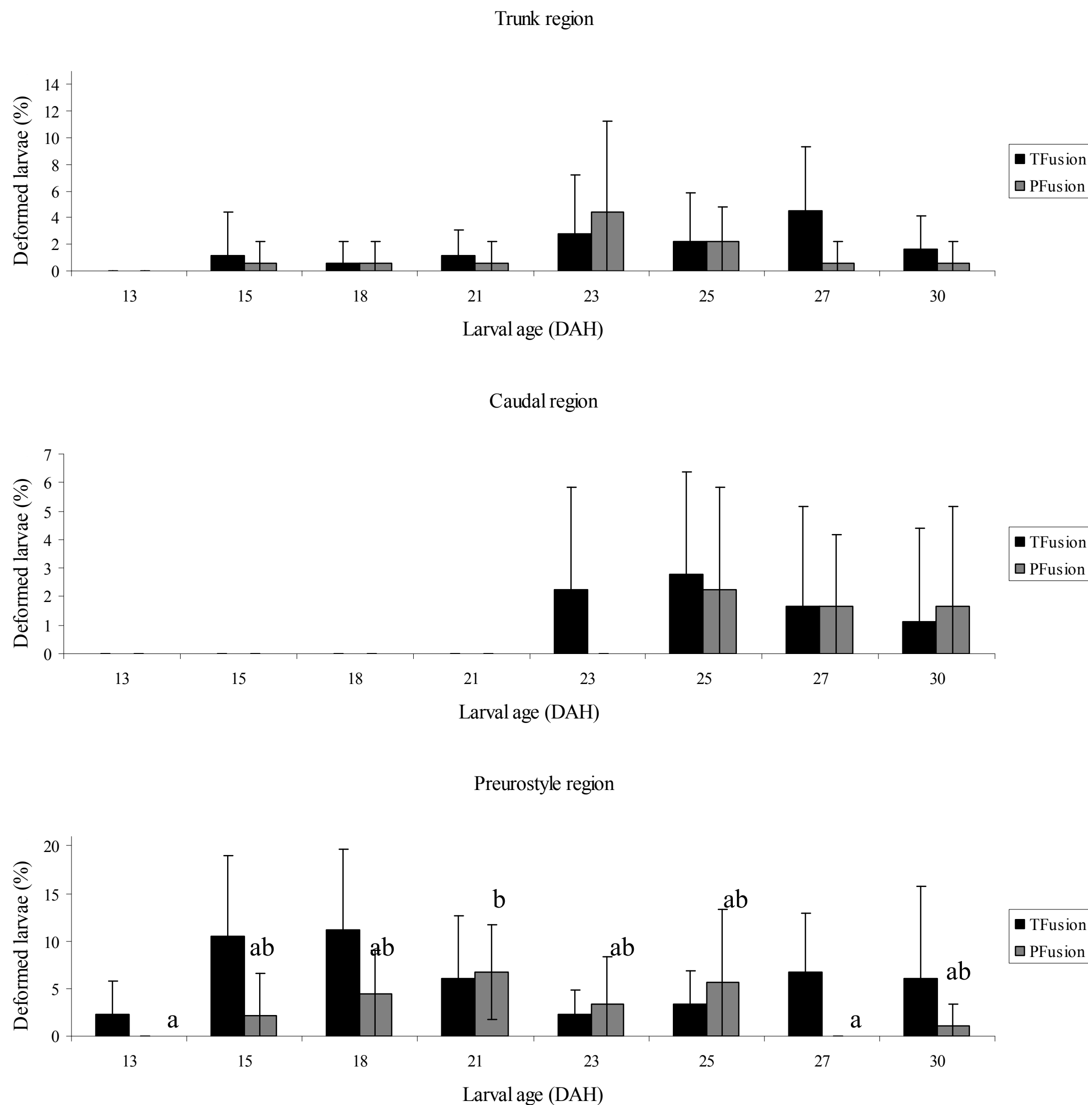

Fig 4. Frequency of *Diplodus sargus* larvae presenting total or partial vertebral fusions in the trunk (vertebrae 1 to 11), caudal (vertebrae 12 to 20) and preurostyle (vertebrae 21 to 23) regions of the vertebral column from 13 to 30 DAH. Values are mean and standard deviation (n=180). Letters correspond to significant differences between larval ages.

Vertebral compressions were observed in larval as early as on 13 DAH (Fig. 5). This skeletal deformity presented high incidence in the preurostyle region where affected larvae had a frequency between 40 and 60%. Significant differences were found for the trunk region ($H_{(7,724)}$=19.2, p=0.008), where on 23 DAH a higher compression percentage was found. The caudal region presented as well significant differences between larval ages in the frequency of vertebral compressions ($H_{(7,72)}$=20.9, p=0.004),

with a higher number of compressions on 25 DAH when compared to 13, 15 or 18 DAH Fig. 5). Abnormal shape vertebrae affected especially the caudal region of the vertebral column and on 23 and 25 the values obtained were above 20%. Although trunk and preurostyle regions are also affected by this malformation, the percentage registered were, on almost all ages, bellow 10%. In this study this deformity was usually located between vertebrae 15 to 21 (Fig. 5). Higher percentages were found on 23 and 25 DAH in the caudal region ($H_{(7,72)}$=40.1, p‹0.001) and for 23 DAH alone in the trunk region ($H_{(7,72)}$=29.1, p‹0.001).

Another common skeletal malformation observed in this study was the presence of hypertrophic vertebrae. Hypertrophic vertebrae were mainly observed in the trunk region between vertebrae 5 and 12. Significant differences were found on 18 DAH in the trunk ($H_{(7,72)}$=25.3, p=0.0007) and preurostyle ($H_{(7,72)}$=28.6, p=0.0002) regions, where, on this age, the frequency of this abnormality was higher. After 18 DAH, in the preurostyle region, no more hypertrophic vertebrae were observed in this work and on 21 DAH it was not observed in any larvae (Fig. 5). Caudal region also presented significant differences for this deformity ($H_{(7,72)}$=16.1, p=0.02) but the post- Hoc tests did not show which ages were different.

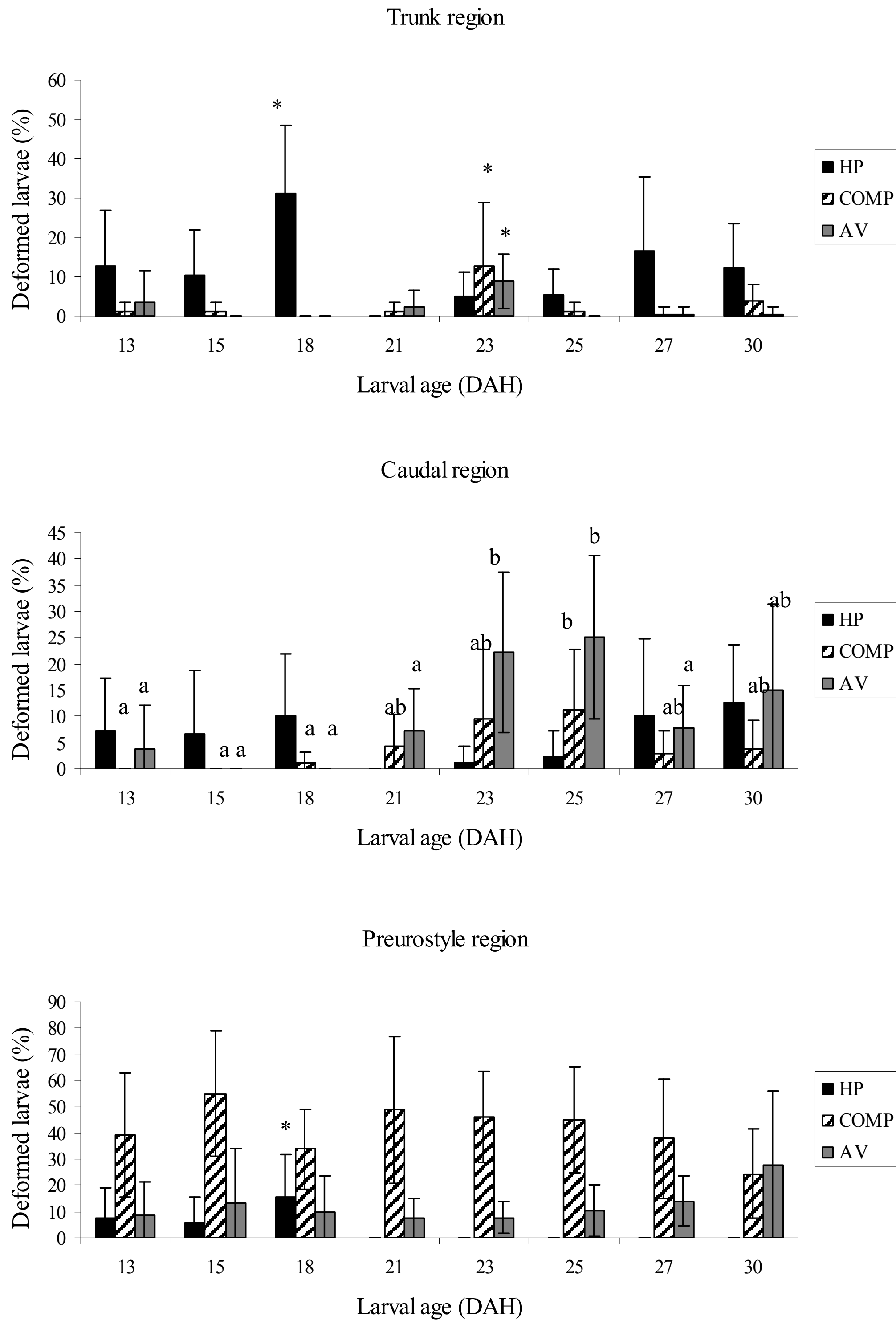

Fig. 5. Frequency of *Diplodus sargus* larvae with hypertrophic vertebrae (HP), vertebral compressions (COMP) and abnormal shape vertebrae (AV) in the trunk (vertebrae 1 to 11), caudal (vertebrae 12 to 20) and preurostyle (vertebrae 21 to 23) regions of the vertebral column from 13 to 30 DAH. Values are mean and standard deviation (n=180). (* represents one age significant difference from all others and letters correspond to significant differences between several larval ages).

Fig. 6 summarises all the deformities found at the vertebral column (sum of frequencies in all vertebral column regions) of *Diplodus sargus* larvae in this study. The most common deformity was vertebral compressions, recording values higher than 60%, and the most seldom was lordosis with levels lower than 10% (fig. 6). Hypertrophic vertebrae often varied frequency, reaching a peak on 15 DAH and then suddenly decreased to zero on 21 DAH. Abnormal vertebrae had higher values after 21 DAH and scoliosis had higher frequency on 23 DAH (Fig. 6).

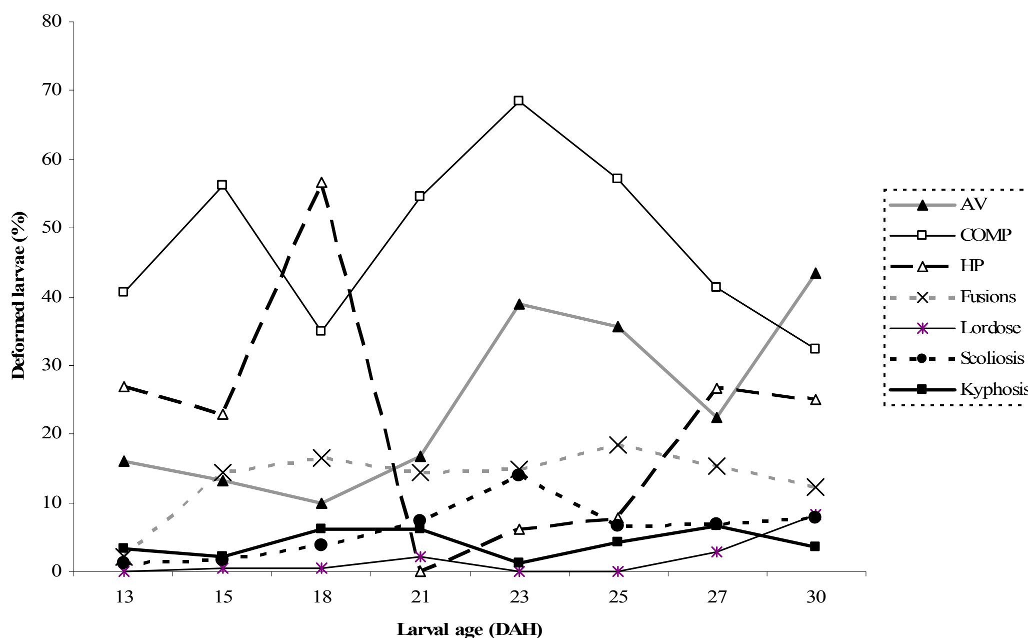

Fig. 6. Frequency of deformities in *Diplodus sargus* larvae from 13 to 30 DAH at the vertebral column (average of the frequency found in all vertebral column regions). AV- Abnormal vertebrae, COMP- vertebral compression, HP- hypertrophic vertebrae. Values are means (n=180).

2.4. DISCUSSION

The aim of this study was to identify the main deformities occurring at the vertebral column of *Diplodus sargus* larvae during their first 30 days of life as well as to determine on which ages they were more frequent. To fulfil these purposes three larval batches from three different spawns were studied. In general, it was observed an

increasing number of deformities with the increasing larval size. The same was stated by the Lewis et al. (2004) for Atlantic halibut larvae and juveniles.

The results obtained show a similar frequency of several skeletal abnormalities as to the ones observed by Saavedra et al. (2007 a, b) for *Diplodus sargus* on 15 and 25 DAH. In early larval stages, until 13 DAH, larvae presented a small percentage of malformations, not higher than 5%. At this stage, the main observed deformity was an abnormal curvature of the vertebral column. This suggests that only a very small proportion of deformities exist in newly hatched larvae and that the transference from the incubators to the rearing tank does not seem to cause much harm as most deformities appear after 8 DAH. On 13 DAH the number of skeletal malformations increased to approximately 50%, being vertebral compressions, mainly in the preurostyle region, the most common observed malformation.

It is only between 8 and 13 DAH that most vertebral abnormalities started appearing. On 15 DAH, more than 80% of the larvae presented some sort of vertebral deformity. On 18 DAH the percentage of larvae with hypertrophic vertebrae in the trunk region was higher than 30% and kyphosis and scoliosis started increasing as well. Scoliosis levels were usually lower than 15%. Kyphosis did not vary much between larval ages and was seldom above 15%. According to Koumoundourous et al. (2002), *Dicentrarchus labrax* starts developing kyphosis when larvae are between 10 and 17 mm but it is earlier (7.5 to 8.5 mm) that vertebrae seem to be more sensitive to potential harmful factors. Similarly, was only after *Diplodus sargus* larvae reached 7 mm that the first cases of kyphosis were recorded in the present study.

Lordosis was mainly observed towards the end of the experiment, on 27 and 30 DAH and with a frequency lower than 10%. Some years ago lordosis was usually pointed as the most common deformity in reared fish (Kranenbarg, 2005) due to

swimbladder insufflations failure and high swimming activity (Chatain, 1994). The implementation of prevention measures such as oil surface removal and decrease of water current decreased lordosis frequency in most farms (Chatain, 1994) and these findings are consistent with results presented here.

Abnormal shape vertebrae were recorded in a higher number of larvae between 21 and 30 DAH. This type of deformities often affects vertebrae 14 to 21, but more commonly vertebrae 16 to 19 and that is the reason why the caudal region showed a higher frequency.

Vertebrae compression was probably the deformity affecting most larval ages, values higher than 80% were observed. Vertebrae compression was most commonly observed in the preurostyle region of the vertebral column. Gavaia et al. (2002) reported that in *Solea senegalensis* more than half of the deformities occurred in the caudal region.

Another frequent vertebral abnormality observed in these *Diplodus sargus* larvae was the presence of hypertrophic vertebrae. This type of deformity has been previously reported in this species by Saavedra et al. (2007b) showing percentages up to 70%, mainly located in the trunk region of the vertebral column of *Diplodus sargus* larvae. Lewis and Lall (2006) reported similar incidences for Atlantic halibut larvae being the most frequent deformity. Hypertrophic vertebrae were mainly observed from 13 to 18 DAH. On 21 DAH no case of hypertrophic vertebrae was recorded and in the preurostyle region the same happen to older ages. Hypertrophic vertebrae are described to be a result of accelerated organogenesis which is not followed by an increase of the body size (Gould, 1977). However, this is not considered a serious abnormality because as development proceeds, the smaller vertebrae increase growth and/or the larger vertebrae decrease growth, resulting in a homogeneous vertebrae size and shape (Lewis

et al., 2004). This is consistent with the results obtain in this study as the higher highest relative growth rate was from 18 to 21 DAH which may explain why on 21 DAH no hypertrophic vertebrae was observed.

When deformities at the vertebral column were expressed as a sum of frequencies found in all vertebral column regions, the larval age with higher incidence of vertebral malformations was on 23 DAH and then on 25 DAH. On these ages, compressions percentages were approximately 70%, vertebrae with abnormal shape were between 30% and 40% and vertebral fusions approximately 20% .The most serious deformities such as kyphosis, scoliosis and lordosis, which probably imply fish discard, were present at lower percentages. This means that although a high frequency of deformities were found in this study, most fish will probably be viable as most malformations found might not have a strong impact on fish shape.

This work does not aim to explain the factors leading to such a high frequency of skeletal abnormalities but some possible reasons can be hypothesised. Recent data showed nutritional components of the diet given to the larvae in early life stages may play an important role in the origin of skeletal malformations (Cahu et al., 2003). Saavedra et al. (2006) compared amino acids (AA) profiles from *Diplodus sargus* larvae and their diet and the results obtained suggested severe nutritional imbalances in live feed, especially in rotifers. When a balanced AA diet was given to the larvae there was a decrease in the number of skeletal deformities (Saavedra et al., 2007a). Tryptophan is thought to be involved in the development of skeletal malformations. Tryptophan deficient diets seem to induce scoliosis in salmonids (Akiyama et al., 1986) however Saavedra et al. (2007b) tested diets supplemented with tryptophan and did not obtain lower percentages of deformities at the vertebral column of *D. sargus*. Vitamin C levels and DAH in the diet have also been shown to play a role in the development of skeletal

structures (Gavaia et al., 2002 and Gapasin et al., 2001). Low levels of vitamin C seemed to conduct to severe deformities in rainbow trout (Madsen and Dalsgaard, 1999) and milkfish larvae fed DHA incorporated diets presented lower opercular deformities (Gapasin et al., 2001). Beside nutritional factors probably other external effects may be influencing the number of deformities in *Diplodus sargus*. Rearing techniques and protocols of *Diplodus sargus* are an adaptation from the ones already applied for *Sparus aurata* and since there are a lack of studies concerning the former species maybe some of the conditions are not ideal for this species farming.

The development of abnormalities at the vertebral column has been a problem not only for *Diplodus sargus* production but for many other species. However, since the origin of these malformations is possibly related to more than one factor, solutions to this problem are still far away. This study provides important information about the development of the deformities at the vertebral column, indicating the phases were they start appearing and its frequencies. This knowledge will reduce the time frame where to search for factors contributing to the incidence of deformities at the vertebral column.

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Chapter 3

Amino acid profiles of *Diplodus sargus* (L., 1758) larvae: implications for feed formulation

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Abstract

The indispensable AA profile of fish carcass has been commonly used as a good indicator of fish amino acids requirements. Amino acid composition of the whole body tissue of *Diplodus sargus* was determined for the larval ages of 0, 2, 5, 8, 12, 17, 25, 35 and 45 days after hatching (DAH). No significant differences were found during this species ontogeny, except for phenylalanine. A comparative analysis of amino acid profiles from larvae and respective diet was performed. Low correlation was found to rotifers (R^2 approximately 0.5), while higher correlations were found for Artemia Nauplli, metanauplii (R^2 approximately 0.8) as well as for the dry feed. These results suggest that *Diplodus sargus* are subjected to higher nutritional imbalances during the first ten days of feeding when larvae are fed on rotifers alone. Arginine, threonine, lysine, cysteine and histidine appeared to be limiting amino acids at 2, 12, 25 and 45 DAH, respectively. Similar results were reported in literature for *Sparus aurata* and *Solea senegalensis,* although *Diplodus sargus* diets seem to have more amino acids in deficiency as well as more severe differences between larval and diet amino acid profiles. To solve these apparent nutritional imbalances, amino acid supplementation should be considered. The use of inert diets in early larvae ages seems to be most adequate as live feed supplementation appears to be more difficult.

Keywords: *Diplodus sargus*, amino acids, limiting amino acid, artemia, rotifers, dry feed

3.1. INTRODUCTION

Marine aquaculture in the South-European countries is based on gilthead seabream (*Sparus aurata),* seabass (*Dicentrachus labrax*) and turbot (*Scophthalmus maximus*) farming. To develop aquaculture in this region an investment in new species must occur (Ortiz-Delgado et al., 2003).

White seabream (*Diplodus sargus*) is considered to be a promising new species with high market price and demand (Pousão-Ferreira et al., 2001, Ozorio et al., 2006, Santos et al., 2006). In the southern coast of Portugal, white sea bream landings decreased from 200.3 t to 75.2 t between 1987 and 2004 (Santos et al., 2006) and therefore, this species is also important for restocking purposes (Santos et al., 2006). Preliminary experimental studies seem to indicate a higher growth rate at the larval stage when compared with *Sparus aurata* (Dores et al., 2000) and a larval survival rate of approximately 20%. However, when approaching the juvenile stage, a decrease of the growth rate (Cejas et al., 2003) and the presence of several skeletal deformities, especially at the dorsal column, are some of the bottlenecks to farming this species. These skeletal deformities might be related to problems of imbalanced nutrition or inadequate handling.

During larval stages growth rate is usually very high and, as growth is mainly related to muscle protein deposition (Conceição et al, 1998a) a high amino acids flow is required from food to growing biomass (Rønnestad et al., 2003). In addition, as amino acids (AA) seem to be the major energy source during the larval stage (Rønnestad et al., 1999) there is a need to determine the amino acid requirements. Furthermore, growth and food conversion efficiency can be maximised by manipulating the composition of the dietary amino acids (Conceição et al., 2003). AA imbalances in the diet will increase

AA oxidation and therefore cause a decrease on the conversion efficiency (Fauconneau et al., 1992).

The indispensable AA profile of fish carcass has been commonly used as a good indicator of fish amino acids requirements (Wilson and Poe, 1985; Watanabe and Kiron, 1994). Using this AA profile a first approach to determine diet imbalances can be achieved (Conceição et al., 2003). Although this comparison can only be seen as a first step, it will provide important information on the possible dietary amino acid deficiencies.

The purpose of this study was to determine the whole body amino acid profiles during development of *Diplodus sargus* larvae and to compare them with amino acid profiles in the diet, in order to identify possible deficiencies during ontogeny. In this study it is also discussed how supplementation could be carried out.

3.2. MATERIALS AND METHODS

3.2.1 Husbandry and experimental set-up

This study was carried out at the Aquaculture Research Station of INIAP/IPIMAR, in Olhão, South of Portugal during 2004.

Diplodus sargus eggs were obtained from a mix of wild and farmed broodstock consisting of 38 fish with an average weight of 869.2 g ± 319.8 g, kept at a density of 4 kg/m^3. Sex ratio was approximately 1/1.

After collected from the egg incubators, newly hatched larvae were transferred to three 200l cylindro conical fibreglass tanks at a density of 100 larvae/ l. The system worked in a closed circuit and water temperature was maintained constant at 18 ºC ± 0.6 º C, oxygen at 6.2 mg /l ± 0.9 mg /l and salinity at 37 ± 1 ppt. Before entering the tank

water passed through a biological filter and then through a mechanical filter and sterilized by a UV. Water flow started at 0.6 l /min and then was slowly increased with larvae age until a maximum of 3 l /min, at 40 DAH. Photoperiod was 14 hours Light: 10 hours Darkness.

Feeding protocol consisted of *Brachionus plicatilis* enriched with Protein Selco (INVE Aquaculture, Belgium) from 3 to 20 DAH. At day 12, larvae started having Artemia nauplii (BE 480, INVE Aquaculture, Belgium) and *Artemia* metanauplii, enriched with Super Selco (INVE, Aquaculture, Belgium) from 17 DAH until 39 DAH. Dry feed (Nippai, Japan) was given from day 25 until the end of the experiment.

3.2.2. Sampling and biochemical analysis

Three larvae samples were taken from the three tanks at 0, 2, 5, 8, 12, 17, 25, 35 and 45 DAH for amino acid quantification. At the beginning, samples consisted of 80 larvae although this number decreased with larvae size (minimum of 20 larvae per sample). These samples were frozen in liquid nitrogen at -80 ºC and then freeze-dried (RVT 400, Savant, NY) and weighed.

3.2.2.1. Amino acids analysis

Protein-bound amino acids samples were hydrolysed in 6M HCL at 108ºC 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).

Results for tryptophane are not reported here since this amino acid is destroyed by acid hydrolysis.

Glutamine is converted to glutamate during acid hydrolysis so the sum of these amino acids is presented. The same occurs for asparagine and aspartate.

3.2.3. Data analysis

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

Indispensable amino acids (IAA) data were expressed in weight percentage of the Indispensable amino acids pool ((weight of one IAA) x (weight of all IAA) $^{-1}$x 100), to enable comparisons between live feedand larval amino acids profiles. Dispensable amino acids (DAA) data were expressed in weight percentage of all amino acids (IAA + DAA) pool ((weight of one DAA) x (weight of all AA) $^{-1}$x 100).

The first limiting amino acid was determined by the formula: (IAA diet- IAA larva)*100*(IAA larva)$^{-1}$ (Conceição et al., 2003). The limiting amino acid is the one presenting the lowest relative difference between its contribution to the diet and larval amino acid profiles. This AA will determine the point from which protein synthesis cannot continue (Aragão et al., 2004a).

The indispensable amino acid index (IAA) was calculated according to the formula: IAA= $((a_1/A_1)* (a_2/A_2)*\ldots(a_n/A_n))^{1/n}$, where a_n is the indispensable amino acid in the diet and A_n is the indispensable amino acid in the larval body (Peñaflorida, 1989).

Differences between amino acids at different ages were determined using one-way Analysis of Variance (ANOVA) at a minimum significance of $p<0.05$ with Statistisca© (v.5.5) for lysine, histidine, tyrosine, glycine and proline.

For all other amino acids which did not satisfy ANOVA parametric assumptions, Kruskal-Wallis non-parametric Anova was performed.

3.3 RESULTS

3.3.1 Growth

During the experimental period larval survival was 18.7 ± 1.5%.

An exponential relationship ($y= 0.018e^{0.1387x}$, $R^2=0.98$) describes *Diplodus sargus* growth, expressed in dry weight, for the 45 days (Fig 1). During the experimental period first feeding larvae weighed 0.026 ± 0.002 mg (dry weight) and measured 3.26 ± 0.20 mm. At 45 DAH their weight was 11.86 ± 1.33 mg and length was 18.82 ± 2.14 mm.

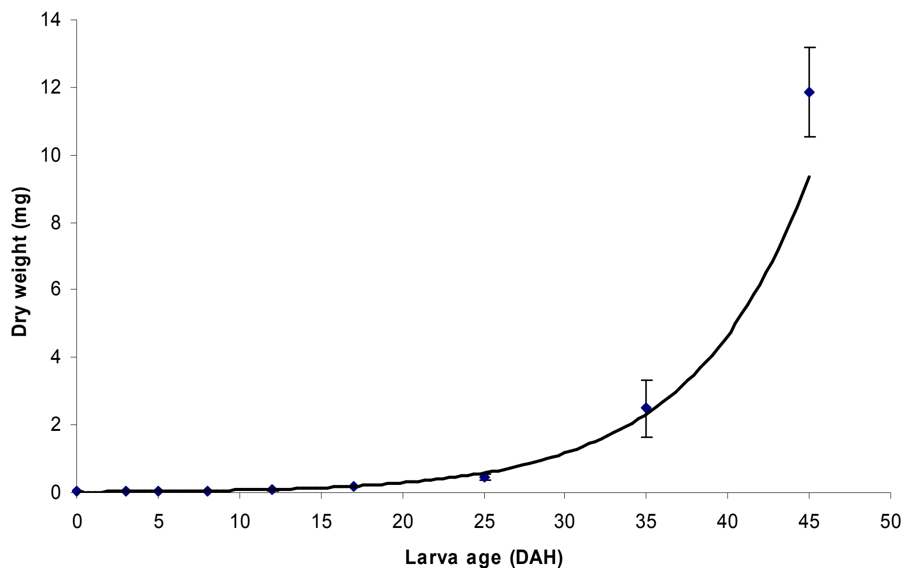

Fig 1. *Diplodus sargus* growth in dry weight (mean and standard deviations) during the first 45 days after hatched.

The relative growth rate was negative, from day two to five, and then increased at 8 DAH (13.17 % DW/day). The highest rate was registered at 17 DAH (20.78 % DW/day). After this stage the RGR was more or less constant between 13 and 19 % DW/day (Table 1).

Table 1. Relative growth rate (RGR) of *Diplodus sargus* from 2 to 45 DAH.

Age	RGR (% DW/day)
2	-16.44
5	3.66
8	13.17
12	15.05
17	20.78
25	13.29
35	16.70
45	18.59

3.3.2. Amino acids larvae content

Indispensable amino acids (IAA) profile did not change significantly during the experimental period with the exception of Phenylalanine (Phe) ($p=0.015$) (Table 2). Lysine, leucine and arginine presented the highest relative AA content whereas cysteine, methionine and histidine presented the lowest relative AA levels.

For the dispensable amino acids (DAA) there were no significant differences on profile during the 45 days studied (Table 2). Glutamate and Glutamine were always the amino acids with highest content, followed by Aspartate and Asparagine (Table 2).

Table 2. Dispensable and dispensable amino acids profile (Mean and standard deviation) of *Diplodus sargus* from 0 days after hatch (DAH) until 45 DAH. IAA data are represented as a percentage of the IAA whereas the DAA are expressed as a percentage of all AA.

	Larva Age (DAH)								
	0	2	5	8	12	17	25	35	45
IAA									
Leu	16.0 ± 0.2	15.2 ± 0.3	15.1 ± 0.1	15.8 ± 0.6	15.2 ± 1.5	15.4 ± 1.1	15.2 ± 0.6	15.6 ± 0.1	15.5 ± 0.2
Lys	15.4 ± 0.5	15.6 ± 0.7	16.4 ± 0.3	17.4 ± 1.9	16 ± 1.5	17.3 ± 2.3	15.7 ± 0.8	15.9 ± 0.2	16.2 ± 0.4
Arg	14.9 ± 0.7	16.3 ± 0.2	16.2 ± 0.8	14.2 ± 0.2	16.7 ± 1.6	15.2 ± 1.5	16.3 ± 2.0	13.8 ± 0.2	13.4 ± 0.4
Val	9.8 ± 0.0	9.6 ± 0.6	9.3 ± 0.2	9.6 ± 0.1	9.7 ± 1.0	9.7 ± 0.6	9.6 ± 0.3	9.1 ± 0.1	8.9 ± 0.0
Thr	10.3 ± 0.6	10.4 ± 1.1	9.8 ± 0.3	7.8 ± 1.6	9.7 ± 2.8	9.4 ± 2.0	10.3 ± 0.6	10.2 ± 0.1	9.9 ± 0.3
Ile	8.2 ± 0.5	7.7 ± 1.0	6.9 ± 0.2	7.5 ± 0.7	7.3 ± 0.9	7.6 ± 1.3	7.2 ± 0.5	7.5 ± 0.1	7.4 ± 0.1
His	4.7 ± 0.3	4.7 ± 0.5	4.9 ± 0.1	4.6 ± 0.4	5.3 ± 0.5	5.0 ± 0.5	5.1 ± 0.3	5.0 ± 0.1	5.1 ± 0.1
Met	3.8 ± 1.2	3.3 ± 0.9	3.5 ± 0.9	3.6 ± 2.6	3.0 ± 1.8	3.1 ± 2.0	3.2 ± 2.4	5.7 ± 0.4	6.3 ± 0.8
Cys	1.8 ± 0.5	1.6 ± 0.9	2.0 ± 0.1	2.2 ± 0.8	1.2 ± 0.2	1.2 ± 0.4	1.5 ± 0.3	1.5 ± 0.0	1.5 ± 0.1
Phe	7.9 ± 0.1	7.8 ± 0.2	8.0 ± 0.1	8.9 ± 0.7	8.0 ± 0.9	8.3 ± 0.7	8.1 ± 0.3	8.3 ± 0.1	8.4 ± 0.1
Tyr	7.4 ± 0.4	7.6 ± 0.4	8.1 ± 0.2	8.4 ± 0.1	8.0 ± 0.8	7.9 ± 0.5	7.9 ± 0.4	7.6 ± 0.2	7.5 ± 0.1
DAA									
Glu + Gln	12.1 ± 0.9	12.1 ± 1.6	11.3 ± 0.3	12.4 ± 2.5	13.4 ± 1.0	13.8 ± 0.4	13.5 ± 1.8	15.5 ± 0.3	15.9 ± 0.2
Ala	7.3 ± 0.3	6.2 ± 0.1	6.2 ± 0.2	5.6 ± 0.8	5.8 ± 0.3	5.8 ± 0.2	6.0 ± 0.2	5.9 ± 0.2	6.0 ± 0.1
Gly	4.5 ± 0.1	5.1 ± 0.4	5.0 ± 0.1	5.1 ± 0.6	5.6 ± 0.2	5.7 ± 0.2	5.5 ± 0.2	5.4 ± 0.1	5.6 ± 0.2
Asp+Asn	7.9 ± 1.2	7.7 ± 2.0	6.4 ± 0.6	7.4 ± 3.5	8.9 ± 1.1	9.5 ± 1.4	8.6 ± 1.7	10.4 ± 0.1	9.8 ± 0.2
Ser	5.1 ± 0.3	5.6 ± 0.7	5.4 ± 0.1	5.6 ± 0.5	6.1 ± 0.4	5.9 ± 0.4	5.7 ± 0.3	5.8 ± 0.1	5.7 ± 0.0
Pro	4.9 ± 0.2	4.8 ± 0.2	4.7 ± 0.1	4.8 ± 0.7	5.0 ± 0.3	4.6 ± 0.6	4.9 ± 0.3	4.8 ± 0.2	4.8 ± 0.2

3.3.3. Comparison between larval and diet amino acid profiles

On 2 DAH the essential amino acids apparently in deficiency were cysteine, histidine, threonine, lysine and arginine (Fig 2). Histidine was the amino acid which, at this stage, presented the highest difference between the amino acid profile from the larva and the diet (Table 3). Valine seemed to present the same differences between larval and diet amino acids profiles but, in this case, the diet had a higher relative content.

On 12 DAH, threonine seemed to be the limiting amino acid, although arginine, cysteine, histidine lysine and tyrosine were also in deficiency. Isoleucine seemed to be in excess.

On 25 DAH cysteine was the AA in most deficiency and arginine, lysine, threonine and tyrosine also had shown lower relative contents in the diet (Table 3). Again, isoleucine was present in excess.

When larvae were adapted to dry feed, on 45 DAH, methionine was apparently in deficiency for the first time, being the first limiting amino acid. Arginine, cysteine, histidine, lysine, threonine and tyrosine were in deficiency as well.

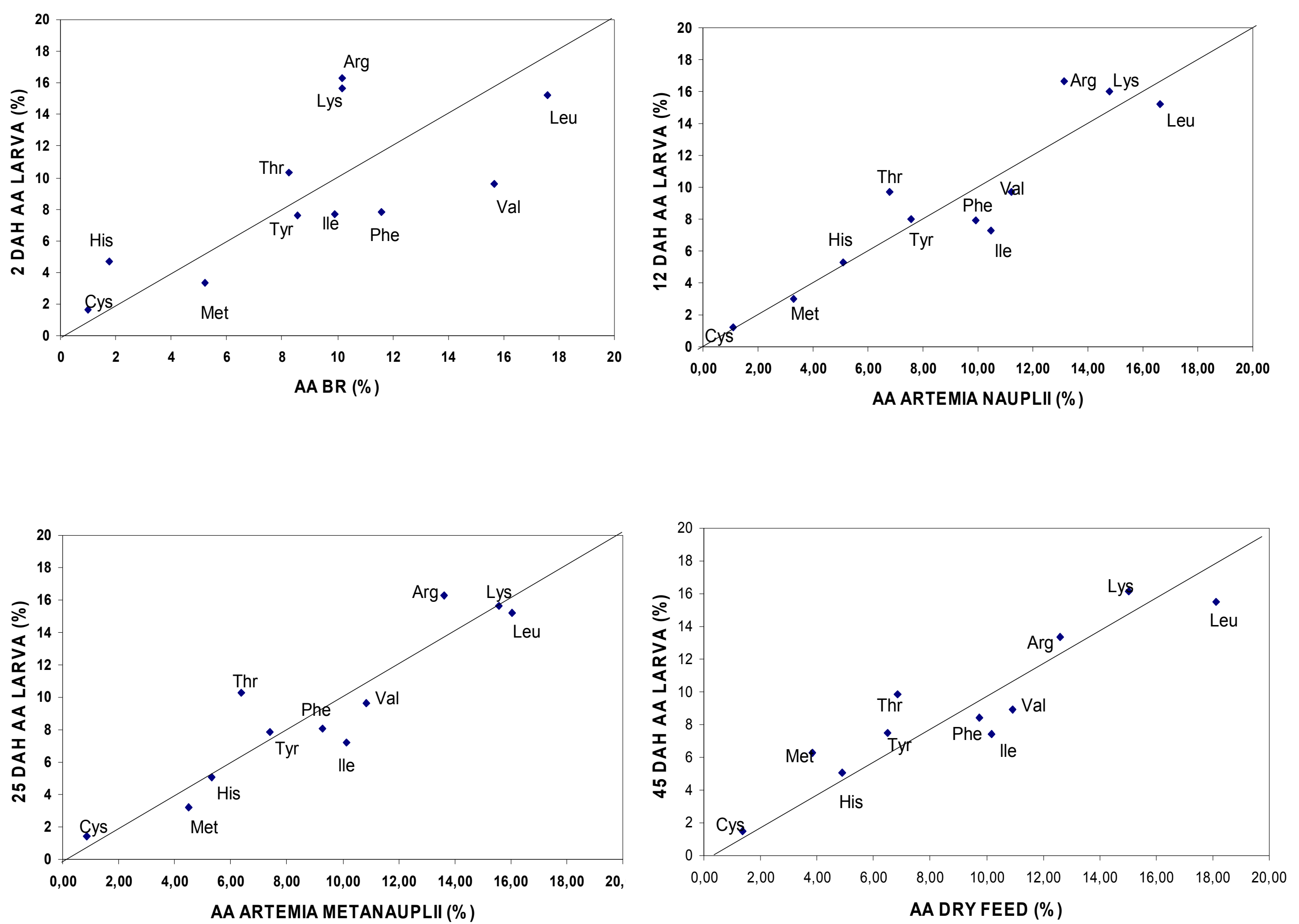

Fig. 2. Comparison between *Diplodus sargus* larvae and diet amino acid profiles on different ages.

Table 3. Relative differences between Indispensable amino acids from the diet and *Diplodus sargus* larvae (apparent first limiting amino acid for each larval age is marked in bold). Diet given to the larvae: Rotifers (2 DAH), Artemia nauplii (12 DAH), Artemia metanauplii (25 DAH), Dry feed (45 DAH).

	Larva age (DAH)			
	2	**12**	**25**	**45**
Arg	-37.7	-21.1	-16.4	-5.9
Cys	-37.5	-7.8	**-40.0**	-8.2
His	**-62.6**	-3.8	4.3	-4.3
Ile	28.7	44.1	40.9	37.2
Leu	15.6	9.3	5.5	17.1
Lys	-34.9	-7.6	-0.6	-7.1
Met	57.3	9.6	39.8	**-39.1**
Phe	47.6	24.7	14.8	16.2
Thr	-20.2	**-29.9**	-37.9	-30.6
Tyr	12.0	-5.4	-5.7	-12.8
Val	62.7	15.4	12.7	22.0

The indispensable amino acid profiles obtained in this study showed low correlations with rotifers (0.42 to 0.5 from 2 DAH to 8 DAH) (Table 4). Results for Artemia, nauplii and metanauplii, as well as dry feed presented high correlations between larval and diet amino acid profiles.

Table 4. Indispensable amino acid index (IAA) for larvae and diet, according to larva age.

Larva age (DAH)	Diet	IAA Index	r2
2	Rotífers	0.94	0.5
5	Rotífers	0.92	0.47
8	Rotífers	0.93	0.42
12	Rotífers	0.96	0.49
12	Artemia Nauplii	1,010	0.85
17	Rotífers	0.96	0.51
17	Artemia Nauplii	1.00	0.88
17	Artemia metanauplii	1.00	0.9
25	Artemia metanauplii	0.98	0.85
25	Dry feed	1.00	0.81
35	Dry feed	0.97	0.85
45	Dry feed	0.96	0.85

3.4. DISCUSSION

Diplodus sargus larvae presented an insignificant growth in dry weight from 0 DAH until 5 DAH and only after eight days after hatching a substantial increase on the growth rate occurred. This is common in species such as *Sparus aurata* (Aragão et al., 2004a) and *Solea senegalensis* (Ribeiro et al., 2003).

Changes in AA profiles were examined in *Diplodus sargus* from first feeding larvae until post-larvae stage (45 DAH), but whole body amino acid composition of *Diplodus sargus* did not change significantly from 0 DAH to 45 DAH, except for phenylalanine. Therefore, this species AA profile seems to remain relatively constant throughout ontogeny which is similar to species such as common dentex (Tulli and Tibaldi, 1997), which had only minor changes in the percentages of amino acids during larval exogenous feeding (12 to 30 DAH), or striped trumpeter (*Latris lineata*) from day 0 to day 12 after hatching (Brown et al., 2005). On contrary, for *Sparus aurata* significant differences were found for early phases of ontogeny, even if they were not

very marked (Aragão et al., 2004a). For *Solea senegalensis* reported changes in amino acid profiles during ontogeny were especially evident at the metamorphosis stage, where this species strongly changes morphologically (Aragão et al., 2004a). Since *Sparus aurata* and *Diplodus sargus* are very close species, both belonging to the Sparidae family, a similar result would be expected. However, in this study, standard deviations were higher than the ones reported in Aragão et al (2004a), especially for threonine, histidine, methionine, isoleucine and phenylalanine. These higher variations could have hidden some potential small but significant differences in the AA profile during larval development, especially in the early stages. Other possible reason to explain the non existence of significant differences could be the higher growth rate registered for *Diplodus sargus.* From 0 DAH to 17 DAH, *Sparus aurata* and *Diplodus sargus* growth rate was approximately the same (9% body weight/day) whereas from 17 DAH to 45 DAH *Diplodus sargus* presented almost the double growth rate (16.1 % body weight/ day compared to 9 % body weight/day), meaning that white sea bream have a faster ontogeny and therefore amino acid profile changes might not be so significant.

In the indispensable amino acid (IAA) profile determined for *Diplodus sargus*, arginine, leucine and lysine showed the highest relative levels whereas cysteine, histidine and methionine presented the lowest relative content. These results are consistent with the ones reported for Atlantic salmon, Rainbow trout, yellowtail flounder (Kim and Lall, 2000), *Sparus aurata* and *Solea senegalensis* (Aragão et al., 2004a).

When larval IAA profiles were plotted against diet IAA profiles, it was found that almost the same amino acids appeared in deficiency, independently of type of food given or larval stage. Arginine, cysteine, histidine, threonine and lysine were always in

deficiency and presented higher relative differences between the diet and larval indispensable amino acids. On the contrary, there were amino acids which were present in excess such as isoleucine, valine and phenylalanine.

The first limiting amino acid was different according to the larva age and diet. A limiting or a deficient AA in the diet will be forwarded to protein synthesis rather than oxidation (Wilson, 1994). The opposite happens when an amino acid is supplied in excess, it will be used mainly for oxidation (Wilson, 1994).

Histidine was apparently the limiting amino acid on 2 DAH. Histidine deficiency may lead to a lower nitrogen loss when comparing with other amino acids because a change of the nature of the synthesised protein to those of lower histidine concentrations may occur (Rollin et al., 2003).

On 12 DAH the limiting AA was threonine whereas on age 25 and 45 the limiting AA was cysteine and methionine, respectively. Methionine seemed to be, on 45 DAH, a deficient AA for the first time. Therefore, the dry feed did not present balanced methionine content.

Comparing larval and diet amino acid profiles, low correlations were found for rotifers (r^2 <0.51) and high correlations were found for Artemia, nauplii and metanauplii ($r^2 \geq 0.85$) and dry feed ($r^2 \geq 0.81$). This suggests that a diet based on rotifers produces major AA nutritional imbalances. In Artemia and dry feed, although some AA are in deficiency, their general AA profile is quite similar to the one determined for the larva. This is consistent with other studies on *Sparus aurata* and *Solea senegalensis* (Ribeiro, 2003).

The implications of the low correlations found for rotifers are higher nutritional imbalances because, on that stage, no other type of food is given to the larvae. This means larvae must spend more time catching preys to compensate the dietary amino

acid losses (Aragão et al., 2004a). This problem probably is attenuated on 12 DAH, when larvae start ingesting Artemia nauplii which seems to present less dietary amino acids deficiencies and showed higher correlations with larval amino acid profiles.

The use of AA imbalanced diets was also reported for turbot (Conceição et al., 2003), african catfish (Conceição et al., 1998b), seabream and senegalese sole (Aragão et al., 2004a). The imbalances between the dietary and larval AA profiles will lead to unavoidable AA losses and increase nitrogen excretion (Conceição et al., 1998b, Aragão et al., 2004a). To overcome this, fish will need to increase their food consumption (Aragão et al., 2004a).

These results raise the question of how these nutritional imbalances could be corrected and how should supplementation be carried out. As mentioned before, dietary AA imbalances will lead to a lower of growth rate and food conversion ratios so the supplementation should be carefully considered. Better feeding efficiencies and higher growth rates were reported for gilthead sea bream juveniles (Gómez-Requeni et al., 2003) when diets were supplemented with IAA to resemble the whole body AA profile. One way of trying to compensate the deficient amino acids could be the direct supplementation of the live feed, enriching rotifers and Artemia with the limiting AA. Live feed AA supplementation could only be done by increasing the free AA pool because protein bounded AA composition is not possible to change. Some studies showed that an increase of 298% of the rotifers free amino acid (FAA) pool when FAA where delivery through liposomes (Barr et al., 2005). However, the effect on the larvae still has to be shown.

Another possible solution could be starting the dry feed at earlier larva stages. Although *Diplodus sargus* larval digestive tract is not fully developed yet (Cara et al., 2003, Ortiz-Delgado et al., 2003), some preliminary experiments with *Diplodus sargus*

first feeding larvae showed encouraging results, even if the survival rate was not high. If the IAA profiles are not taken into account then a poor growth rate of early fish larvae fed on artificial diets might be due to the use of a non-optimal biochemical composition (Langdon, 2003).

The inclusion of FAA in microdiets for larval fish may also have other advantages besides balancing the IAA profile. The incorporation of FAA in microdiets has been reported to increase trypsin secretion in seabass larval (Cahu and Zamboinino Infante, 1995) and trypsin is especially important because it activates other pancreatic proteases (Rønnestad et al., 1999). Also, some amino acids such as arginine and alanine in their free form seem to stimulate ingestion (Kolkovski et al., 1997) and therefore might increase larval feeding rates (Aragão et al., 2004b). However, supplementation of microdiets with FAA should be done with care, as major leaching losses may occur depending on microdiet type (Yúfera et al., 2002), and in high levels FAA may have toxic effects.

Yúfera et al. (1999, 2002) have developed cross-linked casein-walled capsules and concluded that fish larvae are likely to have a greater enzymatic capacity to digest dry feed and obtained a good growth on sea bream larvae fed in co-feeding with rotifers. Therefore, during the first feeding days this balanced diet could be use in co-feeding with rotifers as the free amino acids in the tissues of the live feed may be important in stimulating the release of digestive enzymes and enhancing the ingestion rates by affecting the “smell” and “taste” of the diets (Cahu and Zambonino Infante, 1995).

Considering the importance of *Diplodus sargus* as a potential species for marine aquaculture, the present study estimates the qualitative amino acid requirements for this species and shows that supplementation of diets with FAA may be necessary to optimize larval growth.

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Chapter 4

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

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Abstract

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

4.1. 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) and mortality (Aragão et al, 2007) 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 and 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 and 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 2007ab). The level of AA such as tryptophan is reported to be relevant to prevent such problems (Akiyama et al., 1986; Hseu et al., 2002). 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., 2002).

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.

4.2. MATERIAL AND METHODS

4.2.1. 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 ± 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 ºC. After a 30 minutes 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.

4.2.2. Treatments

For each AA there was a separately trial. Four treatments were carried out for each trial using 14C 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 5µl 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.

4.2.3. 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 two minutes. 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).

4.2.4. Radioactivity counting

Once digestion was completed (6 hours, 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 hours at 30 ºC. 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).

4.2.5. 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%.

4.3. RESULTS

Larval survival rate after 6 h incubation period was 94.1 ± 6.5 %. In the tryptophan experiment, significant differences were found for larval body ($F_{3,38}$= 6.1 and 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).

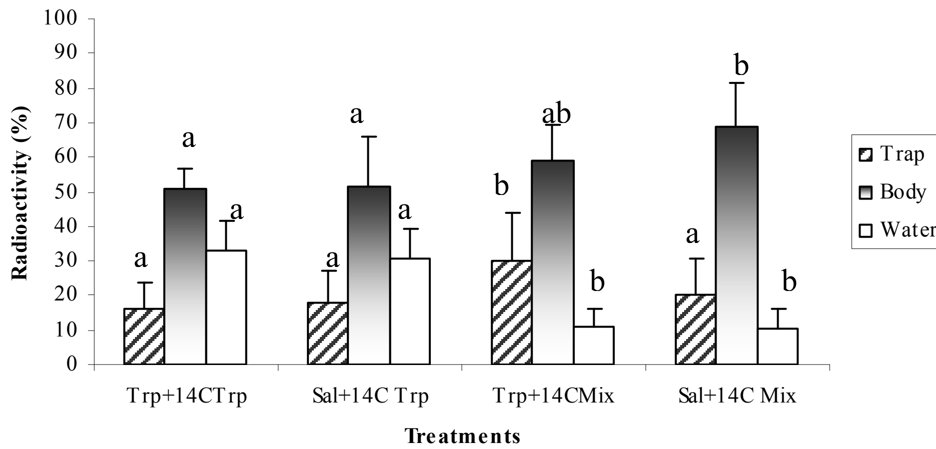

Fig 1. Proportion of the tube-fed ^{14}C amino acid mix (Mix) or ^{14}C tryptophan (Trp) catabolised (black stripes columns), retained in body (grey columns) and evacuated into water (white columns) of *Diplodus sargus* larvae. Trp+ ^{14}C Trp: Trp ^{14}C supplemented with crystalline tryptophan; Sal+ ^{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 ($p< 0.05$).

The absorption efficiency (retention + 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 catabolism proportion was 25% of the absorbed tube-fed ^{14}C AA.

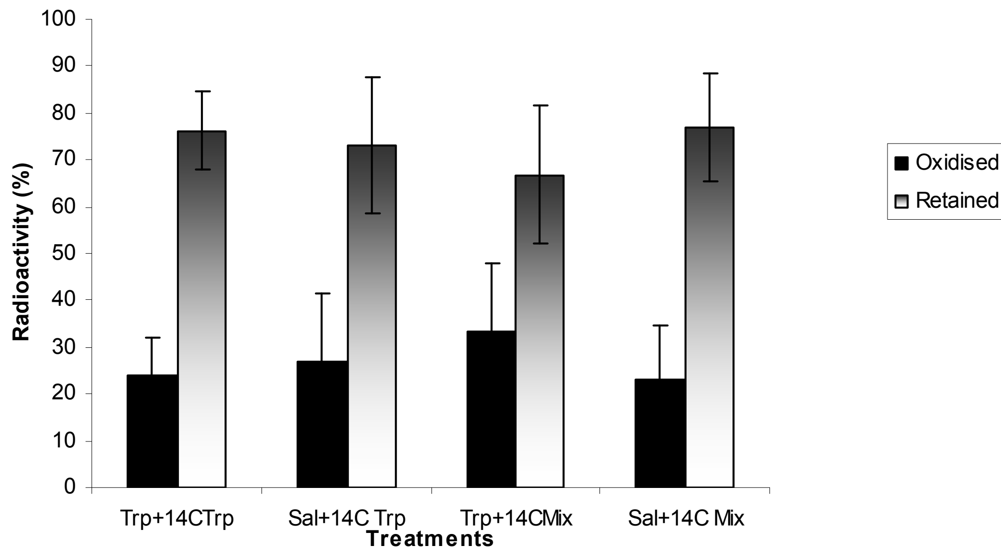

Fig 2. Proportion of catabolised (black columns) and retained in body (grey columns) ^{14}C AA of tube-fed *Diplodus sargus* larvae. Trp+ 14C Trp: Trp 14C supplemented with crystalline tryptophan; Sal+ 14C Trp: Trp 14C without supplementation; Trp+ 14C Mix: 14C Mix supplemented with crystalline tryptophan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

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).

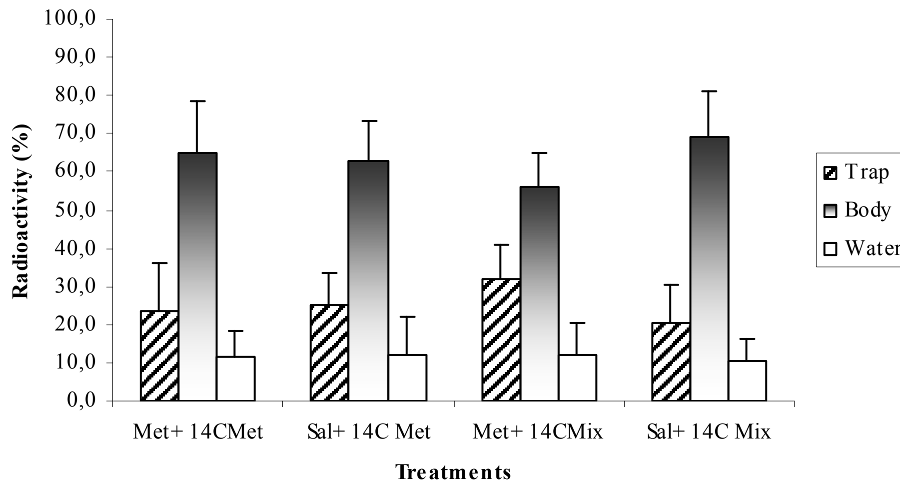

Fig. 3. Proportion of tube-fed ^{14}C amino acid Mix and Methionine catabolised (black stripes columns), retained in body (grey columns) and evacuated into water (white columns) of *Diplodus sargus* larvae. Met + ^{14}C Met: Met ^{14}C supplemented with crystalline methionine; Sal+ ^{14}C Met: Met ^{14}C 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).

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).

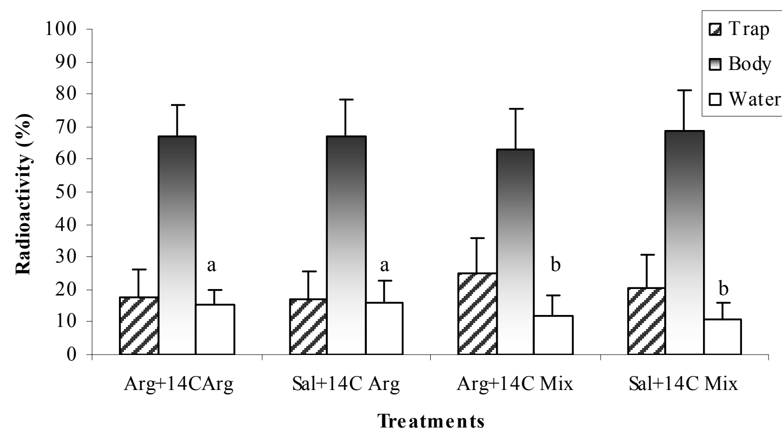

Fig. 4. Proportion of the tube-fed ^{14}C Mix and Arg counted in metabolic trap (black stripes columns), larval body (grey columns) and incubation water (white columns) of *Diplodus sargus* larvae. Arg + ^{14}C Arg: Arg ^{14}C supplemented with crystalline arginine; Sal+ ^{14}C Arg: Arg ^{14}C without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

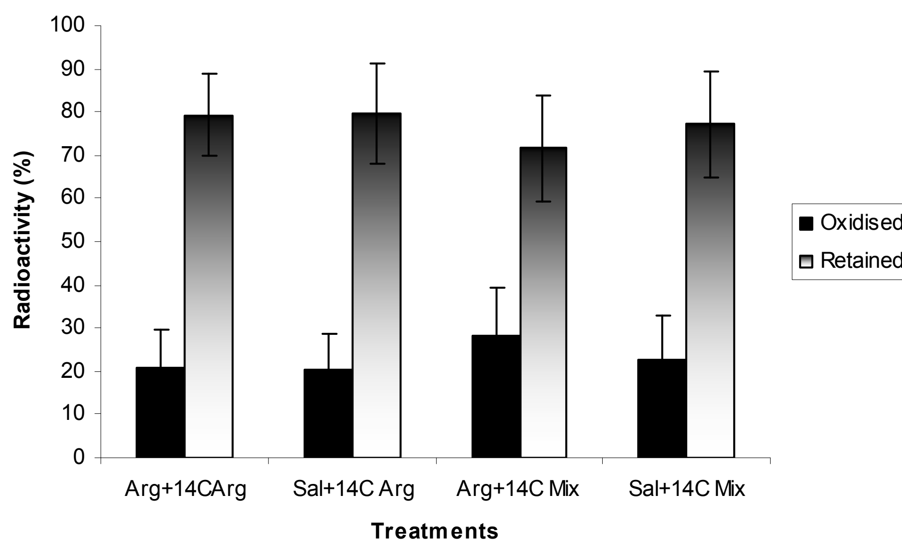

Fig 5. Proportion of catabolised (black columns) and retained (grey columns) ^{14}C of tube-fed *Diplodus sargus* larvae. Arg + ^{14}C Arg: Arg ^{14}C supplemented with crystalline arginine; Sal+ ^{14}C Arg: Arg 14C without supplementation; Arg + ^{14}C Mix: ^{14}C Mix supplemented with crystalline tryptophan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

4.4. 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., 2001) 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. (2006), 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 have strong AA imbalances for this species (Saavedra et al., 2006). 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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Chapter 5

Effect of lysine and tyrosine supplementation in the amino acid metabolism of *Diplodus sargus* larvae fed rotifers

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Abstract

Dietary amino acid imbalances are likely to happen when fish larvae are fed live feed. This can lead to reduced growth as well as decreased larval quality. The tube-feeding technique can be used to assess the effect of free amino acid supplementation at short term and determine whether a given amino acid is deficient in the diet. In this study supplementation of lysine and tyrosine were tested in *Diplodus sargus* larvae fed rotifers in order to determine its effect on the metabolism of these amino acids. Supplementation was done using rotifers enriched with liposomes boosted with free amino acids. Single crystalline ^{14}C amino acids as well as a mixture of ^{14}C amino acids were used as tracers to compare results of individual amino acid metabolism with the average of all amino acids. The results showed low absorption efficiencies for both tyrosine and lysine when compared to the average of all amino acids. A lower relative ^{14}C retention was found when *Diplodus sargus* larvae were fed tyrosine enriched rotifers and tube-fed ^{14}C tyrosine, indicating that tyrosine was not a limiting amino acid. On the other hand, lysine supplementation had a similar retention percentage in the treatments with and without lysine supplementation. Based on the tube feeding studies with lysine and tyrosine supplemented rotifers neither lysine nor tyrosine affected protein synthesis in a way to indicate that these amino acids are insufficient in *Diplodus sargus* larvae fed rotifers.

Keywords: *Diplodus sargus,* lysine, tyrosine, tube-feeding, amino acid metabolism

5.1. INTRODUCTION

Amino acids (AA) seem to be the major energy source during fish larval stages (Rønnestad et al., 1999) and a high and balanced amino acid content in the diet is required in order to maximize growth (Conceição et al., 2003; Rønnestad et al., 2003). Dietary AA deficiencies cause a reduction in growth, decrease protein retention in fish (e.g., Tibaldi et al., 1994; Luzzana et al., 1998) and increase AA oxidation (Fauconneau et al., 1992).

The indispensable AA profile of *Diplodus sargus* carcass was used by Saavedra et al. (2006) as an indicator of *D. sargus* AA requirements. This AA profile can be used as a preliminary approach to determine diet imbalances (Conceição et al., 2003). Although this comparison alone is not sufficient to determine AA requirements, it can provide important information on the possible dietary amino acid deficiencies. Saavedra et al. (2006) compared *Diplodus sargus* larvae and diet AA profiles and suggested lysine as potentially limiting in rotifers and *Artemia*. Lysine is an indispensable AA that reduces nitrogen loss by improving the use of other indispensable amino acids (Kerr and Easter, 1995), therefore promoting a higher growth rate. Lysine is also a precursor of carnitine, which plays an important role in the transport of long-chain fatty acyl groups into the mitochondria for β oxidation (Tanphaichitr et al., 1971).

Tyrosine is an aromatic AA which is produced from phenylalanine. It is therefore often considered as a semi-indispensable AA. Tyrosine is the precursor of thyroid and adrenocortical hormones and of norepinephrine and epinephrine. Dopamine regulates central and peripheric nerve activity and therefore can be related to the control of stress in fish. Tyrosine is also metabolized to catechol derivates which are melanin precursors which play an important role in fish pigmentation.

Live feed is still essential to meet fish larval AA requirements during early life stages (Shields, 2001). Although AA are mainly present in the form of protein, live feed has large AA pools, representing between 10 to 20% of total AA content (Fyhn et al., 1995; van der Meeren et al., 2001). Due to this large pool size, it is possible to significantly increase the AA percentage in rotifers and *Artemia* (Tonheim, 2000; Barr and Helland, 2007). Life feed enrichment can be achieved using liposomes encapsulating free AA (Barr and Helland, 2007). Free AA have been shown to be faster absorbed in the larval gut compared to peptides and protein (Rønnestad et al., 2000, 2001a, b).

The tube-feeding technique, developed by Rust et al. (1993) and later modified by Rønnestad et al. (2001a) can be used to determine if an AA is deficient in the diet and therefore limiting growth in fish larvae (Aragão et al., 2004). This technique also enables the study of the effects of short term supplementation of individual AA in terms of percent AA retention, oxidation and gut absorption (Conceição et al., 2007a). Tube-feeding consists of the use of a ^{14}C tracer nutrient which is deposited into the larval gut by a capillary tube (Rønnestad et al., 2001a) and after a digestion period the fate of the tube-fed ^{14}C nutrient can be determined. In tube-feeding AA trials, it is possible to determine the supplementation efficiency of a certain AA by comparing AA retention in AA enriched and unenriched treatments, using the supplemented AA as the tracer. An efficient supplementation should be reflected in a similar or higher AA retention (% basis). With this technique, it is also possible to determine if a certain AA is limiting larval growth. However, if a limiting AA is used as the tracer, its retention is not expected to improve, as it is already being used close to maximum efficiency. In this case, the comparison of a mixture of tracer AA, both with and without target AA supplementation, is advised (Aragão et al., 2004). If the target AA is limiting, its

supplementation will increase overall protein synthesis, and therefore retention of an AA mixture will be higher compared to the treatment without supplementation.

The purpose of this study was to test if lysine and tyrosine supplementation affects the metabolism of these AA in *Diplodus sargus* larvae and determine if either of these AA were limiting protein synthesis (and growth). AA supplementation was done using rotifers enriched with liposomes boosted with tyrosine or lysine.

5.2. MATERIAL AND METHODS

5.2.1. Larvae

Diplodus sargus larvae were obtained from IPIMAR Aquaculture Research Station (EPPO, Olhão, Portugal) and reared under standard conditions (Pousão-Ferreira and Dores, 2000). At 24 days after hatching (DAH), *Diplodus sargus* larvae (7.97 ± 0.55 mm, 0.59 ± 0.11 mg, mean ± s.d., n=20) larvae were transferred to CCMAR (University of Algarve, Faro, Portugal) facilities where the tube-feeding trials were performed. Before being tube-fed, larvae were kept in small white trays at a room temperature of 25 ºC and were fed a meal of rotifers (enriched or non-enriched rotifers, according to treatment). After a 1 h period of acclimation and feeding, larvae were ready to be tube-fed. Larvae were tube-fed with fairly full stomachs to avoid regurgitation (Rønnestad et al., 2001a).

5.2.2. Treatments

Four treatments, each one with twelve larvae, were carried out using L-[1-^{14}C] lysine (ARC, USA) and L-[U-^{14}C] tyrosine (ARC, USA) as target AA tracers: 1) ^{14}C tracer AA (tyrosine or lysine) + rotifers enriched with FAA (tyrosine or lysine); 2) ^{14}C tracer AA (tyrosine or lysine) + non-enriched rotifers; 3) L-[U-^{14}C] protein hydrolysate

(obtained by hydrolysis of an algal protein, Amersham Pharmacia, UK) that contains 19 of the AA used for protein synthesis (except Trp) as a tracer (tracer mix AA) + rotifers enriched with FAA (tyrosine or lysine); 4) L-[U-^{14}C] protein hydrolysate AA tracer mix + non-enriched rotifers. The first two treatments provide information about supplementation efficiency. Comparing the four treatments, it is possible to know if either tyrosine or lysine are deficient and therefore if they are limiting *Diplodus sargus* growth (Aragão et al., 2004).

Before being given to the larvae, rotifers were enriched for 1h with liposomes boosted with either crystalline tyrosine or lysine (0.003 g of crystalline tyrosine/million of rotifers or 0.055 g of crystalline lysine/million of rotifers) (Table 1 and 2). This protocol was carried out according to Barr and Helland (2007). The quantity of free AA in the liposomes was based on rotifers and larval AA profiles (Saavedra et al., 2006) in order to correct the lysine and tyrosine percentages in rotifers. Tyrosine incorporation in the liposome was limited by its low solubility.

Table 1. Free amino acid profiles of rotifers with and without lysine enrichment. Values are mean and standard deviations. Individual amino acids are expressed as g/ 100g Indispensable AA. (Br- Rotifers)

	Br non-enriched	Br Lys enriched
Leu	3.0 ± 0.1	3.4 ± 0.3
Lys	**25.1 ± 0.3**	**32.5 ± 0.3**
Arg	18.8 ± 0.0	17.8 ± 0.1
Val	4.8 ± 0.1	5.3 ± 0.0
Thr	13.3 ± 0.1	10.4 ± 0.4
Ile	3.4 ± 0.0	3.5 ± 0.2
His	18.6 ± 0.4	14.2 ± 0.2
Met	7.8 ± 0.1	6.1 ± 0.1
Cys	0.7 ± 0.1	1.0 ± 0.2
Phe	0.8 ± 0.1	1.1 ± 0.2
Tyr	3.8 ± 0.1	4.5 ± 0.1

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Table 2. Free amino acid profiles of rotifers with and without tyrosine enrichment. Values are mean and standard deviations. Individual amino acids are expressed as g/ 100g Indispensable AA. (Br- Rotifers).

	BR non-enriched	Br Tyr enriched
Leu	2.65±0.00	2.87±0.02
Lys	17.33±0.04	16.95±0.14
Arg	16.65±0.82	15.12±0.20
Val	4.05±0.01	4.87±0.07
Thr	11.50±0.25	11.22±0.10
Ile	3.59±0.00	4.21±0.14
His	15.63±0.29	15.84±0.09
Met	4.38±0.07	4.29±0.23
Phe	18.20±0.44	17.29±0.06
Tyr	**6.00±0.18**	**7.34±0.04**

5.2.3. Tube-feeding set-up

These experiments followed the methodology described by Rust et al. (1993) and later modified by Rønnestad et al. (2001a). Larvae were tube-fed using a 0.19 mm diameter plastic capillary tube (Sigma-Aldrich) inserted in a nanoliter injector (World Precision Instruments, Sarasota, FL, USA). Before being tube-fed, larvae were lightly anathetized with phenoxyethanol (Sigma-Aldrich) (400 µl/ l) for 2 min. Two consecutive injections of 13.6 nl were done into the larval gut. Due to difficulties in obtaining sufficient radioactivity in some compartments in previous *Diplodus sargus* tube-feeding trials, the injection volume was increased from 18.4 nl (Saavedra et al., 2008) to 27.2 nl in the present study. This injection volume is in the range of that used for other species: 13.8 nl for 36 DAH *Solea* senegalensis (Aragão et al., 2004), and 32nl for 4 DAH herring (Conceição et al., 2002). It should be noted that in all these studies a tracer quantity of ^{14}C-AA was used, with no nutritional significance (Conceição et al., 2002). The nutritional effect in the present study comes from the meal supplied to the larvae prior to tube-feeding. After tube-fed, larvae were rinsed twice in seawater and then transferred to a sealed incubation well (8 ml seawater), which was connected to a

metabolic trap (5 ml 0.1 M KOH) by an air tube. For further details see Rønnestad et al. (2001a).

5.2.4. Radioactivity counting

Larval digestion was estimated by observing the time that the larval needed to assimilate or evacuate the total contents of their stomach. Once digestion was completed (6 h), larvae were removed from the incubation well and placed in a 6 ml scintillation plastic vial. Then 0.5ml of Solvable (Packard Instruments) was added in order to dissolve the larvae during a period of 24 h at 30 ºC. In order to obtain the results for AA catabolism the protocol of Rønnestad et al. (2001a) was followed for collecting CO_2 from the incubation water. The incubation and metabolic trap vials were collected. Ultima Gold scintillation liquid (Packard Instruments) was added to all larvae (representing ^{14}C-AA retention), metabolic trap (representing the labelled CO_2 from AA oxidation) and incubation (unabsorbed, evacuated ^{14}C-AA) vials before counting (DPM) in a Beckman LS 6000IC liquid scintillation counter (Fullerton, CA).

5.2.5. Data analysis

Differences in the percentage of ^{14}C among treatments were assessed using both two-way and one-way ANOVA for larvae and metabolic chamber data from the tyrosine experiment All the remaining data were analysed by a one-way non-parametric ANOVA, Kruskal-Wallis. Post-Hoc tests used in this study were Scheffé for parametric data and Nemeyi for non-parametric data. All data were calculated by subtracting the blanks (just scintillation liquid) and expressed as a percentage of the tracer fed, i.e. the sum of the DPM counted in the three vials (incubation water, metabolic trap and larvae). When there were differences in the incubation chamber, retention and catabolism

percentages, the data were also calculated using the metabolic trap and larvae sum as 100%.

5.3. RESULTS

Larval survival after the 6 h incubation period was 98 and 100% for the lysine and tyrosine trials, respectively. In the tyrosine experiment, significant differences were found in the incubation chamber (water) ($F_{3,36}$= 5.7 and P= 0.004). In the treatment where larvae were tube-fed labelled tyrosine and fed tyrosine enriched rotifers, there was a higher percentage of ^{14}C in the water than the AA mixture. Unenriched rotifers with ^{14}C Tyr were intermediate. Larval body also showed significant differences ($F_{3,36}$= 17.1, P‹0.001) among treatments. Larvae fed tyrosine enriched rotifers and tube-fed ^{14}C tyrosine had the lowest ^{14}C incorporation whereas larvae fed non-enriched rotifers and tube-fed the AA mixture showed the highest retention (Fig. 1). The metabolic chambers (traps) were also found to be significantly different among treatments ($F_{3,36}$=4.5, P=0.009) (Fig. 1).

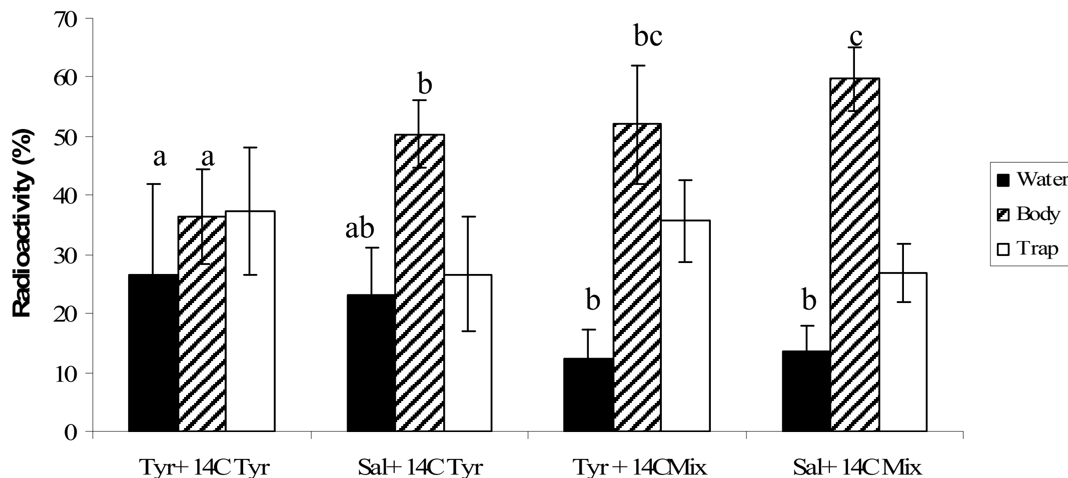

Fig 1. Proportion of the tube-fed ^{14}C amino acid mixture (Mix) or ^{14}C tyrosine (Tyr) catabolised (white columns), retained in body (black stripes columns) and evacuated into water (black columns) of *Diplodus sargus* larvae. Tyr+ ^{14}C Tyr: larvae tube fed ^{14}C Tyr and Tyr enriched rotifers; Sal+ ^{14}C Tyr: tube fed Tyr ^{14}C and un-enriched rotifers; Tyr+ ^{14}C Mix: tube fed ^{14}C Mix and Tyr enriched rotifers; Sal+ ^{14}C Mix: tube fed ^{14}C Mix and un-enriched rotifers. Values are mean and s.d. (n=12). Different letters in the same compartment indicate significant differences (P< 0.05).

Significant differences were found among the different treatments for retention and oxidation percentages in the tyrosine experiment ($F_{3,36}$=10.2, P‹ 0.001) (Fig. 2). The lowest retention was observed for the treatments where larvae where fed either tyrosine enriched rotifers or tube-fed ^{14}C tyrosine which had approximately the same percentage of retention and catabolism (Fig. 2). The treatment fed tyrosine un-enriched rotifers had the lowest oxidation percentages and the highest retentions (Fig. 2). Treatments fed tyrosine enriched rotifers and tube fed the AA mixture had intermediate retention and oxidation percentages.

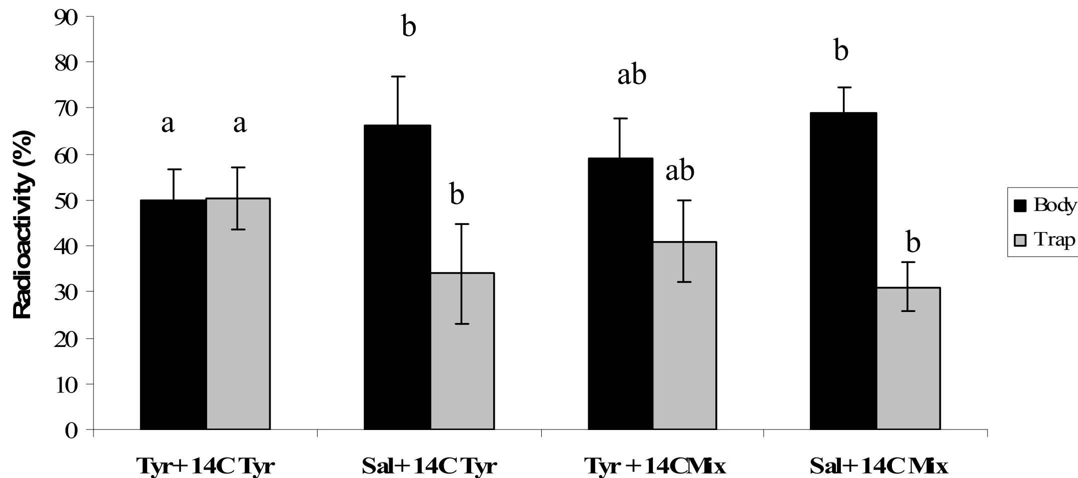

Fig 2. Proportion of catabolised (black columns) and retained in body (black columns) ^{14}C AA of tube-fed *Diplodus sargus* larvae. Tyr+ ^{14}C Tyr: larvae tube fed ^{14}C Tyr and Tyr enriched rotifers; Sal+ ^{14}C Tyr: tube fed Tyr ^{14}C and un-enriched rotifers; Tyr+ ^{14}C Mix: tube fed ^{14}C Mix and Tyr enriched rotifers; Sal+ ^{14}C Mix: tube fed ^{14}C Mix and un-enriched rotifers. Values are mean and s.d. (n=12). Different letters in the same compartment indicate significant differences (P< 0.05).

In the lysine tube-feeding trials, significant differences were found in the incubation chambers ($F_{3,39}$=24.3, P‹0.001) where ^{14}C lysine treatments had more than three times the radioactivity than the ^{14}C AA mixture treatments (Fig 3). Significant differences were also found for larvae bodies (F3,39=5.3, P=0.02). The treatment with larvae fed un-enriched rotifers and tube-fed the ^{14}C AA mixture showed the highest ^{14}C retention whereas treatment where larvae were fed lysine enriched rotifers and tube-fed ^{14}C lysine showed the lowest ^{14}C retention (Fig.3). Catabolism percentage showed significant differences among treatments ($F_{3,39}$=18.0, P‹0.001) (Fig.3). The ^{14}CAA mixture treatments had higher catabolism rates than ^{14}C lysine treatments.

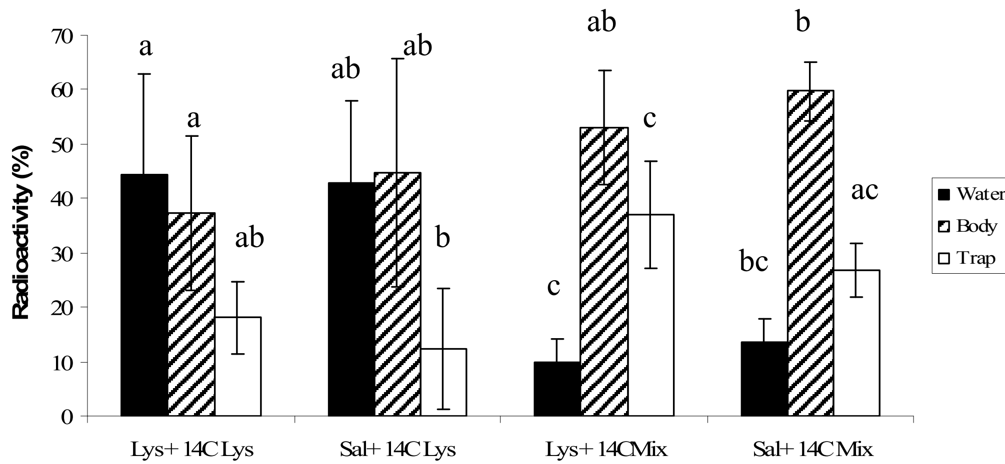

Fig 3. Proportion of the tube-fed ^{14}C amino acid mix (Mix) or ^{14}C Lysine (Lys) catabolised (white columns), retained in body (black stripes columns) and evacuated into water (black columns) of *Diplodus sargus* larvae. Lys+ ^{14}C Lys: larvae tube fed ^{14}C Lys and Lys enriched rotifers; Sal+ ^{14}C Lys: tube fed Lys ^{14}C and un-enriched rotifers; Lys+ ^{14}C Mix: tube fed ^{14}C Mix and Lys enriched rotifers; Sal+ ^{14}C Mix: tube fed ^{14}C Mix and un-enriched rotifers. Values are mean and s.d. (n=12). Different letters in the same compartment indicate significant differences ($P < 0.05$).

When larval body and trap data were analysed alone significant differences were found in retention and catabolism between treatments ($F_{3,39}=2.7$, $p=0.03$) (Fig. 4) although the post-hoc test could not determine which treatments were significant different. Slightly higher retention percentages were observed for ^{14}C lysine treatments (Fig. 4). No additional differences were found when data were analysed when tracer and supplements were both included as independent factors (2-way ANOVA).

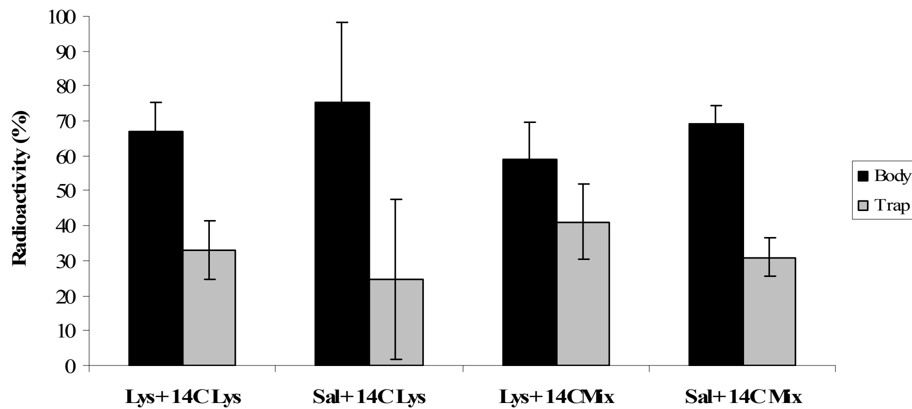

Fig 4. Percentage of ^{14}C catabolised (grey columns) and ^{14}C retained in the larval body (black columns) of tube-fed *Diplodus sargus* larvae. Lys+ ^{14}C Lys: larvae tube fed ^{14}C Lys and Lys enriched rotifers; Sal+ ^{14}C Lys: tube fed Lys ^{14}C and un-enriched rotifers; Lys+ ^{14}C Mix: tube fed ^{14}C Mix and Lys enriched rotifers; Sal+ ^{14}C Mix: tube fed ^{14}C Mix and un-enriched rotifers. Values are mean and s.d. (n=12).

5.4. DISCUSSION

The aim of this work was to study tyrosine and lysine metabolism in *Diplodus sargus* larvae and determine if either of these AA were limiting protein synthesis. The results obtained showed very high standard deviations, especially in the ^{14}C lysine tube fed larvae. It is difficult to explain why this treatment, specifically, had such a high variation. Possible explanations include an effect of larval size dispersion, different tube-fed doses in the larval gut, different rotifer intake by larvae, and high biological variation in response to dietary lysine. As previously reported by Saavedra et al. (2008), some of the radioactive dosage is lost by regurgitation after *Diplodus sargus* is tube-fed. This effect was minimized by tube feeding larvae with fairly full stomachs as suggested by Rønnestad et al. (2001a). In this study a higher dosage was used compared to Saavedra et al. (2008) (2 x 13.6 versus 2 x 9.8 nl, respectively). This change in dosage volume was due to the lower DPM counts registered by Saavedra et al. (2008) and failure to be able to obtain more than 50% of the larvae injected with radio-labelled AA since much was lost due to regurgitation.

Unabsorbed labelled lysine and tyrosine (percentage of ^{14}C in the water) in larvae fed enriched rotifers and tube-fed tyrosine or lysine was significantly higher than for the average of all AA. In the lysine treatment, the evacuation percentage reached almost 50%. Saavedra et al. (2008) reported high evacuation values for tryptophan when conducting a similar trial with *D. sargus* larvae. Similarities between tryptophan and tyrosine structures, both with a large heterocyclic group, may be related with these similar results. Kolkovski and Tandler (2000) suggested that the larval digestive tract may not be able to handle large amounts of free AA and that the majority will pass unabsorbed from the digestive tract. However, this was not observed in studies by Applebaum and Rønnestad (2004) and Rønnestad et al. (2001b), where unabsorbed percentages were less than 10%, the same as observed in this study for the larvae tube-fed the AA mixtures and by Saavedra et al. (2008). Therefore, the high evacuation rates for lysine and tyrosine might be related to the transporters of these AA in the brush border membrane possibly becoming saturated. Gut absorption of AA is quite complex and involves a large number of transporters, some specific for particular AA, on the brush border membrane (Jürss and Bastrop, 1995; Mailliard et al., 1995). AA can cross the enterocyte membrane by carrier-independent diffusion, facilitated transport or active transport (Mailliard et al., 1995). The absorption efficiency can be affected by several factors such as relative and total concentrations of specific AA in the gut (Hirst, 1993; Rønnestad et al., 2007).

When analysing retention in the tyrosine trial a lower incorporation of ^{14}C was found in larvae fed rotifers and tube-fed ^{14}C tyrosine. This means, when larvae were given a higher amount of tyrosine, it was not incorporated (as retention decreased when compared to the unenriched treatment tube fed tyrosine) and therefore tyrosine supplementation was not efficient. The extra tyrosine given to this treatment seemed to

have been oxidized since a higher percentage in catabolism was observed. This is consistent with Benevenga et al. (1993) who suggested that a decrease in protein synthesis usually results in an increase in AA catabolism. Saavedra et al. (2006) also found a good correlation between the tyrosine content in rotifers and in larval bodies, meaning that this AA was probably not deficient in the diet. The same was not observed in the lysine experiment, where larvae fed either lysine enriched rotifers or un-enriched rotifers then tube-fed ^{14}C lysine, showed similar retention percentages and therefore a higher quantity of lysine was retained, suggesting that the supplemented lysine was efficiently supplemented. However, the results obtained for lysine retention (approximately 50%) are quite different from those reported by Rønnestad et al. (2001b), where a retention of 80% was achieved when tube-feeding free ^{14}C lysine in 32 DAH *Solea senegalensis*. These differences may be explained by the more advanced ontogenic stage of sole used in the previous study, and/or by the peculiar digestive physiology of sole (Conceição et al., 2007b). Lysine retention may have been affected by the low absorption rates observed in the current study. When the absorption efficiency (oxidation+retention) was analysed alone, percentages between 70 and 80% were found, and these values were slightly higher than those observed for the AA mixture.

In both the tyrosine and lysine experiments, a lower retention was not observed when larvae were tube-fed the ^{14}C-AA mixture and fed unenriched rotifers. This suggests that both of these AA were probably not limiting growth in *Diplodus sargus* larvae at this age. If an AA is deficient or limiting in the diet, it will mainly be used in protein synthesis and only a small percentage will be oxidized to carbon dioxide (Wilson, 1994). These findings differ from those of Saavedra et al. (2006) who suggested that lysine was potentially deficient in *Diplodus sargus* larvae fed rotifers.

However, a recent study on lysine supplementation in zootechnic trials using *D. sargus* first feeding larvae did not improved growth, suggesting that this AA is not deficient in *D. sargus* larval diets (unpublished results).

In conclusion, tyrosine supplementation in *Diplodus sargus* diets does not seem necessary for a larval age of 24 DAH since a lower retention percentage was found when larvae were fed tyrosine enriched rotifers and tube-fed ^{14}C tyrosine. On the other hand, lysine supplementation seemed to be efficient. Both tyrosine and lysine seemed to have lower absorptions efficiencies compared to the average of all AA and none of these AA appeared to limit *Diplodus sargus* growth when larvae are fed rotifers.

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Chapter 6

A balanced amino acid diet improves *Diplodus sargus* larval quality and reduces nitrogen excretion

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Abstract

Fish larvae present high amino acid requirements due to their high growth rate. Maximizing this growth rate depends on providing a balanced amino acid diet which can fulfil larval amino acid nutritional needs. In this study two experimental microencapsulated casein diets were tested: one presenting a balanced amino acid profile and another presenting an unbalanced amino acid profile. A control diet, live feed based, was also tested. Trials were performed with larvae from 1 to 25 DAH. Microencapsulated diets were introduced at 8 DAH in co-feeding with live feed and at 15 DAH larvae were fed the microencapsulated diets alone. Results showed a higher survival for the control group (8.6 ±1.3% versus 4.2 ± 0.6% and 3.2 ± 1.8%) although dry weight and growth were similar in all treatments. The proportion of deformed larvae as well as the ammonia excretion was lower in the group fed a balanced diet than in the unbalanced or control groups (38.3% deformed larvae in control, 30% in larvae fed unbalanced diet and 20% on balanced diet group). Furthermore, larvae fed the microencapsulated diets presented higher DHA and ARA levels. This study demonstrates that dietary amino acid profile may play an important role in larval quality. It also shows that balanced microencapsulated diets may improve some of the performance criteria, such as skeletal deformities, compared to live feeds.

Keywords: *Diplodus sargus*, amino acids, balanced diet, ammonia, deformities, microencapsulated diet

6.1. INTRODUCTION

White seabream (*Diplodus sargus*) is considered to be a promising new species to fish farming in Southern Europe, having a high market price and demand (Pousão-Ferreira et al., 2001, Ozorio et al., 2006, Santos et al., 2006). It is also involved in restocking programmes in the southern coast of Portugal, where landings decreased from 200.3 t to 75.2 t between 1987 and 2004 (Santos et al., 2006).

Saavedra et al. (2006) reported some promising results of *Diplodus sargus* larvae rearing, indicating a higher growth rate at the larval stage when compared to *Sparus aurata.* However, when approaching the juvenile stage, a decrease on the growth rate (Cejas et al., 2003) and the presence of several skeletal deformities, especially at the dorsal column, are bottlenecks to this species farming (Saavedra et al., 2006). Recent studies suggest that the nutritional composition of the diet may play an important role in the frequency of skeletal deformities (Cahu et al., 2003) and the deficiency of some amino acids may increase this percentage (Akiyama et al., 1986). Skeletal deformities can have an economical impact as they can affect both size and shape of the fish therefore decreasing their economical value (Favaloro and Mazzola, 2000; Boglione et al., 2001). They can also make commercialization difficult as the consumers tend to choose fish with standard shape (Koumoundourous et al., 1997, Boglione et al., 2001).

Ten amino acids (AA) are considered indispensable for normal fish growth (Wilson, 1989) and the AA profile of the diet affects larval growth (Conceição et al., 2003). In order to maximize larval growth rate the AA profile of the diet should be as close as possible to the larval AA requirements (Akiyama et al., 1995, Conceição et al., 2003). In fact, juvenile rainbow trout, in presence of several AA diets, selected

preferentially balanced diets (Yamamoto et al., 2000). Balanced diets increase protein synthesis as well as decrease nitrogen excretion (Aragão et al., 2004a).

Manipulation of rotifers and *Artemia* AA profiles is difficult (Aragão et al., 2004b) and a balanced AA diet can be better achieved using a microencapsulated feed. However, weaning Sparidae larvae in early stages can compromise survival and growth as there is still a high dependence on live feed (Yúfera et al., 1996, Fernández-Díaz and Yúfera, 1997). Yúfera et al. (1999, 2002) have developed cross-linked casein-walled capsules and showed this type of microencapsulated diet was able to substitute with some success live feed in *Sparus aurata* early larval stages. During the first feeding days a microencapsulated diet used in co-feeding with rotifers can increase the enzymatic capacity to digest the inert diet (Yúfera et al., 1999, 2002). This suggests that free AA in the tissues of the live feed may be important in stimulating the release of digestive enzymes and enhancing the ingestion rates by affecting the attractability of the diets (Cahu and Zambonino Infante, 1995).

The purpose of this study was to determine if a balanced AA diet would promote growth as well as high quality larvae. The balanced diet was given to the fish larvae in the form of cross-linked casein-walled capsules.

6.2. MATERIALS AND METHODS

6.2.1. 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. *D. sargus* eggs were obtained from a mix of wild and farmed broodstock consisting of 38 fish with an average weight of 869.2 g ± 319.8 g and tank density of 4 kg/m^3. After collected from the incubators, first feeding larvae

were transferred to 200l conical cylindrical fibreglass tanks at a density of 80 larvae/ l. Before entering the tank water passed through a biological filter and then through a mechanical and sterilized by a UV filter. The system worked in a semi-closed circuit and water temperature was maintained at 19.1 ± 0.7 º C, oxygen at 6.5 ± 0.4 mg /l and salinity at 37 ± 1 ppt. Water flow started at 0.4 l /min and then was slowly increased with larvae age until a maximum of 1 l /min, on 12 DAH. Photoperiod was 24 hours light. The trial lasted 25 days and three treatments, randomly distributed, were used in triplicate: larvae fed a balanced amino acid microencapsulated diet, larvae fed an unbalanced amino acid microencapsulated diet and a control fed live feed.

6.2.2. Cross-linked casein-walled capsules formulation

Cross-linked casein-walled capsules were used in this study due to the lower free amino acid leaching when compared to other kind of inert diets (Yúfera et al., 2002). To formulate the balanced diet, 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). The microencapsulated diet consisted on 70% protein, of which 31.1% was casein (minimum required to obtain this type of capsules) (table 1.) The microencapsulated diet was formulated in order to obtain an AA diet profile as close as possible to the larval carcass profile (see Saavedra et al., 2006). AA in deficiency in the diet were supplemented with crystalline indispensable AA in the balanced diet. The same percentage of free indispensable AA was added to the unbalanced AA diet in the form of crystalline dispensable AA, in order to keep digestible nitrogen comparable in the two diets. 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 percentages was added (*eg.* if the leaching percentage of a certain free AA is 50% its content in the diet was increased 50%). Microencapsulated diets were prepared according to Yúfera et al. (1999). Fatty acid profiles of the diets are presented in table 2.

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

Ingredients (g.kg^{-1})	BALANCED	UNBALANCED
Fish Meal[1]	159	159
Squid Meal[2]	73	73
Hydrolised Protein Concentrate[3]	73	73
Casein[4]	311	311
Fish Oil[5]	159	159
Vitamin+Mineral Complex[6]	35	35
Agar[7]	05	5
Vitamin E[8]	7	7
Vitamin C[9]	14	14
Threonine[*]	55	-
Arginine[*]	55	-
Phenylalnine[*]	10	-
Cystein[*]	14	-
Lysine[*]	42	-
Serine[+]	-	55
Alanine[+]	-	38
Glutamate[*]	-	38
Proline[*]	-	38

Table 2. Fatty acid profile of *Diplodus sargus* microencapsulated diet. Values are expressed g. kg^{-1} of total fatty acids.

Fatty acid	g. kg^{-1}
14:0	39.1
16:0	138.3
18:0	29.4
Total – SFA	217.7
16:1	54.0
18:1	187.2
Total – MUFA	415.5
18:2n6	107.6
18:3n3	18.7
20:4n6	4.0
20:5n3	46.9
22:5n3	21.3
22:6n3	75.6
Total – PUFA	342.2
Sn3	190.0
Sn6	148.9
Sn3/Sn6	12.8
n-3 HUFA	143.8
DHA/EPA	16.1
EPA/ARA	117.8

6.2.3. Feeding Protocol

Feeding protocol for the control treatment consisted of *Brachionus plicatilis* (5/ ml) enriched with Protein Selco (INVE Aquaculture, Belgium) from 3 to 20 DAH. At day 12, larvae started having *Artemia* nauplii (BE 480, INVE Aquaculture, Belgium) (0.125/ ml) and *Artemia* metanauplii (0.25/ ml), enriched with Rich (Rich®, Greece) from 17 DAH until the end of the experiment. Twice every day *Tetraselmis* spp. and *Isochrysis galbana* was added to the tanks in order to obtain an average concentration of 18 x 10^3 and 82x 10^3 cells/ ml, respectively.

The feeding protocol for balanced and unbalanced diet treatments was the same as the control until the 11 DAH. From 12 to 14 DAH the live feed was cut to half of the control and afterwards *Brachionus plicatilis* and *Artemia* were only given at 10% of the control. Microencapsulated diets were introduced on 8 DAH. From 8 to 10 DAH 0.5 g

was given to each tank, on 11 and 12 DAH 1 g was given and from 13 to the end of experiment 1.5 g. The micro encapsulated diets were provided to the fish larvae by hand during the day at 10 am, 12 am, 3 pm and 6 pm and in during the night by a automatic feeder at 9 pm, 3 am and 9 am. Before given to the larvae, the micro encapsulated diets were quickly hydrated in 100 ml of fresh water and then spread around the tanks. Being hydrated, the microencapsulated diets remained at the surface for a shorter period and then settled at the bottom. Bottom water was hand flushed three to four times a day. The automatic feeder was equipped with a plastic tube and a water inlet in order for the hydration occur.

6.2.4. Ammonia excretion

Ammonia excretion was determined using trials of 10 fish larvae 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. Two replicates from each tank were used (five replicated *per* treatment) as well as three blanks *per* treatment. Trials were carried out at a temperature of 19 ºC 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 µmol g DW^{-1} h^{-1}) was calculated as follows:

$MNH_4^+ = \Delta[NH_4^+]\ V_{H20}\ DW^{-1} \Delta T^{-1}$, where $\Delta[NH_4^+]$ is the difference between the sample and the blank water, V_{H2O} is the volume of water in the spherical glass, DW is the larvae dry weight, and ΔT is the time duration of the trial.

6.2.5. Deformity analysis

On 15 and 25 DAH, 30 larvae were taken from each tank to observe 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.

6.2.6. Sampling and biochemical analysis

Larvae samples were taken from every tank on 0, 15 and 25 DAH for dry weight determination. Each sample consisted of 40 and 20 larvae on 25 DAH. On 20 DAH, 20 larvae were taken from each tank for fatty acid analysis. These samples were frozen in liquid nitrogen at -190 ºC, then freeze-dried (RVT 400, Savant, NY) and weighed.

Ingestion of microencapsulated diet in the larval gut was monitored from 8 DAH to 15 DAH by observation of 30 larvae *per* treatment.

6.2.6.1. Amino acids analysis

Protein-bound amino acids samples were hydrolysed in 6M HCL at 108ºC 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).

6.2.6.2. Fatty acid analysis

After freeze drying, fatty acid composition was determined using the transesterification method by basic catalysis (Park et al., 2001). Fatty acid methyl esters (FAME) were separated and quantified using a Varian CP 3800 gas chromatograph equipped with a flame ionization detector (250 ºC) and a DB-WAX Polyetilene Glicol column (30mx0.25 mm ID.0.25 µm). Injector temperature was maintained constant at 250 ºC over the 40 minutes of the analysis. The column was submitted to a temperature gradient, 5 minutes at 180 ºC, an increase of 4 $ºC.min^{-1}$ for 10 minutes and 220 ºC for 25 minutes.

6.2.7. Data analysis

The relative growth rate (RGR, % DW day^{-1}) was calculated using the following formula: $RGR = (e^{(\ln DWt - \ln DW0) / t} - 1)*100$, being DWt and DW_o the final and initial dry weights respectively and t the trial duration. Survival and ammonia excretion data were analysed for significant differences P<0.05 by one-way ANOVA. Post-Hoc analyses were performed using Scheffé test. Growth, fatty acids, larval deformities were analysed by a non-parametric one-way analysis of variance Kruskal-Wallis at a significance level of $P<0.05$.

6.3. RESULTS

Amino acid profiles of the diets presented the expected results, a balanced diet with a higher level of indispensable amino acids and an unbalanced diet with higher dispensable amino acid proportion (table 3).

Table 3. Amino acid profiles of balanced and unbalanced *Diplodus sargus* microencapsulated diets. Values are mean and standard deviation and expressed as percentage (g. kg^{-1} of AA).

	BALANCED	UNBALANCED
IAA		
Leu	84.3 ± 3.7	87.0 ± 4.4
Lys	79.9 ± 1.7	75.3 ± 9.8
Arg	74.7 ± 5.9	56.1 ± 1.2
Val	52.3 ± 5.1	55.1 ± 3.9
Thr	56.0 ± 4.4	43.8 ± 2.4
Ile	43.6 ± 5.2	46.3 ± 4.5
His	25.0 ± 1.0	26.6 ± 1.1
Met	24.7 ± 3.1	18.3 ± 5.4
Cys	9.1 ± 1.4	3.0 ± 0.4
Phe	55.2 ± 1.8	48.1 ± 2.4
Tyr	46.2 ± 2.0	48.0 ± 2.7
DAA		
Glu+Gln	161.7 ± 1.0	174.0 ± 7.3
Ala	43.5 ± 1.9	51.5 ± 2.1
Gly	42.3 ± 4.1	39.3 ± 3.2
Asp+Asn	69.0 ± 1.6	70.3 ± 1.5
Ser	52.7 ± 2.3	68.0 ± 6.1
Pro	79.8 ± 0.7	89.1 ± 3.8

When larva AA profiles are compared to AA profiles of rotifers, balanced and unbalanced microencapsulated diets, a higher similarity is observed between larva and the balanced diet (Fig 1). This is especially evident for arginine, cysteine and valine (Fig 1). The unbalanced diet also seems to have a closer AA composition to the larvae than the control.

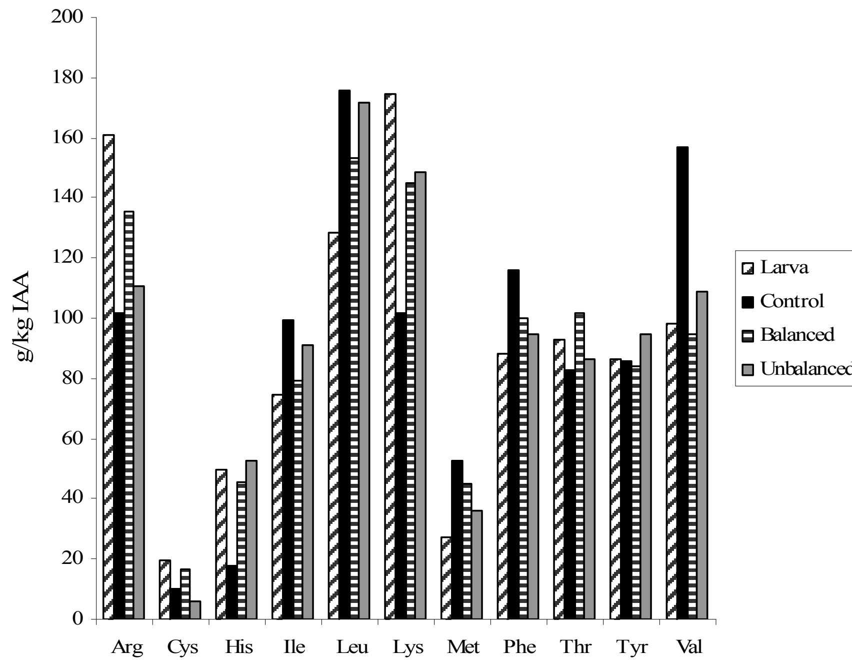

Fig. 1. Amino acid profiles from 8 DAH larva, control (rotifers), balanced and unbalanced microencapsulated diet. Amino acids are expressed as g. kg^{-1} of indispensable AA (IAA). Larva and rotifers AA profiles from Saavedra et al. (2006).

Survival rate was significantly higher in the control group ($F_{2,6}$=13.8, p=0.006), almost the double of balanced and unbalanced diet groups (table 4). Growth rate was approximately the same between treatments, both from 8 to 15 days after hatching (DAH) and from 15 to 25 DAH (table 4). No significant differences were found for larval dry weight between treatments.

Table 4. Growth and survival of *Diplodus sargus* fed with a balanced diet and unbalanced diet. Values are mean and standard deviations (for growth n=4 of 5 larvae pools).

	CONTROL	BALANCED	UNBALANCED
DRY WEIGHT (mg)			
8 DAH	0.04± 0.00	0.05 ± 0.02	0.04 ± 0.02
15 DAH	0.25 ± 0.03	0.28 ± 0.04	0.28 ± 0.05
25 DAH	0.87 ± 0.14	0.78 ± 0.16	0.68 ± 0.07
RGR (%/DAY)			
0-8 DAH	4.3 ± 1.0	8.1 ± 4.1	6.2 ± 5.4
8-15 DAH	32.5 ± 1.6	29.4 ± 7.6	32.2 ± 9.4
15-25 DAH	13.3 ± 0.6	10.8 ± 2.8	9.3 ± 1.1
SURVIVAL (%)	8.6 ± 1.3[a]	4.2 ± 0.6[b]	3.2 ± 1.8[b]

Larval guts were observed with a binocular microscope during the first eight days of microencapsulated diet introduction and an increase in the number of larvae with microcapsules in their digestive tract was found (Fig. 2). At the beginning, the percentage of larvae with micro capsules in the gut was approximately 20 % whereas towards the end the proportion increased up to 90% (Fig. 2). It was also possible to observe that most larvae gut ranged from half-full to full and larvae were able to disintegrate the microencapsulated diet.

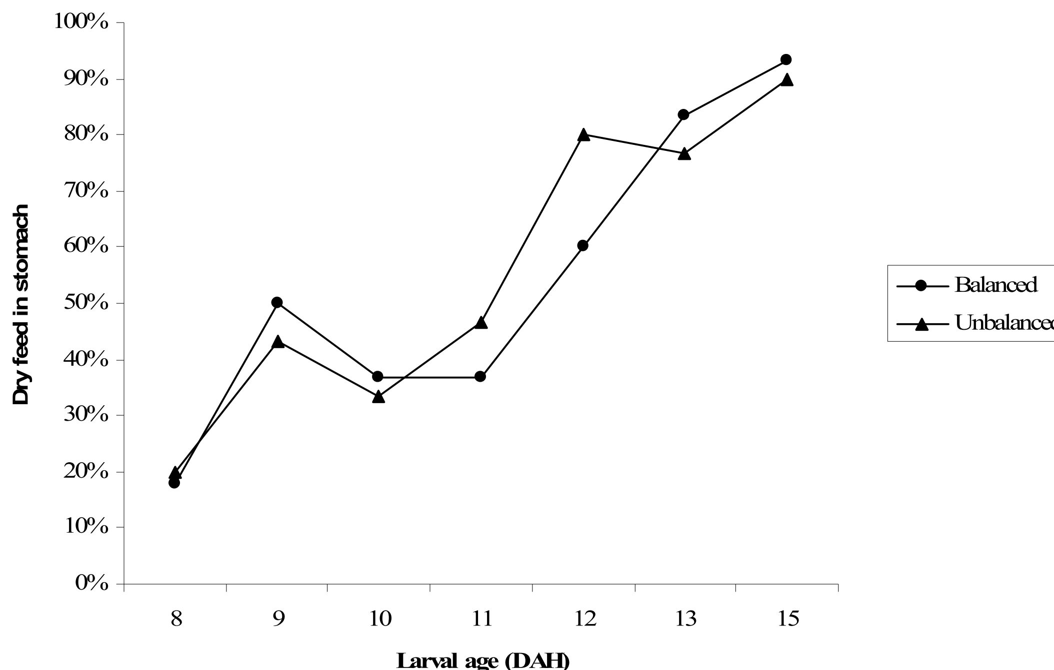

Fig 2. Percentage of larvae with the microcapsules-filled gut during the first 8 days after the microencapsulate diet was introduced (n=20 larvae *per* tank, three tanks *per* treatment).

Significant differences were found for all fatty acids except for total saturated fatty acids (SFA) (table 5). Larvae from control and balanced diets presented significant differences for the arachidonic acid (ARA) ($F_{2,24}$=4.1, p‹0.001). For eicosapentaenoic acid (EPA) as well as docosahexaenoic acid (DHA), larvae presented significant differences between control diet and both balanced and unbalanced diets (($F_{2,24}$=104.6, p‹0.001) and ($F_{2,24}$=127.4, p‹0.001), respectively) (table 5). Larvae from control diet presented higher EPA levels and lower DHA levels when compared with the inert diets. As a result, DHA/EPA ratios of larvae fed balanced and unbalanced diets were three times higher than control.

Table 5. Fatty acid profiles of *Diplodus sargus* larvae on 25 DAH fed with a balanced diet, an unbalanced diet and a control diet (n=90 larvae *per* tank, three tanks *per* treatment). Different letters represent significant differences for $p < 0.05$.

$g.kg^{-1}$	**Unbalanced**	**Balanced**	**Control**
14:0	11.7 ± 1.6^{a}	10.7 ± 1.1^{a}	7.6 ± 0.5^{b}
16:0	152.5 ± 6.3^{a}	150.9 ± 6.8^{a}	142.2 ± 5.3^{b}
18:0	66.1 ± 2.8^{a}	61.3 ± 3.2^{a}	78.5 ± 5.4^{b}
Others	14.7 ± 1.3^{a}	11.4 ± 1.6^{b}	14.1 ± 0.4^{b}
Total - SFA	244.9±9.9	234.3±8.5	242.4±8.7
16:1	30.7 ± 2.7^{a}	26.8 ± 1.4^{b}	27.8 ± 2.2^{b}
18:1	156.0 ± 21.8^{a}	135.7 ± 3.6^{b}	203.0 ± 4.0^{c}
20:1	24.9 ± 4.9^{a}	21.6 ± 1.4^{ab}	21.1 ± 1.5^{b}
Others	21.0 ± 5.3^{a}	16.7 ± 2.4^{ab}	16.1 ± 2.1^{b}
Total - MUFA	232.6 ± 33.2^{a}	200.7 ± 3.4^{b}	268.0 ± 8.9^{c}
18:2n6	58.8 ± 8.2^{a}	71.7 ± 4.0^{b}	76.8 ± 2.2^{b}
18:3n3	31.7 ± 26.0^{a}	12.5 ± 1.4^{a}	109.6 ± 9.4^{b}
18:4n3	7.9 ± 6.1^{a}	2.9 ± 0.4^{a}	20.5 ± 2.7^{b}
20:4n6 - ARA	12.1 ± 5.5^{ab}	15.0 ± 0.8^{a}	10.8 ± 0.8^{b}
20:4n3	8.2 ± 1.2^{a}	6.9 ± 0.8^{a}	14.7 ± 0.8^{b}
20:5n3 - EPA	68.2 ± 5.8^{a}	66.0 ± 3.8^{a}	96.5 ± 5.2^{b}
22:5n6	4.9 ± 1.2^{a}	5.5 ± 0.5^{a}	3.6 ± 0.6^{b}
22:5n3	26.9 ± 3.4^{a}	29.6 ± 1.7^{a}	15.8 ± 1.4^{b}
22:6n3 - DHA	190.1 ± 31.0^{a}	208.4 ± 7.8^{a}	77.5 ± 6.5^{b}
Others	26.9±7.1	26.3±6.5	29.3±4.2
Total - PUFA	435.7 ± 12.6^{a}	444.9 ± 11.0^{ab}	455.0 ± 3.8^{b}
Sn3	340.1 ± 11.7^{a}	329.9 ± 6.7^{b}	352.3 ± 4.7^{c}
Sn6	95.6 ± 11.4^{a}	115.0 ± 9.1^{b}	102.7 ± 6.0^{a}
Sn3/Sn6	36.1 ± 5.2^{a}	28.9 ± 2.4^{b}	34.4 ± 2.2^{a}
n-3 HUFA	285.2 ± 30.9^{a}	304.0 ± 7.9^{a}	189.8 ± 12.0^{b}
DHA/EPA	28.3 ± 6.3^{a}	31.7 ± 2.5^{a}	8.0 ± 0.5^{b}
EPA/ARA	84.5±73.3	43.9±2.5	90.0±5.4
Non identified	86.9 ± 49.0^{a}	120.2 ± 14.9^{a}	34.7 ± 5.0^{b}

The ammonia excretion was not significantly different between treatments in fasted larvae (Fig. 3). However, when larvae were fed significant differences were found between control and balanced diet treatments ($F_{2,6}$=9.1, p=0.02). Balanced diet seems to present almost no difference between fed and fasted larvae and registered the lowest ammonia excretion of all treatments.

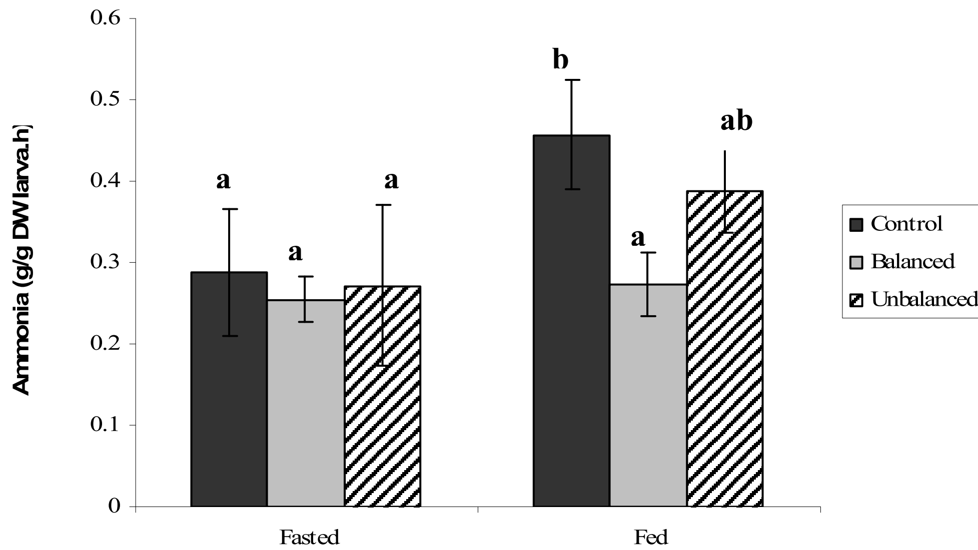

Fig. 3. Mass-specific ammonia excretion of fasted and fed 25 DAH *Diplodus sargus* from control, balanced and unbalanced diets. Values are mean and standard deviations (n=5 of pool of 10 larvae *per* treatment). Different letters represent significant differences for $p < 0.05$.

At 15 DAH the proportion of deformed larvae was lower than 5%, most of the observed deformities were abnormal shape vertebrae (Fig. 4). At 25 DAH the total frequency of deformed larvae was approximately 40% in the control group, 30% in the unbalanced diet group and 20% on the balanced diet group (Fig. 4). A significant ($F_{2,6}$=0.01, p=0.04) high number of vertebrae fusions were found in the control treatment whereas in the unbalanced diet group the highest frequency of skeletal deformity was vertebral compression although not significantly different. Lordosis was found in control and unbalanced diet groups but none in the balanced diet group (Fig. 4).

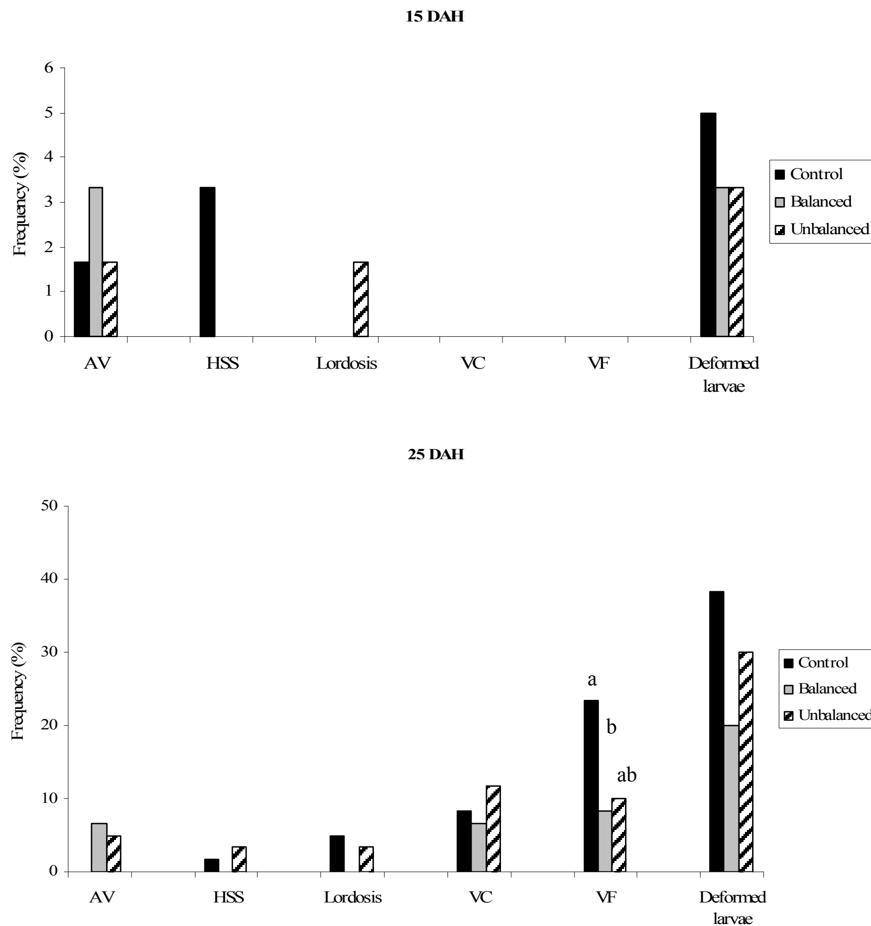

Fig. 4. Deformities observed at the dorsal column in *Diplodus sargus* fed on control, a balanced and unbalanced diets (n=60 larvae *per* treatment). AV- Abnormal shape vertebra, HSS- Supranumeric haemal process, VC- Vertebral compression, VF-Vertebral fusion. Different letters represent significant differences for $p < 0.05$.

6.4. DISCUSSION

The substitution of live feed by a compound diet in most marine fish larvae is still difficult and usually performed some weeks after hatching (Cahu and Zambonino Infante, 2001). In this study a casein microencapsulated diet was introduced at 8 DAH and at 15 DAH the live feed was reduced to 10% of the control treatment.

D. sargus larvae acceptance of the microencapsulated diet was quite high achieving almost 100% seven days after its introduction, meaning microencapsulated diet was given in enough quantities and that larvae were able to ingest it. In previous trials, capsules ingestion by larvae was difficult to achieve and only with a pre-

hydration of the microencapsulated diet was possible to observe active feeding. The supplementation with free amino acids in the microencapsulated diet was reflected in the HPLC analysis indicating the expected losses during production and a successful incorporation of these free amino acids.

D. sargus survival rate was higher in the control group which was an expected result as first feeding larvae are commonly dependent on live feed. This difference in the survival rate could be due to an adaptation phase to the microencapsulated diet where mortality rates in the microencapsulated diet treatments were higher. Low success of weaning marine fish larvae in early phases have been reported before (Kolkovski et al., 1997, Cahu and Zambonino Infante, 2001). A lower survival rate could suggest that the 10% of live feed given to the larvae, after the introduction of microencapsulated diet, were not enough to obtain a similar survival rate between dry and live feed treatments. However, survival results from the microencapsulated diet treatment are quite encouraging even if additional work is required until a full weaning is possible in early life stages for this species.

D. sargus larval growth in this study was higher than the reported by Saavedra et al. (2006) for the same period. Larvae from the different treatments presented no significant differences in dry weight which suggests that although live feed promotes a higher survival it does not promote a higher growth rate. In fact, live feed may not be fulfilling larval nutritional needs. Saavedra et al. (2006) reported unbalanced amino acid profiles when rotifers and *Artemia* are used as diet of *D. sargus*. This is consistent with the results obtained in this study for nitrogen excretion. Larvae from the control group, fasted or fed, presented higher ammonia excretion indicating higher nitrogen losses. When fed, the ammonia excretion seemed to increase significantly in both unbalanced and control treatments. This suggests that *D. sargus* larvae catabolize more protein for

energy production after being fed unbalanced dietary AA profiles. When fasted, other nutrients such as lipids might be used in order to spare protein. Still, larvae fed the balanced diet presented lower nitrogen losses, which is in line with previous works (Conceição et al., 2003), and agrees with the higher nitrogen retention for a AA balanced diet observed for *Solea senegalensis* using an in-vivo tube-feeding method (Aragão et al., 2004a). However, the lower nitrogen loss observed in larvae fed the balanced diet did not reflect a higher growth rate. This may be explained by larvae fed the unbalanced diets (microencapsulated and live fed control) compensating for the AA imbalance through a higher feed intake or that AA were used for metabolic processes other than growth.

Fish larvae have a high requirement of AA for energy (e.g., Rønnestad and Conceição, 2005). Therefore, it might be argued that AA imbalances will be negligible in larvae fed a diet containing approximately 70% crude protein, as happened in the present study. However, this is probably not the case considering the growth rates of up to 30%/day and the low digestive capacity of marine fish larvae (Rønnestad and Conceição, 2005; Tonheim et al. 2005; Conceição et al. 2007). The low capacity to digest proteins and the AA requirements for energy production and growth of marine fish larvae, means AA requirements are likely to be very high and that dietary imbalances will have a burden in terms of nitrogen utilisation (Aragão et al., 2004a) and eventually growth (Conceição et al., 2003).

Concerning fatty acids analysis, a similar pattern between larvae fed a balanced and an unbalanced amino acid microencapsulated diets was observed. This was expected because both diets had the same fatty acid constituents. For ARA and DHA the microencapsulated diets presented higher larval percentages which were translated into higher DHA/EPA ratios. This ratio is often used to measure eggs and larval quality

(Bromage and Roberts, 1995). Larval fatty acid profiles also suggest that the microencapsulated diets used in this study were more efficient in delivering the essential fatty acid to the larvae than enriched rotifers and *Artemia*. A higher level of DHA in the inert diet treatments could also explain the lower proportion of deformed larvae in these groups. Some studies in milkfish evidence that dietary incorporation of DHA induced a decrease of opercular deformities (Gapasin and Duray, 2001). Also, highly unsaturated fatty acids may indirectly regulate some genes involved in skeleton development during ontogenesis (Cahu et al., 2003). Skeletal deformities are one of the constraints to *D. sargus* farming and a frequency of 40% and the control group proves it. Larvae fed the AA balanced diet were almost 50% less affected by deformities than larvae fed live feed only and presented significant lower frequency of vertebral fusions and no case of lordosis. This suggests dietary AA profile might have a role in normal skeleton development.

In conclusion, this study shows that *D. sargus* larvae are still dependent on live feed in order to obtain a high survival rate but when larval quality is concerned, microencapsulated diets seem to promote higher larval quality. Larvae fed rotifers and *Artemia* alone showed very low similarities to the larval AA composition. In contrast, the microencapsulated diets balanced in AA used in this study produced larvae with reduced skeletal deformities, higher DHA levels and lower nitrogen losses.

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Chapter 7

Supplementation of tryptophan and lysine in *Diplodus sargus* larval diet: effects on growth and skeletal deformities

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Abstract

Amino acids are the building blocks for growth and the major energy source during fish larval stages. Deficient amino acids in the diet can be supplemented in early larval stages directly in rotifers or, in later phases, in the dry feed. This might help to overcome the problems of low growth rates and skeletal deformities in fish larvae. In this study, three experimental diets were tested: a balanced diet supplemented with lysine, a balanced diet supplemented with tryptophan and, as control, a balanced diet with no supplementation. Trials were performed with *Diplodus sargus* larvae from 1 to 25 days after hatching (DAH). Dry feed was introduced on 15 DAH in co-feeding with live feed and from 20 DAH larvae were fed only inert diet. The effect of the supplemented diets was assessed in terms of survival, growth rate, skeletal deformities, ammonia excretion and activity of amino acid catabolism enzymes. Results showed a similar survival in all treatments. However, larvae given tryptophan supplementation had lower weight on 25 DAH. This could be related to an increase in the serotonin levels which might have decreased feed intake. No significant differences were found for ammonia excretion, level or type of deformities or enzymatic activity. Larval free amino acid profiles were not significantly different between treatments but there were significant differences between larval ages. Similar changes in the free amino acid profiles were observed from 15 DAH to 25 DAH for all treatments, suggesting larval have a tight regulation of amino acid metabolism. Tryptophan and lysine supplementation failed to improve larval growth, survival, or larval quality.

Keywords: *Diplodus sargus*, amino acids, tryptophan, lysine, ammonia, deformities, growth

7.1. INTRODUCTION

White seabream (*Diplodus sargus*) is a commercially exploited species with a high market price and demand in the Mediterranean countries (Harmelin-Vivien et al., 1995). *Diplodus sargus* is successfully bred (Mazzola et al., 1985) and its larval growth rates are higher than the ones observed for *Sparus aurata* (Saavedra et al., 2006). The main problem related of this species farming has been the high incidence of deformities, especially at the vertebral column (Dores et al., 2006). Vertebral deformities are the most important skeletal malformations as they are usually present in high frequencies in captive fish (Divanach et al., 1996). Vertebral abnormalities such as scoliosis, lordosis and kyphosis are often associated to a poor hatchery performance, lower survival and growth rates and higher susceptibility to stress and disease (Boglione et al., 2001). Deformities at the vertebral column in reared fish are common (Kranenbarg et al., 2005) and can be caused by abnormal swim bladder insuflation (Chatain, 1994), dietary deficiencies (Lim and Lovell, 1978, Baeverfjord et al., 1998, Helland et al., 2005, 2006), high-current velocity (Divanach et al., 1997), among others. Skeletal malformations can have an important economical impact as they can affect both size and shape of the fish and therefore decrease their economical value (Favaloro and Mazzola, 2000; Boglione et al., 2001). In salmonids, scoliosis has been shown to be associated to tryptophan deficiency (Akiyama et al., 1986, Hseu et al., 2003). Halver and Shanks (1960) and Kloppel and Post (1975) found accentuated scoliosis and some lordosis in sockeye salmon and scoliosis in rainbow trout, respectively, in tryptophan-deficient fish.

Another bottleneck to white seabream intensive farming is a decrease in the growth rate when approaching the juvenile stage (Cejas et al., 2003). This could be due to an amino acid (AA) imbalance in the diet which increases AA oxidation and

therefore decreases the food conversion efficiency (Fauconneau et al., 1992). Using an AA diet composition similar to the AA larval requirements is possible to maximize growth and food conversion (Conceição et al., 2003). Saavedra et al. (2006) determined the AA profile of *D. sargus* and observed that when comparing the AA composition of larval carcass and the AA composition of the diet low correlation was found for lysine. For that reason, Saavedra et al. (2006) suggested lysine as a possible limiting amino acid in the commonly used *D. sargus* larval diets. Supplementation of this AA in the diet may enhance protein synthesis as well as reduce the nitrogen losses by improving the use of indispensable amino acid (Kerr and Easter, 1995; Conceição et al. 2003), promoting growth.

The aim of the present study was to investigate effects of dietary tryptophan and lysine supplementation on growth, survival, and skeletal of deformities in *D. sargus* larvae.

7.2. MATERIAL AND METHODS

7.2.1 Husbandry and experimental set-up

The present study was done at the Aquaculture Research Station of IPIMAR, in Olhão, South of Portugal during 2006.

Diplodus sargus eggs were obtained from spontaneous spawning of a mix of wild and farmed broodstock consisting of 38 fish with an average weight of 869 g ± 320 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 200 L conical cylindrical fibreglass tanks at a density of 80 larvae/ L. Three tanks per treatment were used. Before entering the tank, water passed through a biological filter and then through a cartridge filter and a UV filter. The system worked in a semi-closed circuit and water

temperature was maintained constant at 19.1 ± 0.7 º C, oxygen at 6.5 ± 0.4 mg /L and salinity at 37 ± 1 ppt. Water flow started at 0.4 L /min and then was slowly increased with larval age until a maximum of 1 L /min, on 12 DAH. The photoperiod was 24 hours light.

7.2.2. Cross-linked casein-walled capsules formulation

Cross-linked casein-walled capsules were used in this study because of its lower free amino acid leaching properties when compared to other kind of microencapsulated diets (Yúfera et al., 2002). Indispensable AA profiles from larval carcass (Saavedra et al., 2006) were used to formulate the balanced diet, control, as they are commonly used as a good indicator of fish amino acids requirements (Wilson and Poe, 1985; Watanabe and Kiron, 1994). The microencapsulated diet (Table 1) consisted on 70% protein, 31.1% of which was casein (minimum required to obtain this type of capsules). The percentages of the other protein sources in the diet were manipulated in order to get an AA diet profile as similar as possible to the larval carcass profile. AA presumed to be in deficiency were supplemented to the balanced diet in the form of crystalline AA. Once the balanced diet was formulated, tryptophan and lysine were supplemented to the tryptophan and lysine diets, respectively, in the form of crystalline AA. Diets were formulated to be isonitrogenous at intake, taking into account the amino acid leaching estimates by Yúfera et al. (2002) meaning that for all free AA in the diet a leaching allowance was added (*eg.* if the leaching percentage of a certain free AA is 50% its content in the diet was increased by 50%). Microencapsulated diets were prepared according to Yúfera et al. (1999).

7.2.3. Feeding protocol

Feeding protocol consisted of rotifers, *Brachionus plicatilis,* enriched with Protein Selco (INVE Aquaculture, Belgium). One hour before being administrated to the larvae, rotifers were short time enriched with liposomes encapsulating crystalline AA. From 3 to 14 DAH 5 rotifers /ml were fed 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 on 15 DAH. On the first five days 1.5 g was given to each tank and 2g from 20 DAH until the end of the experiment.

Liposomes encapsulating crystalline AA were done according to Barr and Helland (2007), using the crystalline amino acid quantities detailed in Table 2. Inclusion of AA other than Trp and Lys was done to balance the indispensable amino acids (IAA) profile of rotifers according to what was proposed by Saavedra et al. (2006).

Table 1. Ingredient composition of balanced and unbalanced diets (%). [1]AgloNorse, Norway [2] Rieber & Son, Norway [3]CPSP 90, Sopropêche (Boulogne sur Mer, France), [4]Acid hydrolysate, Sigma- Aldrich (Germany),[5] Maialab (Portugal) [6] PREMIX (Portugal) [7]Acofarma (Spain), [8]DL-alpha-Tocopherol Acetate Powder, ICN (USA), [9] Rovimix Stay C-35, Roche [10]Sigma-Aldrich, purity ≥ 98%.

Ingredients (%)	Control	Lysine	Tryptophan
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[10]	5.5	5.5	5.5
Arginine[10]	5.5	5.5	5.5
Phenylalnine[10]	1.0	1	1
Cystein[10]	1.4	1.4	1.4
Lysine[10]	4.2	6.3	4.2
Tryptophan [10]	-	-	0.4

Three experimental diets were prepared according to Yúfera et al. (1999). A balanced diet formulated according to Saavedra et al. (2006) amino acids profiles for *D. sargus* was used as control. This balanced diet has been used in a previous study (Saavedra et al., 2007) and was thought to decrease ammonia excretion as well as slightly reduce skeletal deformities. Tryptophan and lysine diets had the same composition although a supplement of crystalline tryptophan and lysine was added, respectively. The composition of the microencapsulate diet is given in table 2.

Table 2. Amino acid quantities (g AA/million Rotifers) added to the liposomes used to enrich rotifers of the control, lysine (Lys) and tryptophan (Trp) treatments.

G AA/million Br	His	Lys	Arg	Thr	Met	Trp
Control	0.086	0.344	0.129	0.086	0.043	0
Lys	0.086	0.516	0.129	0.086	0.043	0
Trp	0.086	0.516	0.129	0.086	0.043	0.043

7.2.4 Ammonia excretion

Ammonia excretion was determined using pools of 10 fish larvae enclosed in 45 ml spherical glass vials for two hours. The vials were filled with oxygen saturated sea water and sealed without any air. Two replicates from each tank were used as well as three blanks (trials without larvae) *per* treatment. The trials were done at a temperature of 19 ºC and 38 ppt salinity and done for starved and fed larvae.

When the trials were finished, larvae were rinsed in distilled water and frozen for dry later weight determination. Sea water was filtered, 20 µl of sulphuric acid 25% were added and then frozen in 20 ml plastic flasks. Ammonia concentration was determined according to Berthelot (Grasshoff, 1983). Samples were treated with alkaline citrate, sodium hypochloride and phenol in the presence of sodium nitroprussiate which catalyzes the reaction. The blue colour formed by indofenol plus ammonia reaction was measured at 630 nm.

7.2.5 Deformity analysis

On 15 and 25 DAH, 30 larvae were taken from each tank to observe deformities in the vertebral column. Cartilage was stained with Alcian blue and ossified bone was stained with Alizarin Red, according to Gavaia et al. (2000). Deformities were determined using Koumoundouros et al. (2001) development descriptors as a standard. In order to simplify the abnormalities, vertebral column was divided into three regions: trunk vertebrae (1 to 11), caudal vertebrae (12 to 20) and preurostyle vertebrae (21 to 23).

7.2.6 Enzymatic analysis

On 25 DAH, 50 larvae were collected and immediately frozen at -190 ºC to determine the metabolic activity of the following enzymes: alanine amino-transferase (ALAT), aspartate aminotransferase (ASAT) and glutamate dehydrogenase (GDH).

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 ºC. The supernatant was collected and centrifuged again at 15000 g for 20 min at 4 ºC. Enzyme activities were assayed on the supernatant at 37 ºC 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, 1.1 mM NAD, 1mM ADP, 5 mML leucine, started by 100 mML 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.

7.2.7 Sampling and biochemical analysis

Larvae samples were taken in triplicates from every tank on 15 and 25 DAH for free amino acid quantification and on 0, 15 and 25 DAH for dry weight determination. Each sample consisted of 40 on 0 and 15 DAH and 20 larvae on 25 DAH. These samples were frozen in liquid nitrogen at -190 ºC and then freeze-dried (RVT 400, Savant, NY) and weighed.

7.2.7.1 Amino acids analysis

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

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).

7.2.8. Data analysis

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

All amino acids data are expressed in weight percentage of the indispensable amino acids (IAA) pool ((weight of one IAA) x (weight of all IAA) $^{-1}$x 100), to enable comparisons between live feed and larval amino acids profiles. Growth, enzymes and larval deformity were analysed by a non-parametric one-way analysis of variance, Kruskal-Wallis, at a significance level of $P<0.05$. Ammonia and FAA profiles were analysed by a parametric ANOVA using Statistica 5.0.

7.3. RESULTS

7.3.1 Free amino acids of rotifers and microencapsulated diet

The results of rotifers free amino acid analysis showed higher percentages of lysine, arginine, threonine, histidine and methionine in all treatments (Table 3) compared to non-enriched rotifers. Rotifers from lysine treatment had the highest lysine content whereas the ones from tryptophan treatment had the highest tryptophan content (Table 3).

Table 3. Free amino acid profile of *Brachionus plicatilis* (g /100 g AA). Non-enriched rotifers (Blank), rotifers enriched with balanced amino acid liposomes (control), rotifers enriched with balanced amino acid liposomes plus lysine supplementation (Lys) and rotifers enriched with balanced amino acid liposomes plus tryptophan supplementation. Values are mean and standard deviation (n=3).

g/ 100g AA	**Blank**	**Control**	**Lys**	**Trp**
Leu	11.8 ± 0.6	3.0 ± 0.1	3.4 ± 0.3	2.7 ± 0.2
Lys	**11.4 ± 3.0**	**25.1 ± 0.3**	**32.5 ± 0.3**	**31.7 ± 0.7**
Arg	14.2 ± 0.3	18.8 ± 0.0	17.8 ± 0.1	16.8 ± 0.1
Val	16.3 ± 0.4	4.8 ± 0.1	5.3 ± 0.0	4.6 ± 0.1
Thr	7.2 ± 0.9	13.3 ± 0.1	10.4 ± 0.4	12.5 ± 0.2
Ile	11.9 ± 0.3	3.4 ± 0.0	3.5 ± 0.2	3.1 ± 0.2
His	8.7 ± 1.2	18.6 ± 0.4	14.2 ± 0.2	17.3 ± 0.3
Met	3.2 ± 0.1	7.8 ± 0.1	6.1 ± 0.1	6.4 ± 0.2
Cys	1.1 ± 0.2	0.7 ± 0.1	1.0 ± 0.2	0.7 ± 0.0
Phe	2.5 ± 0.1	0.8 ± 0.1	1.1 ± 0.2	0.4 ± 0.3
Tyr	11.4 ± 0.0	3.8 ± 0.1	4.5 ± 0.1	3.4 ± 0.2
Trp	**0.3 ± 0.3**	**0.2 ± 0.0**	**0.3 ± 0.0**	**0.7 ± 0.0**

7.3.2. Growth, survival and larval free amino acid content

The relative growth rate (RGR) did not differ significantly between treatments at the age of 15 DAH ($F_{(2,6)}$ =2.3, p= 0.17). However, significant differences were found on 25 DAH ($F_{(2,6)}$ =9.3, p= 0.04), when larvae fed on the diet supplemented with tryptophan had lower dry weight as well as lower RGR (Table 4). At the end of the experiment no significant differences were found in survival (Table 4).

Table 4. Dry weight, relative growth rate and survival of *Diplodus sargus* fed a balanced diet, a balanced diet supplemented with lysine (Lys) and a balanced diet supplemented with Tryptophan (Trp). Values are mean and standard deviations (n=3 tanks, except for growth; n=20 larvae).

	Control	Lys	Trp
Dry weight (mg)			
0	0.04± 0.00	0.04 ± 0.00	0.04 ± 0.00
15	0.26 ± 0.01	0.31 ± 0.02	0.30 ± 0.00
25	0.97 ± 0.15[a]	0.95 ± 0.12 [a]	0.77 ± 0.17 [b]
RGR (%/day)			
15	13.27 ± 0.30	14.56 ± 1.17	14.43 ± 0.74
25	14.52 ± 0.45[a]	14.19 ± 0.93 [a]	11.80 ± 1.02 [b]
Survival (%)	6.2 ± 0.2	5.0 ± 1.2	4.7 ± 1.4

No significant differences were found in larval free amino acid content between treatments on the age of 15 and 25 DAH (Table 5). However, significant differences were found for almost all free amino acids when 15 and 25 DAH larvae contents were compared, with the exceptions of arginine, threonine, methionine and cysteine.

Table 5. Free indispensable amino acid profile (g IAA/ 100g all IAA) of *Diplodus sargus* larvae on the age of 15 DAH and 25 DAH. Values are mean and standard deviation (n=3).

	15 DAH			25 DAH		
	Control	**Lys**	**Trp**	**Control**	**Lys**	**Trp**
Leu	7.1 ± 0.4	7.1 ± 1.0	6.7 ± 0.7	5.7 ± 0.8	5.7 ± 0.9	4.9 ± 0.8
Lys	8.5 ± 0.7	8.0 ± 1.4	8.8 ± 0.6	13.4 ± 0.9	12.5 ± 1.3	12.1 ± 1.4
Arg	9.2 ± 0.5	9.9 ± 0.6	10.4 ± 1.2	5.9 ± 0.9	8.1 ± 1.7	7.9 ± 1.5
Val	5.8 ± 0.3	6.0 ± 0.4	5.5 ± 0.4	2.7 ± 0.1	3.8 ± 0.6	3.6 ± 0.7
Thr	10.5 ± 0.6	10.7 ± 0.4	10.7 ± 0.7	11.7 ± 1.0	13.0 ± 1.0	12.5 ± 1.3
Ile	4.4 ± 0.4	4.6 ± 0.2	4.3 ± 0.7	2.6 ± 0.6	2.7 ± 0.3	2.7 ± 0.5
His	2.2 ± 1.0	2.3 ± 0.7	1.8 ± 0.9	2.1 ± 0.7	1.3 ± 0.2	1.0 ± 0.2
Met	8.2 ± 0.3	8.4 ± 0.4	8.6 ± 0.6	7.1 ± 1.6	8.9 ± 0.9	8.7 ± 0.5
Cys	10.7 ± 0.8	10.1 ± 0.3	10.5 ± 0.4	10.2 ± 2.0	12.1 ± 1.4	11.4 ± 0.7
Phe	12.7 ± 0.3	12.6 ± 0.1	12.3 ± 0.8	5.8 ± 1.3	7.6 ± 0.7	7.2 ± 0.5
Tyr	2.4 ± 0.5	2.3 ± 0.1	2.2 ± 0.3	1.2 ± 0.2	1.1 ± 0.2	1.1 ± 0.1
Trp	18.3 ± 0.8	18.2 ± 0.2	18.1 ± 0.5	31.7 ± 5.5	23.1 ± 2.1	27.1 ± 2.2

7.3.3. Larval deformities

A high frequency of deformities in the vertebral column was found in this study. On 15 DAH there was almost no serious malformations such as scoliosis, lordosis or kyphosis. On 25 DAH, kyphosis was mainly found in the trunk vertebrae, and scoliosis in the caudal vertebrae (Figure 1).

Hypertrophic vertebrae were the most common abnormal feature on both ages, reaching almost 70% on 25 DAH larvae. This malformation affected especially the trunk and preurostyle vertebrae on 15 DAH although on 25 DAH the incidence was high in all vertebrae categories. The presence of fusions in the last three vertebrae was also registered in all treatments on 15 DAH. In some larvae, on 25 DAH, a supplementary vertebra was observed (Fig. 1).

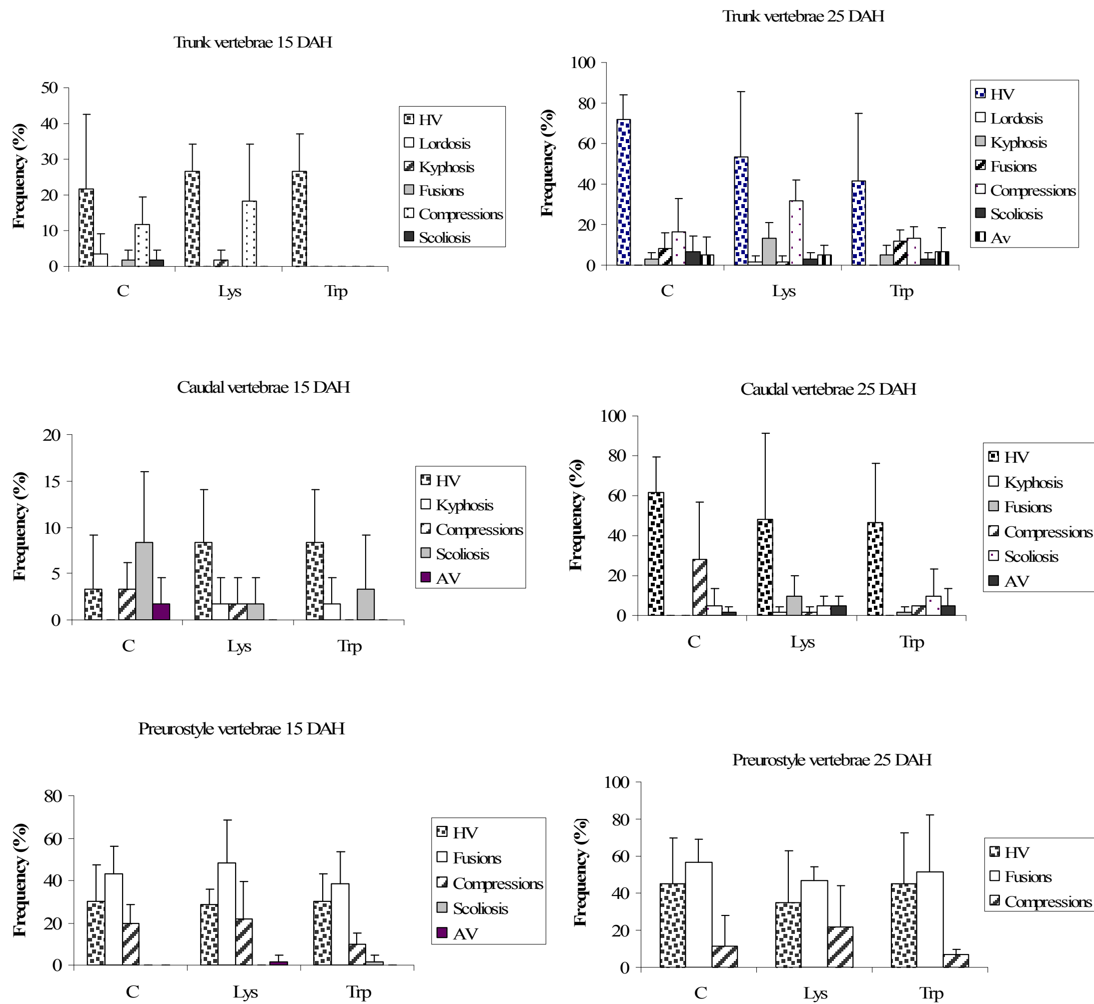

Fig. 1. Frequency of deformities at the vertebral column of *Diplodus sargus* observed in larvae from control, lysine and tryptophan treatments (n=60 larvae *per* treatment) on 15 DAH and 25 DAH (trunk vertebrae- 1 to 10, caudal vertebrae- 11-20, preurostyle vertebrae-21-23.HP-hypertrophic vertebrae, AV- abnormal shape vertebrae).

7.3.4. Enzymatic activity

There were no significant differences in enzymatic activity between treatments. Aspartate aminotransferase showed the highest activity and glutamate dehydrogenase had the lowest activity for all treatments (Table 6).

Table 6. Specific activity (IU/ g protein) of amino acid metabolic enzymes in 25 DAH *Diplodus sargus* larvae from control, lysine and tryptophan diets. Values are mean and standard deviations (n=3)

	CONTROL	LYS	TRP
ALANINE AMINOTRANSFERASE (ALAT)	405.8 ± 41.5	479.5 ± 93.2	502.0 ± 165.5
ASPARTATE AMINOTRANSFERASE (ASAT)	1131.0 ± 319.8	1055.3 ± 241.3	1079.3 ± 145.8
GLUTAMATE DEHYDROGENASE (GDH)	84.5 ± 7.5	88.1 ± 18.0	98.5 ± 46.1

7.3.5. Ammonia excretion

No significant differences between treatments were found for the mass specific ammonia excretion between treatments within the same stage (Fig. 2). However, differences were found for ammonia excretion between fasted and fed larvae from control ($F_{1,4}$=9.1, p=0.04) and lysine ($F_{1,4}$=21.1, p=0.01) treatments (Fig.2).

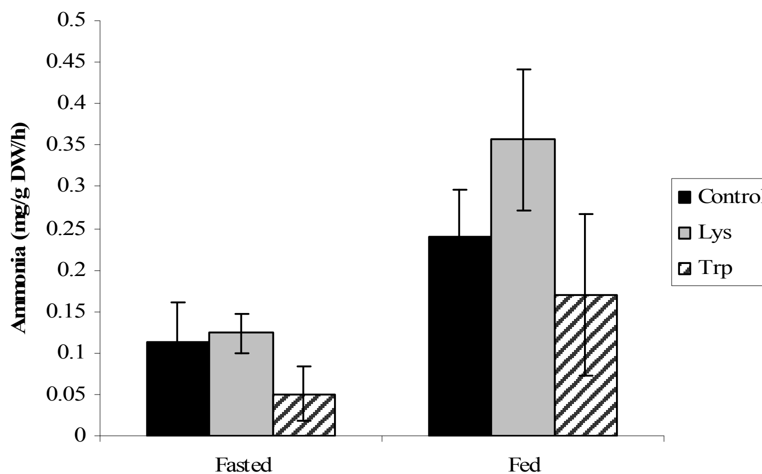

Fig. 2. Mass-specific ammonia excretion of fasted and fed from control, lysine and tryptophan treatments. Values are mean and standard deviations (n=5 of pools of 10 larvae).

7.4. DISCUSSION

Substitution of live feed for compound diets in early larval stages has been difficult to achieve with success in terms of survival and growth (Cahu and Infante, 2001). Weaning marine fish larvae is usually done some weeks after hatching (Cahu and Infante, 2001). In the present study, weaning started on 15 DAH and survival and growth rates were quite promising, although the survival was low compared to that found in later weaned larvae. Growth was similar to that reported by Saavedra et al. (2006) except for the tryptophan treatment. Larvae given a supplement of tryptophan had lower dry weight as well as lower RGR. Similar effects of trypthophane supplemantation have been reported by Papoutsoglou et al. (2005a) for rainbow trout juveniles and Papoutsoglou et al. (2005b) for white sea bass juveniles. In both studies a depression of growth rate and food conversion ratios was observed but rainbow trout showed higher feed intake whereas sea bass and a lower feed consumption (Papoutsoglou et l., 2005a,b). In the current study, larval guts were observed in order to make sure larvae were ingesting the microencapsulated diet but feed intake was not quantified. Tryptophan is the precursor of serotonin, which controls appetite. An excessive dose of tryptophan might increase the serotonin levels in a way that decreases the food intake. Hseu et al. (2003) reported a positive effect on survival and growth in juvenile grouper *Epinephelus coioides* when the diet was supplement up to 1% with tryptophan. *D. sargus* diet had 1.4% of tryptophan and it was 2% of the total AA components. Thus, tryptophan supplementation might have been slightly excessive. Another possible explanation is a negative impact of tryptophan in the taste of the microencapsulated diet. Tryptophan seems to have a deterrent gustatory property for fish such as carp (Kasumyan and Morsi, 1996) which can lead to a decrease in the food consumption.

Ingestion of the microencapsulated did not seem to be a limiting factor of the casein diet because the observed *D. sargus* guts were full with microencapsules. This is in accordance with Fernández-Díaz and Yúfera (1997) who reported good ingestion rates for the casein-based diets for sea bream. Digestion, however, might have been difficult (Cahu and Infante, 2001) as *D. sargus* do not have a fully developed digestive tract with functional stomach until approximately 30 days after hatching (Cara et al., 2003, Ortiz-Delgado et al., 2003)). Due to this and in order to increase larval survival, rotifers were given to larvae in co-feeding with the dry feed as proposed by Yúfera et al. (1999, 2002).

Successful supplementation was done using liposomes encapsulating free amino acid fed to rotifers. This is an important step in amino acid live feed supplementation which until now has been difficult to achieve (Aragão et al., 2004), leaving dry feed as the only possible amino acid delivery system to early larval stages. This AA supplementation did not increase the levels of tryptophan or lysine in the larval whole body AA pool. This might have been because AA pool results from dietary amino acid supply does not always reflect the dietary amino acid profile (Conceição et al. 2003), as larval amino acid metabolism also has an impact on the AA pool. However, some studies reported high correlations between diet and free amino acid profiles in juveniles of rainbow trout and European eel (Ogata et al. 1985). In this study this was not observed possibly due to the high protein turnover in fish larvae (Conceição et al. 1997, Jarvis and Ballantyne, 2003). Absorbed dietary AA can be immediately used for protein synthesis, especially if they are in deficiency, or used for catabolism if they are in excess.

An interesting result recorded in this study was the similar changes in the free amino acid profiles from 15 to 25 DAH in all treatments tested. It seems that larvae,

already on this early stage, regulate within a narrow range their free pool of amino acids.

The activity of amino acid catabolism enzymes was quantified in the present study. If a higher AA catabolism had occurred in same treatment then a high enzymatic activity would be expected. This was not observed in the present study. Also ammonia excretion was approximately the same in all treatments. These results suggest that neither lysine nor tryptophan were limiting protein synthesis in *D. sargus* larvae (Conceição et al., 2003; Aragão et al., 2003). However, significant differences were found in the present study in ammonia excretion between fed and starved larvae. When larvae were fed the excreted ammonia levels increased up to three times. This agrees with the findings for juveniles of *Dicentrachus labrax* (Robaina et al., 1990) and *Sparus aurata* (Gómez-Requeni et al., 2003) that has a high increase in ammonia excretion few hours after being fed, resulting from postprandial metabolism.

A high frequency of skeletal malformations was observed in larvae at 15 and 25 DAH, and was not affected by lysine or tryptophan supplementation. On these ages, vertebral thickening, called hypertrophic vertebrae, was the most common abnormality, reaching 70% in control treatment on 25 DAH in the trunk vertebrae. Lewis and Lall (2006) reported similar results for Atlantic halibut larvae where the presence of hypertrophic vertebrae was the malformation with highest frequency. In Atlantic halibut this type of malformation seems to occur more often in earlier larval stages and as development proceeds, the smaller vertebrae increases and result in similar size and shape as normally found in later phases (Lewis et al., 2004). The same has been found in previous studies for *D. sargus* larvae (Saavedra et al., unpublished results). When the growth rate increases the incidence of this malformation seems to decrease.

Approximately half of the observed larvae had fusions in the preurostyle vertebrae on 25 DAH, slightly less on 15 DAH. In general, a higher proportion of abnormalities was registered from 15 to 25 DAH. This corresponds with findings of Lewis and Lall (2006) for Atlantic halibut and those of Witten and al. (2006) for Atlantic salmon. Major vertebral abnormalities such as lordosis, kyphosis and scoliosis were registered in small percentages and only on 25 DAH. Lordosis is usually pointed as the most frequent deformity in reared fish (Kranenbarg, 2005) but in this study the highest registered percentage was 3%. Hattori et al. (2003) reported similar incidence of lordosis for *Pagrus major*. This is possibly because measures have been taken to prevent problems regarding swimbladder inflation failure and high swimming activity (Chatain, 1994). Almost no such deformities were observed in the preurostyle vertebrae and the highest incidence was in the caudal vertebrae (approximately 7% scoliosis) and in the trunk vertebrae (approximately 8% kyphosis). Nevertheless, the presence of vertebrae fusions and compressions may have a negative effect on the viability of these larvae.

In conclusion, tryptophan supplementation failed to prevent skeletal deformities in *D. sargus* larvae as well as it seemed to have a negative effect on larval growth, probably either by inhibiting feed consumption or by decreasing the palatability of the diet. Lysine supplementation did not seem to bring any advantage when compared to the balanced AA diet.

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Chapter 8

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 enriched with liposomes encapsulating free AA in order to obtain a balanced AA profile until 20 DAH. The effects of the supplemented diets were assessed in terms of larval survival, growth rate, skeletal deformities, enzyme activities of AA catabolism, ammonia excretion and resistance to a temperature stress test. The experimental diets resulted in similar larval survival, growth, enzyme activities of AA catabolism and nitrogen excretion in all treatments. High levels of skeletal deformities were registered and significant differences were found between control and the phenylalanine treatment for the percentage of vertebral compressions 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 found for the larvae fed the diet supplemented with phenylalanine and tyrosine. The results found in the present study suggest that supplementation of phenylalanine / tyrosine in fish diets might be useful, in order to reduce skeletal deformities, and to reduce mortalities caused by stress. The present study confirms AA requirements sufficient for covering growth and survival may be insufficient to cover other metabolic processes.

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

8.1. INTRODUCTION

White seabream, *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 most common in Greece and in Portugal there is already some small scale production (Sá, 2007). *D. sargus* has also been in involved in restocking programmes in the southern coast of Portugal where the landings decreased from 200 to 75 tonnes 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, the sole 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 can therefore be related to

the control of stress in fish (Lehnert and Wurtman, 1993). Diets supplemented with tyrosine have been shown to reduce acute stress such as cold exposure in rodents (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 larval stages using live feed. Inert diets are still more efficient in terms of supplementation because a larger change in the AA profile is possible (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 and production. Yúfera et al. (1999, 2002) developed cross-linked casein-walled capsules and showed this diet was able to substitute with some success live feed in early larval stages of *Sparus aurata*. Saavedra et al. (2007a,b) presented interesting results introducing this microencapsulated diet in *D. sargus* early larval feeding protocol. Larvae fed the microencapsulated diet had a lower survival rate when compared to the live feed protocol but the growth rate was similar and when larvae were fed an AA balanced diet they had higher larval quality and lower nitrogen excretion (Saavedra et al., 2007a).

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

8.2. MATERIAL AND METHODS

8.2.1. Husbandry and experimental set-up

This study was done at the Aquaculture Research Station of 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 g ± 319.8 g, a biomass and tank density of 33 kg and 4 kg/m^3, respectively.

After being 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, then through a cartridge and sterilized by a UV filter before entering the larval tank. The system worked in a semi-closed circuit and water temperature was maintained constant at 20.3 ± 0.3 º C, oxygen at 6.3 ± 0.4 mg /l and salinity at 37 ± 1 ppt. Water flow started at 0.4 l /min and was then slowly increased with larvae age until a maximum of 1 l /min, on 20 DAH. The photoperiod was 24 hours light.

The trial lasted 25 days and three dietary treatments were tested: a control diet that represented a balanced AA profile, a balanced diet supplemented with phenylalanine and a balanced diet supplemented with tyrosine.

8.2.2. 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 fair indicator of fish amino acids requirements (Wilson and Poe, 1985; Watanabe and Kiron, 1994;

Conceição et al., 2003). The microencapsulated diets consisted of 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 were 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).

8.2.3. 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.

The algae *Tetraselmis sp.* ($12x10^3 \pm 2x10^3$ cells/ml) and *Isochrysis galbana* ($73x10^3 \pm 14x10^3$cells/ml) were added to the larval rearing 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 so that the supplemented amount would be the same in both treatments.

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. [1]AgloNorse, Norway [2] Rieber & Son, Norway [3]CPSP 90, Sopropêche (Boulogne sur Mer, France), [4]Acid hydrolysate, Sigma- Aldrich (Germany),[5] Maialab (Portugal), [6] PREMIX (Portugal) [7]Acofarma (Spain), [8]DL- alpha-Tocopherol Acetate Powder, ICN (USA), [9] Rovimix Stay C-35, Roche [*]Sigma-Aldrich, purity ≥ 98%, [+] Fluka (Germany) ≥ 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
Phenylalnine[*]	1	2	1
Cystein[*]	1.4	1.4	1.4
Lysine[*]	4.2	4.2	4.2
Tyrosine[*]	-	-	4.7

8.2.4. Ammonia excretion

Ammonia excretion trials were done using 10 fish larvae per tank enclosed in 45 ml spherical glass vials for two hours. Trials were done 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 done at a temperature of 20 ºC and 38 g/l salinity. When the trials were finished, larvae were rinsed in distilled water and frozen for dry weight determination. The sea water was filtered and 20 µl of sulphuric acid 25% was added and the sample was then frozen in 20ml plastic flasks for later 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 µmol g DW^{-1} h^{-1}) was calculated as follows:

$MNH_4^+ = \Delta[NH_4^+]\ V_{H20}\ DW^{-1} \Delta T^{-1}$, where $\Delta[NH_4^+]$ is the difference between the sample and the blank water, V_{H2O} 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.

8.2.5. Deformity analysis

On 25 DAH, 30 larvae were taken from each tank to quantify 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, the development descriptions by Koumoundouros et al. (2001) 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).

8.2.6. Enzymatic analysis

On 25 DAH, 50 larvae per tank were collected and immediately frozen at -196 ºC 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 ºC. The supernatant was collected and centrifuged again at 15000 g for 20 min at 4 ºC. Enzyme activities were assayed on the supernatant at 37 ºC 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 mML leucine, started by 100 mML 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.

8.2.7. Stress Test

On 25 DAH a temperature stress test was done. 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 sea water volume to 20 ml at water temperature of approximately 21ºC. The larvae were then transferred into a 2 l bucket containing 400 ml of sea water at a temperature of 10 ºC. Five minutes later, water at room was added until a final volume of 1.8 l was reached. After waiting five more minutes the number of dead larvae is counted thereby obtaining the survival rate. This trial was repeated three times for each tank (nine replicates *per* treatment).

8.2.8. Sampling and biochemical analysis

On day 0, 15 and 25 DAH 40, 40 and 25 larvae per tank were sampled for dry weight determination, respectively. These samples were frozen in liquid nitrogen at -196 ºC and then freeze-dried (RVT 400, Savant, NY) and weighed.

8.2.8.1 Amino acids analysis

Protein-bound amino acids samples (microencapsulated diet) were hydrolysed in 6M HCL at 108ºC 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).

8.2.9. Data analysis

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

All amino acid 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 feed 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 Statistisca© (v.5.5). Data from the stress test were transformed (arcsin) to satisfy ANOVA parametric assumptions. Post-Hoc analysis was done using Scheffé test.

8.3. RESULTS

8.3.1 Free Amino acids profile of rotifers and microencapsulated diet

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

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

	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

The microencapsulated diet supplemented with tyrosine had higher tyrosine content whereas the diet supplement with phenylalanine had a higher relative content of phenylalanine when compared to the other treatments (Table 4).

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	**PHE**	**TYR**
IAA			
Leu	15.3 ± 0.5	14.4 ± 0.4	12.4 ± 0.4
Lys	14.5 ± 0.2	12.3 ± 0.8	11.3 ± 0.6
Arg	13.6 ± 1.2	11.3 ± 0.3	11.0 ± 0.9
Val	9.5 ± 0.8	8.8 ± 0.3	8.0 ± 0.3
Thr	10.2 ± 1.0	8.9 ± 0.3	8.4 ± 0.7
Ile	7.9 ± 0.8	7.2 ± 0.4	6.6 ± 0.1
His	4.5 ± 0.1	4.3 ± 0.0	3.8 ± 0.2
Met	4.5 ± 0.6	4.8 ± 0.4	3.3 ± 0.9
Cys	1.7 ± 0.3	9.9 ± 2.8	7.4 ± 2.6
Phe	10.0 ± 0.2	10.2 ± 0.4	7.9 ± 0.3
Tyr	8.4 ± 0.2	7.8 ± 0.3	20.0 ± 1.3
DAA			
Glu+Gln	16.2 ± 0.1	16.6 ± 0.2	15.4 ± 0.5
Ala	4.4 ± 0.2	4.1 ± 0.2	3.6 ± 0.1
Gly	4.2 ± 0.4	4.3 ± 0.0	3.8 ± 0.1
Asp+Asn	6.9 ± 0.2	8.0 ± 0.0	7.3 ± 0.2
Ser	5.3 ± 0.2	5.4 ± 0.0	4.9 ± 0.1
Pro	8.0 ± 0.1	8.2 ± 0.2	7.6 ± 0.3

8.3.2. Growth and larval survival

There were no significant differences in the relative growth rate (RGR) between treatments on the ages 15 and 25 DAH (Table 5). Nor were differences found in larval dry weight from the different treatments (Table 5).

At the end of the experiment the survival rate was 6.0 ± 1.4 % for the control treatment, 4.8 ± 1.4 % for the tyrosine supplemented diet and 4.8 ± 0.9 % for the phenylalanine supplemented diet (Table 5). No significant differences were found for survival.

Table 5. Growth and survival of *Diplodus sargus larvae* fed a balanced diet (control), a balanced diet supplemented with tyrosine (Tyr) and a balanced diet supplemented with phenylalanine (Phe). Values are mean and standard deviations (for weight n=3).

	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

8.3.3. Enzymatic activity

Alanine aminotransferase (ALAT) had 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.

Table 8. 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 ± 253.3	910.6 ± 229.7	1059.4 ± 114.7
ASPARTATE AMINOTRANSFERASE (ASAT)	2396.2 ± 384.0	2404.5 ± 509.7	2242.1 ± 195.3
GLUTAMATE DEHYDROGENASE (GDH)	324.2 ± 46.0	328.0 ± 49.5	300.4 ± 26.5

8.3.4. Ammonia excretion

There were no significant differences in ammonia excretion between treatments or between fasted and fed larvae (Fig. 1).

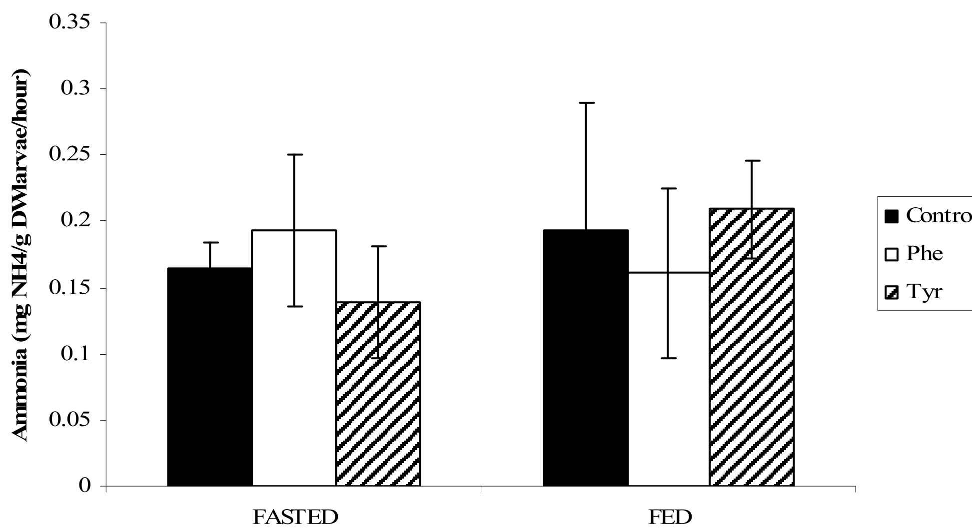

Fig. 1. Mass-specific ammonia excretion of fasted and fed from control, tyrosine and lysine treatments on 25 DAH. Values are mean and standard deviations (five replicates *per* treatment).

8.3.5. 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 on 25 DAH, larvae fed a diet supplemented with tyrosine had significantly higher survival rate when compared with the other treatments (Fig. 2).

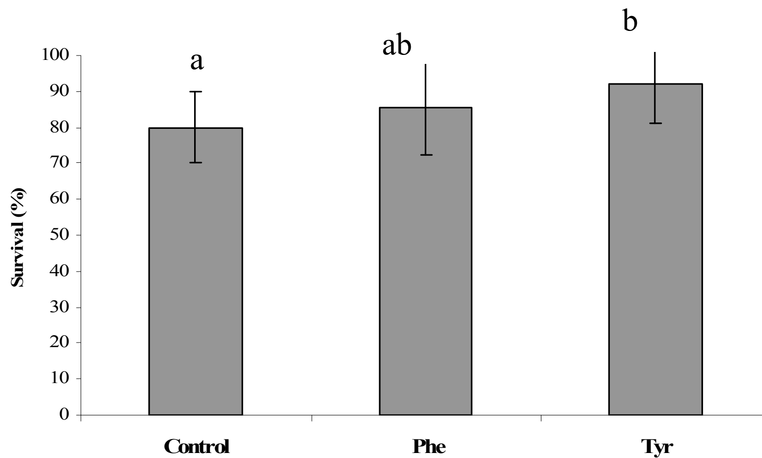

Fig 2. *Diplodus sargus* larval survival rate after being subjected to a temperature stress test. Values are mean and standard deviations (n=9). Different letters represent significant differences.

8.3.6. Larval deformities

The incidence of deformities in the vertebral column was 85 ± 22% in the control, 72 ± 7 % in phenylalanine treatment and 78 ± 6 % in the tyrosine treatment (Fig.3). Scoliosis was found in a maximum of 8 ± 14 % in the control, kyphosis was 3 ± 7 % in the phenylalanine treatment and no lordosis cases were found in the present study (Fig. 3). Most deformities were located in the preurostyle region, mainly total or partial vertebrae fusions and hyperthropic vertebrae. 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). The caudal region had the lowest deformity incidence. In this region mainly abnormal shape vertebrae were found, which were usually between the 16 and

the 20 vertebrae. In all vertebral column regions there were 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).

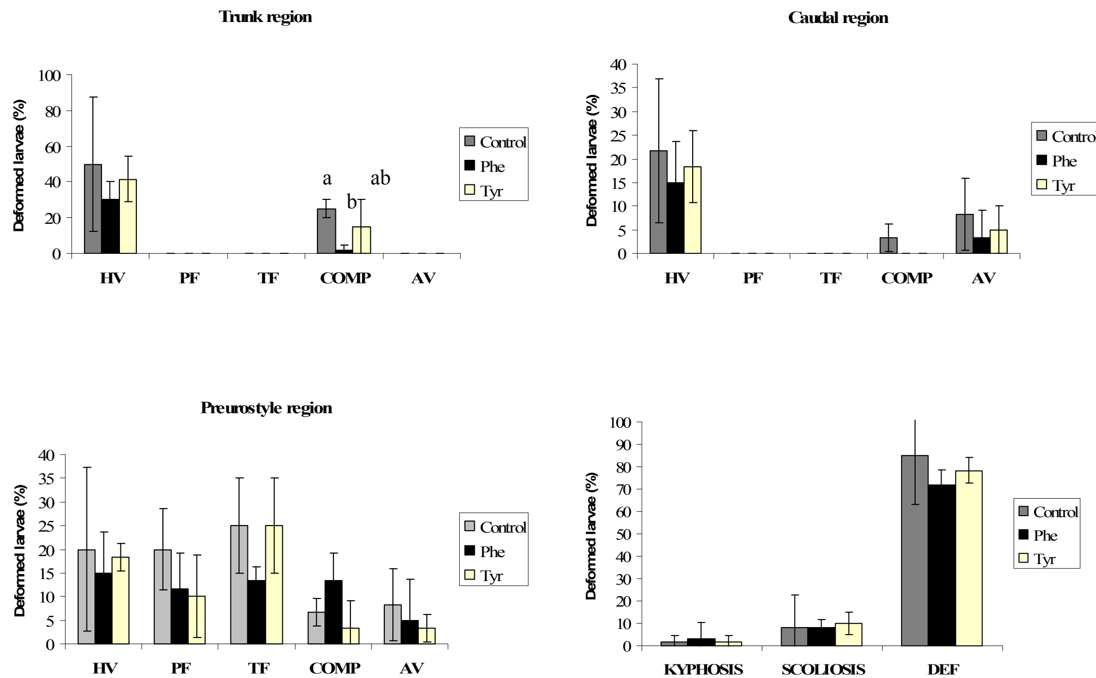

Fig 3. Percentage of deformities in the vertebral column of 25 DAH *D. sargus* larvae. from control, phenylalanine and tyrosine treatments Values are mean and standard deviations (n=60 larvae *per* treatment). Different letters represent significant differences. HV- hypertrophic vertebrae, PF- partial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

8.4. DISCUSSION

The aim of the present study was to find the effects of diets supplemented either with phenylalanine or tyrosine on *D. sargus* larval growth, survival, and quality. Supplementation was done using enriched rotifers on early larval stages and a microencapsulated diet in later larval stages. FAA supplementation of rotifers was done successfully using the method descried by Barr and Helland (2007). Although this technique has been a major advance, survival rates were not higher compared to other

D. sargus studies (Saavedra et al., 2007a) where a casein microencapsulated diet replaced 90% of the live feed on 15 DAH. The survival rates were approximately the same to previous work with *D. sargus* weaning on 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 (co-feeding period) was probably a critical period, especially to the smaller larvae, and may have led to an increase in the mortality rate that does not occur using 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 limitations faced 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. That 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 on 25 DAH, ranging from 72% to 85% of the fish. However, only a small proportion of larvae had serious skeletal malformations such as kyphosis and scoliosis which affected less than 10% of the larvae. 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, Sfakianakis et al., 2006). In the present study the preurostyle region was most affected by deformities in all the treatments. In *Solea senegalensis*, Gavaia et al. (2002) observed that more than half of the deformities occurred in the caudal region.

The most common skeletal abnormality in the present study was vertebral fusions, where approximately 25% of the fish in the control and tyrosine treatments and 15% in the phenylalanine treatment were affected. Elevated incubation temperature, either as temperature shock or as long term effects has shown to induce vertebral fusions (Baeverfjord et al., 1999, Ørnsrud et al., 2004 and . Other causal factor that has been shown or discussed to induce vertebral malformations are handling stress, vaccination, sub-optimal environmental conditions, and malnutrition (Baeverfjord et al., 1998 and

The only significant difference found for deformities was in the number of compressed vertebrae in the trunk region of the vertebral column. The control group had 30% compressed vertebrae, which was significantly higher than found 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 marginally deficient or less available in the microencapsulated diet and that 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, which could explain the results of the present study.

Hypertrophic vertebrae were the most common deformity observed in this study. 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 found for 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 the capacity of fish larvae to convert phenylalanine to tyrosine may be insufficient or that higher dietary concentration of phenylalanine is need in order to achieve higher tyrosine availability in the fish.

In conclusion, tyrosine and phenylalanine supplementation in *Diplodus sargus* diet did not seem to cause any effect on growth or survival. However, phenylalanine decreased 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 that may be higher than the requirements sufficient for growth.

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Chapter 9

General discussion

9.1. Amino acid profile of *Diplodus sargus* is stable throughout larval ontogeny and there are likely dietary amino acid imbalances in live feed

Diplodus sargus amino acid (AA) profile is relatively constant throughout its larval development as no changes were observed between larval ages (Chapter 3). This is similar to other species such as common dentex (Tulli and Tibaldi, 1997) and striped trumpeter (Brown et al., 1995). However, a similar species such as seabream (*Sparus aurata*) has been shown to present changes its AA profiles during ontogeny (Aragão et al., 2004). Constant AA profiles of *D. sargus* might be related to pattern of growth of the different body parts close to isometry, which means that maybe its high growth rate and a light metamorphosis do not imply changes in AA profile on different larval ages. Several AA were found to be in potential deficiency when larval AA profile was compared to dietary AA profiles. These AA were arginine, cysteine, histidine, threonine and lysine as they presented the highest relative differences between the diet and larval AA profiles. These apparently deficient AA were almost the same, independently on the live prey considered.

Live feed showed several AA imbalances, especially rotifers. This was also observed for seabream (Aragão et al., 2004), Senegalese sole (Aragão et al., 2004), turbot (Conceição et al., 2003) and African catfish (Conceição et al., 1998). Imbalances between dietary and larval AA profiles are likely to lead to unavoidable AA losses and increase nitrogen excretion (Conceição et al., 1998, Aragão et al., 2004). To overcome these AA imbalances supplementation should be considered in order to obtain better growth rates as well as higher food conversion ratios. Supplementation can be done directly on live feed using free AA liposome enrichment (Barr and Helland, 2007), or live feed can be replaced by inert diets with a balanced AA profile. Although early fish larvae weaning is generally difficult (Kolkovski et al., 1997; Cahu and Zambonino

Infante, 2001), Yúfera et al. (1999, 2002) have developed cross-linked casein-walled capsules which seem to present successful results for seabream. However, in order to obtain better larval survival results it is advisable to use the microencapsulate diet in co-feeding with live feed (Yúfera et al., 1999, 2002).

9.2. A balanced amino acid diet improves *Diplodus sargus* larval quality and reduces nitrogen excretion

Nutritional imbalances between dietary and larval AA profiles decrease larval growth and food conversion rates. AA imbalances will lead to unavoidable AA losses and increase nitrogen excretion (Conceição et al., 1998, Aragão et al., 2004). Better feeding efficiencies and higher growth rates were reported for gilthead seabream juveniles when fed diets supplemented with indispensable AA, resembling larval AA whole body (Gómez-Requeni et al., 2003). In this thesis, the AA imbalances described in chapter 2 were overcome using two approaches: with direct supplementation of the live feed (Chapter 7 and 8), enriching rotifers and *Artemia* with the potentially limiting AA; and with the inclusion of free AA in the inert diet (chapter 6, 7 and 8). *Diplodus sargus* larval stages are still live feed dependent and therefore the use of an inert balance diet in an earlier larval stage had a negative impact on larval survival (Chapter 6). Difficulties in early weaning have been reported before for several fish species (Kolkovski et al., 1997, Cahu and Zambonino Infante, 2001). The higher mortality observed in the larval groups fed the inert diet may be related to an adaptation period to the diet and/or to the digestive tube of larvae which might not be fully developed yet (Cara et al., 2003) and therefore might not be able to digest completely the diet. It is also possible that a co-feeding with a percentage higher than 10% of live feed may make a difference on larval survival.

The use of a balanced AA inert diet improved several larval quality parameters such as the incidence of certain skeletal deformities and decreased nitrogen excretion (Chapter 6). The reduction on the nitrogen excretion was observed in previous studies (Conceição et al., 2003) and agrees with the higher nitrogen retention observed in *Solea senegalensis* fed an AA balance diet using an in-vivo tube-feeding method (Aragão et al., 2004). However, in *Diplodus sargus* larvae, the higher nitrogen retention did not reflect a higher growth rate (Chapter 6). This could be explained by the use of AA for metabolic processes other than growth or that larvae fed the unbalanced diet might have ingested more food to compensate the dietary AA imbalances. Growth was not affected by the change into a microencapsulated diet suggesting that although live feed is crucial to achieve a higher larval survival is not promoting a better larval growth.

An AA balanced inert diet positively affected larval quality by reducing the percentage of skeletal deformities, which are constraining *Diplodus sargus* farming. Larvae fed the AA balanced diet were almost 50% less affected by deformities than larvae fed live feed (Chapter 6). It especially reduced the frequency of vertebral fusions and lordosis suggesting that dietary AA profile may have a role in normal skeleton development. The frequency of deformities in all groups fed a balanced diet, supplemented or not (Chapter 6, 7 and 8), was also lower compared to the estimated *D. sargus* larval deformity pattern (Chapter 2), with the exception of the hypertrophic vertebrae. A high incidence of this skeletal malformation has also been observed for Atlantic halibut post larvae (Lewis and Lall, 2006). This type of deformity is not very serious as it results from an uneven vertebral growth and as larvae grow, the vertebrae shape and size get more homogeneous (Lewis and Lall, 2004).

Finally, the use of a casein microencapsulated diet showed advantages on larval fatty acids composition. Larvae fed these diets showed higher content of docohexaenoic

acid (DHA) and arachidonic acid (ARA) as well as higher DHA/ EPA ratios. This ratio is often used to measure egg and larval quality (Bromage and Roberts, 1995). These diets were also more efficient in delivering the essential fatty acid to the larvae than enriched rotifers and *Artemia*.

9.3. Skeletal deformities are a bottleneck for *Diplodus sargus* farming: Amino acid supplementation may reduce its incidence

Vertebral deformities are the most important skeletal malformations and a high frequency has been observed in several species wild or reared in captivity (Divanach et al., 1996). These vertebral malformations are often associated with reduced hatchery performance including lower survival rates, poor growth and increased susceptibility to stress and disease (Boglione et al., 2001). Skeletal deformities have been pointed to be bottlenecking *D. sargus* farming and when a skeletal deformities pattern was estimated throughout larval ontogeny, frequencies of approximately 50% were observed, starting as early as 13 DAH (Chapter 2). Before this larval age the most common deformity was an abnormal curvature of the vertebral column. This suggests that only a very small proportion of deformities exist in newly hatched larvae and most malformations start appearing with the development of more skeletal structures. In general, frequency of deformities in *D. sargus* increased with larval size. The same was reported in Atlantic halibut larvae and juveniles (Lewis and Lall., 2004).

From all skeletal deformities, vertebral compressions were the most common, occurring throughout ontogeny. Serious skeletal deformities such as scoliosis, lordosis and kyphosis were usually present in percentages lower than 15 % and were observed after 18 DAH (Chapter 2). In *Dicentrarchus labrax* it was observed that kyphosis would start developing when larvae were between 10 and 17 mm although it was earlier when

they were more sensitive to potential harmful factors (Koumoundourous et al., 2002). Lordosis was seldom observed possibly due to the implementation of prevention measures such as oil surface removal which reduced the lordosis frequency in several fish species (Chatain, 1994).

Nutritional components of larval diets may play an important role in the genesis of skeletal malformations (Cahu et al., 2003). Vitamin C levels and DHA in the diet have been shown to be involved in the development of skeletal structures (Gavaia et al., 2002; Gapasin et al., 2001). Low levels of vitamin C lead to severe deformities in rainbow trout (Madsen and Dalsgaard, 1999) and milkfish larvae fed DHA incorporated diets presented lower opercular deformities (Gapasin et al., 2001). In this thesis, several AA compositions were tested in order o look for a positive effect on fish larvae skeletal deformities prevention. *D. sargus* present several AA nutritional imbalances in live feed, especially in rotifers. When a balanced AA diet was given to the larvae a decrease in the number of skeletal deformities was observed (Chapter 6). Larvae fed an AA balanced diet had almost 50% less deformities than larvae fed live feed or an AA unbalanced inert diet and were less affected by vertebral fusions and lordosis.

Tryptophan is believed to be involved in the development of skeletal malformations. Tryptophan deficient diets seem to induce scoliosis in salmonids (Akiyama et al., 1986). However, an inert diet supplemented with tryptophan did not decrease the frequency of deformities in *D. sargus* larvae (Chapter 7). Phenylalanine supplementation was also tested and further reduced the development of skeletal deformities compared to the diet balanced in AA. A decrease in the frequency of vertebral compressions was observed (Chapter 8). The prevention of this specific deformity in *Diplodus sargus* is extremely important as it is the most frequent skeletal malformation and observed in most larval stages (Chapter 2).

9.4. Supplementation of the amino acids studied did not improve larval growth. Supplementation of tryptophan decreased *D. sargus* larval growth

One of the aims of this study was to verify wether AA supplementation could improve growth. However, no increase on growth rate was found in any of the supplemention trials using the different AA studied. In fact, a decrease on dry weight and growth rate was observed when larvae were fed a supplement of tryptophan. Similar results have been reported before for rainbow trout juveniles (Papoutsoglou et al., 2005a) and white sea bass juveniles (Papoutsoglou et al., 2005b). This negative effect of tryptophan might have been caused by a decreased on the feed intake. Tryptophan is the precursor serotonin, which is involved in the regulation of appetite (Papoutsoglou et al., 2005a). An excessive dose of tryptophan might have increased the serotonin levels and therefore food intake decreased. Other possible explanation is a negative impact of tryptophan on the taste of the microencapsulated diet. This AA has been reported to have a deterrent gustatory property in fish such as carp (Kasumyan and Morsi, 1996), leading to a decrease in the food consumption. Tryptophan was also shown not to be limiting larval growth as in the tube feeding trials its supplementation was not translated into an increase on the AA retention.

In the beginning of this thesis several AA were identified as potential limiting AA (Chapter 3). From these, lysine was supplemented using the in vivo tube-feeding technique (Chapter 5) and a long-term zootechnique trial (Chapter 7). In both, a similar retention/ growth to the unsupplemented groups was observed. Therefore, this AA does not seem to be limitng *D. sargus* larval growth. All remaining AA studied in this thesis were thought not to be deficient in *D. sargus* diet and therefore they were not expected to increase larval growth.

9.5. Amino acids have important roles other than growth

This thesis shows interesting results concerning AA effect on the development of certain body structures, such as skeleton, and effect on stress resistance. As mentioned previously, a balanced diet decreased the number of skeletal deformities and phenylalanine supplementation prevented a higher incidence of vertebral compressions. Tyrosine supplementation also played an important role increasing larval resistance when subjected to a temperature stress test, by increasing larval survival rate. Tyrosine is precursor of catecolamines and this AA seems to enhance its synthesis (Lehnert and Wurtman, 1993) and may prevent stress-induced noradrenaline depletion in the animal brain (Deijen et al., 1999). There is few published information on this matter related to fish but laboratory studies strongly suggest that tyrosine supplementation may serve to reduce cognitive and behavioural effects of exposure to stress (Deijen et al., 1999). Larval stress is a relevant factor as it can affect the dynamic in the tank, promoting hierarchic relationships and can affect larval appetite and therefore growth.

9.6. Towards the future

This thesis aimed to estimate the AA requirements of *D. sargus* larvae and study the effects of supplementation of several AA in the diet to help solving some of the problems *D. sargus* larval rearing is facing nowadays. An AA balanced diet based on the AA profiles of larval carcass was formulated and was observed to reduce by half the incidence of skeletal deformities, one of the most serious problems in *D. sargus* aquaculture. The estimated AA requirements will contribute to the formulation of a specific diet for this species at larval stage, which, at the moment, does not exist. However, this study is only a first step to the knowledge of *Diplodus sargus* nutritional requirements. In order to formulate a completely balanced diet, it is important carry out

further studies focusing on other nutrients such as fatty acids, vitamins and minerals as these may also affect larval survival, growth and quality. Following AA studies should determine AA bioavailability for *D. sargus* as different AA absorption rates may lead to different AA requirements, modifying AA balance diet formulation. Further studies are also required to determine bioavailability of individual AA for *D. sargus.* Individual AA may have different absorption and catabolism rates which may lead to different AA requirements. To would be also interesting to study the supplementation of methionine, cysteine and taurine, as these AA are crucial to the metabolism of several molecules such as CoA. Taurine has an important role in cellular ion-balance and is also involved on emulsion of dietary lipids, promoting digestion.

As already mentioned, this thesis contributes to solve one of the main constraints for *D. sargus* farming: skeletal deformities developing during larval stages. Besides an AA balanced diet, phenylalanine was relevant in preventing some skeletal malformations. Still, incidence of skeletal deformities was still quite high, suggesting that further work is needed in nutrition and possibly other areas of research.

This thesis was centered on the importance of AA during *D. sargus* larval stages and during this study another result was obtained: Tyrosine supplementation increased stress resistance, making larvae less sensitive to temperature stress test. It would be interesting to know more about this effect of tyrosine as litle information is available concerning fish. *D. sargus* shows some agonist behaviour and hierarchic relationships triggered by high densities, food distribution or light incidence (Caballero and Castro, 1999, 2003; Castro and Caballero, 1998, 2004) and stress levels may affect this.

D. sargus has a high potential for aquaculture and it is a fairly easy species to culture. For this reason, it is important to continue research to find solutions for the problems still faced when farmimg this species.

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