U.PORTO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

Stress mitigation in sole (*Solea senegalensis*) through improved nitrogen nutrition: amino acid utilization, disease resistance and immune status

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Tese de Doutoramento em Ciência Animal

2011

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Tese de Candidatura ao grau de Doutor em Ciência Animal, especialidade em Morfologia e Fisiologia, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientador – Prof. Doutor António Manuel dos Santos Afonso

Categoria – Professor Associado

Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Co–orientador – Doutor Luís Eugénio Castanheira da Conceição

Categoria – Investigador Auxiliar

Afiliação – Centro de Ciências do Mar da Universidade do Algarve.

This thesis also includes four scientific papers published in international journals originating from part of the results obtained in the experimental work referenced to as:

Costas, B., Aragão, C., Mancera, J.M., Dinis, M.T., Conceição, L.E.C., 2008. High stocking density induces crowding stress and affects amino acid metabolism in Senegalese sole *Solea senegalensis* (Kaup 1858) juveniles. Aquaculture Research 39, 1 – 9.

Costas, B., Conceição, L.E.C., Aragão, C., Martos, J.A., Ruiz-Jarabo, I., Mancera, J.M., Afonso A., 2011. Physiological responses of Senegalese sole (*Solea senegalensis* Kaup, 1858) after stress challenge: effects on non-specific immune parameters, plasma free amino acids and energy metabolism. Aquaculture 316, 68 – 76.

Costas, B., Conceição, L.E.C., Dias, J., Novoa, B., Figueras, A., Afonso, A., 2011. Dietary arginine and repeated handling increase disease resistance and modulate innate immune mechanisms of Senegalese sole (*Solea senegalensis* Kaup, 1858). Fish & Shellfish Immunology 31, 838 – 847.

Costas, B., Aragão, C., Soengas, J.L., Míguez, J.M., Rema, P., Dias, J., Afonso, A., Conceição, L.E.C., 2011. Effects of dietary amino acids and repeated handling on stress response and brain monoaminergic neurotransmitters in Senegalese sole (*Solea senegalensis* Kaup, 1858) juveniles. Comparative Biochemistry and Physiology, Part A, *in press*. DOI: 10.1016/j.cbpa.2011.08.014.

"All thruths are easy to understand
once they are discovered; the point
is to discover them"

Galileo Galilei

Cover design:
Ana C.P. Simões Ozório

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Acknowledgements

This Thesis results from years of hard work and enthusiasm, to which many other people have contributed in one way or another. During this long walk I also had the opportunity to learn so many things, in both professional and personal life, and the chance to meet so many people that by mention all I would require another Thesis! Though, I would like to gather all those who really contributed to this work and supported me, express my gratitude, and of course give them credit.

I would like to express my most sincere appreciation and respect to Luís Conceição. Many years have passed now since I was taken as his Masters student. Since then, I became first technician and then PhD student. It must not be by chance that we have been working together for 6 years already! He greatly contributed to the establishment of this Thesis from the very first draft. His strong scientific personality, encouragement and friendship have always been a motivation force for me! I only have words of gratitude for all the trust, guidance and great availability shown throughout these years. But most of all, I am deeply grateful to Luís for believing in me!

A special word to Prof. António Afonso. He took the risk of accepting me as his student without knowing me! I really appreciate his consent to give me autonomy to work all this time, always under his supervision. Thank you for the support and encouraging given during these years.

I want to thank Fundação para a Ciência e Tecnologia (FCT) for the financial support. This work was partially developed under the project OPTISOLE – QREN IDT Co-Promoção Nº 1605, which was co-funded by the National Strategic Reference Framework – *QREN* under the Regional Operational Programme North – ON2, with 343751.84 € originating from the European Regional Development Fund – *FEDER*. I am also grateful to the Management Committee of the COST Action 867, who agreed to finance a Short Term Scientific Mission within the COST scientific programme on Welfare of fish in European aquaculture, which granted me a research stay at the Universitat Autonoma de Barcelona, Spain.

Two other people had major roles in this Thesis. I would like to express my gratitude to Jorge Dias and Cláudia Aragão for important advices, availability, kindness and critical scientific revisions in the chapters they co-author. In particular, I will always be thankful to Cláudia for showing me the right basis at the lab many years ago. Now I always try to do everything "bonitinho" and "direitinho" ;o). Thanks a lot Cláudia! Wisdom and sympathy are only two of the many virtues of Jorge Dias. He was responsible for diets formulation in this Thesis, a basic and essential tool for the success of this research. I will always be grateful to Jorge for his priceless help.

I had the great opportunity of collaborating with Juan Miguel Mancera. His strong scientific knowledge has significantly contributed to the establishment of this research. Thank you very much, not only for the scientific support, but also for being always available for doubts and corrections. I would also like to acknowledge Ignacio Ruiz-Jarabo for all the backup work at the lab and friendship. A big hug goes also to Paco and Lucho for their sympathy, frienship and great hospitality when visiting their lab.

I also had the honour of visiting the lab of Antonio Figueras and Beatriz Novoa. I am deeply grateful, not only for the scientific support, but also for showing me the "molecular world" and let me feel at home during those months in my stay abroad. I would also like to acknowledge Patricia Díaz and Laura Poisa for all the instructions and advise in the Molecular Biology field. I also appreciate all the kindness of everyone at the Genomics and Immunology Group. Coffee breaks were always an entertainment. Thanks Alex, Sonia, Pablo, Rebeca Rodríguez, Rebeca Moreira, Mónica, Patricia Pereiro, Patricia Díaz, Mari Mar, Noelia and Raquel for your sympathy. A special hug goes to Rubén, Pep and Rosi for coffee breaks, cortos and laughs!

Collaboration in this Thesis also extended to the lab of José Luis Soengas and Jesús Míguez. Their strong scientific personality and sympathy made possible my stay at the Universidade de Vigo. Thank you for your accessibility and support always I needed it. I would also like to acknowledge Manuel Gesto and Sergio Polakof for all the instructions and advise with the HPLC and glucose and lactate assays. I also appreciate all the kindness showed by Rosi, Arnau and Pepe. All of them were always ready to help!

I would like to extend my thanks to Lluis Tort. My short stay at the Universitat Autonoma de Barcelona was valuable and of guidance for the successful accomplishment of the complement and *in vitro* techniques. Thank you very much for giving me this opportunity. I would also like to acknowledge all the people that I met there and that made my stay easy and practical. Thanks Simon, Sonia, Carmen, Mauri, Sebastián and Mariana for your kindness.

I would like to thank A. Coelho & Castro, Lda (Póvoa de Varzim, Portugal) for gently conceding fish for the experiments.

I am also grateful to all the CIIMAR members. I would like to specifically acknowledge Prof. Luísa Valente for her availability and help with analytical procedures with diets. Special thanks go to BOGA team (Hugo, Ricardo and Olga), Paulo César, Rodrigo Ozório, Joana Silva, Cláudia Silva, Pedro Borges, Pedro Reis, Leonor, Ralph, Catarina, Silvia Soares, Begoña, and so many others who were always around for lunch, coffee and of course scientific discussion. When I was in Faro, it was always enjoyable time thanks to all members of the Aquaculture Research Group. I would like to specifically acknowledge Prof. Maria Teresa Dinis, for accepting me as a member many years ago and for her words of wisdom during all this time. Special thanks go to Maria Filipa, André and Lena, who actively contributed for the accomplishment of this Thesis. I would like to extend my thanks to the Ramalhete people, in particular to João Reis for the hard work with the metabolic chambers, and also to Sofia, Ana Ramalho, Vera, François and Alberto, who were around from time to time.

During all these years some of my friends accompanied my work in one way or another and I would like to show them my gratitude for their interest, support and friendship. Rodrigo Ozório and Paulo César Rêgo were two essential elements during my life in Porto, both at work and outside CIIMAR walls. Thanks for all advices, discussions and beer! A special thank to PC for showing me the "hematology world", and to Ana Ozório for her friendship and help with the cover design. In Faro, Wilson Pinto, Luís Figueira and João Sendão were my backup team. Wilson and Luís were extraordinary lab colleagues. For instance, good understanding was essential during work at the radioactivity room. In addition to that, Wilson is my *soul mate* in research. João also had a major contribute in the

essential “backstage work” that allowed conducting some of the experiments. Thanks to all for the valuable advice, support, friendship and great wine!

A big hug goes to my dear *Metal friends* Seve and João. I finally made it fellas!!! Although thousands of miles take us apart, METAL will always be with us \m/ Missing a lot our metal-nights!

A special word to my friends who, despite of hundreds of km away, will always be present! So kisses and big hugs to André, Joni, Ana Luísa, Rita, Cheila, Leon, João Lima, Liliana, Mario, Catarina Oliveira, Eviña, Su, Sonia, Pablo, Susana, Pilin, Berto, David, Nipur, Olivitas, Iria, Silviña, Juanilho, Michi, Oscar, Javi and Suso. I hope I have mentioned everyone!

This Thesis is dedicated to my whole family, to whom I would like to express my appreciation with some words in my own language.

A mis suegros, cuñados y cuñadas por momentos en familia que siempre me han dado motivos de alegría. Gracias por estar ahí y por preocuparos por mí. Y a mis sobrinos y ahijado, por ser unos enanos guapísimos e inteligentes que no paran de sorprenderme!

A mis padres Toñi y Min, que son los mejores padres del mundo. Por todos los sacrificios y preocupaciones que les he hecho padecer, por todo el apoyo y cariño que siempre me han dado. Por estar siempre ahí.

A mi hermana Rebe por todo el apoyo y cariño. Por estar siempre preocupada por mí. Por creer en mí!

A Bea. Por ser una parte esencial de mi vida. Por los buenos momentos y por todo lo que compartimos. Por haber hecho un esfuerzo tan grande en el tramo final de esta tesis y haber aguantado mi ausencia y falta de apoyo como la mujer fuerte que es. Por haber creído en mí hasta el final y no haberme dejado abandonar cuando ya no había “luz al final del túnel”. Porque sin ti no habría sido posible.

A mis hijos, Lucía y Adrián. Por ser lo mejor que me haya pasado en la vida. Por quererme incondicionalmente a pesar de mi ausencia y falta de atención en estos últimos 3 meses! Por hacerme querer llegar más lejos.

Summary

Scientific and technical knowledge on the cultivation of the Senegalese sole (*Solea senegalensis* Kaup, 1858) have evolved slow, but gradually, until reaching nowadays a level of acceptable risk for commercial culture. However, growth and survival from juvenile to market-size fish is still not fully controlled with regard to rearing technology and husbandry conditions, playing stress a pivotal role during Senegalese sole ongrowing. Moreover, stressful rearing conditions that induce high plasma cortisol levels also modify fish amino acid metabolism in several teleost species, including Senegalese sole. In fact, it has been suggested that fish under stressful conditions present additional amino acid requirements, due to increased energy demands or for the synthesis of stress-related proteins and other compounds related with the stress response. In addition, several studies reported that the effects of dietary amino acids in fish go beyond meeting the requirement for protein synthesis. Moreover, dietary amino acids have been shown to have effects on innate immunity in mammals, and stress response has been documented to interact with immune system. However, the role of dietary amino acids in fish under aquaculture-related stressful conditions is lacking and requires further attention.

The main goal of this study is to allow a better understanding of the role of arginine and other AA in relation to the stress response and disease resistance in fish. This Thesis will focus mainly on the role of indispensable AA due to the need of meeting its requirements through dietary sources. The possibility of mitigating the negative effects of stress and disease susceptibility of fish by altering dietary AA levels is proposed. Ultimately, this study should contribute to the improvement of commercial diets for sole farming, and thereby for better welfare and reduced disease susceptibility of this species.

The effects of stressful practices frequently occurring in aquaculture on amino acid metabolism, stress and immune responses of Senegalese sole are assessed in **Chapters 2 and 3**. High stocking density and air exposure were chosen as chronic and acute stressors, respectively. Particularly, **Chapter 2** tested 3 different stocking densities (low, medium and high) and results pointed out to high stoking density as a stressful rearing condition which increased cortisol levels, and suggested that crowding stress may affect amino acid requirements. Although no immune parameters were assessed in this study, disease outbreaks

observed in fish under high stocking density contributed to confirm that chronic stress situations may impair immune system and increase the susceptibility of Senegalese sole to infectious diseases. On the other hand, **Chapter 3** describes physiological responses after an acute handling stress and their subsequent effects on innate immune parameters and plasma free amino acids during 24 h. This time-course study suggests that Senegalese sole presents a stress response comparable to that observed in other teleosts, with plasma cortisol, glucose, lactate and osmolality levels peaking at 1 h. While some indispensable amino acids may be used for the synthesis of compounds related to the stress response or fatty acid transport, dispensable amino acids were probably mainly employed either as energy sources or in gluconeogenesis. Moreover, results from the non-specific immune parameters assessed suggest that cortisol may act as regulator of the innate immune system.

Chapter 4 assessed how individual indispensable amino acids are utilized in Senegalese sole juveniles. The experiments demonstrated differences in digestibility, retention and catabolism between individual indispensable amino acids. While tryptophan was significantly less absorbed (59 %), the other ingested indispensable amino acids showed values for digestibility around 90 %. Moreover, lysine was the most catabolised indispensable amino acids and seems to have an important role as an energy substrate in this species. This information allowed a more accurate feed formulation for the studies assessing the effects of amino acid supplements on stress and immune responses.

In **Chapter 5**, the effects of increased availability of dietary amino acids on the metabolic processes resulting from stressful situations in fish due to repeated handling stress were assessed. For this purpose, amino acids were provided through a high protein (HP) diet, which was formulated to contain 51.2 % digestible protein. A reference diet (46.9 % digestible protein) served as control. Repeated handling was effective in inducing a chronic stress response after 14 and 28 days in air exposed fish fed both diets, whereas HP diet decreased post-stress plasma glucose and lactate levels only at 14 days. Furthermore, both repeated handling and HP diet increased plasma lysozyme activity after 14 and 28 days. It was concluded that feeding sole a diet with an increase in some indispensable amino acids, for instance arginine, phenylalanine and tryptophan,

may contribute to minimize the extra energy costs attributed to cortisol release at least after 14 days of feeding, and therefore may improve welfare in farmed sole.

Chapter 6 aimed to investigate leucocyte responses to inflammation as well as some innate immune parameters of Senegalese sole following challenge with two strains of *Photobacterium damselae* subsp. *piscicida*. This study showed for the first time leucocyte responses to inflammation in both peripheral blood and peritoneal cavity of flatfish, presenting Senegalese sole, an inflammatory response similar to that observed for other teleosts. Pathogenicity assays were performed in order to assess the virulence of each bacterial strain for sole, and validated the infection model for use in future experiments assessing disease resistance following chronic stress and dietary treatments.

In **Chapter 7**, repeated handling was employed as a chronic stressor in order to verify whether its attributed immunosuppressive effects could be minimized by dietary arginine supplementation. Therefore, Senegalese sole were air exposed daily for 3 min during 14 days (handling) or left undisturbed (control). Cellular responses increased parallel to dietary arginine supplementation, whereas HIF-1, HAMP-1, MIP1-alpha and gLYS expression values and some humoral parameters augmented in control specimens fed arginine supplements. Interestingly, chronic stress increased both resistance to *Photobacterium damselae* subsp. *piscicida* and some innate immune mechanisms. The role of dietary arginine and repeated handling on Senegalese sole innate immunity and disease resistance is discussed.

The effect of partial or total replacement of fish meal by plant-protein ingredients balanced with indispensable amino acids on some innate immune parameters was assessed in **Chapter 8**. Results suggest that the enhancement of the innate immune parameters assessed may be related with both indispensable amino acids dietary balance and partial fish meal replacement by plant-protein sources. However, the effects of such long-term immunomodulatory pattern associated to changes in dietary protein quality and its effectiveness on promoting disease resistance require further investigation.

In **Chapter 9**, the overall AA metabolism and physiological responses to different stressors, as well as subsequent changes on innate immune parameters

in stressed Senegalese sole are discussed. Although with some particularities, Senegalese sole appears to present stress and immune responses similar to that find in other teleosts. Additionally, the low digestibility of tryptophan observed in sole juveniles must be considered during formulation of feeds containing amino acid supplements. This Thesis also suggests the possibility of mitigating the increased metabolic cost attributed to cortisol release through diets with an increase in the availability of some key indispensable amino acids. Ultimately, dietary arginine and possibly branched-chain amino acids, threonine, lysine or methionine are proposed as potential nutrients for use in functional feeds in aquaculture.

Resumo

O conhecimento científico e técnico que suporta o cultivo do linguado Senegalês (*Solea senegalensis* Kaup, 1858) evoluiu de um modo lento mas gradual, até chegar, hoje em dia, a um nível de risco aceitável para o seu cultivo à escala comercial. No entanto, a tecnologia e condições de cultivo para uma boa sobrevivência e crescimento do linguado até um tamanho comercial, ainda não estão completamente controlados. Durante a fase de engorda, o stress poderá ser um dos factores mais relevantes no crescimento e sobrevivência do linguado Senegalês. Além disso, foi já verificado que condições de cultivo casuadoras de stress que aumentaram os níveis de cortisol no plasma, também modificam o metabolismo de aminoácidos em várias espécies de teleósteos, incluindo o linguado Senegalês. De facto, estudos recentes sugerem que peixes cultivados em condições de stress apresentam requisitos adicionais de aminoácidos, o que poderá ser devido ao aumento das suas necessidades energéticas ou para a síntese proteínas relacionadas com o stress ou outros compostos relacionados com a resposta ao stress. Por outro lado, vários estudos reportaram que os efeitos dos aminoácidos na dieta vão para além da sua utilização para síntese de proteínas. Porém, o papel que os aminoácidos da dieta poderão ter em peixes que são cultivados em condições de stress é desconhecido e é necessário dar-lhe uma maior atenção.

O principal objectivo de este estudo é possibilitar um melhor entendimento do papel da arginina e outros aminoácidos em relação à resposta ao stress em peixes, e da sua resistência a doenças. Esta Tese irá focar apenas na função dos aminoácidos indispensáveis devido à necessidade de chegar ao seu requisito através da dieta. Ainda é proposta a possibilidade de mitigar os efeitos negativos do stress e da susceptibilidade à doenças através de alterações dos níveis de aminoácidos na dieta. Por último, esta Tese deverá contribuir para o melhoramento de rações comerciais para o linguado, assim como dos resultados no cultivo, através de um melhor bem-estar e uma redução da susceptibilidade à doença.

Os efeitos de situações de stress frequentemente verificadas em aquacultura, no metabolismo de aminoácidos e nas respostas imune e ao stress foram estudados nos **Capítulos 2 e 3**. Densidades de cultivo elevadas e exposição ao ar foram as escolhas representativas de stress crónico e agudo,

respectivamente. Em particular, no **Capítulo 2** foram testadas 3 densidades de cultivo (baixa, média e alta). Os resultados apontaram para a densidade alta como a condição de cultivo causadora de maior stress, a qual aumentou os níveis de cortisol no plasma e afectou o metabolismo dos aminoácidos de uma forma significativa. Mesmo que não tenham sido analisados parâmetros imunológicos, os surtos patológicos verificados nos peixes da densidade alta sugerem que situações de stress crónico poderão inibir o sistema imune e aumentar a susceptibilidade do linguado Senegalês a doenças infecciosas. Por outro lado, o **Capítulo 3** descreve as respostas fisiológicas após um stress agudo por manuseamento, e os seus efeitos subsequentes em parâmetros do sistema imune inato e aminoácidos livres no plasma durante 24 horas. Este estudo sugeriu que o linguado Senegalês apresenta uma resposta ao stress semelhante à verificada em outros peixes teleósteos, com níveis máximos de cortisol, glucose, lactato e osmolaridade no plasma após 1 hora. Foi ainda verificado que alguns aminoácidos indispensáveis poderão ter sido utilizados na síntese de compostos relacionados com a resposta ao stress ou com o transporte de ácidos gordos. Relativamente aos aminoácidos dispensáveis, provavelmente foram oxidados e utilizados como fonte de energia, ou foram usados como fonte de carbono na gliconeogênese. Por último, os resultados dos parâmetros imunes não-específicos analisados sugerem que o cortisol poderá ter actuado como regulador do sistema imune inato.

No **Capítulo 4** foi investigado como os aminoácidos indispensáveis são individualmente utilizados em juvenis de linguado Senegalês. Estas experiências mostraram diferenças na digestibilidade, retenção e catabolismo entre os aminoácidos indispensáveis. Se o triptofano foi significativamente menos absorvido (59 %), os outros aminoácidos indispensáveis ingeridos mostraram valores de digestibilidade de aproximadamente 90 %. Além disso, a lisina foi o aminoácido indispensável mais catabolisado e poderá apresentar uma função importante como substrato energético. A informação compilada neste capítulo permitiu ainda formular com mais precisão as dietas utilizadas nas experiências dos capítulos 5 e 7, podendo ter melhorado a eficiência dos seus efeitos nas respostas imunológica e ao stress.

No **Capítulo 5** foram avaliados os efeitos que um aumento na disponibilidade de aminoácidos na dieta poderão ter tido nos processos

metabólicos resultantes de situações de stress crónico, como por exemplo o manuseamento repetido. Para isto, os aminoácidos foram fornecidos através de uma dieta rica em proteína, a qual foi formulada para conter 51,2 % de proteína digerível. Como controlo foi utilizada uma dieta de referência com 46,9 % de proteína digerível. O manuseamento repetido resultou numa resposta crónica nos peixes submetidos ao stress e alimentados com as duas dietas depois de 14 e 28 dias. No entanto, a dieta rica em proteína levou a um abaixamento dos níveis de glucose e lactato no plasma após 14 dias. Além disso, quer o manuseamento repetido, quer a dieta rica em proteína levaram a um aumento da actividade da lisozima no plasma após 14 e 28 dias. Foi concluído que a alimentação de linguados com uma dieta suplementada com alguns aminoácidos indispensáveis, por exemplo arginina, fenilalanina e triptofano, poderá contribuir para a minimização dos custos de energia atribuídos à libertação de cortisol, pelo menos após a sua alimentação durante 14 dias. Este protocolo experimental poderá vir a melhorar o bem-estar do linguado Senegalês nas pisciculturas.

O principal objectivo do **Capítulo 6** foi investigar as respostas dos leucócitos a um processo de inflamação, assim como avaliar alguns parâmetros imunológicos do linguado Senegalês após um desafio com duas estirpes de *Photobacterium damselae* subsp. *piscicida*. Este estudo mostrou pela primeira vez em peixes planos, as respostas dos leucócitos a um processo de inflamação quer no sangue periférico quer na cavidade peritoneal. Verificou-se que o linguado Senegalês apresenta uma resposta inflamatória semelhante à observada noutros peixes teleósteos. Experiências de patogenicidade foram feitas para averiguar a virulência que cada estirpe da bactéria tem contra o linguado, servindo ainda para validar o modelo de infecção para uso em futuras experiências de avaliação da resistência à doença após um stress crónico e a alimentação com dietas experimentais.

No **Capítulo 7**, o manuseamento repetido foi utilizado como modelo de stress crónico, de modo a averiguar se os seus conhecidos efeitos immunossupressores poderão ser minimizados através da suplementação de arginina na ração. Para isso, linguados do Senegal foram expostos ao ar (stress) todos os dias durante 3 minutos até 14 dias ou mantidos sem stress (controlo). As respostas dos leucócitos aumentaram em paralelo à suplementação com arginina. O nível de expressão dos genes HIF-1, HAMP-1, MIP1-alpha e gLYS,

assim como alguns parâmetros humorais, aumentaram nos peixes controlo alimentados com ração suplementada com arginina. Surpreendentemente, o stress crónico aumentou a resistência à *Photobacterium damselae* subsp. *piscicida*, juntamente com alguns mecanismos imunológicos. Neste capítulo são discutidas as funções da suplementação com arginina e do manuseamento repetido na imunidade inata e na resistência à doença do linguado Senegalês.

O efeito da substituição parcial ou total de farinha de peixe por fontes de proteína vegetal balançados com aminoácidos indispensáveis em alguns parâmetros imunológicos foi verificado no **Capítulo 8**. Os resultados sugerem que o aumento dos parâmetros imunológicos avaliados poderá ser devido ao balanceamento com aminoácidos indispensáveis e à substituição parcial de farinha de peixe por fontes de proteína vegetal. No entanto, os efeitos de um padrão imuno-modulador de tão longo prazo associado a alterações na qualidade da proteína na dieta, assim como a sua eficiência em promover resistência a doenças, requerem mais investigação.

No **Capítulo 9**, o metabolismo de aminoácidos em geral e as respostas fisiológicas a diferentes factores de stress são discutidos, assim como as alterações subsequentes nos parâmetros da imunidade inata de linguados cultivados em condições de stress. Ainda que com algumas particularidades, o linguado Senegalês parece apresentar respostas imunológicas e ao stress semelhantes ao verificado noutros peixes teleósteos. Alem disso, a baixa digestibilidade do triptofano observada em juvenis de linguado deverá ser tida em conta durante a formulação de dietas com suplementação de aminoácidos. Esta Tese sugere também a possibilidade de mitigar o aumento do custo metabólico associado à liberação de cortisol, através de dietas com um aumento na disponibilidade de alguns aminoácidos chave. Por último, a arginina e possivelmente os aminoácidos de cadeia ramificada, a treonina, a lisina ou a metionina são propostos como nutrientes potenciais para serem suplementados em dietas funcionais para uso em aquacultura.

Chapter 1

General Introduction

1.1. Senegalese sole

Aquaculture is the only alternative to the world-wide collapse of commercial fisheries stocks and represents nowadays about 45 % of the world fish food production. Aquaculture thus plays a vital role in supplying products known to have a high biological value to humans (Kaushik and Seiliez, 2010). Scientific and technical knowledge on the cultivation of the Senegalese sole (*Solea senegalensis* Kaup, 1858) have evolved slow, but gradually, until reaching nowadays a level of acceptable risk for commercial culture. This species became an important research focus for production in the aquaculture industry, particularly in Spain and Portugal, due to both a market saturation of European sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) in the Mediterranean region, and a high economic and commercial potential (Imsland et al., 2003). The current market price in the European Union is around 8-14 euros / kg and is being exploited semi-extensively and extensively in earthen ponds and land-based facilities along the coasts of the Iberian Peninsula (Dinis et al., 1999; Imsland et al., 2003). Particularly in Portugal, sole is also being exploited intensively the North region, which is the more important area of inland aquaculture where freshwater farms, distributed by several rivers, represent 96% of rainbow trout (*Oncorhynchus mykiss*) production. Despite being the region with the lower surface use and with the less number of farms, it is the third production area in terms of volume, due to the use of intensive systems (Ramalho and Dinis, 2010). Nevertheless, there are still some aspects regarding Senegalese sole rearing procedures that require optimisation before this species can be produced in a large scale intensive regime (Imsland et al. 2003).

Senegalese sole is a benthonic flatfish marine species living up to 100 m depth, in sandy or muddy bottoms of the continental shelf from the Gulf of Biscay to the coasts of Senegal (Quéro, 1984; Whitehead et al., 1986). This species feeds basically on benthonic invertebrates, such as polychaets, bivalves, other molluscs and small crustacea. Sexual maturity is reached at age 3+ with a total length of around 32 cm (Dinis, 1986). Sole are batch spawners and the spawning season occurs mostly between the months of March and July, with most of the batches normally happening during May (Dinis, 1986). In culture conditions, eggs are normally obtained from natural spawns of wild broodstocks kept in captivity (Dinis et al. 1999). Larvae hatch after 24-48 h depending on the water

temperature that might vary between 16-18ºC. Sole are usually fed with rotifers for a few days at first feeding in order to allow enrichment with highly unsaturated fatty acids and thereafter they eat *Artemia* nauplii and metanauplii (Dinis et al., 1999). These live feeds are used instead of natural plankton because of their constant availability, cost-efficiency and versatility in application (Coutteau and Sorgeloos, 1997). Although the weaning has traditionally been considered the major bottleneck of Senegalese sole rearing (Dinis et al., 1999), this has been largely overcome in recent years (Engrola, 2008). Moreover, Engrola et al. (2009) have recently shown that offering inert diet to sole at mouth opening in a co-feeding regime promotes growth and better quality juveniles.

Grow-out is one of the most important steps with regard to economic viability and the least well-studied phase for sole intensive aquaculture rearing. In intensive culture systems, profitability depends on the rate of production, which is a function of both the mean growth rate of the fish and the stocking density, which has been demonstrated as a crucial variable regarding growth performance of cultured fish. The effects of density on growth are variable, usually showing a negative correlation in several teleosts species such as Atlantic cod (*Gadus morhua*), turbot (*Psetta maxima*), Dover sole (*Solea solea*) and Senegalese sole (Irwin et al., 1999; Lambert and Dutil, 2001; Schram et al., 2006; Sánchez et al., 2010). In contrast, Senegalese sole juveniles stocked in the range of 30-45 kg m^{-2} did not reveal differences in terms of growth between the assayed densities (Salas-Leiton et al., 2008, 2010). However, high stocking density may decrease the expression levels of g-type lysozyme and hepcidin antimicrobial peptide in Senegalese sole and thus susceptibility to opportunistic pathogens may increase (Salas-Leiton et al., 2010). Furthermore, natural variations in temperature and salinity usually influence physiologic and metabolic aspects of the Senegalese sole when reared in earthen ponds (Dinis et al., 1999; Imsland et al., 2003). In fact, changes in both ambient water temperature and salinity elicit osmoregulatory, thyroidal and metabolic responses in Senegalese sole that can be interpreted to meet the altered requirements of metabolic and osmoregulatory systems following activation of the stress response (Aragão et al., 2010; Arjona et al., 2007, 2008, 2010). Moreover, high stocking density and abrupt variations in both temperature and salinity usually induce a stress response, which to a large extent may have an impact on appetite reduction, stimulated catabolism and/or individual endurance (Pickering, 1998). Therefore,

growth and survival from juvenile to market-size fish is still not fully controlled with regard to rearing technology and husbandry conditions, playing stress a pivotal role during Senegalese sole ongrowing.

1.2. The stress response in fish

The stress response is a vitally important normal response of all living organisms. In fish, stress can be defined as a condition in which the dynamic equilibrium called homeostasis is threatened or disturbed as a result of the actions of intrinsic or extrinsic stimuli, commonly defined as stressors (Wendelaar Bonga, 1997). Nowadays, there is a sophisticated appreciation of the complex nature of the response and the manner in which it influences, and is itself influenced by, a range of factors, including the immune and reproductive systems, and behavioural and cognitive processes (Wendelaar Bonga, 1997, Barton, 2002, Øverli et al., 2005; Verburg-van Kemenade et al., 2009). The context, severity, and duration of the stress challenge, and of the resultant response, must all be taken into account when assessing whether an adverse outcome is likely for the individual (Pottinger, 2008). Acute stress is usually considered of short duration allowing the fish to respond to the stressor and recover (i.e., return to homeostasis), whereas chronic stress is long-term with more severe effects (Barton et al. 2002). Furthermore, within a population there are a range of individual coping styles that encompass variation in the neuroendocrine, physiological and behavioural responses to stress (Martins, 2005; Portz et al., 2006). Differences in social rank among individuals may cause differences in feeding behaviour and stress response. For instance, aggressive behaviour tends to increase with increased fish densities to a maximum at intermediate densities, and decrease as densities increase further (Portz et al., 2006). Densities and social hierarchies can markedly affect the growth, health, and stress of fish of different social status (Jørgensen et al., 1993). In addition, capture, handling and transport are known factors that also elicit behavioral and physiological stress responses (Masuda and Ziemann, 2003).

Confinement, high stocking density, handling (e.g., air exposure due to grading procedures or vaccination) and transport are stress inducers highly relevant to aquaculture and have received considerable attention (Barton and Iwama, 1991; Wendetaar Bonga, 1997, Barton, 2002, Pottinger, 2008).

Physiological responses of fish to these stressors have been grouped broadly as primary, secondary and tertiary (Figure 1). The primary stress response represents the perception of an altered state and initiates a neuroendocrine response that forms part of the generalized stress response in fish (Barton, 2002). This response includes the activation of brain-sympathic-chromaffin cells (BSC) axis with enhancing of plasma catecholamines (e.g., norepinephrine, epinephrine) as well as stimulation of the hypothalamic-pituitary-interrenal (HPI) axis, resulting in the release of corticosteroids into circulation. Therefore, catecholamines are firstly released from the chromaffin tissue situated in the head-kidney of teleosts, and also from the endings of adrenergic nerves (Randall and Perry, 1992). And secondly, corticosteroids (e.g., cortisol) are released from the interrenal tissue, also located in the head-kidney, in response to several pituitary hormones, but most potently to adrenocorticotrophic hormone (Balm et al., 1994). The secondary stress response comprises the various biochemical and physiological effects associated with stress, and mediated to a large extent by the mentioned stress hormones. A number of metabolic pathways are activated, resulting in alterations in blood chemistry and haematology (Mommsen et al., 1999). Secondary responses to stress include measurable changes in blood haemoglobin and hematocrit as well as in plasma glucose, lactate and major ions (e.g., chloride, sodium, and potassium). The measurement of plasma glucose concentration has been used as an indicator of stressed states and probably is the most commonly measured secondary (metabolic) response to stressors in fish (Barton, 2002). The production of glucose with stress assists the animal by providing energy substrates to tissues such as brain, gills and muscles, in order to cope with the increased energy demand (Mommsen et al., 1999). Tertiary responses are on the level of whole organisms and populations, including inhibition of growth and immune response as well as changes in metabolic rate, behaviour and survival (Barton and Iwama, 1991; Wendelaar Bonga, 1997; Mommsen et al., 1999; Barton, 2002). The extent of tertiary responses may be directly related to the severity and duration of the stressor. While physiological compensation to stress may be achieved, chronic stress may impair performance by diverting energy resources that might otherwise be used for regular biosynthesis (Barton and Iwama, 1991; Barton et al., 2002).

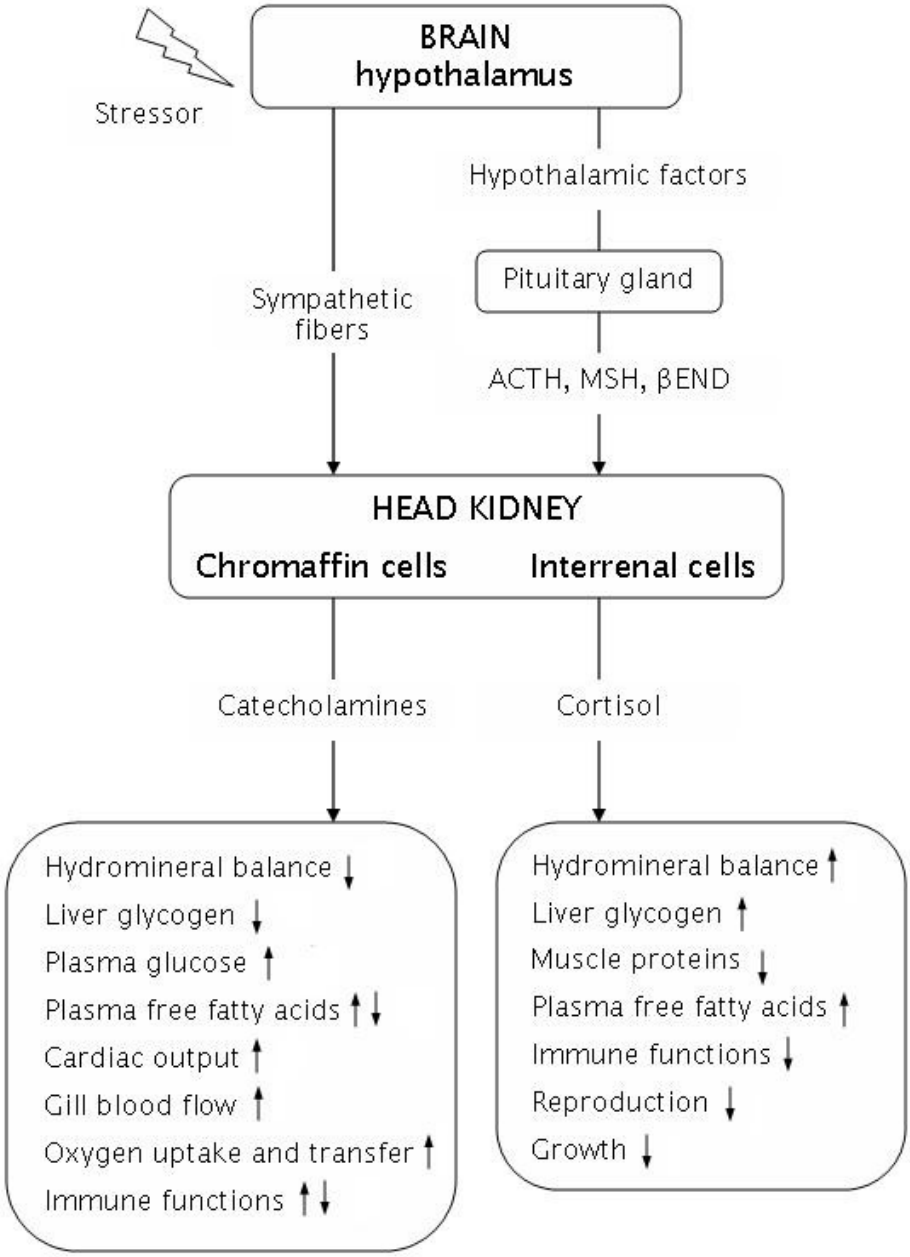

Figure 1. Generalized diagram of the stress response in fish. ACTH, adrenocorticotropic hormone; MSH, melanophore-stimulating hormone; β-END, β-endorphin; ↑ stimulatory; ↓ inhibitory (adapted from Wendelaar Bonga, 1997).

Plasma cortisol level is widely used as a general indicator of stressful situations in fish (Wendelaar Bonga, 1997; Mommsen et al., 1999). High stocking density is one of the most common sources of stress in fish farms, and together with other handling procedures like net confinement or grading (air exposure), induce a significant increase in plasma cortisol levels in teleosts (Arends et al., 1999; Acerete et al., 2004; Salas-Leiton et al., 2010). Cortisol synthesis and

release from interrenal cells presents a lag time of several minutes, unlike catecholamines, therefore, proper sampling protocol can allow measurement of basal levels of this hormone in fish (Gamperl et al., 1994). Fish held at high stocking densities are often considered to be exposed to chronic stressor situations that predispose the fish to infection, reproductive impairment, and may impose severe energy demands (Vijayan et al., 1990; Rotllant et al., 2000, 2001). In addition, increasing the number of fish per culture unit is one of the most common practices under farm conditions to increase profitability. Although high stocking densities may be good for the revenue of the farm, stocking density is known to have a strong influence on growth and survival in fish (Almazán-Rueda, 2004). Most studies reporting cortisol effect on carbohydrate metabolism in fish rely on plasma glucose and liver glycogen content as indicators of metabolism (Mommsen et al., 1999). In fact, hepatic metabolic changes associated to stressful conditions (e.g. hypoxia, high density or osmotic challenge) have been reported in fish (Vijayan et al., 1990; Dalla Via et al., 1994; Sangiao-Alvarellos et al., 2005, 2006; Arjona et al., 2009). In addition, both catecholamines and cortisol have been shown to mobilize hepatic glycogen stores in fish (Wright et al., 1989; Vijayan et al., 1994). Furthermore, plasma glucose levels usually augment following stressful situations such as handling, crowding or salinity transfer (Waring et al., 1996; Arends et al., 1999; Arjona et al., 2007). This mobilization of glucose in response to stress is generally accepted as a means of providing extra energy resources, enabling the animal to overcome the physiological disturbance (Barton and Iwama, 1991; Wendelaar Bonga, 1997). Similarly, plasma lactate concentrations increase significantly in several fish species following severe exercise (Milligan, 1996) or as a result of hypoxia (Arends et al., 1999). Stimulatory actions of cortisol on hepatic glucose production may be limited to gluconeogenesis (Vijayan et al., 1991), contributing to the weight loss that may occur during chronic stress (Wendelaar Bonga, 1997). Liver is an important site of gluconeogenesis in fish, being amino acids the main source of carbon (Ballantyne, 2001). However, the relative importance of amino acids for gluconeogenesis may vary depending on the availability of lactate (French et al., 1981). Still, species differences are substantial and the glucocorticoid actions of cortisol may vary depending on life-style and habitat as suggested by Vijayan and Moon (1994). For instance, glucose production by isolated hepatocytes of sea raven (*Hemitripterus americanus*) can be stimulated

by both cortisol and catecholamines, in contrast to salmonids, where only catecholamines have this activity (Randall and Perry, 1992).

1.3. Amino acid metabolism

Amino acids (AA) are present in living organisms either polymerised in protein or free in body fluids. The AA turnover between the free and protein pools is constant, allowing among other functions to renew or repair tissue proteins (Conceição, 1997). The 10 AA considered indispensable for fish are arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (Wilson, 2002). Cysteine and tyrosine are considered semi-indispensable, since they can only be synthesised from methionine and phenylalanine, respectively. AA are either used for the synthesis of proteins or used otherwise (Houlihan et al., 1995). Protein synthesis requires a righteous balance of all indispensable AA (Carter and Houlihan, 2001). If one indispensable AA is deficient at the protein synthesis site, the remaining indispensable AA will be in excess and will be deaminated for energy production or used in lipogenesis and gluconeogenesis (Ballantyne, 2001). Therefore, a reduced supply of any indispensable AA relatively to its demand will limit protein synthesis and growth. As shown in Figure 2, AA which are not polymerised into proteins can be used for energy production (catabolised), transaminated into another AA, used in gluconeogenesis or lipogenesis, or used in the synthesis of other nitrogen-containing molecules (e.g., purines, pyrimidines, hormones).

Dietary AA are mostly absorbed as free AA (FAA). The absorption of individual AA in the gut of fish depends on different transport systems (Storelli et al., 1989; Vilella et al., 1990; Collie and Ferraris, 1995) which may proceed at different rates (Dabrowski, 1983). Variation in the rates of absorption of individual AA may lead to transitory AA imbalances, and thus to an increase in AA catabolism. Different enzymes are involved in transamination and catabolism of AA (Cowey and Walton, 1989; Jürss and Bastrop, 1995), allowing for the differential use of individual AA in these processes. The liver is the main organ for AA catabolism in fish. The primary mechanism for catabolism of AA in fish liver is transdeamination in which the amino group of a variety of AA is transferred to α-ketoglutarate to from glutamate. Then, the metabolic fate of glutamate varies among species (Ballantyne, 2001).

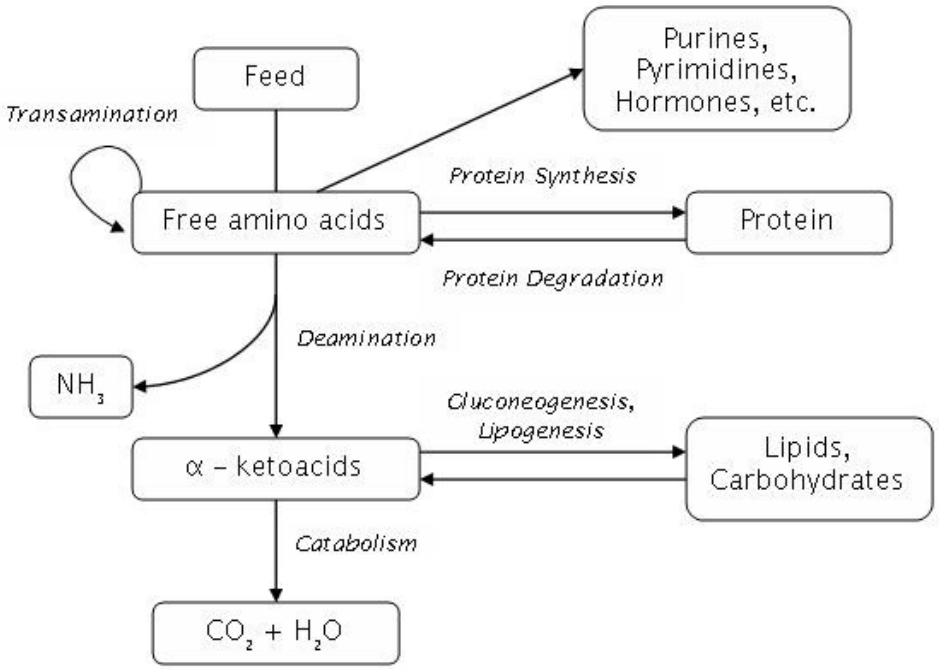

Figure 2. Generalized overview of the metabolism of amino acids (adapted from Conceição, 1997).

Although the major fate of AA is towards protein synthesis, emerging evidence shows that many AA are not just crucial for growth and energy production (Walton, 1985; Cowey and Walton, 1989), but also have important roles in immunity (Li et al., 2009), osmoregulation (Yancey, 2001; Aragão et al., 2010) and reproduction (Li and Gatlin, 2006). Moreover, stressful rearing conditions that induced high plasma cortisol levels also modified fish amino acid metabolism in several teleost species (Milligan, 1997; Vijayan et al., 1997), including Senegalese sole (Pinto et al., 2007; Aragão et al., 2008, 2010; Costas et al., 2010). As reviewed by Mommsen et al. (1999), stress affects several metabolic pathways in fish and some studies have assessed the relation between cortisol and AA (mostly FAA) concentrations in fish. The mode of cortisol application (e.g., single or repeated injection, intraperitoneal implants or minipumps) and treatment time employed in those experiments make complex the comparison of the results, being sometimes contradictory. Nevertheless, cortisol is known to affect protein and AA metabolism. In fact, it has been suggested that fish under stressful conditions even present additional AA requirements, due to increased energy demands or for the synthesis of stress-related proteins and other compounds related with the stress response (Aragão et al., 2008, 2010). Therefore, the expected increase in cortisol due to both acute or chronic stress challenges and the augmented energy requirements during these

processes will probably have a great impact on AA metabolism in fish. Figure 3 summarizes some physiologically active nitrogen-containing compounds derived from AA, which may have important roles during stress and immune responses in fish.

The aforementioned increase in plasma cortisol levels due to stressful rearing conditions (e.g., Aragão et al. 2008, 2010) induced most changes in the metabolism of individual indispensable (and semi-indispensable) AA. Particularly, changes in plasma arginine, branched-chain amino acids (BCAA: leucine, isoleucine and valine), glutamine, lysine, methionine, tryptophan and tyrosine were commonly observed.

Arginine is a particularly versatile AA. Besides its importance as dietary indispensable AA for fish, its involvement in a number of metabolic pathways is well recognised (Mommsen, 2001). Hydrolysis by arginase leads to production of ornithine, which can be used for the synthesis of urea and proline, glutamate, glutamine or polyamines (Gouillou-Coustans et al., 2002). In addition, arginine can stimulate the release of various hormones such as insulin, growth hormone and glucagon, and the insulinotropic action of arginine stimulates AA uptake and protein synthesis in fish (Plisetskaya et al., 1991). Arginine can also be cleaved to produce nitric oxide (NO; Buentello and Gatlin, 1999). While physiological levels of NO play an important role in cellular signalling, a high concentration of NO can result in AA and protein modification, oxidative stress, apoptosis, and killing of target cells (Galli, 2007; Mannick, 2007). Inducible NO production in macrophages is affected by extracellular arginine availability or nutrition of channel catfish (*Ictalurus punctatus*; Buentello and Gatlin, 1999). Furthermore, Buentello and Gatlin (2001) demonstrated a positive effect of an arginine-enriched diet on the resistance of channel catfish to infection with *Edwardsiella ictaluri*. Therefore, the effects of dietary arginine on growth and health beyond meeting the requirement for protein synthesis have been reported (Arndt et al., 1994; Buentello and Gatlin, 2001; Mommsen, 2001). A substantial decrease in plasma concentrations of arginine and ornithine has been observed for Senegalese sole (*Solea senegalensis*) subjected to chronic stressful conditions (Aragão et al., 2008), suggesting the requirement of arginine may increase when fish is chronically stressed. In addition, since arginine and ornithine metabolism

converge on that of glutamate (Brosnan, 2000), they are easily transaminated into glutamate which can be readily utilized for ammonia detoxification.

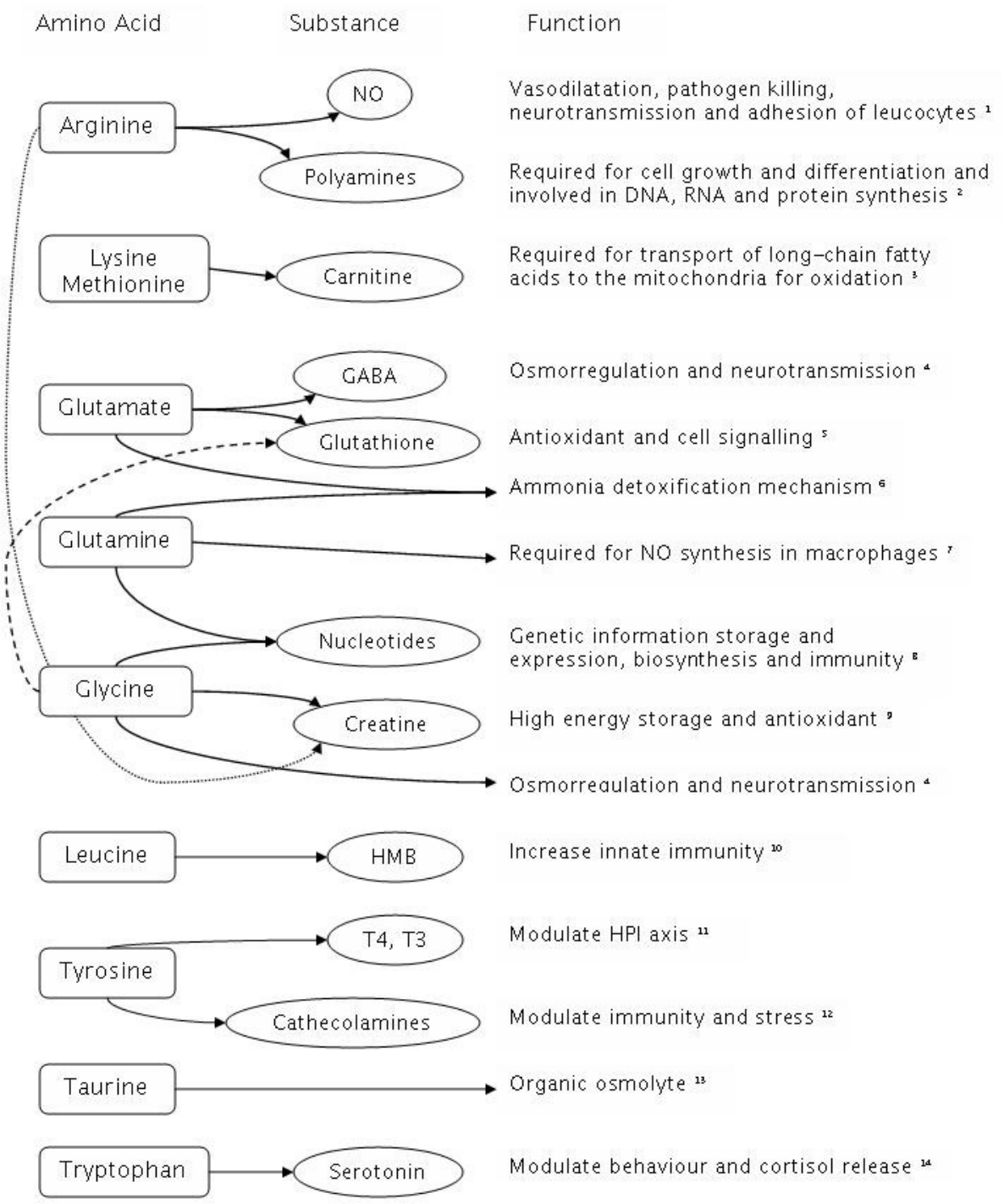

Figure 3. Overview of some important physiologically active substances which need amino acids to be synthesised and may be utilized during immune and stress responses. [1]Wu and Morris, 1998; [2]Soda et al., 2005; [3]Harpaz, 2005; [4]Schaarschmidt et al., 1999; Ballantyne, 2001; [5]Wu et al., 2004; [6]Ip et al., 2001; [7]Li et al., 2007; [8]Li and Gatlin, 2006; [9]Bystriansky et al., 2007; [10]Siwicki et al., 2003, 2006; [11]Geven et al., 2006; [12]Verburg-van Kemenade et al., 2009; [13]Yancey, 2001; [14]Lepage et al., 2002. GABA, γ-Aminobutyric acid; HMB, β-hydroxy-β-methylbutyrate; T3, triiodothyronine; T4, thyroxine.

Glutamine is one of the most versatile AA in cellular metabolism and physiology. Glutamine not only acts as a precursor for protein synthesis, but is also an important intermediate in a large number of metabolic pathways. Glutamine synthesis is an important pathway for ammonia detoxification in several fish species (Ip et al., 2001), and an increase in plasma glutamine levels in chronically stressed Senegalese sole has been previously observed (Pinto et al., 2007; Aragão et al., 2010; Costas et al., 2010). Therefore, this pathway may assume a special importance in fish exposed to exogenous ammonia, prolonged starvation and osmotic challenge. Glutamine presents also important roles in improving gastrointestinal tract maturation and health, maintaining mucosal integrity and the gut-associated lymphoid tissues, and enhancing the synthesis of secretary immunoglobulin (Wu et al., 2007; Lobley et al., 2001). Glutamine is also linked to functional activities of immune system cells, such as proliferation, antigen presentation, phagocytosis, cytokine, nitric oxide and superoxide production (Li et al., 2007). As the precursor for the synthesis of glutathione, glutamine is also essential for defending cells from oxidative stress (Wu et al., 2007).

Several studies in animals indicate that an inadequate intake of BCAA results in immune impairment and that leucine appears to exert a greater effect on immune function than isoleucine and valine (Li et al., 2007). Moreover, BCAA have an important role in regulating protein synthesis in skeletal muscle, being leucine the most effective in the regulation of this process (Yoshizawa, 2004). In fish, it has been proposed that a rise in plasma cortisol concentrations results in an increased proteolysis activity in liver leading to an increased BCAA oxidation in white muscle (Milligan, 1997). Increased plasma levels of BCAA correlated well with increased plasma cortisol levels, being consistent with the proteolytic properties attributed to cortisol in fish (Milligan 1997; Vijayan et al. 1997; Costas et al., 2010). Furthermore, some studies have recently analysed the effects of dietary supplemental β-hydroxyl-β-methyl-butyrate (a leucine metabolite) on growth and immune responses in fish. This dietary supplementation resulted in a non-specific immune enhancement in several fish species as rainbow trout (*Oncorhynchus mykiss*), pikeperch (*Sander lucioperca*) or tench (*Tinca tinca*). Moreover, no significant effects on the immune response of hybrid stripped bass (*Morone chrysops* x *M. saxatilis*) were observed (Siwicki et al., 2003, 2005, 2006;

Li and Gatlin, 2007), suggesting that further attention is still needed on this subject.

Tryptophan can be converted to serotonin and melatonin (Lepage et al., 2002). The influence of tryptophan in behaviour and stress response has gained interest in the recent years, since brain serotonin is involved in the control of aggression and HPI axis in fish (Winberg et al., 1997; Winberg and Lepage, 1998). Additional requirements for tryptophan may be expected in fish under stressful husbandry conditions, since a decrease in their plasma concentrations was observed when compared to non-stressed fish (Aragão et al. 2008). Dietary tryptophan supplementation can prevent a stress-induced cortisol surge (Lepage et al., 2002), reduce stress-induced anorexia and cannibalism (Höglund et al., 2007) and inhibit aggression in fish (Hseu et al., 2003).

Phenylalanine is the sole precursor of tyrosine. Tyrosine is involved in the synthesis of several hormones and neurotransmitters, several of those with important roles during stress response in fish, as cathecolamines (epinephrine, norepinephrine and dopamine) or thyroid hormones (triiodothyronine and thyroxine). Plasma free phenylalanine and tyrosine concentrations increased during the first 24 h of confinement in fish (Vijayan et al., 1997), whereas in chronic stress experiments no significant changes in tyrosine plasma levels were found (Pinto et al., 2007; Aragão et al., 2008). This may be indicative of an increased requirement of tyrosine during acute stress response in fish.

Lysine and methionine are the substrates for the synthesis of L-carnitine, which in fish is required for the transport of long-chain fatty acids to mitochondria for oxidation (Harpaz, 2005). Therefore, L-carnitine may be involved in fatty acids mobilization to prepare the fish to face increased energy demands due to the stress challenge imposed. Moreover, methionine is the precursor of cysteine, which in turn is required for the synthesis of glutathionine and taurine. In fact, methionine and cysteine supplementation have been shown to be beneficial for the immune system in chickens (Li et al., 2007). Therefore, these indispensable AA may play important roles in energy balance, oxidative stress, osmorregulation and immune status in animals (Li et al., 2007). Some studies reported changes in plasma levels of lysine and/or methionine in stressed fish compared with unstressed groups (Vijayan et al., 1997; Pinto et al., 2007;

Aragão et al., 2008, 2010; Costas et al., 2010), suggesting an increased usage of these particular indispensable AA due to an augmented synthesis of L-carnitine, glutathionine and/or taurine. Taking all together, a possible increase in the requirement of lysine and methionine in fish under stressful conditions deserves further attention.

1.4. Innate immunity in fish

The immune system protects an organism against diseases by identifying and eliminating the pathogen and suppressing the emergence of neoplasic cells. Another important role of the immune system is to participate in processes that maintain stable conditions (homeostasis) during development and growth and following inflammatory reaction or tissue damage (Magnadottir, 2010). The classical division of the immune system is into the innate and the adaptive systems. The importance of the innate defense mechanisms is three-fold. Firstly, the protection is non-specific and does not depend upon recognition of the distinctive molecular structure of the invading species. Secondly, there is no or little time lag for them to act. And thirdly, they are relatively temperature independent. In contrast, adaptive defense mechanisms in ectothermic vertebrates take a considerable time to respond and are very temperature dependent.

Therefore, the innate defense mechanisms are more important in fish than in endothermic vertebrates (Ellis, 2001). These non-specific defense reactions in fish represent both humoral and non-lymphoid cellular components of the immune system. The first line of defense against microorganisms is represented by the epithelia covering gills, skin and gut. These epithelia secrete a layer of mucus that contains non-specific defense factors such as complement, lysozyme and peroxidase activities, produced by cells of the immune system or epithelial cells (Iger and Wendelaar Bonga, 1994; Shephard, 1994; Ellis, 2001). When epithelial barriers are breached by microorganisms, an inflammatory response is induced with the ultimate influx of phagocytes which have potent bactericidal properties (Ellis, 2001). The involvement of neutrophils and macrophages in phagocytosis, killing and degradation of invading microoganisms, as well as different patterns of localization and mobilization into the infected areas are well

documented in fish (Steinhagen and Jendrysek 1994; Afonso et al. 1998; do Vale et al. 2002).

Furthermore, several humoral substances and cell secretions also contribute to the natural resistance of fish to pathogenic and infectious agents. These include complement, transferrins, anti-proteases, various lytic enzymes (e.g. lysozyme), lectins, C-reactive protein, interferon and enzyme inhibitors. Moreover, some of these factors, such as lysozyme and complement, appear to be more potent in fish than in mammals (Ellis, 1999, 2001). The complement system can be activated by antigen-antibody reactions or by the so called alternative route, via binding to microbial cell wall polysaccharides, which results in opsonization and/or lysis of foreign cells (Sakai, 1992). Furthermore, a group of molecules that initiate and regulate inflammatory events, the cytokines, have also been extensively studied in fish (Secombes et al., 2001).

1.5. Neuroendocrine – immune interactions in fish

It is now recognized that immune and neuroendocrine systems interact in a bi-directional way (Verburg-van Kemenade et al., 2009). The influence of stressors on immune competence have been studied in teleosts, including effects of handling, confinement, transport, crowding, subordinate position and heat shock on several immune parameters (Barton and Iwama, 1991; Schreck, 1996; Wendelaar Bonga, 1997). The well-established negative effects of stress on immune competence are thought to be maladaptive responses (tertiary stress responses) to chronic or severe stressors (Wendelaar Bonga, 1997). Stress-related physiological changes affect metabolism and cell processes (including the immune cells), compromising the innate defence mechanisms and thereby increasing the outcome of diseases (Espelid et al., 1996; Ellis, 2001). For instance, cortisol may decrease the number of lymphocytes, selectively suppress phagocytic and complement activities in head-kidney and blood and increase susceptibility to infection in teleosts (Pickering and Duston, 1983; Pickering, 1984; Law et al., 2001; Ortuño et al., 2001). A variety of different stressors (e.g. transport, anoxia, social conflict, handling, injection, crowding) have been reported to result in decreased numbers of circulating B-lymphocytes and increased numbers of circulating neutrophils in several fish species (Pickering and

Pottinger, 1987; Pulsford et al., 1994; Espelid et al., 1996). These effects are also mimicked by corticosteroid treatment *in vivo* (Ellsaesser and Clem, 1987; Espelid et al., 1996; Weyts et al., 1998a). Furthermore, macrophages isolated from Atlantic salmon (*Salmo salar*) submitted to repeated handling for 3 and 4 weeks showed decreased survival when exposed to *Aeromonas salmonicida* (Fast et al., 2008).

On the other hand, *in vitro* cortisol stimulation significantly depressed phagocytosis and respiratory burst activity of head-kidney leucocytes in several teleosts (Law et al., 2001; Esteban et al., 2004). Similarly, cortisol inhibited chemotaxis, phagocytosis and respiratory burst activity in a goldfish macrophage cell line in a dose dependent manner (Wang and Belosevic, 1995). Additionally, several *in vitro* studies demonstrated that cortisol affects the cytokine production of immune cells. For instance, cortisol inhibits LPS-induced expression of acute phase protein serum amyloid S and pro-inflammatory cytokines interleukin-1β, tumor necrosis factor-α, interleukin-11 and inducible nitric oxide synthase (Saeij et al., 2003; Huising et al., 2005; Fast et al., 2008; Stolte et al., 2008). Cortisol also affects apoptosis and proliferation of immune cells (Verburg-van Kemenade et al., 2009). For instance, cortisol inhibited proliferation of a rainbow trout monocyte/macrophage cell line (Pagniello et al., 2002). Oral administration of cortisol also induced apoptosis of lymphocytes present in the skin epithelium of rainbow trout (Iger et al., 1995).

Interestingly, neutrophilic granulocytes are protected by stress. Cortisol can induce apoptosis in activated B lymphocytes (Weyts et al., 1998b) but not in neutrophils (Weyts et al., 1998c). Similarly, repeated temperature shocks reduced the number of circulating B lymphocytes whereas the relative percentage of circulating granulocytes nearly doubled (Engelsma et al., 2003). As neutrophilic granulocytes are of great importance to the first line of defense, it may be beneficial in situations of acute stress (and possible injury) to prolong their life-span and maintain higher numbers in circulation (Verburg-van Kemenade et al., 2009). However, little attention has been paid to the effects of acute stress and catecholamines, which seem to have both stimulatory and inhibitory actions on immune functions in fish (Wendelaar Bonga, 1997). In fact, an acute increase of catecholamines and cortisol levels may signal the immune system to prepare for

possible consequences of a stressor and thus serve as an adaptive function (Verburg-van Kemenade et al., 2009).

1.6. This Thesis

Several studies have shown that the effects of dietary AA in fish go beyond meeting the requirement for protein synthesis. However, the role of dietary AA in fish under aquaculture-related stressful conditions is lacking and requires further attention. The main goal of this study is to allow a better understanding of the role of arginine and other AA in relation to the stress response and disease resistance in fish. This Thesis will focus mainly on the role of indispensable AA due to the need of meeting its requirements through dietary sources. The possibility of mitigating the negative effects of stress and disease susceptibility of fish by altering dietary AA levels is proposed. Ultimately, this study should contribute to the improvement of commercial diets for sole and farming results, through in better welfare and reduced disease susceptibility of this species.

A general overview of AA metabolism and stress and immune responses in fish, with particular reference to stressful rearing conditions is given in **Chapter 1**. The effects of both acute and chronic stressors on AA metabolism and stress and immune responses of Senegalese sole are assessed in **Chapters 2 and 3**. Particularly, **Chapter 2** evaluates the metabolic response of this species to a chronic stressor, whereas **Chapter 3** describes metabolic and immune responses to an acute stressor. In **Chapter 4** digestibility and retention efficiency of individual indispensable AA are described. The effects of increased dietary AA availability on the stress response and AA metabolism are assessed in **Chapter 5**. **Chapter 6** deals with the immune and inflammatory responses of Senegalese sole following challenge with *Photobacterium damselae* subsp. *piscicida* (*Phdp*) and validates the infection model. **Chapter 7** assesses the effects of arginine supplementation on the immune response and resistance of chronically stressed Senegalese sole to *Phdp*. In **Chapter 8,** the effects of fish meal replacement by plant protein sources and indispensable AA supplementation on some innate immune parameters are assessed in a contribution to the development of practical, environmentally sustainable and cost-effective plant protein based diet for Senegalese sole juveniles. Finally, the overall AA metabolism and physiological

responses to different stressors, as well as subsequent changes on innate immune parameters in stressed Senegalese sole will be discussed in **Chapter 9**. Additionally, this chapter also discusses the effects of dietary AA supplementation on the stress response, immune status and disease resistance, and the use of dietary AA as nutraceutics for fish is proposed.

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Chapter 2

High stocking density induces crowding stress and affects amino acid metabolism in Senegalese sole *Solea senegalensis* (Kaup 1858) juveniles

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Costas, B., Aragão, C., Mancera, J.M., Dinis, M.T., Conceição, L.E.C., 2008. High stocking density induces crowding stress and affects amino acid metabolism in Senegalese sole *Solea senegalensis* (Kaup 1858) juveniles. Aquaculture Research 39, 1 – 9.

High stocking density induces crowding stress and affects amino acid metabolism in Senegalese sole *Solea senegalensis* (Kaup 1858) juveniles

Abstract

Fish held at high stocking densities are generally exposed to chronic stress situations that impose severe energy demands and may predispose the fish to infection. Senegalese sole *Solea senegalensis* (Kaup) juveniles (78.8 ± 18.9 g body weight) were maintained at low (LSD; 4 kg m^{-2} at the end of the experiment), medium (MSD; 9 kg m^{-2}) and high (HSD; 14 kg m^{-2}) stocking densities during a period of 63 days. Although disease outbreaks were observed in fish reared at HSD, growth and food consumption did not vary among different treatments. Results from plasma cortisol and free amino acids showed significant differences among different rearing densities pointing to HSD as stressful rearing condition. However, higher plasma glucose and osmolality levels indicated that fish held at MSD may also be under stress. The higher usage of free amino acids from HSD group may be due to higher demand for energy production in order to cope with stressful rearing conditions, higher rate of protein synthesis or due to synthesis of other important metabolites related to stress response. Therefore, results from the present study point out to HSD as a stressful rearing condition and suggest that crowding stress may affect amino acid requirements.

Keywords: Amino acids, cortisol, density, glucose, Senegalese sole, stress response

2.1. Introduction

Senegalese sole *Solea senegalensis* (Kaup) is a species highly valued for its taste, flesh quality and price, and has been introduced as a new species in aquaculture since early 1980s (Ramos 1982; Dinis 1986). Reproduction control and successful spawning, hatching and larval rearing under captivity have all been already studied and achieved (Dinis, Ribeiro, Soares & Sarasquete 1999). Nowadays, some Spanish and Portuguese companies are carrying out broodstock management and ongrowing culture of Senegalese sole (Rodríguez & Souto 2003). Nevertheless, growth and survival from juvenile to market-size fish revealed deficiencies with regard to rearing technology and husbandry

conditions, feeding behaviour, and nutritional requirements. Among the different factors that may induce high mortality during juvenile stage, stress might be one of the key issues.

The stress response is vitally important for all living organisms. In response to a stressor a fish will undergo a series of biochemical and physiological changes in an attempt to compensate for the challenge imposed upon it, and thereby cope with the stress (Wendelaar Bonga 1997; Iwama, Afonso & Vijayan 2004). Various stressors, such as grading, transport, crowding and vaccination, are necessary components of modern intensive fish culture. High stocking density has been considered as an aquaculture-related chronic stressor producing a chronic elevation of plasma cortisol (Pickering & Pottinger 1989; Barton & Iwama 1991; Mommsen, Vijayan & Moon 1999). Plasma cortisol level is widely used as a general indicator of stressful situations in vertebrates and particularly in fish. An elevation of plasma cortisol induces a wide variety of secondary physiological responses (Barton & Iwama 1991; Wendelaar Bonga 1997), including a decrease in immune response (Tort, Sunyer, Gómez & Molinero 1996), which will increase disease susceptibility, and may decrease growth and survival rates (Vijayan, Ballantyne & Leatherland 1990; Rotllant, Arends, Mancera, Flik, Wendelaar Bonga & Tort 2000, Rotllant, Balm, Pérez-Sánchez, Wendelaar Bonga & Tort 2001; Tort 2005; Portz, Woodley & Cech 2006). At metabolic level amino acids are an important energy source in fish (Cowey & Walton 1989), contrasting to that observed in homeotermic animals. Under a stress situation, cortisol will influence amino acid metabolism, leading to a decrease in plasma amino acid concentrations (Aragão, Corte-Real, Costas, Dinis & Conceição in press), which are subsequently funnelled into key metabolic pathways, including glycogenesis, gluconeogenesis and possibly protein synthesis (Mommsen *et al.* 1999).

This study was designed with three aims: i) to investigate how does density have influence upon growth performance in Senegalese sole juveniles; ii) to study whether density induces stress or Senegalese sole physiologically copes with different rearing densities; and iii) to determine if stress changes amino acid requirements, using as indicator plasmatic free amino acid concentrations.

2.2. Materials and methods

2.2.1. Experimental procedures

Fish originated from natural spawning of wild broodstock and were reared according to standard larval and juvenile rearing protocols (Dinis *et al.* 1999). Prior to the experiment, they were maintained in one flat-bottomed fibreglass tank for at least two months at a density of 6 kg m^{-2}, in open seawater circulation system with natural temperature and photoperiod. The experiment was carried out at the Ramalhete Research Station (CCMAR, Faro, Portugal). One hundred and forty-four Senegalese sole juveniles of 78.8 ± 18.9 g (mean ± standard deviation) were anaesthetized with 2-phenoxyethanol (Sigma-Aldrich, Germany; 300 ppm) and marked individually with intramuscular colour marks in the abdomen. Fish were measured, weighed and distributed in order to have a mean weight around 80 g per fish per tank, over nine 42 L yellowish flat-bottomed fibreglass tanks (70 cm length x 30 cm width x 20 cm depth) filled with 20 L of seawater. Three densities were tested in triplicate: low stocking density (LSD) with 8 fish per tank (3.1 kg m^{-2} at initial density); medium stocking density (MSD) with 16 fish per tank (6.1 kg m^{-2} at initial density); and high stocking density (HSD) with 24 fish per tank (9.1 kg m^{-2} at initial density). Stocking densities were chosen in order to span the range currently used in intensive systems for Senegalese sole. Fish were kept during 63 days in a partial-recirculated seawater system. Water flows varied among treatments with the aim of maintaining oxygen saturation over 80% and avoid ammonia and nitrite accumulation in the tanks. Thus, water supply rate was 171 L h^{-1} for HSD tanks, 114 L h^{-1} for MSD tanks and 54 L h^{-1} for LSD tanks. Water temperature ranged between 19-21 ºC, salinity was 36 ‰ and the photoperiod was 9L:15D. Fish were fed every day by hand four times a day (9:30 h; 12:30 h; 15:00 h and 17:30 h) to apparent satiety (based on the assessment of the remaining food) with the 3 mm commercial diet "DourasojaExtra3" (Aquasoja, SORGAL, S.A., Ovar, Portugal). At the beginning of the experiment fish were fed with 2% of the biomass. Ration size was daily adjusted (± 0.5%) based on the assessment of the non consumed food, with an average ration of 1.7 % biomass day^{-1}. Fish from each tank were fed with the same food amount per kg of fish. Furthermore, fish were not fed 24 h before sampling since plasma cortisol and glucose levels may be affected by feeding (Arends, Mancera, Muñoz, Wendelaar Bonga & Flik 1999).

At end of the experiment (day 63), individual sampling of each tank was carried out. Firstly, anaesthetic 2-phenoxyethanol (Sigma; 500 ppm) was added into each target tank in order to avoid stress due to handling. After one minute, five fish were quickly taken out from each tank at a time and anaesthetized with 2-phenoxyethanol (Sigma; 1500 ppm). Blood samples were withdrawn from the caudal vein by puncturing with a heparinised syringe. The blood collection lasted less than 3 min. in order to avoid cortisol increase due to manipulation during sampling. After each tank sampling, stored blood was centrifuged at 1500 x *g* during 2 min. at room temperature. The collected plasma was stored at -25 ºC until assayed. In addition, all fish from each tank was weighed and measured. In fish used to collect blood, liver was subsequently dissected on a glass placed over an ice bed, weighed, frozen in liquid nitrogen and kept at -80 ºC for further enzymatic analysis.

2.2.2. Analytical procedures

Plasma cortisol was determined by radioimmunoassay (RIA) as described by Rotllant, Ruane, Dinis, Canário & Power (2006). Briefly, 50 µL of plasma samples were diluted in 950 µL phosphate buffer containing 1 g L^{-1} gelatin, pH 7.6 and denatured at 80°C for 1 h. Duplicate aliquots (100 µL) of diluted denatured plasma were then used in the assay.

Glucose analysis was performed on plasma samples using a commercially available kit (Boehringer Mannheim, R-Biopharm AG., Darmstadt, Germany), after deproteinisation using Carrez reagents. Plasma osmolality was determined using a cryo-osmometer (Osmomat 030).

Plasmatic 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, Meys & Tarvin (1989). Resulting peaks were analysed with the Breeze software (Waters, USA).

Livers were individually assayed for enzyme activities of amino acid catabolism. Crude extracts were obtained by homogenisation of frozen tissue in ice-cold buffer (30 mM Hepes, 0.25 mM saccharose, 0.5 mM EDTA, 5 mM K_2HPO_4, 1 mM dithiothreitol; pH 7.4), followed by centrifugation at 1000 x_*g* at 4 ºC for 10 min. The supernatants were then treated by ultrasound and centrifuged again at 15000 x *g* at 4 ºC for 20 min. Activities of alanine aminotransferase (ALAT, EC 2.6.1.2), aspartate aminotransferase (ASAT, EC 2.6.1.1) and glutamate dehydrogenase (GDH, EC 1.4.1.2) were measured on supernatants at 37 ºC using spectrophotometric procedures, according to Aragão, Conceição, Dias, Marques, Gomes & Dinis (2003). Enzyme activity units (IU), defined as µmoles of substrate converted to product per minute at assay temperature, are expressed per gram of liver (total activity). Due to technical constraints, not all analytical procedures were performed for all samples.

2.2.3. Data analysis

For each fish, relative growth rate (RGR), condition factor (K) and hepatosomatic index (HSI) were calculated as follows:

$$RGR\ (\%\ day^{-1}) = (e^{g} - 1) \times 100$$

with:

$$g = [\ln (W_2) - \ln (W_1)]\ days^{-1}$$

where, W_1 and W_2 are the initial and final wet weights, respectively;

$$K\ (g\ cm^{-3}) = \text{wet weight} / \text{total length}^3 \times 100;$$

$$HSI\ (\%) = \text{liver weight} / \text{final wet weight} \times 100.$$

Statistical analysis was performed using the computer package SPSS for Windows 15.0. Biometrical results were expressed as mean ± standard deviation (SD) and analytical results as mean ± standard error of the mean (SEM). Data were analysed for normality (Kolomogorov-Smirnov test) and homoscedasticity of variance (Levene's test) and, when necessary, they were log-transformed before being treated statistically. Data was analysed by one-way analysis of variance (ANOVA). When significant differences were obtained from the ANOVA, multiple comparisons were carried out performing Tukey – HSD mean comparison test.

The level of significance used was $P \leq 0.05$ for all statistical tests. All results expressed as a percentage were previously arcsine transformed (Zar 1999). In addition, Dixon's criteria for testing extreme observations was performed in order to exclude values regarded as outliers (Snedecor & Cochram 1989).

2.3. Results

2.3.1. Growth and survival

During the experiment fish held at LSD and MSD did not present any illness symptom or mortality, while in fish held at HSD disease outbreaks where observed. Four fishes from one replicate of the HSD treatment died by the end of the first month of the experiment. Therefore, this tank was excluded from the experiment because of infection risk for the remaining tanks. In addition, in day 53 of the experiment some fish from another replicate of the HSD treatment showed the same symptoms that the ones previously excluded. To avoid possible spread of the disease a treatment with antibiotic was performed to all fish in that tank. Since antibiotics may alter physiological parameters in mammals (Barrero González, Sánchez Miranda & Cruz Padrino 2004; Carroll, Fangman, Hambach & Wiedmeyer 2004), only data from fish held at HSD in the untreated tank was used in this study, hence, ten fish from this tank were analysed in order to have enough data for physiological parameters, and all fish from both HSD tanks were used for biometric data.

At end of the experiment (day 63), final densities were respectively 4.3 ± 0.1, 9.2 ± 0.3 and 13.6 ± 0.5 kg m^{-2} for LSD, MSD and HSD. Table 1 showed fish growth parameters assessed (RGR, wet weight, K and HSI) and no significant ($p>0.05$) differences among LSD, MSD and HSD treatments were observed.

2.3.2. Stress indicators

Fish held at HSD had significantly higher cortisol levels than fish held at MSD and LSD (Figure 1), with no significant differences between fish held at MSD and LSD. Plasma glucose and osmolality levels were significantly higher in fish held at MSD and HSD with respect to fish held at LSD (Figures 2 and 3). However, these parameters did not vary significantly between fish held at HSD and MSD.

Table 1. Relative growth rate (RGR), mean wet weight, condition factor (*K*) and hepatosomatic index (HSI) in *Solea senegalensis* held at different stocking densities.

		Wet weight (g)			
Treatments	RGR (% day^{-1})	Initial	Final	*K* (g cm^{-3})	HSI (%)
Low density	0.63 ± 0.15	76.7 ± 18.1	113.1 ± 24.6	1.4 ± 0.3	1.97 ± 0.74
Medium density	0.66 ± 0.16	80.5 ± 18.6	121.3 ± 27.7	1.2 ± 0.4	2.15 ± 0.79 ‡
High density	0.69 ± 0.14	77.8 ± 19.0	116.8 ± 27.0	1.6 ± 0.4	2.26 ± 0.72 ‡

Values are means±SD (n=24 for low density; n=48 for medium and high densities, except ‡ n=24). Differences were not significant among groups.

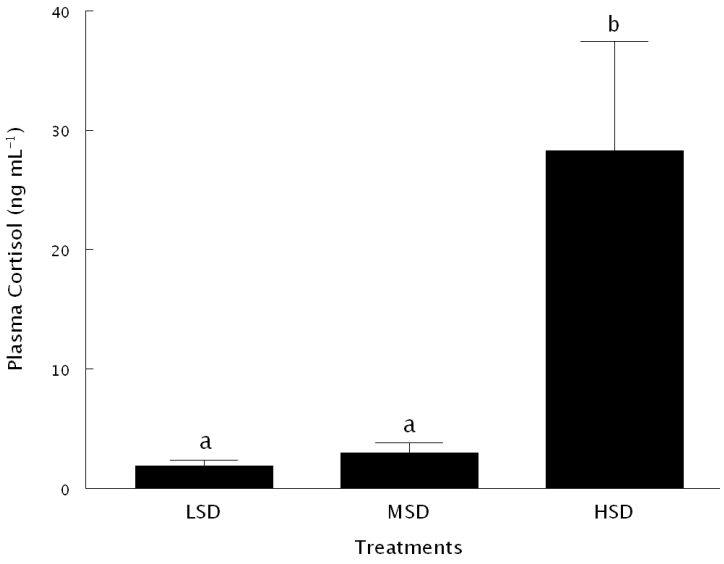

Figure 1. Plasma cortisol levels in *Solea senegalensis* juveniles held at LSD, MSD or HSD. Values are means ± SEM (n=7 for LSD; n=13 for MSD; n=9 for HSD). Different letters indicate significant differences among treatments ($p < 0.05$).

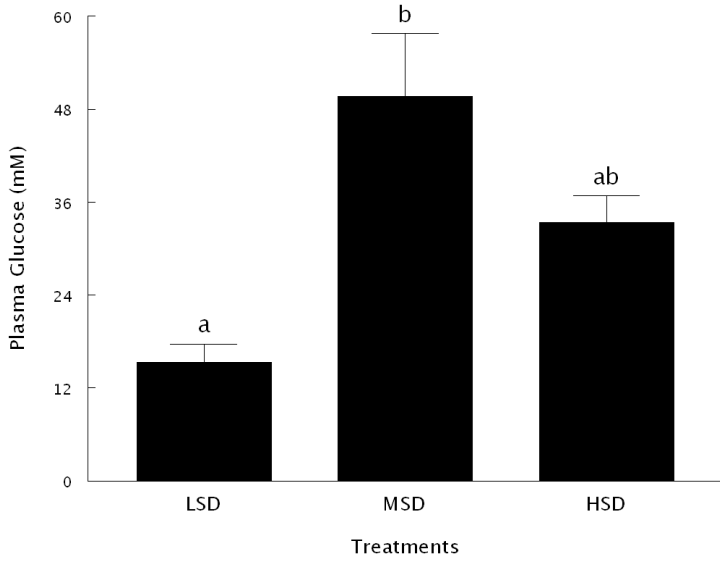

Figure 2. Plasma glucose levels in *Solea senegalensis* juveniles held at LSD, MSD or HSD. Values are means ± SEM (n=7 for LSD; n=15 for MSD; n=10 for HSD). Different letters indicate significant differences among treatments ($p < 0.05$).

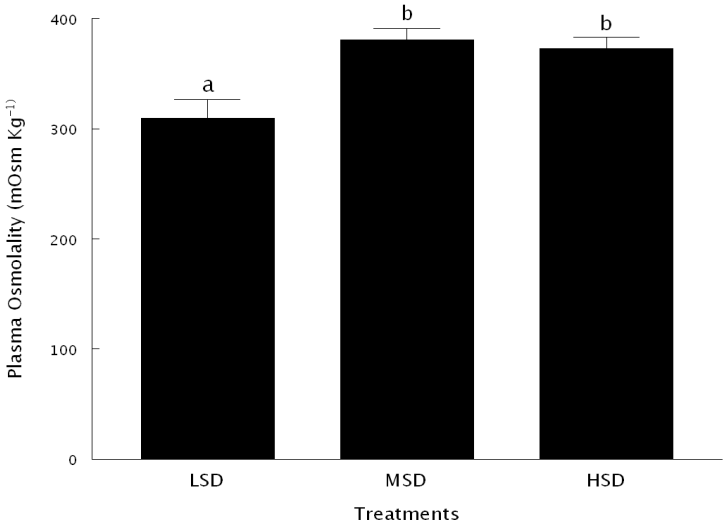

Figure 3. Plasma osmolality levels in *Solea senegalensis* juveniles held at LSD, MSD or HSD. Values are means ± SEM (n=7 for LSD; n=15 for MSD; n=9 for HSD). Different letters indicate significant differences among treatments ($p < 0.05$).

2.3.3. Free amino acid concentrations and enzymatic activities

Total plasma free amino acid (FAA) concentrations (Figure 4) showed an inverse linear relationship respect to stocking density, with significant differences between fish held at LSD and HSD. A similar trend was also observed for the sum of both dispensable amino acid (DAA) and indispensable amino acid (IAA) concentrations in plasma.

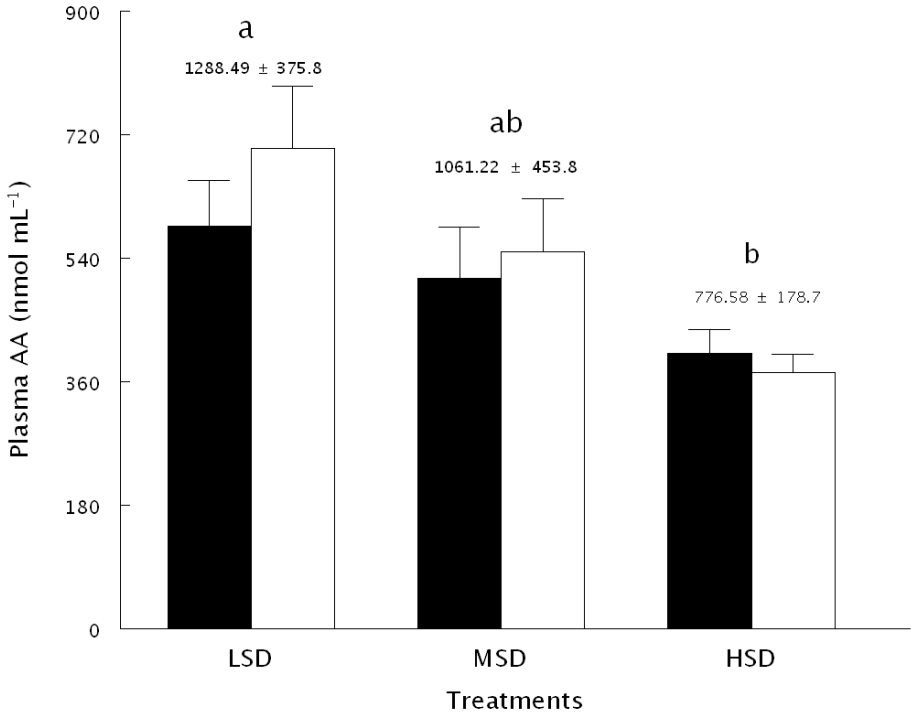

Figure 4. Total dispensable (■) and indispensable (□) amino acid plasmatic concentrations in *S. senegalensis* juveniles held at LSD, MSD or HSD. Values are means ± SEM (n=6 for LSD; n=9 for MSD and HSD). Values above bars indicate total free amino acids for each treatment. Different letters indicate significant differences among treatments both for DAA and IAA ($p<0.05$).

Regarding DAA concentrations (Figure 5A), significant differences were found for citrulline, glutamine, hydroxyproline, ornithine, and serine. Respect to IAA concentrations (Figure 5B), significant differences were found for arginine, histidine, isoleucine, leucine, lysine, valine and tryptophan. Similarly to total plasma FAA an inverse linear relationship was observed in several plasma AA levels respect to stocking density (Figures 5A and B).

Data on hepatic activities of the three main enzymes involved in amino acid catabolism (alanine aminotransferase, ALAT; aspartate aminotransferase, ASAT; glutamate dehydrogenase, GDH) are shown in Table 2. Total enzymatic activities did not vary significantly among treatments. The same tendency was observed when the results are expressed per whole liver weight per 100 g of fish or per gram of protein (results not shown).

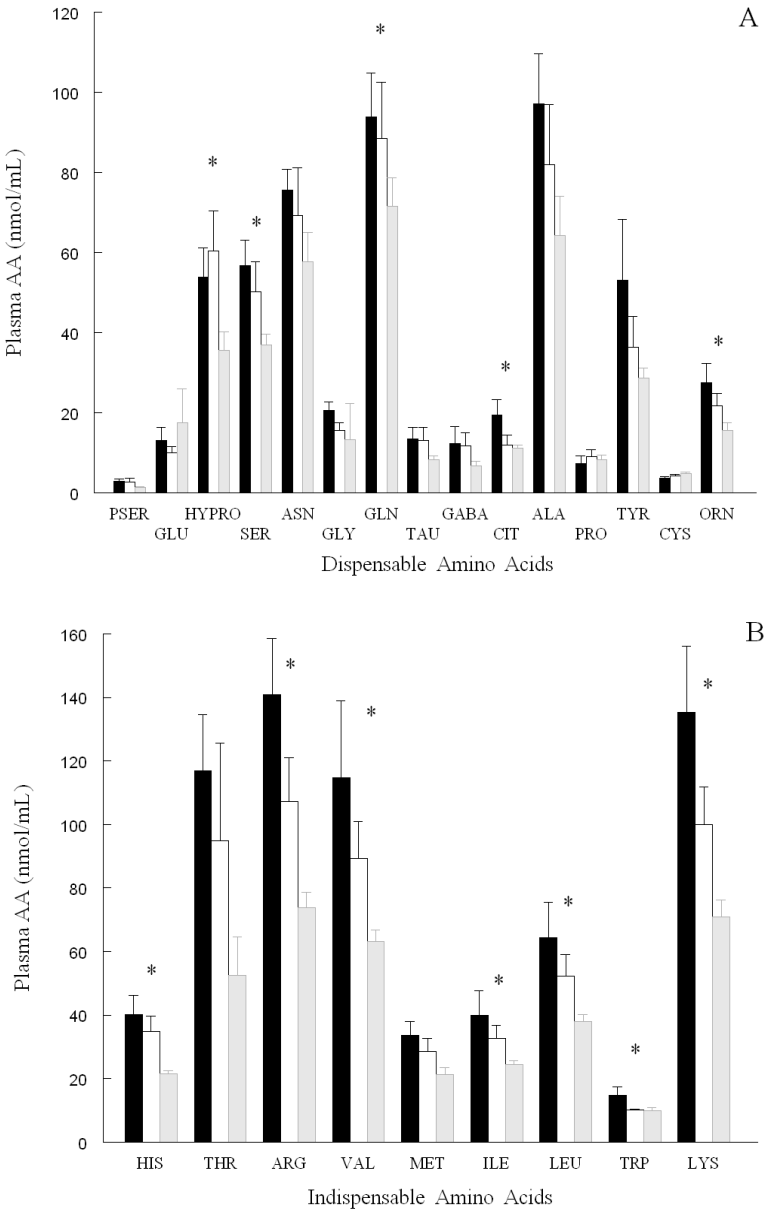

Figure 5. Dispensable (A) and indispensable (B) amino acid plasmatic concentrations in *S. senegalensis* juveniles held at LSD (■), MSD (□) or HSD (▩). Values are means ± SEM (n=6 for LSD; n=9 for MSD and HSD). Asterisk indicate significant differences between LSD and HSD ($p<0.05$). Alanine (ALA), asparagine (ASN), arginine (ARG), citrulline (CIT), cystine (CYS), glutamine (GLN), glutamic acid (GLU), glycine (GLY), histidine (HIS), hydroxyproline (HYPRO), isoleucine (ILE), leucine (LEU), lysine (LYS), methionine (MET), ornithine (ORN), proline (PRO), phosphoserine (PSER), serine (SER), taurine (TAU), threonine (THR), tryptophan (TRP), tyrosine (TYR), valine (VAL), γ-aminobutyric acid (GABA).

Table 2. Effect of different stocking densities on activities of amino acid catabolic enzymes in liver of *Solea senegalensis* held at different stocking densities.

Treatments/Enzymes	ALAT (IU g^{-1} liver)	ASAT (IU g^{-1} liver)	GDH (IU g^{-1} liver)
Low density	31.2 ± 1.3	111.5 ± 8.4	4.6 ± 0.3
Medium density	27.9 ± 1.2	112.7 ± 8.1	4.0 ± 0.3
High density	25.9 ± 2.2	108.8 ± 8.7	4.3 ± 0.2

Values are means ± SEM (n=15 for low and medium densities; n=10 for high density). Differences were not significant among groups. ALAT = alanine aminotransferase, ASAT = aspartate aminotransferase, GDH = glutamate dehydrogenase.

2.4. Discussion

Cortisol in plasma is considered to be a good indicator of stress levels in fish (Wendelaar Bonga 1997; Barton & Iwama 2001). In the present study, fish held at HSD showed significantly higher plasma cortisol levels when compared with LSD and MSD groups. These findings are in agreement with that observed for seabream *Sparus aurata* L. (Montero *et al.* 1999; Sangiao-Alvarellos, Guzmán, Laiz-Carrión, Míguez, Martín del Rio, Mancera & Soengas 2005), Atlantic salmon *Salmo salar* L. (Mazur & Iwama 1993) and brown trout *Salmo trutta* L. (Pickering & Pottinger 1985), suggesting that Senegalese sole is under stress when reared at HSD (9 kg m^{-2}). It has been shown that high plasma cortisol activate gluconeogenesis stimulating glucose production and increasing plasma glucose levels (Vijayan, Foster & Moon 1993; Laiz-Carrión, Sangiao-Alvarellos, Guzmán, Martín del Rio, Míguez, Soengas & Mancera 2002; Laiz-Carrión, Martín del Rio, Míguez, Mancera & Soengas 2003). Results from this study indicated as fish held at MSD and HSD presented significantly higher levels of plasma glucose respect to LSD, other accepted indicator of stress in fish (Wendelaar Bonga 1997; Barton & Iwama 2001). Since fish held at MSD showed significantly lower plasma cortisol levels, it is possible that they adapted to stress maintaining plasma glucose at high levels in order to cope with the high-energy demand due to stressful conditions, while HSD group was not able to adapt to the new stressful situation. It is also necessary to remark the great variability in plasma cortisol and glucose values within treatments observed in this study that may be due to different stress responses among individual sole.

The physiological response of a fish to chronic confinement results in modifications to cardiorespiratory and metabolic functions, which carry with them a temporary side effect of osmotic and ionic disturbance. In salt water fish this is seen as a diffusional loss of osmotic water efflux and the ions influx (Pickering 1998). In the present study, fish held at MSD and HSD showed significantly higher levels of plasma osmolality respect to LSD, suggesting an osmotic and ionic disturbance due to the induced stressful condition, as it has been demonstrated in other teleosts (Arends *et al.* 1999; Varsamos, Flik, Pepin, Wendelaar Bonga & Breuil 2006) including Senegalese sole (Aragão *et al.* in press).

Previous studies have shown that stocking density may alter growth rate (Pickering & Stewart 1984), feeding behaviour (Almazán-Rueda 2004), and disease resistance of fish (Mazur & Iwama 1993). However, high stocking densities did not affect feed intake in gilthead seabream after a period of 15 weeks (Montero *et al.* 1999). The results from this study did not show significant differences among the three stocking densities used with regard to RGR, wet weight, condition factor and HSI. Therefore, fish gained the same body weight regardless of treatment in which they were reared during the experimental period. These findings suggest that crowding stress, at least under the present experimental conditions, do not alter growth in Senegalese sole.

Stress is known to increase susceptibility of fish to infectious diseases, and challenges with various bacterial and protozoan pathogens have been used to assess its effects (Varsamos *et al.* 2006). Furthermore, a study carried out by Pulsford, Crampe, Langston & Glynn (1995), reported that cortisol suppressed phagocytic activity and proliferative activity of lymphocytes in some species of marine flatfish, like flounder *Platichthys flesus* L. and sole *Solea solea* L. Cortisol has also been shown to induce apoptosis in carp B-lymphocytes (Kemenade, Nowak, Engelsma & Weyts 1999). In this study, several disease events observed in fish under HSD, which presented higher plasma cortisol levels (see above), contribute to confirm that chronic stress situations increase the susceptibility of Senegalese sole to infectious diseases. Thus, susceptibility to infections seems to increase only at HSD, suggesting optimal rearing densities below 9 kg m^{-2} for Senegalese sole juveniles around 100 g.

Under stressful conditions an animal needs to provide energy substrates to tissues such as brain, gills and muscles, in order to cope with the increased energy demand (Iwama *et al.* 2004). In addition, Medale, Parrent & Vellas (1987) suggested that a decrease in plasma amino acids might be due to an increase in tissue energy utilization. The results from the current study showed that some DAA and IAA plasma concentrations were significantly lower in fish held at HSD respect to fish under LSD, suggesting that fish under HSD condition used more FAA as a source of energy facing the increased energy demand due to stressful conditions. Under this situation it would be expected an increase in liver enzymatic activities involved in amino acid catabolism (i.e. ALAT, ASAT), since several studies have provided evidence that alanine constitutes a good hepatic gluconeogenic precursor in fish (Medale *et al.* 1987). However, significant differences in these activities in liver of Senegalese sole maintained under different stocking densities were not observed. In addition, higher rate of protein synthesis, eventually in particular of stress-related proteins, could explain the lower plasmatic concentrations of most FAA in fish held at HSD. A similar decrease in plasma FAA concentration has been associated to an increase of protein synthesis in rainbow trout *Oncorhynchus mykiss* (Walbaum) (Carter, He, Houlihan, McCarthy & Davidson 1995) and in mammals (Schreurs, Koopmanschap & Boekholt 1997).

Another possible explanation for the higher usage if IAA in fish held at HSD, may be increased requirements of individual IAA involved in metabolic pathways that could be important during stressful conditions. For instance, arginine and histidine concentrations were significantly lower in fish under HSD respect to LSD. In mammals arginine presents an effect as immunological modulator (Park 1993). Moreover, when catabolised, arginine can be cleaved to produce ornithine or nitric oxide (NO), a molecule with bactericidal properties (Strasser, McCarron, Ishii, Stanimirovic & Spatz 1994; DiLorenzo, Bass & Krantis 1995). In addition, an important metabolite of histidine is the chemical messenger histamine, a powerful component of many allergic and inflammatory reactions (Massey, Blakeslee & Pitkow 1998). Therefore, fish held at HSD may use arginine and histidine in order to cope with the increased susceptibility to infections due to the chronic stress situation induced by HSD condition.

Results from the present study indicated that fish held at HSD had significantly lower tryptophan concentrations. This AA may be converted into two products: niacin and serotonin, being this the precursor of melatonine (Shibata 1995). It has been demonstrated that a chronic stress situation, where high cortisol levels are expected, can induce an elevation of plasma melatonine levels (Relkin 1989; Larson, Winberg, Mayer, Lepage, Summers & Øverlif 2004). All these data suggested an increase in tryptophan metabolism in order to increase plasma melatonin levels. However, further analyses are necessary to confirm this hypothesis, for instance, serotonin quantification in brain or melatonin in plasma.

In summary, while growth and food consumption in Senegalese sole were not significantly affected by different stocking densities, mortalities and disease events observed in fish held at HSD suggest that Senegalese sole around 100 g should be reared, under conditions comparable to the ones tested, at densities below 9 kg m^{-2}. Senegalese sole may not be able to adapt when reared at this husbandry condition. This study also suggests the existence of a wide intraspecific variability to stress response in Senegalese sole, which might define if a fish will or not adapt to different stocking densities. Furthermore, the lower plasma FAA concentrations observed in fish under HSD may be due to either increased demand for energy production and/or due to synthesis of proteins or other important metabolites related to stress response, what suggests that crowding stress may affect IAA requirements.

2.5. Acknowledgements

This work was supported by project STRESSAA - POCTI/CVT/49324/2002 (FCT, Portugal and FEDER). Cláudia Aragão benefited from a grant by Fundação para a Ciência e Tecnologia, Portugal (SFRH/BPD/14835/2003).

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Chapter 3

Physiological responses of Senegalese sole (*Solea senegalensis* Kaup, 1858) after stress challenge: effects on non-specific immune parameters, plasma free amino acids and energy metabolism

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Costas, B., Conceição, L.E.C., Aragão, C., Martos, J.A., Ruiz-Jarabo, I., Mancera, J.M., Afonso A., 2011. Physiological responses of Senegalese sole (*Solea senegalensis* Kaup, 1858) after stress challenge: effects on non-specific immune parameters, plasma free amino acids and energy metabolism. Aquaculture 316, 68 – 76.

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Abstract

Physiological responses after an acute handling stress and their subsequent effects on innate immune parameters, plasma free amino acids (AA) and liver energy substrates were assessed in Senegalese sole (*Solea senegalensis*). Eight groups of six specimens (136.1 ± 58.4 g wet weight) were maintained undisturbed, while other eight groups of six specimens were used for acute stress challenge (air exposed during 3 minutes). A group of six specimens was sampled for blood and head-kidney collection immediately after air exposure (time 0), while the remaining groups were sampled at 5 and 30 minutes, 1, 2, 4, 6 and 24 h. Undisturbed fish were sampled at the same times and used as control. Plasma cortisol, glucose, lactate and osmolality levels increased immediately after stress peaking at 1 h in air exposed fish. Changes in plasma free AA were also observed at 1 and 24 h after stress. In liver, glycogen levels significantly decreased at times 30 min and 1 h, while triglycerides values significantly increased at times 1, 2 and 4 h in air exposed fish. In addition, total AA levels in liver augmented significantly at 2 h holding high until 24 h in air exposed specimens. The respiratory burst of head-kidney leucocytes from air exposed fish was significantly higher than that from control groups at 2 and 6 h after air exposure. On the other hand, plasma lysozyme activity significantly decreased at 4 h after acute stress in air exposed fish, while plasma alternative complement pathway followed an inverse linear relationship with respect to cortisol showing the lowest value at 1 h after air exposure. The present study suggests that Senegalese sole presents a stress response comparable to that observed in other teleosts. While some indispensable AA may be used for the synthesis of compounds related to the stress response or fatty acid transport, dispensable AA were probably mainly employed either as energy sources or in gluconeogenesis. Moreover, results from non-specific immune parameters assessed suggest that cortisol may act as regulator of the innate immune system.

Keywords: amino acids; cortisol; plasma metabolites; respiratory burst activity; stress response; Sengalese sole

3.1. Introduction

Fish in aquaculture are often exposed to husbandry-related acute (e.g. handling, temperature changes) and chronic (e.g. rearing density, water quality) stressors, which induce physiological alterations in response to the stress imposed. Plasma cortisol level is widely used as a general indicator of stressful situations in fish (Wendelaar Bonga, 1997; Mommsen et al., 1999). Several stressors, such as air exposure or net confinement, induce a significant increase in plasma cortisol levels in teleosts (e.g., Arends et al., 1999; Acerete et al., 2004). Most studies relating cortisol effect on carbohydrate metabolism in fish rely on plasma glucose and liver glycogen content as indicators of metabolism (Mommsen et al., 1999). In fact, hepatic metabolic changes associated to stressful conditions (e.g. hypoxia, high densities, osmotic challenge) have been reported in fish (Vijayan et al., 1990; Dalla Via et al., 1994; Sangiao-Alvarellos et al., 2005, 2006; Arjona et al., 2009). Furthermore, plasma glucose levels usually augment following stressful situations such as handling, crowding, salinity transfer or acute stress (Waring et al., 1996; Arends et al., 1999; Arjona et al., 2007; Costas et al., 2008). Similarly, plasma lactate concentrations increase significantly in several fish species following severe exercise (Milligan, 1996) or as a result of hypoxia (Arends et al., 1999). In addition, stress conditions that induced high plasma cortisol levels also modified fish amino acid (AA) metabolism in several teleost species (Milligan, 1997; Vijayan et al., 1997; Pinto et al., 2007; Aragão et al., 2008, 2010; Costas et al., 2008). In fact, it has been suggested that fish under stressful conditions present additional AA requirements, due to higher energy demands or for the synthesis of stress-related proteins and other compounds related with the stress response (Aragão et al., 2008, 2010; Costas et al., 2008). Therefore, the expected increase in cortisol due to an acute stress challenge and the increased energy requirements during this process will probably have a great impact on AA metabolism in fish.

Stress-related physiological changes affect metabolism and cell processes (including the immune cells), compromising the innate defence mechanisms and thereby increasing the outcome of diseases (Espelid et al., 1996; Ellis, 2001). It is now recognized that the neuroendocrine and immune systems interact in a bi-directional way (Verburg-van Kemenade et al., 2009). In fish, the well-established negative effects of stress on immune competence are thought to be maladaptive

responses (tertiary stress responses) to chronic or severe stressors. For instance, cortisol may decrease the number of lymphocytes, selectively suppress phagocytic and complement activities in head-kidney and blood and increase susceptibility to infection in teleosts (Pickering and Duston, 1983; Pickering, 1984; Law et al., 2001; Ortuño et al., 2001). However, an acute increase of cortisol levels may signal the immune system to prepare for possible consequences of a stressor and thus serve as an adaptive function (Verburg-van Kemenade et al., 2009).

Senegalese sole (*Solea senegalensis*) constitutes a new option in aquaculture. The few existing studies focusing on stress response of this species relate to chronic stressors, pointing to an elevation of plasma cortisol values in fish submitted to chronic handling (Aragão et al., 2008), high stocking densities (Costas et al., 2008; Salas-Leiton et al., 2010), osmotic challenge (Arjona et al., 2007, 2009; Aragão et al., 2010) or temperature (Arjona et al., 2010), and lower values in specimens exposed to chronic ammonia (Pinto et al., 2007), when compared to control fish. Furthermore, there is no data available regarding either changes on plasma AA levels or innate immune parameters after an acute stress challenge for this species. Therefore, this study aimed to evaluate the primary and secondary stress responses of Senegalese sole after an acute stress challenge, and to assess to what extent the subsequent stress response may influence plasma AA levels, liver energy substrates, and some innate cellular and humoral immune parameters.

3.2. Material and Methods

3.2.1. Experimental procedures

The experiment was carried out at the CIIMAR facilities (Porto, Portugal), where 96 Senegalese sole (136.1 ± 58.4 g wet weight) were randomly distributed in two separate recirculated seawater systems (temperature: 18-20 ºC; salinity: 34 ‰; photoperiod: 14h light / 10h dark; dissolved oxygen: above 90 % saturation level). In one of the systems, 48 fish were maintained in eight flat-bottomed rectangular tanks (60 cm length x 35 cm width x 40 cm depth; bottom

surface = 0.21 m^2, volume 84 L, water flow rate 114 L/h, n = 6 fish/tank, density = 3.8 Kg/m^2) and remained undisturbed except for daily tank cleaning procedures. The remaining 48 fish were maintained in three flat-bottomed round tanks (r = 45 cm; bottom surface = 0.64 m^2, volume 300 L, water flow rate 114 L/h, n = 16 fish/tank, density = 3.4 Kg/m^2) and used for acute stress challenge. Fish were acclimated for 14 days (April 2008) and fed twice a day by hand to apparent satiety (based on the assessment of feed remaining in the tanks) with a 3 mm commercial diet (Alpis, A. Coelho e Castro Lda., Póvoa de Varzim, Portugal). After this period, specimens from round tanks were air exposed during 3 min at a time and redistributed in groups of six individuals into seven new tanks (60 cm length x 35 cm width x 40 cm depth; bottom surface = 0.21 m^2, volume 40 L, n = 6 fish/tank, density = 3.8 Kg/m^2) independently set up from each other. A group of six specimens was sampled for blood and tissues collection immediately after air exposure (time 3 min), while the other groups were sampled after 5 and 30 min, 1, 2, 4, 6 and 24 h. Undisturbed fish were sampled at the same times and used as control. Fish were fasted for 24 h prior to air exposure and during the subsequent 24 h sampling period in order to avoid any influence of feeding on stress plasmatic parameters (Arends et al., 1999).

For sampling procedures, all individuals were quickly removed from each tank at a time and anaesthetized with ethyl 3-aminobenzoate methanesulfonate (MS-222, 200 mg/L; Sigma – Aldrich, Germany). Blood was withdrawn from the caudal vein of every sampled fish using heparinised syringes. Blood collection lasted less than 3 min in order to avoid a cortisol increase due to manipulation during sampling. Plasma was obtained by centrifugation (10000 x *g* for 10 min at 4 ºC) and stored at -80 ºC for further analysis. After blood collection, fish were individually weighed and head-kidney and liver were subsequently dissected over an ice bed. Liver was weighed and kept at -80 ºC for further analysis.

Leucocytes from head-kidney were collected from control and air exposed fish at times 0, 2, 4, 6 and 24 h, isolated and maintained essentially as described by Secombes (1990). Briefly, the head-kidney was removed under aseptic conditions, pushed through a 100 µm nylon mesh and suspended in Leibovitz L-15 medium (L-15: Gibco, Scotland, UK) supplemented with 2% foetal calf serum (FCS; Gibco), penicillin (100 IU/mL; P, Gibco), streptomycin (100 µg/mL; S, Gibco) and heparin (20 units/mL; Sigma). The suspensions were then loaded onto a

34:51 % Percoll (Sigma) density gradient and centrifuged at 400 x *g* and 4 ºC for 40 min. The band of cells laying at the interface of the Percoll gradient was collected and washed three times at 400 x *g* and 4 ºC for 5 min in L-15, 0.1 % FCS, P/S and heparin. The viable cell concentration was determined by the Trypan blue exclusion test. Cells were counted in a haemocytometer and adjusted to 1 x 10^7 cells/mL in L-15, 0.1 % FCS, P/S and heparin. Afterwards, cells were plated in 96 well plates at 100 µL per well. After overnight incubation at 18 ºC, the non-adherent cells were washed off and the monolayers were maintained with L-15 supplemented with 5% FCS, until the respiratory burst assays were conducted after 24 h of incubation at 18 ºC.

3.2.2. Analytical procedures

Plasma cortisol was measured by radioimmunoassay as described by Metz et al. (2005), which was already performed in Senegalese sole (Arjona et al., 2007, 2009). Plasma osmolality was measured with a vapour pressure osmometer (Fiske One-Ten Osmometer, Fiske, VT, USA) and expressed as mOsm/kg. Plasma glucose, lactate and triglycerides were assessed using commercially available Spinreact kits (Glucose HK Ref. 1001200; Lactate Ref. 1001330; Triglycerides Ref. 1001311), adapted for 96-well microplates. Plasma total proteins were determined in 1:50 (v/v) diluted plasma samples using the bicinchoninic acid (BCA) Protein Assay Kit (Pierce #23225, Rockford, USA) for microplates. Bovine serum albumin served as a standard. These assays were run on a Bio Kinetics EL-340i Automated Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA) using DeltaSoft3 software for Macintosh (BioMetallics Inc., NJ, USA).

Plasma samples from either 0, 1 or 24 h were pooled to one sample per sampling time and used for free AA analysis. All pools were run in triplicates. Due to technical constrains, pool from control samples at 1 h was analysed only once. All samples were deproteinised by centrifugal ultrafiltration (10 kDa cut-off, 2500 x *g*, 20 min, 4 ºC). After deproteinisation, samples were pre-column derivatised with phenylisothiocyanate (PITC; Pierce), using the PicoTag method (Waters, USA) described by Cohen et al. (1989). External standards were prepared along with the samples, using physiological AA standard solutions (acid/neutral and basics from Sigma) and a glutamine solution. Norleucine was used as an internal standard. Samples and standards were analysed by High Performance Liquid

Chromatography (HPLC) in a Waters Reversed-Phase Amino Acid Analysis System equipped with a PicoTag column (3.9 x 300 mm), using the conditions described by Cohen et al. (1989). Resulting peaks were analysed with the Breeze software (Waters).

Frozen liver was finely minced on an ice-cold petri dish, vigorously mixed and homogenized by ultrasonic disruption in 7.5 vol. ice-cold 6% (w/v) perchloric acid. The homogenate was then neutralized using the same volume of 1 M $KHCO_3$ and centrifuged (13000 x *g* for 30 min at 4 °C). The supernatants were stored in different aliquots at -80 °C until use in the different metabolite assays. Liver triglyceride levels were determined spectrophotometrically using a commercially available kit (Spinreact SA, Girona, Spain) adapted to 96-well microplates. Liver glycogen concentrations were assessed using the method described by Keppler and Decker (1974). Glucose obtained after glycogen breakdown (after subtracting free glucose levels) was determined with a commercially available kit (Spinreact SA). Total α-amino acid levels were assessed in liver using the nynhidrin method described by Moore (1968) adapted to 96-well microplate format. Spectrophotometric determinations were performed with a Power-Wave™ 340 microplate spectrophotometer (BioTek Instruments) using KCjunior Data Analysis Software for Windows.

Respiratory burst activity of head-kidney leucocytes was based on the reduction of ferricytochrome C method for the detection of O_2^- (Secombes, 1990). Briefly, the leucocytes monolayers were washed twice with L-15 and 100 µL suspension of ferricytochrome C solution (2 mg ferricytochrome C/mL diluted in phenol red-free HBSS) were added. Ferricytochrome C solution containing 10 µg/mL phorbol myristate acetate (PMA, Sigma) was added as a soluble stimulant of the respiratory burst. Ferricytochrome C with PMA and 0.725 mg/mL superoxide dismutase (SOD, Sigma) was used to confirm the specificity of the reaction. For each parameter 3 or more wells of leucocytes per fish were assayed. Plates were read immediately after addition of reagents to the leucocytes and readings were then taken every 60 s for 60 min on a Power-Wave™ microplate spectrophotometer (BioTek) at 550 nm. Data were expressed as the Vmax rate of the response in mOD/min.

Lysozyme activity was measured using a turbidimetric assay based on the method described by Ellis (1990) with some modifications (Wu et al., 2007). Briefly, a solution of *Micrococcus lysodeikticus* (0.5 mg/mL 0.05 M sodium phosphate buffer; pH 6.2) was prepared. In a microplate, 15 µL of plasma and 250 µL of the above suspension were added. The reaction was carried out at 25 ºC and the absorbance at 450 nm was measured after 0.5 and 4.5 min. Lyophilized hen egg white lysozyme (Sigma) was serially diluted in sodium phosphate buffer (0.05 M; pH 6.2) and used to develop a standard curve. The amount of lysozyme in the sample was calculated using the formula of the standard curve.

Alternative complement pathway (ACP) was estimated as described by Sunyer and Tort (1995). The following buffers were used: GVB (Isotonic veronal buffered saline), pH 7.3, containing 0.1% gelatin; EDTA-GVB, as previous one but containing 20 mM EDTA; and Mg-EGTA-GVB, which is GVB with 10 mM Mg^{+2} and 10 mM EGTA. Rabbit red blood cells (RaRBC; Probiologica Lda, Portugal) were used for ACP determination. RaRBC were washed four times in GVB and resuspended in GVB to a concentration of 2.5×10^8 cells/mL. 10 µL of RaRBC suspension were then added to 100 µL of serially diluted plasma in Mg- EGTA-GVB buffer. Samples were incubated at room temperature for 100 min with occasional shaking. The reaction was stopped by adding 100 µL of cold EDTA-GVB. Samples were then centrifuged and the extent of haemolysis was estimated by measuring the optical density of the supernatant at 414 nm. The ACH50 units were defined as the concentration of plasma giving 50% haemolysis of RaRBC. All analyses were conducted by triplicates.

3.2.3. Data analysis

Hepatosomatic index (HSI) was calculated as follows:

HSI (%) = (liver weight) / (final wet weight) x 100

Plasma free AA (FAA) ratios were calculated, for a better understanding of changes in FAA due to stress condition, by dividing the concentration of each AA from air exposed fish by the mean concentration of the same AA from control specimens, minus one. Therefore, ratios higher than 0 express an increase in

plasma FAA concentrations from air exposed fish relative to control specimens, while values lower than 0 express a decrease. Statistical analysis were performed using the computer package SPSS for WINDOWS 15.0. All results are expressed as means ± standard error of the mean (SEM), except for FAA data where results are expressed as means ± standard deviation (SD). Data among treatments were analyzed by one-way, repeated measures analysis of variance (ANOVA). In the case of fold calculations, data was analysed with original values. When significant differences were obtained from the ANOVA, multiple comparisons were carried out performing Tukey – HSD mean comparison tests. The level of significance used was $p \leq 0.05$ for all statistical tests.

3.3. Results

Plasma cortisol, glucose and osmolality levels increased significantly to peak levels at 1 h in air exposed fish. Moreover, those levels were significantly higher with respect to control groups from 5 min to 6 h for cortisol (Figure 1A) and from 30 min to 2 h for glucose and osmolality (Figures 1B and 1C, respectively). In addition to that, cortisol values also presented linear relationships with both glucose ($y = 0.0076x + 5.4639$; $R^2 = 0.6657$; $p = 0.013$) and osmolality ($y = 0.0769x + 321.2$; $R^2 = 0.8424$; $p = 0.001$). Plasma lactate levels augmented significantly from time 0 min until peak levels at 1 h decreasing to control levels at 4 h. Moreover, these levels were significantly higher with respect to control groups from 0 min to 2 h (Figure 1D). Plasma proteins and triglycerides levels from air exposed specimens presented significantly higher values with respect to control groups from time 0 to 1 h (Figure 1E), and from 30 min to 2 h (Figure 1F), respectively.

Plasma total free AA (FAA) levels were not significantly different along the different sampling times in control fish (Table 1). However, in air exposed fish, plasma total FAA levels increased significantly along time. Furthermore, those values were only significantly higher than in control fish at 24 h (Table 1). A similar outcome was observed in indispensable AA (IAA) levels, being also significantly lower at time 0 min in air exposed sole than in control specimens. In addition, dispensable AA (DAA) levels were significantly higher at times 1 and 24

h in air exposed fish, while only levels from 1 h were significantly higher than control fish (Table 1).

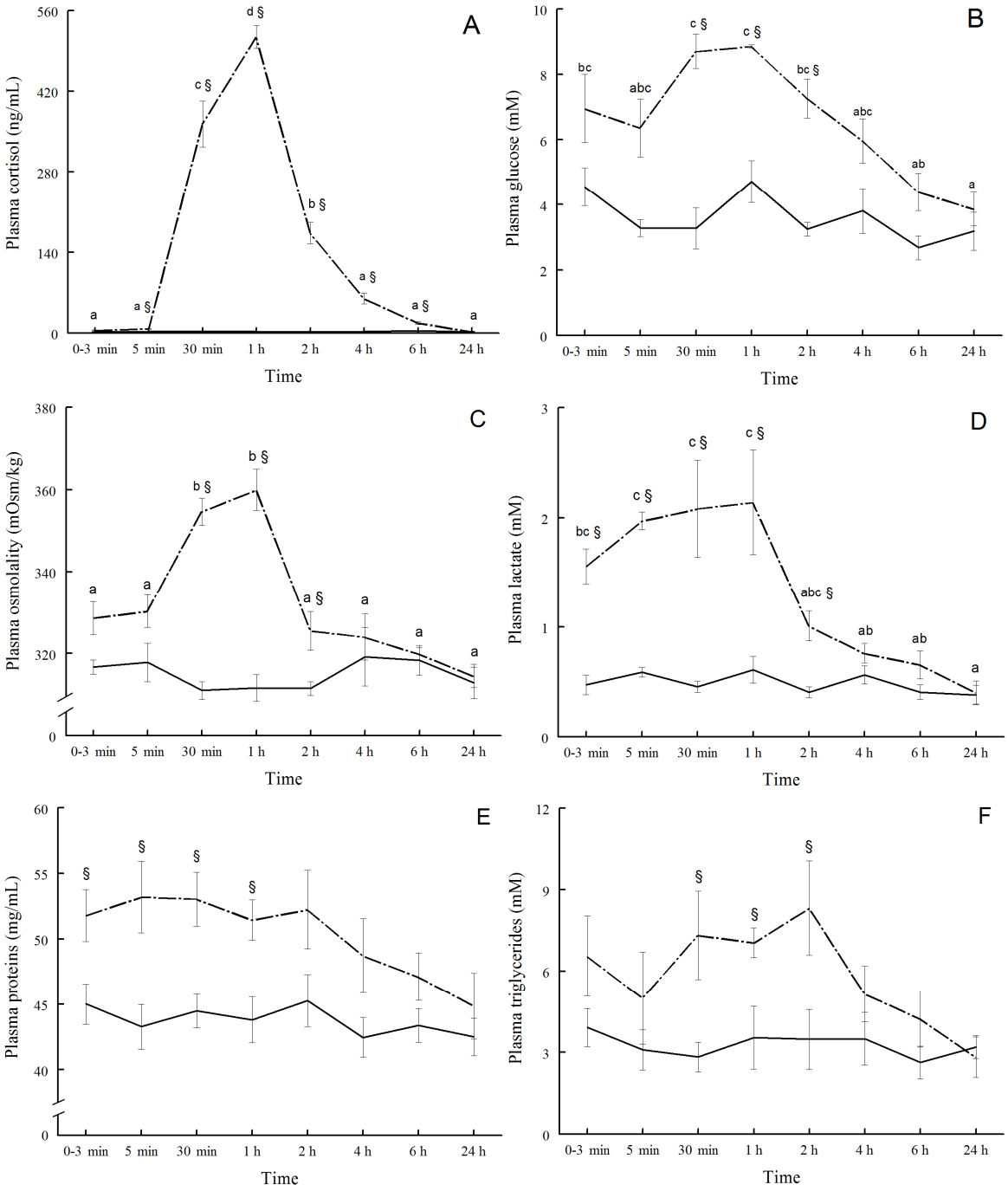

Figure 1. Plasma cortisol (A), glucose (B), osmolality (C), lactate (D), protein (E) and triglycerides (F) levels in *S. senegalensis* either air exposed (dotted line) or undisturbed (solid line). Data are expressed as means ± SEM (n = 6). Different letters stand for significant differences within the same group, and § for significant differences between groups at the same time (One-way repeated measures ANOVA; $p \leq 0.05$).

Table 1. Plasma total indispensable (IAA), dispensable (DAA) and free amino acid (FAA) levels in *Solea senegalensis* either air exposed or undisturbed (Control).

Plasma FAA (µM)	Control			Air exposed		
	0 min	1 h	24 h	3 min	1 h	24 h
Total IAA	1025.4 ± 3.7	1016.8	1001.7 ± 16.4	797.8 ± 56.3^{a,§}	1033.3 ± 18.8^{b}	1148.3 ± 9.9^{c,§}
Total DAA	571.9 ± 6.9	571.1	524.8 ± 46.1	446.8 ± 51.5^{a}	630.5 ± 4.2^{b,§}	602.4 ± 20.2^{b}
Total FAA	1597.3 ± 10.5	1587.9	1526.5 ± 29.7	1244.6 ± 107.6^{a}	1663.8 ± 18.7^{b}	1750.7 ± 16.6^{c,§}

Data are expressed as means ± SD (n = 3, except for control 1 h n = 1). Different letters stand for significant differences among time within the same group, and § for significant differences between air exposed and control specimens at the same time (One-way repeated measures ANOVA; $p \leq 0.05$).

Regarding individual plasma FAA levels, no significant changes were observed along time in control specimens, while in air exposed fish, only citruline and tyrosine were not affected along time (Table 2). However, drastic changes occur in the later when compared with control fish (Figure 2A and 2B). Regarding individual DAA, citruline, glutamine, glycine, proline and tyrosine levels were significantly lower in air exposed fish than in control specimens at time 0 h, while γ-Aminobutyric acid (GABA) level was significantly higher. Moreover, glutamine and proline values remained significantly lower in air exposed than in control fish together with ornithine and taurine levels at time 1 h, while GABA, alanine, aspartic acid, glutamic acid, serine and tyrosine levels increased significantly. In addition, while glutamine, ornithine and taurine values remained significantly lower in air exposed than in control fish at time 24 h, aspartic acid, glutamic acid, glycine, serine and tyrosine levels augmented significantly (Figure 2A). Regarding individual IAA, arginine, histidine, lysine and methionine values were significantly lower in air exposed fish than in control specimens at time 0 h, while isoleucine, phenylalanine and tryptophan levels were significantly higher. Moreover, methionine level remained significantly lower in air exposed than in control fish together with isoleucine, leucine and valine levels at time 1 h, while lysine, phenylalanine, threonine and tryptophan values were significantly higher. In addition, while isoleucine level remained significantly lower in air exposed than in control fish at time 24 h, lysine, methionine, phenylalanine, threonine and tryptophan levels increased significantly (Figure 2B).

No changes in HSI were observed during the experiment (data not shown). Hepatic glycogen concentrations significantly decreased at 30 min and 1 h after air exposure in stressed specimens. However, glucose levels did not change through experimental time. Triglycerides levels significantly increased until peak levels at 2 h after air exposure and remained elevated until 4 h post-air exposure. Total AA augmented significantly at 2 h holding high until the last sampling point (24 h) in air exposed fish, being significantly higher with respect to control groups (Table 3).

Table 2. Individual plasma free amino acid (FAA) levels of *Solea senegalensis* either air exposed or undisturbed (Control).

Plasma FAA (μM)	Control			Air exposed		
	0 min	1 h	24 h	3 min	1 h	24 h
Alanine	143.3 ± 4.2	144.3	138.7 ± 8.6	125.9 ± 10.4^{a}	182.5 ± 3.1^{b,§}	132.4 ± 2.8^{a}
Asparagine	50.7 ± 1.1	50.9	54.2 ± 6.6	42.0 ± 7.9^{a}	50.3 ± 0.3^{a}	63.1 ± 3.0^{b}
Aspartic acid	7.5 ± 2.0	8.8	6.4 ± 0.8	9.1 ± 6.6^{a}	23.8 ± 5.9^{b,§}	16.1 ± 4.3^{ab,§}
Arginine	210.3 ± 11.6	211.4	202.7 ± 6.2	94.0 ± 6.3^{b,§}	193.1 ± 7.4^{a}	201.4 ± 3.6^{a}
Citruline	13.8 ± 3.5	15.4	13.0 ± 1.6	11.4 ± 0.3§	14.9 ± 6.2	7.9 ± 2.2
γ-Aminobutyric acid	7.3 ± 2.1	7.7	7.3 ± 0.4	8.7 ± 0.3^{a,§}	15.9 ± 0.9^{b,§}	8.2 ± 1.6^{a}
Glutamine	107.1 ± 3.9	106.1	111.5 ± 4.0	46.4 ± 6.4^{a,§}	57.0 ± 1.0^{b,§}	76.0 ± 1.3^{c,§}
Glutamic acid	24.7 ± 3.6	21.5	22.4 ± 2.8	34.3 ± 8.6^{a}	60.5 ± 4.6^{b,§}	62.5 ± 6.2^{b,§}
Glycine	36.5 ± 2.6	35.4	32.6 ± 0.9	19.9 ± 1.5^{a,§}	40.9 ± 2.5^{c}	36.1 ± 0.2^{b,§}
Histidine	25.5 ± 0.8	26.7	24.9 ± 0.9	21.6 ± 0.7^{a,§}	23.5 ± 0.9^{b}	25.2 ± 0.4^{b}
Isoleucine	54.8 ± 4.2	66.1	58.0 ± 3.1	64.1 ± 1.6^{c,§}	40.3 ± 1.1^{a,§}	48.1 ± 0.6^{b,§}
Leucine	61.8 ± 1.9	59.3	63.4 ± 1.9	65.1 ± 2.8^{b}	49.8 ± 1.5^{a,§}	63.9 ± 1.0^{b}
Lysine	413.4 ± 10.2	397.2	401.9 ± 5.1	235.5 ± 29.2^{a,§}	445.9 ± 8.7^{b,§}	508.5 ± 7.1^{c,§}
Methionine	38.8 ± 3.2	35.2	37.4 ± 1.4	18.6 ± 1.3^{a,§}	31.2 ± 0.5^{b,§}	43.7 ± 1.0^{c,§}
Ornithine	10.3 ± 0.2	9.9	10.1 ± 0.1	13.4 ± 1.3^{c}	6.8 ± 0.5^{b,§}	3.7 ± 0.2^{a,§}
Phenylalanine	33.5 ± 1.7	31.0	30.1 ± 5.3	48.6 ± 0.4^{b,§}	39.6 ± 1.2^{a,§}	47.6 ± 0.6^{b,§}
Proline	22.1 ± 0.8	22.7	21.0 ± 0.5	12.6 ± 0.2^{a,§}	17.5 ± 0.8^{b,§}	17.6 ± 1.3^{b}
Serine	85.4 ± 5.9	86.5	83.6 ± 0.1	69.5 ± 9.1^{a}	101.6 ± 1.3^{b,§}	121.4 ± 4.3^{c,§}
Taurine	17.4 ± 0.1	23.7	21.2 ± 0.7	16.4 ± 0.3^{b}	16.7 ± 1.0^{b,§}	9.6 ± 0.3^{a,§}
Threonine	88.5 ± 0.3	88.4	86.5 ± 2.7	129.2 ± 10.8^{b}	117.3 ± 1.3^{ab,§}	103.7 ± 2.1^{a,§}
Tryptophan	8.8 ± 0.5	9.4	8.5 ± 0.2	14.8 ± 0.5^{b,§}	11.4 ± 0.2^{a,§}	11.9 ± 0.1^{a,§}
Tyrosine	32.7 ± 2.2	23.1	25.8 ± 0.9	23.9 ± 0.4§	25.8 ± 0.4§	38.1 ± 1.4§
Valine	89.8 ± 1.5	91.9	95.0 ± 3.3	106.1 ± 3.7^{c}	81.1 ± 2.2^{a,§}	94.3 ± 2.0^{b}

Data are expressed as means ± SD (n = 3, except for control 1 h n = 1). Different letters within the same row indicate significant differences among time in air exposed fish, and § stands for significant differences between air exposed and control specimens at the same time (One-way repeated measures ANOVA; $p \leq 0.05$).

Head-kidney leucocytes from control groups showed significant differences in the respiratory burst, being significantly higher at 4 and 6 h than at 0 and 24 h. However, the experimental protocol used in this study significantly increased this respiratory burst from air exposed specimens at 2, 4 and 6 h with respect to 24 h fish, being significantly higher with respect to control groups at 2 and 6 h (Figure 3). On the other hand, plasma lysozyme activity and plasma ACH50 values due to ACP haemolytic activity followed a similar pattern of change in air exposed specimens, decreasing significantly from 1 to 4 h (Figure 4) and from 30 min to 4 h (Figure 5), respectively. Plasma ACH50 values also presented an inverse linear relation with respect to cortisol levels ($y = -0.4429x + 345.64$; $R^2 = 0.8185$; $p = 0.002$), being the lowest value found at 1 h after air exposure.

Table 3. Glycogen, glucose, triglycerides and total α-amino acids levels in liver of *Solea senegalensis* either air exposed or undisturbed (Control).

Treatment/Paramenter		Glycogen (µmol glycosyl units/g wet weight)	Glucose (µmol/g wet weight)	Triglycerides (µmol/g wet weight)	Total α-amino acids (µmol/g wet weight)
0-3 min	Control	59.9 ± 6.5	35.4 ± 3.5	3.2 ± 0.3	61.1 ± 6.0
	Air exposed	46.9 ± 6.8 ^{a}	33.1 ± 1.8	2.7 ± 0.2 ^{a}	53.8 ± 6.4 ^{a}
5 min	Control	67.9 ± 4.3	34.6 ± 4.8	2.5 ± 0.1	63.9 ± 9.4
	Air exposed	46.4 ± 12.9 ^{ab}	39.5 ± 1.5	2.8 ± 0.4 ^{a}	60.5 ± 8.6 ^{a}
30 min	Control	61.1 ± 12.5	34.7 ± 5.1	2.9 ± 0.2	66.4 ± 10.3
	Air exposed	30.9 ± 6.6 ^{a,§}	39.5 ± 3.1	2.7 ± 0.4 ^{a}	58.1 ± 7.2 ^{a}
1 h	Control	70.1 ± 10.3	32.9 ± 3.5	2.7 ± 0.2	56.0 ± 10.2
	Air exposed	26.3 ± 9.6 ^{a,§}	39.3 ± 2.3	4.3 ± 0.5 ^{ab,§}	53.0 ± 6.5 ^{a}
2 h	Control	65.8 ± 7.4	29.6 ± 2.6	3.1 ± 0.3	69.1 ± 5.9
	Air exposed	44.7 ± 6.6 ^{ab}	36.2 ± 3.3	5.2 ± 0.9 ^{b,§}	109.8 ± 5.9 ^{b,§}
4 h	Control	66.9 ± 4.3	31.8 ± 3.8	3.1 ± 0.2	63.0 ± 8.3
	Air exposed	58.7 ± 4.0 ^{ab}	30.6 ± 2.9	4.9 ± 0.6 ^{b,§}	155.8 ± 13.9 ^{c,§}
6 h	Control	60.4 ± 5.7	28.1 ± 3.6	2.9 ± 0.2	67.3 ± 6.0
	Air exposed	70.7 ± 13.9 ^{b}	30.3 ± 3.4	3.3 ± 0.3 ^{ab}	155.1 ± 10.0 ^{c,§}
24 h	Control	61.7 ± 11.9	31.6 ± 3.9	2.6 ± 0.5	67.5 ± 11.3
	Air exposed	71.0 ± 7.0 ^{b}	30.6 ± 2.1	3.1 ± 0.1 ^{ab}	155.6 ± 11.7 ^{c,§}

Data are expressed as means ± SEM (n = 6). Different letters stand for significant differences within the same group, and § for significant differences between groups at the same time (One-way repeated measures ANOVA; $p \leq 0.05$).

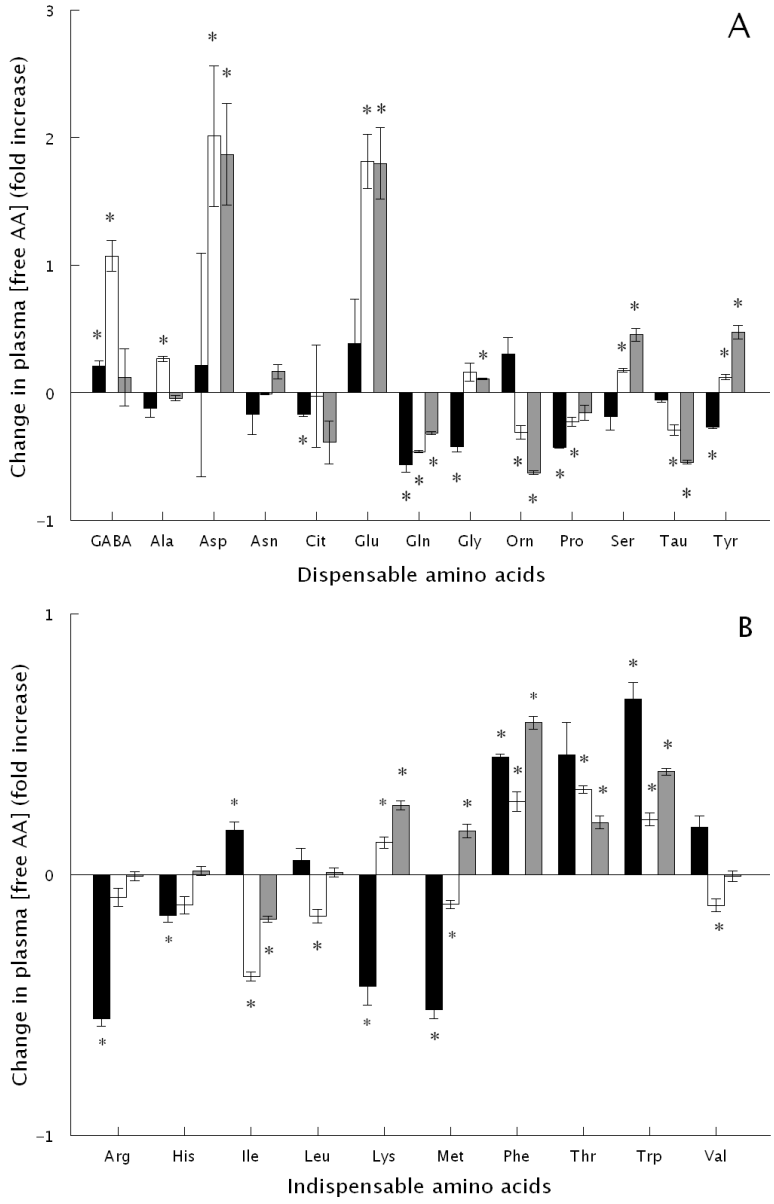

Figure 2. Plasma dispensable (A) and indispensable (B) free amino acid ratio between air exposed and undisturbed *S. senegalensis* at 0-3 min (■), 1 h (□) and 24 h (▩) after air exposure. Values (means ± SD) were calculated by dividing each amino acid concentration from air exposed fish by the mean concentration of the same amino acid from control specimens, minus one (n = 3). * stands for significant differences between air exposed and control fish at the same time (One-way repeated measures ANOVA; $p \leq 0.05$).

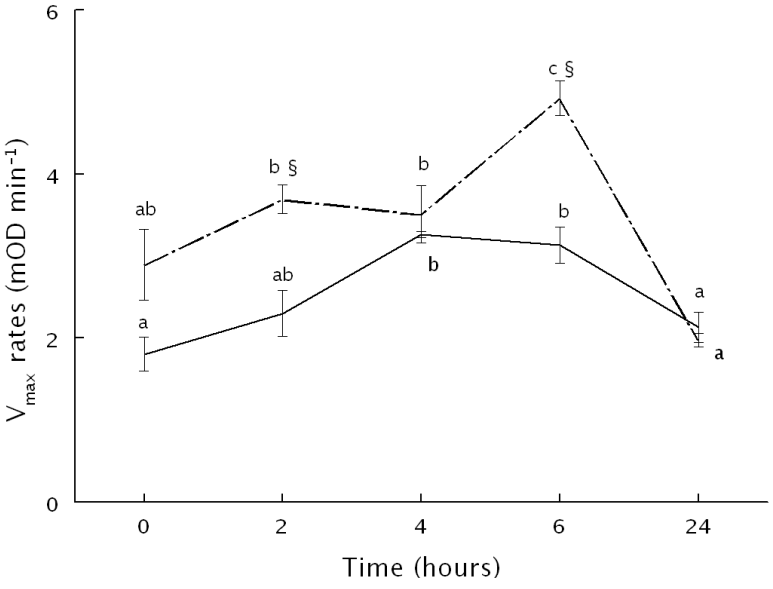

Figure 3. Respiratory burst activity of head-kidney leucocytes from *Solea senegalensis* either air exposed (dotted line) or undisturbed (solid line). Data are presented as V_{max} rates of ferricytochrome C reduction, expressed as means ± SEM (n = 3). Different letters stand for significant differences within the same group, and § for significant differences between groups at the same time (One-way repeated measures ANOVA; $p \leq 0.05$).

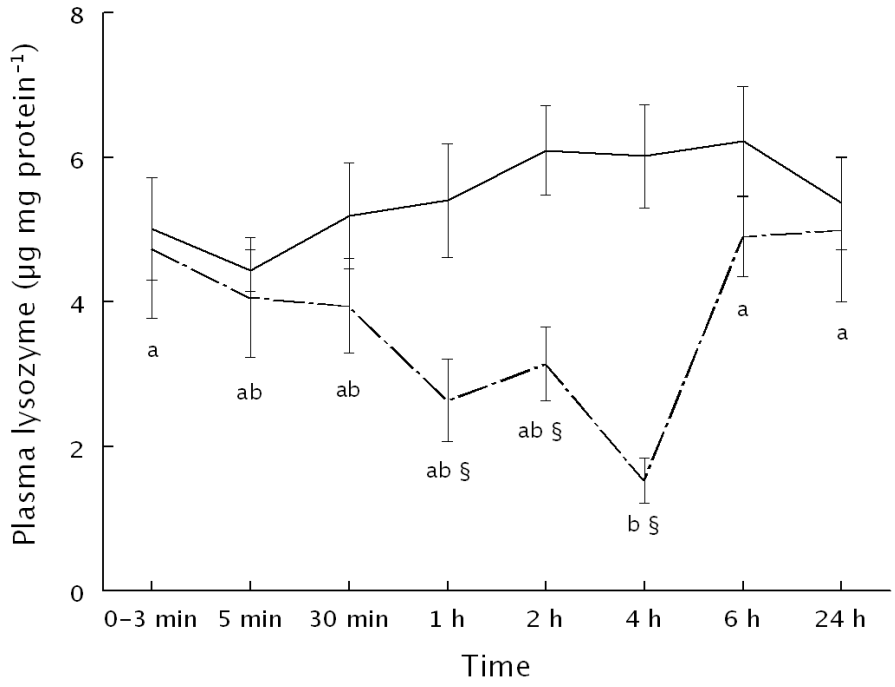

Figure 4. Plasma lysozyme activity of *S. senegalensis* either air exposed (dotted line) or undisturbed (solid line). Data are expressed as means ± SEM (n = 6). Different letters stand for significant differences within the same group, and § for significant differences between groups at the same time (One-way repeated measures ANOVA; $p \leq 0.05$).

3.4. Discussion

3.4.1. Endocrine and metabolic responses to acute stress

Experimental acute stressor used in this study (air exposure) induced a significant increase (165-fold after 1 h) of plasma cortisol levels in Senegalese sole specimens, a typical primary stress response, which is considered to be a good indicator of the stress levels in fish (Barton and Iwama, 1991; Wendelaar Bonga, 1997; Barton, 2002). Similar results have been reported in others species under similar stressful conditions: gilthead seabream (*Sparus aurata*) augmented more than 50-fold the values of this hormone within 30 min after 3 min of air exposure (Arends et al., 1999), while cobia (*Rachycentron canadum*) showed either a 7-fold or a 10-fold increase of plasma cortisol levels after 1 min of air exposure at 30 min and 1 h, respectively (Cnaani and McLean, 2009; Trushenski et al., 2010). Therefore, results from this study suggest that Senegalese sole presents a comparable primary stress response to that observed in other teleosts.

Furthermore, it was also detected a strong and rapid increase in plasma glucose levels of Senegalese sole, a typical secondary stress response, comparable to the one observed in rainbow trout (*Oncorhynchus mykiss*) and gilthead seabream (Barton et al., 1987; Arends et al., 1999). These plasma glucose levels increased parallel to those of cortisol, suggesting an activation of hypothalamic-pituitary-interrenal axis, probably attributed to cortisol that enhanced glycogenolytic potential, gluconeogenic capacity and glucose export capacity in liver of Senegalese sole submitted to air exposure (Vijayan et al., 1994b; Laiz-Carrión et al., 2002). This is in agreement with the reduction in glycogen content observed in this group at 30 min and 1 h. However, it is necessary to remark the possible contribution of catecholamines to the plasma glucose augmentation observed in air exposed fish. During acute stress situations brain-sympathic-chromaffin cells axis is activated increasing plasma catecholamine levels (Wendelaar Bonga, 1997; Arends et al., 1999).

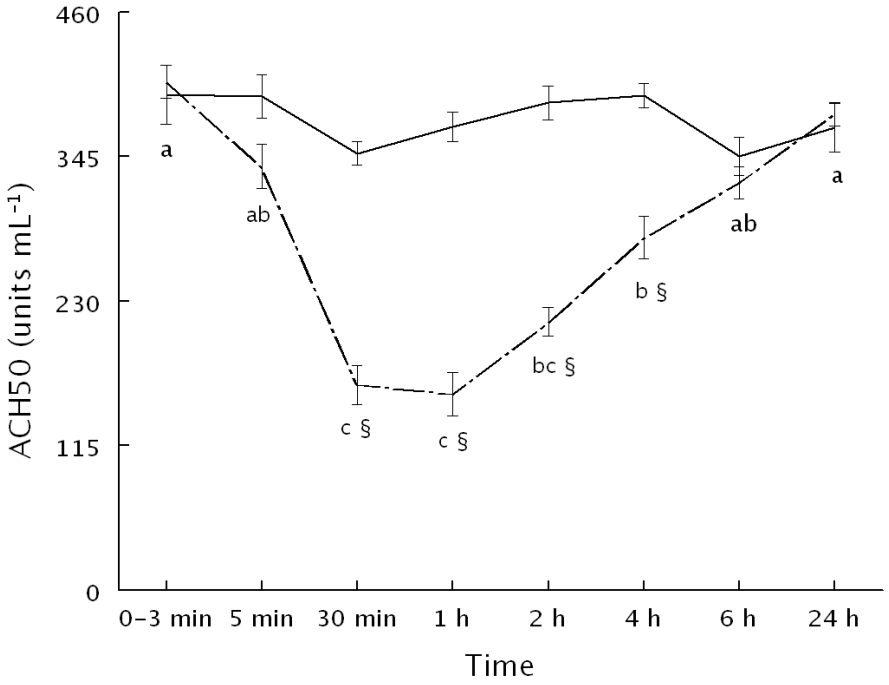

Figure 5. Plasma alternative complement pathway activity of *S. senegalensis* either air exposed (dotted line) or undisturbed (solid line). Data are presented as ACH50 values, expressed as means ± SEM (n = 6). Different letters stand for significant differences within the same group, and § for significant differences between groups at the same time (One-way repeated measures ANOVA; $p \leq 0.05$).

Plasma osmolality levels increased parallel to cortisol values, being in agreement with that observed for other teleosts submitted to similar acute stressful conditions (Waring et al., 1996; Arends et al., 1999). This could enhance gill permeability and, because specimens are maintained in hyperosmotic environment, increase ions and water permeability with an influx of ions and an efflux of water (McDonald and Milligan, 1992; Evans et al., 2005). In addition, this loss of water could also explain the higher protein and triglycerides levels observed during the first hours after air exposure. However, other metabolic changes also could contribute to these changes (see below).

An increase in plasma lactate levels induced by air exposure has been described in gilthead seabream and cobia at 30 min and 1 h after handling, respectively (Arends et al., 1999; Trushenski et al., 2010). However, results from this study show an earlier increase of this metabolite (just after 3 min of air exposure) suggesting a faster lactate metabolism in Senegalese sole. Due to the absence of a starting sample from stressed fish (taken prior to air exposure), it must be assumed that pre-stress lactate levels in air exposed specimens were identical to those from control fish. Still, given the very stable pattern evident in plasma lactate levels from control fish and the fact that those levels from air exposed specimens returned to identical levels to those of control fish after 24 h, this assumption seems likely. This rapid augmentation of plasma lactate levels could be due primarily to muscle glycolysis immediately after stress (Milligan and Girard, 1993) and are associated with hypoxia (Fabbri et al., 1998). Since lactate and AA have been shown to be the preferred substrates for gluconeogenesis in fish (Mommsen et al., 1999), increased lactate levels observed in this study may have been used for glucose production and/or glycogen repletion in the liver of air exposed Senegalese sole, suggesting that the hepatic capacity for lactate utilization may be enhanced in stressed specimens. In this way, it will be interesting the study of hepatic enzymatic activities (i.e. lactate dehydrogenase, phosphoenolpyruvate carboxykinase, aspartate aminotransferase, or glycogen synthase) related to glycogen and lactate metabolism during the first moment after air exposure.

The acute stress induced in the present study lead to several changes in the plasma AA levels of air exposed Senegalese sole, in line with what has been observed under chronic stressful conditions for this species (Pinto et al., 2007;

Aragão et al., 2008, 2010; Costas et al., 2008). Results from the current study show a significant decrease of total IAA levels at time 3 min in air exposed fish when compared to control specimens at time 0 min, which is mainly due to lower arginine and lysine levels. Arginine serves as the precursor for the synthesis of nitric oxide (NO) in terrestrial animals (Wu and Morris, 1998). Physiological levels of NO stimulate glucose uptake and oxidation as well as fatty acid oxidation in liver and muscle in mammals (Jobgen et al., 2006). In addition, lysine and methionine are the substrates for the synthesis of L-carnitine, which in fish is required for the transport of long-chain fatty acids to the site of oxidation (Harpaz, 2005). Therefore, lower arginine, lysine and methionine levels observed in the present study at time 3 min may suggest an increased usage of these IAA due to an augmented synthesis of NO and L-carnitine. These molecules are probably involved in glucose and fatty acids mobilization to prepare the fish to face increased energy demands due to the stress challenge imposed. In fact, plasma glucose levels were significantly higher at time 30 min in air exposed fish than in control specimens. A similar increase was observed in plasmatic and hepatic triglycerides values, supporting previous hypothesis. Moreover, this hypothesis correlates well with the lower glutamine levels observed at time 3 min, an AA required for NO synthesis in macrophages and monocytes (Li et al., 2007). The highest total DAA levels observed in air exposed fish at time 1 h, when highest plasma cortisol values were detected, may result from an increased proteolysis due to cortisol action (Mommsen et al. 1999). These changes may be due to increased energetic costs of stress challenge, and some of these AA can be either used directly as energetic substrates or as carbon sources for hepatic gluconeogenesis. This could explain the lowest glycogen contents at hepatic level, which support hyperglycemia detected at this time, but without any changes in glucose values. In fact, plasma alanine, aspartic acid and serine levels increased significantly at this sampling time, suggesting their role frequently assigned as important glucogenic AA in fish (Ballantyne, 2001). On the other hand, not all changes in DAA at time 1 h are related to energy supply. For instance, the observed increase of GABA and glutamic acid could be related to their higher usage in the brain of air exposed specimens, since these DAA are neurotransmitters present at high concentrations in fish brain during periods of anoxia (Ballantyne, 2001; Soengas and Aldegunde, 2002; Li et al., 2009). Moreover, the increased GABA levels observed in air exposed fish at time 3 min support this hypothesis. Similarly, tryptophan is the precursor of serotonin and

phenylalanine can be converted to tyrosine, which is the precursor of dopamine (Li et al., 2009). Since serotonin and dopamine are involved in the control of the HPI axis in fish and stressful conditions can induce the elevation of these monoamine neurotransmitters levels in brain of rainbow trout (Øverli et al., 2005; Gesto et al., 2008), the high tryptophan and phenylalanine levels at times 3 min and 1 h post air exposure also suggests its uptake in brain of stressed fish due to HPI axis activation after acute stress. In addition, results from the present study showed a significant increase of both total IAA and total FAA levels in plasma of air exposed fish when compared to control specimens at time 24 h. This augmentation in plasmatic total FAA levels was mainly due to increased lysine, methionine, phenylalanine, tryptophan, serine and tyrosine levels, AA involved in the synthesis of compounds related to the stress response, fatty acid transport or used either as energy sources or in gluconeogenesis in liver (Mommsen et al. 1999; Ballantyne, 2001; Harpaz, 2005; Li et al., 2009). In fact, this outcome correlates well with the higher total AA levels observed at 24 h in the liver of air exposed specimens. Moreover, this enhanced AA mobilization at 24 h was also observed in tilapia (*Oreochromis mossambicus*) submitted to confinement stress (Vijayan et al., 1997).

In the present study, the post-stress decrease of hepatic glycogen levels in air exposed fish was probably due to increased glycogenolysis, as already observed in fish submitted to an acute stress (Vijayan et al., 1994a, 1997). In fact, the decrease in liver glycogen levels at 30 min translated in an immediate increase in plasma glucose levels in stressed specimens. On the other hand, results from this study showed an increase of total AA and triglycerides levels in liver at 2 h post-stress, suggesting an increased availability of AA and glycerol from peripheral protein catabolism and lipolysis, respectively. The relative importance of AA for gluconeogenesis may vary depending on the availability of lactate (Ballantyne, 2001). In addition, one mechanism operating to conserve glycogen during stress appears to be an altered sensitivity of hepatocytes to hormones (Vijayan et al., 1994a). Thus, it appears that cortisol shifted the preferred gluconeogenic substrate from lactate to AA and lipids at 2 h after air exposure, since plasma lactate and triglycerides returned to resting levels after this time.

3.4.2. Non-specific immune response to acute stress

In the present study, head-kidney leucocytes from unstressed specimens showed significantly higher respiratory burst at 4 and 6 h after the beginning of the experiment. This peculiar response may be explained due to the existence of a circadian rhythm of immune parameters in Senegalese sole. In fact, a circadian rhythm in the gilthead seabream humoral non-specific immune system was previously observed, and a modulatory role of melatonin on immune responses proposed (Esteban et al., 2006). However, results from this study also showed a significant increase of the leucocytes respiratory burst from air exposed specimens, suggesting that this particular acute stress can initially be stimulatory, at least for leucocytes respiratory burst activity under these experimental conditions. In fact, the increased respiratory burst from stressed specimens at 2 h after air exposure with respect to control fish may indicate a stimulatory action of cortisol. Interestingly, a similar activation of respiratory burst was observed in kidney cells from dab (*Limanda limanda*) after acute handling stress (Pulsford et al., 1994). However, a short-term crowding stress, which induced a 27-fold increase in cortisol values, did not affect head-kidney leucocytes respiratory burst from gilthead seabream (Ortuño et al., 2001).

On the other hand, significant decreases in lysozyme and complement activities were observed in stressed specimens from this study, suggesting a short-term immunosuppressive action by acute handling stress in Senegalese sole. However, the non-specific humoral immune response appears to depend on the species and type and duration of the stress imposed. For instance, changes on lysozyme activity in response to a stressor present contradictory results in different studies. In some cases, lysozyme activity decreases or no consistent effects are observed (Möck and Peters, 1990; Olsen et al., 1993; Cnaani and McLean, 2009). However, in many other studies this parameter significantly increased in stressed specimens (Fevolden and Roed, 1993; Demers and Bayne, 1997; Rotllant et al., 1997; Caipang et al., 2009). Taking into account that fish leucocytes form the first line of defence against invading microorganisms (Ellis, 2001), mobilisation and/or activation of these cells in conditions of acute stress may be important for survival. Nevertheless, it remains to be clarified whether these temporary effects of acute stress challenge on innate immunity may lead to increased susceptibility to disease in Senegalese sole. In fact, the observed

increase of respiratory burst activity in air exposed specimens from the present study shows that cortisol do not suppress all aspects of the fish innate immune system. These data provide further information that the neuroendocrine responses in flatfish can affect the activity of the innate immune system.

3.5. Conclusions

The present study shows that air exposure translates in the increase of the primary (cortisol) and secondary (glucose) stress responses of Senegalese sole, and the resulting hypoxia appears to induce an increase in plasma lactate levels immediately after air exposure. Furthermore, this metabolite appears to have a role as a substrate for liver gluconeogenesis in a way to maintain liver glycogen and glucose levels due to the increased energetic demand immediately after air exposure. Changes in plasma FAA were also observed through out 24 h after stress. While some IAA were likely used for the synthesis of compounds related to the stress response or fatty acid transport, DAA were probably mainly used either as energy sources or in gluconeogenesis. In addition, data point to cortisol as main regulator in the liver gluconeogenic pathway by shifting the preferred substrate from lactate to AA and lipids 2 h after handling. Furthermore, lysozyme and complement activities decreased after air exposure, and hence, the stress-related physiological changes seem to impair the non-specific humoral immune response. However, the increase of respiratory burst observed in leucocytes from stressed specimens may imply an enhancement of the non-specific cellular immune response and suggests that cortisol may act as a regulator of the Senegalese sole innate immune system. This balance between non-specific cellular and humoral immune parameters in response to an imposed stressful condition, and its relation to the sensitivity of sole to opportunistic pathogens, deserves further research.

3.6. Acknowledgements

This work was developed under the project OPTISOLE – QREN IDT Co-Promoção Nº 1605, which was co-funded by the National Strategic Reference Framework – *QREN* under the Regional Operational Programme North – ON2, with 343751.84 € originating from the European Regional Development Fund – *FEDER*.

This study was also supported by projects AGL2007-61211/ACU (Ministerio de Educación y Ciencia, Spain) and Proyecto de Excelencia PO7-RNM-02843 (Consejería de Innovación, Ciencia y Empresa. Junta de Andalucía) to J.M.M. Cláudia Aragão and Benjamín Costas were supported by FCT, Portugal (SFRH/BPD/37197/2007 and SFRH/BD/38697/2007, respectively).

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Chapter 4

Digestibility and retention efficiency of individual indispensable amino acids are variable in Senegalese sole (*Solea senegalensis* Kaup, 1858) juveniles: a study using ^{14}C tracers

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Costas, B., Castanheira, M.F., Conceição L.E.C., 2011. Digestibility and retention efficiency of individual indispensable amino acids are variable in Senegalese sole (*Solea senegalensis* Kaup, 1858) juveniles: a study using ^{14}C tracers. Submitted.

Digestibility and retention efficiency of individual indispensable amino acids are variable in Senegalese sole (*Solea senegalensis* Kaup, 1858) juveniles: a study using ^{14}C tracers

Abstract

This work aims to study how individual indispensable amino acids (IAA) are utilized in Senegalese sole (*Solea senegalensis*) juveniles. Senegalese sole juveniles were reared following standard procedures. The experiments were done by tube-feeding Senegalese sole juveniles with compound feed containing ^{14}C-labelled IAA as tracers. A setup to determine the digestive and metabolic fate of ^{14}C-labelled IAA was used, in order to quantify the digestibility, catabolism and retention of the IAA fed to Senegalese sole juveniles. The reliability of the experimental setup has been validated and tested, and provides 96.0 ± 2.2 % (mean ± SD) recovery. The experiments demonstrated differences in digestibility, retention and catabolism between individual IAA. While tryptophan was significantly less absorbed (59 %), the other ingested IAA showed values for digestibility around 90 %. Histidine, leucine, methionine and threonine were the most retained in muscle, suggesting a sparing of these IAA for muscle growth, while threonine was the most retained IAA in the whole body. Moreover, lysine was the most catabolised IAA and seems to have an important role as an energy substrate in this species. Bioavailabilities relative to lysine for individual IAA were also calculated. High relative bioavailabilities were found for histidine, leucine, isoleucine, valine, methionine, threonine, phenylalanine and arginine, meaning these IAA are retained more efficiently than lysine. Tryptophan had the lowest relative bioavailability among IAA. This information, together with the carcass IAA profile, also allowed determining the ideal dietary IAA profile for juvenile sole.

Keywords: Bioavailability, catabolism, gut absorption, ideal AA profile

4.1. Introduction

Amino acids (AA) are building blocks for protein deposition which also play important and versatile roles in fish metabolism. Emerging evidence show that AA are not just crucial for growth and energy production (Walton, 1985; Cowey and Walton, 1989), but also have important roles in immunity (Li et al., 2009),

osmoregulation (Yancey, 2001; Aragão et al., 2010), and stress response in fish (Aragão et al., 2008, 2010; Costas et al., 2008). Growth and feed conversion efficiencies in fish can be optimised by manipulating the dietary AA composition (Tacon and Cowey, 1985; Fauconneau et al., 1992). This is of particular importance when trying to replace fish meal by plant ingredients, often deficient in one or more AA.

Indispensable amino acid (IAA) profiles of whole body tissue of a given species of fish can be used as a rough indicator of the qualitative IAA requirements of the fish (Mambrini and Kaushik, 1995). Differences between fish and dietary AA profiles will tend to bring an unavoidable AA loss, which in turn will be reflected in higher AA requirements. However, the precise knowledge of the ideal AA profile implies taking into account the relative bioavailabilities, i.e. the rates of absorption and catabolism, for the individual AA (Conceição et al., 2003a). The absorption of individual AA in the gut of fish depends on different transport systems (Storelli et al., 1989; Vilella et al., 1990; Collie and Ferraris, 1995) which may proceed at different rates (Dabrowski, 1983). Therefore, variation in the rates of absorption of individual AA may attenuate or exacerbate AA imbalances. Furthermore, AA are a major energy source in fish, and fish are able to spare IAA at the expenses of dispensable amino acids (Cowey and Sargent, 1979; Kim et al., 1992; Rønnestad et al., 2001a; Conceição et al., 2002; Applebaum and Rønnestad, 2004). Therefore, the dietary AA profile that will allow for optimal growth will depend on the efficiency of absorption of each AA, on the AA profile of proteins being synthesised and on the relative use of individual AA for energy or other purposes. This ideal AA profile may change among species, but also within species depending on developmental stage (Conceição et al., 1998; Conceição et al., 2003b).

Senegalese sole (*Solea senegalensis*, Kaup 1858) is a marine teleost increasingly reared in recirculating and other land-based systems in Portugal and Spain, and with a high price and market demand. Nutrition physiology of larvae of this species has been intensively studied (Rønnestad et al., 2001a; Aragão et al., 2004a,b; Morais et al., 2004, 2005), including changes in IAA profiles during ontogeny (Aragão et al., 2004a). Information on the nutrition of juveniles is increasingly available, including ability to utilize lipids and carbohydrates (Dias et al., 2004), optimal dietary protein level (Rema et al., 2007), optimal lipid level

(Borges et al., 2009) and ability to perform on plant protein based diets (Silva et al., 2009). However, precise estimates of IAA requirements for sole are not available. Therefore, this work aims to study how the ten AA considered indispensable for fish (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine; Wilson, 2002) are utilized, by quantifying gut absorption, oxidation and retention in tissues of Senegalese sole juveniles, using ^{14}C-labelled IAA.

4.2. Materials and Methods

4.2.1. Fish

Experiments were directed by trained scientists (following FELASA category C recommendations) and were conducted according to the guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE of European Parliament and of the Council of European Union. Senegalese sole originated from the natural spawning of wild broodstock maintained at the Ramalhete station facilities (University of Algarve, Faro, Portugal), and were reared according to standard larval and juvenile rearing protocols (Dinis et al., 1999). Before the experiments, Senegalese sole juveniles with an average initial body weight of 22.6 (SD 3.6) g were maintained in a 200 litres flat-bottomed tank at an initial density of 1.5 kg/m^2 for at least 2 weeks, in a recirculated seawater system (temperature: 18-19 ºC; flow rate: 110 l/h; dissolved oxygen: above 90% saturation level; photoperiod: 12 h dark:12 h light, and the light intensity at water surface was 200 lux). Fish were fed every day semi-continuously (cycles of 2 h of feeding followed by 1 h break) with a commercial feed (Alpis, A. Coelho e Castro Lda., Póvoa de Varzim, Portugal; 2 mm; proximate composition: 53% protein, 12% lipid and 10% ash) supplied by automatic feeders 24 h a day. Diets were analysed for total AA contents. After hydrolysis, samples were analysed by High Performance Liquid Chromatography (HPLC) in a Waters Reversed-Phase Amino Acid Analysis System equipped with a PicoTag column (3.9 x 300 mm), using norleucine as an internal standard and according to the procedures described by Cohen et al. (1989). Resulting peaks were analysed with the Breeze software (Waters). Since asparagine is converted to aspartic acid and glutamine to glutamic acid during acid hydrolysis, the reported

values for these AA represent the sum of the respective amide and acid. AA composition (g/100 g AA) in the diet was thus as follows: alanine, 6.48; aspartic acid + asparagine, 5.03; cystein, 0.55; glutamic acid + glutamine, 0.96; glycine, 6.99; proline, 5.16; serine, 5.49; tyrosine, 4.50; histidine, 4.11; leucine, 8.81; isoleucine, 4.42; valine, 4.88; lysine, 10.51; metionine, 3.10; threonine, 5.08; phenilalanine, 4.75; arginine, 8.40. Tryptophan was not analysed since it is destroyed by acid hydrolysis. Prior to the experiments fish were fasted for 24 h, sedated (0.05 ml/l 2-phenoxyethanol; Sigma-Aldrich, Germany) and transported to the radioactivity room.

4.2.2. Treatments

The experiments were carried out at the CCMAR facilities (Faro, Portugal), and the 10 IAA (Wilson, 2002) were tested. Each tracer IAA was previously diluted in a saline solution (NaCl 0.9 %) in order to obtain a specific activity of 15.9 MBq/ml. The same experimental feed used during rearing was then hydrated with each tracer IAA solution ("hot" pellets) and afterwards dried in an oven at 50 ºC during 15 min. Final specific activity for each "hot" pellet was approximately 0.1 MBq. The tracer molecules used were: L-[U-^{14}C] Histidine (His – American Radiolabeled Chemicals, USA); L-[U-^{14}C] Leucine (Leu – American Radiolabeled Chemicals); L-[U-^{14}C] Isoleucine (Ile – American Radiolabeled Chemicals); L-[U-^{14}C] Valine (Val – American Radiolabeled Chemicals); L-[1-^{14}C] Lysine (Lys – Amersham Biosciences, UK); L-[1-^{14}C] Methionine (Met – Amersham Biosciences); L-[U-^{14}C] Threonine (Thr – Amersham Biosciences); L-[U-^{14}C] Phenylalanine (Phe – American Radiolabeled Chemicals); L-[1-^{14}C] Tryptophan (Trp – Amersham Biosciences); L-[U-^{14}C] Arginine (Arg – American Radiolabeled Chemicals). In addition, experimental feed was also hydrated with a non-radioactive saline solution (NaCl 0.9 %; "cold" pellets) containing a blue food colorant to monitor complete gut evacuation and possible feed regurgitation. Moreover, precision of sample delivery into the fish gut was assessed in preliminary trials. All fish were tube-fed with 2 "hot" and 1 "cold" pellets. The last pellet to be delivered into the gut was always the "cold" pellet. These 3 pellets represented an average protein intake of 0.4 g/kg average body weight.

4.2.3. Tube-feeding and incubation setup

The experimental setup for tube-feeding Senegalese sole juveniles is an up-scaling of the one described by Rust et al. (1993) and modified by Rønnestad et al. (2001b). The setup comprises a handmade plastic tube (inner diameter of 2.5 mm) with a plastic stick (diameter of 2 mm) placed inside. The plastic tube is flexible and can adapt to the mouth and oesophagus diameter of Senegalese sole juveniles from 10 to 50 g (based on preliminary trials). Before tube-feeding, fish were anaesthetised (0.2 ml/l 2-phenoxyethanol) and gently placed on a plastic tray. This is an important step and must be accurate in order to allow handling and feed supply, while maintaining continuous opercular movements (Rust et al., 1993), and allowing a rapid fish recovery for a proper physiological function of the digestive system (Rønnestad et al., 2001b). When a fish was in position, the plastic tube loaded with 2 "hot" pellets from one IAA plus 1 "cold" pellet was gently passed through the mouth into the oesophagus area. With the tube in place, the pellets were deposited into the oesophagus and stomach by pushing them with the inner plastic stick. Six fish were tube-fed for histidine, leucine, isoleucine, valine, methionine, threonine, phenylalanine and arginine, while for lysine and tryptophan nine fish were used. After withdrawal of the tube, fish were gently rinsed for residual anaesthetic in skin and gills and transferred to the incubation chambers containing 1 litre of clean seawater. With appropriate operator training, the total handling time per fish is less than 1 min. After tube-feeding, fish were monitored for eventual pellet regurgitation, which was never recorded.

In order to quantify the assimilation and catabolism of IAA fed to Senegalese sole juveniles by the method described above, a setup for determining the fate of ^{14}C-labelled IAA was used following the method described by Rønnestad et al. (2001b) with minor modifications. Two CO_2 traps permitted the label in the water due to unabsorbed ^{14}C-labelled AA (defecated, evacuated) to be separated from catabolised AA (^{14}C-CO_2). After tube-feeding and rinsing, sole juveniles were transferred to individual cylindrical incubation chambers (r = 7 cm), which were sealed (within 15 min after tube-feeding) and a gentle oxygen flow (3-4 ml/min) was induced into the incubation water. The oxygen/CO_2 flow was then directed by a capillary to a battery of two CO_2 traps (10 ml, 0.5 mol/l KOH per trap) build in series. The oxygen flow permits any ^{14}C-labelled CO_2

produced by the fish during the incubation time to diffuse from the water into the oxigen to be entrapped by conversion to HCO_3^-. According to Dias et al (2010) and preliminary studies using the present methodology, it was observed that total evacuation of faeces corresponding to a single meal (3 pellets) is achieved at 24 hours following that meal. Therefore, once the set incubation period was over (24 h) and the fish had been removed, the chamber was resealed and acid (130 ml, 0.1 mol/l HCl) was introduced into the incubation water using the stepwise procedure suggested by Rønnestad et al. (2001b). The lowered pH led to rapid diffusion of any remaining CO_2 from the water and into the traps. Finally, both KOH from the CO_2 traps and chamber incubation water were transferred to 20 and 6 ml scintillation vials, and 15 and 4 ml of scintillation cocktail (Ultima Gold XR; PerkinElmer, USA) was added, respectively. The samples were then counted in a Beckman LS 6000IC liquid scintillation counter (Fullerton, CA, USA). Due to the high volume that the incubation water represents for scintillation counting (1 litre), three aliquots of 5 ml were collected after stirring the incubation water. Afterwards, the mean value obtained after counting was used to calculate the total unabsorbed ^{14}C-labelled AA that remained in the incubation water. It is important to emphasize that faeces from sole are liquid, and visual inspection suggested they were well solubilized in the incubation water.

After the incubation time, oxygen flow was stopped and fish were killed inside the chambers by a lethal dose of MS-222 (300 mg/l; Sigma-Aldrich, Germany). Afterwards, fish were lifted by the tail using a forceps and transferred to a dissection tray. Liver, gut and about 10 % of total fillet muscle were dissected, weighed and transferred to 20 ml scintillation vials containing 4 ml of tissue solubilizer (Solvable, PerkinElmer, USA). It was previously verified that total fillet muscle of Senegalese sole around 10 - 50 g is equivalent to 50 % of total weight. When the solution was clear after 36 - 48 h at 40 ºC, 15 ml of scintillation cocktail was added. In addition, for each IAA 2 "hot" and 1 "cold" pellets (representing ^{14}C-labelled IAA tube-fed) were transferred to 6 ml scintillation vials (n = 3 vials) containing 1 ml of tissue solubilizer following the same procedure described in the experimental setup. When the solution was clear after 24 h at 40 ºC, 5 ml of scintillation cocktail was added.

All samples were mixed and stored for at least 60 min at room temperature before being counted for 10 min.

4.2.4. Metabolic budget of the IAA

For each tube-fed fish, the following samples were counted (DPM) by liquid scintillation: incubation water (assumed to be unabsorbed defecated ^{14}C-labelled IAA), KOH trap (CO_2 produced by ^{14}C-labelled IAA oxidation), and liver, gut and muscle (representing retained IAA). Moreover, the fraction other tissues was calculated as indicative of retained IAA in the remaining fish tissues.

4.2.5. Validation of the method

In order to validate the experimental setup, the efficiency of this tube-feeding methodology in the recovery of the ^{14}C-labelled IAA tube-fed was assessed. Therefore, Senegalese sole juveniles (n = 2 for histidine, leucine, isoleucine, valine, methionine, threonine, phenylalanine, tryptophan and arginine; n = 3 for lysine) were incubated in the experimental setup described above. After 24 hours, fish were removed from the incubation chambers, completely dissected and the following fractions were collected: blood, liver, gut, skin, dorsal and ventral muscle (fillet), skeleton, fins, head, brain, gills, kidney and mucus. The sum of the fractions blood, skin, skeleton, fins, head, brain, gills, kidney and mucus stand for the other tissues fraction calculated in the remaining of the present study.

4.2.6. Data and statistical analysis

The metabolic budgets were calculated after subtraction of blanks (scintillation liquid alone) and correction for counting efficiency. The fraction other tissues was calculated by subtracting the sum of all other fractions (incubation water, CO_2, liver, gut and muscle) to the respective IAA tube-fed. Each fraction (incubation water, CO_2, liver, gut, muscle and other tissues) was expressed as a percentage of the tracer fed (i.e. DPM counted for a given IAA tube-fed).

Bioavailability was calculated relative to lysine, the putative first limiting AA. Relative bioavailability for each IAA was then calculated (Conceição et al., 2003a):

B_i = (rIAA$_i$ + retained IAA$_i$)/retained lysine

where i stands for a given individual IAA; rIAA means relative difference (%) between the contribution of a given IAA to the diet ($_{diet}$pIAA) and to the Senegalese sole carcass ($_{carcass}$pIAA) IAA profiles. rIAA was calculated as: ($_{diet}$pIAA$_i$ – $_{carcass}$pIAA$_i$) x 100/($_{carcass}$pIAA$_i$). Therefore, the absolute value of the rIAA$_i$ is here considered as an estimate of the unavoidable AA loss (percent of total AA absorbed) for the diet used in this study caused by its deviation to the ideal IAA profile. IAA contents in the sole carcass (g/100 g IAA) were obtained by HPLC analysis from another study (Silva et al., 2010).

The ideal dietary IAA profile was calculated by dividing the contribution of each IAA to the Senegalese sole carcass IAA profile (g of each IAA per 100 g of all IAA considered in this study) by the relative bioavailability of that same IAA (Conceição et al., 2003a).

The results were expressed as mean ± standard deviation (SD). All data were tested for homogeneity of variances by Levene's tests, and then submitted to one-way analysis of variance (ANOVA) using the computer package SPSS for Windows 15.0 (SAS Institute, Inc., Cary, NC, USA), followed by Tukey's post hoc tests. Differences among treatment groups were considered significant when $P < 0.05$. All results expressed as percentage were previously arcsine transformed to avoid problems of heterogeneity of variance and non-additivity (Zar, 1999).

4.3. Results

4.3.1. Validation of the method and recovery test

The efficiency of this methodology in the recovery of the ^{14}C-labelled IAA tube-fed was determined to be 96.0 ± 2.2 % (*n* 2-3). Table 1 shows the results obtained when individual ^{14}C-labelled IAA were tube-fed and retained IAA was completely quantified by dissecting each fish in 13 fractions as described in the validation of the method section. The sum of blood, skin, skeleton, fins, head, brain, gills, kidney and mucus fractions is strongly correlated ($y = 1.2977x - 5.3306$; $R^2 = 0.8365$; $p = 0.0002$) to the estimated amount of retained IAA in the fraction regarded as other tissues in the present study. Moreover, liver, gut and the sum of dorsal and ventral muscle (fillet) fractions were in line with values observed in major tissues (liver, gut and muscle) in the remaining of the present study (Figure 1).

Table 1. Validation of IAA metabolic budgets methodology in Senegalese sole juveniles. Proportion (%) of the total tube-fed ^{14}C-labelled IAA that was evacuated, oxidised or retained in blood, liver, gut, skin, dorsal and ventral muscle, skeleton, fins, head, brain, gills, kidney and mucus of Senegalese sole juveniles.

Fraction (% DPM)	His	Leu	Ile	Val	Lys	Met	Thr	Phe	Trp	Arg
Water	7.4 ± 1.0 ^{a}	13.7 ± 5.3 ^{ab}	4.9 ± 0.8 ^{a}	10.8 ± 3.3 ^{a}	15.1 ± 0.7 ^{ab}	9.3 ± 0.3 ^{a}	8.0 ± 4.7 ^{a}	12.7 ± 7.2 ^{ab}	49.8 ± 4.3 ^{c}	28.0 ± 4.6 ^{b}
CO_2 trap	10.9 ± 0.3 ^{ab}	11.0 ± 3.7 ^{ab}	25.0 ± 0.5 ^{c}	17.5 ± 0.6 ^{bc}	41.7 ± 3.0 ^{d}	13.4 ± 3.8 ^{ab}	5.2 ± 0.7 ^{a}	11.4 ± 0.4 ^{ab}	5.9 ± 1.0 ^{a}	11.9 ± 6.4 ^{ab}
Blood	1.4 ± 0.1 ^{c}	1.4 ± 0.2 ^{c}	1.5 ± 0.1 ^{c}	0.1 ± 0.0 ^{a}	0.8 ± 0.1 ^{b}	0.3 ± 0.1 ^{a}	1.0 ± 0.2 ^{bc}	1.6 ± 0.4 ^{c}	1.0 ± 0.1 ^{bc}	1.6 ± 0.0 ^{c}
Liver	8.1 ± 4.7 ^{b}	4.8 ± 1.1 ^{ab}	5.0 ± 2.0 ^{ab}	3.5 ± 1.6 ^{ab}	1.9 ± 0.4 ^{a}	2.1 ± 0.2 ^{a}	3.6 ± 1.1 ^{ab}	9.4 ± 2.2 ^{b}	4.2 ± 0.1 ^{ab}	3.9 ± 1.2 ^{ab}
Gut	9.5 ± 2.0 ^{ab}	10.1 ± 2.8 ^{ab}	15.2 ± 0.5 ^{b}	8.6 ± 2.9 ^{ab}	4.5 ± 1.2 ^{a}	11.1 ± 0.2 ^{b}	9.0 ± 1.4 ^{ab}	15.1 ± 1.4 ^{b}	13.5 ± 1.4 ^{b}	13.7 ± 2.1 ^{b}
Skin	2.0 ± 0.0	1.9 ± 0.6	2.6 ± 0.4	1.4 ± 1.0	1.3 ± 0.4	1.6 ± 0.2	2.8 ± 0.7	2.4 ± 0.6	1.2 ± 0.2	2.4 ± 0.0
Dorsal muscle	17.3 ± 0.5 ^{d}	16.0 ± 0.1 ^{d}	8.8 ± 0.3 ^{c}	10.2 ± 0.3 ^{c}	4.8 ± 0.2 ^{ab}	17.6 ± 0.2 ^{d}	20.7 ± 1.2 ^{d}	9.2 ± 0.4 ^{c}	2.9 ± 0.2 ^{a}	6.7 ± 1.1 ^{bc}
Ventral muscle	16.2 ± 0.5 ^{d}	16.5 ± 0.3 ^{d}	9.2 ± 0.3 ^{bc}	10.6 ± 0.4 ^{c}	6.6 ± 0.4 ^{ab}	17.5 ± 0.3 ^{d}	19.0 ± 0.9 ^{d}	9.7 ± 1.0 ^{bc}	4.5 ± 0.3 ^{a}	7.7 ± 0.6 ^{ab}
Skeleton	6.6 ± 0.5 ^{ab}	4.9 ± 0.6 ^{ab}	4.6 ± 0.2 ^{ab}	7.2 ± 0.6 ^{b}	3.7 ± 0.2 ^{a}	6.6 ± 0.3 ^{ab}	5.7 ± 2.1 ^{ab}	6.6 ± 1.8 ^{ab}	3.7 ± 0.1 ^{a}	4.6 ± 0.7 ^{ab}
Fins	5.8 ± 0.2 ^{bc}	5.0 ± 0.0 ^{ab}	5.8 ± 0.2 ^{bc}	9.6 ± 1.2 ^{d}	5.0 ± 0.5 ^{ab}	5.8 ± 0.3 ^{bc}	9.6 ± 1.5 ^{d}	5.8 ± 0.7 ^{bc}	3.3 ± 0.4 ^{a}	8.0 ± 0.4 ^{cd}
Head	7.0 ± 0.3 ^{b}	6.1 ± 1.7 ^{ab}	6.7 ± 0.4 ^{ab}	7.9 ± 1.8 ^{b}	5.6 ± 0.4 ^{ab}	7.7 ± 1.1 ^{b}	5.4 ± 1.6 ^{ab}	8.2 ± 1.2 ^{b}	3.0 ± 0.2 ^{a}	4.4 ± 1.4 ^{ab}
Brain	1.0 ± 0.3 ^{b}	0.5 ± 0.4 ^{ab}	0.3 ± 0.0 ^{ab}	0.2 ± 0.0 ^{a}	0.1 ± 0.1 ^{a}	0.2 ± 0.0 ^{a}	0.2 ± 0.0 ^{a}	0.2 ± 0.0 ^{a}	0.4 ± 0.2 ^{ab}	0.2 ± 0.0 ^{a}
Gills	2.8 ± 0.7 ^{ab}	2.2 ± 0.0 ^{ab}	3.7 ± 0.1 ^{b}	3.2 ± 0.2 ^{ab}	2.4 ± 0.3 ^{ab}	1.8 ± 0.1 ^{a}	4.1 ± 1.7 ^{b}	4.0 ± 0.6 ^{b}	1.4 ± 0.2 ^{a}	2.6 ± 0.0 ^{ab}
Kidney	0.9 ± 0.2 ^{ab}	2.6 ± 1.0 ^{b}	2.0 ± 0.3 ^{ab}	0.7 ± 0.3 ^{ab}	0.6 ± 0.0 ^{a}	1.3 ± 0.3 ^{ab}	1.7 ± 0.5 ^{ab}	1.3 ± 0.9 ^{ab}	0.9 ± 0.3 ^{ab}	2.1 ± 1.2 ^{ab}
Mucus	0.08 ± 0.0 ^{a}	0.14 ± 0.0 ^{a}	0.12 ± 0.0 ^{a}	0.03 ± 0.0 ^{a}	1.51 ± 0.3 ^{b}	0.03 ± 0.0 ^{a}	0.12 ± 0.0 ^{a}	0.03 ± 0.0 ^{a}	0.07 ± 0.0 ^{a}	0.07 ± 0.0 ^{a}
Total recovery	97.0 ± 1.0	96.9 ± 0.8	95.6 ± 0.9	91.6 ± 0.9	95.6 ± 3.4	96.3 ± 1.6	96.1 ± 0.4	97.6 ± 0.4	98.2 ± 0.4	97.8 ± 1.6

Data are presented as means ± SD (n = 2 for His, Leu, Ile, Val, Met, Thr, Phe, Trp and Arg; n = 3 for Lys), expressed as a percentage (%) of the total ^{14}C - labelled IAA counted. Mean values within a column with unlike superscript letters were significantly different ($P < 0.05$).

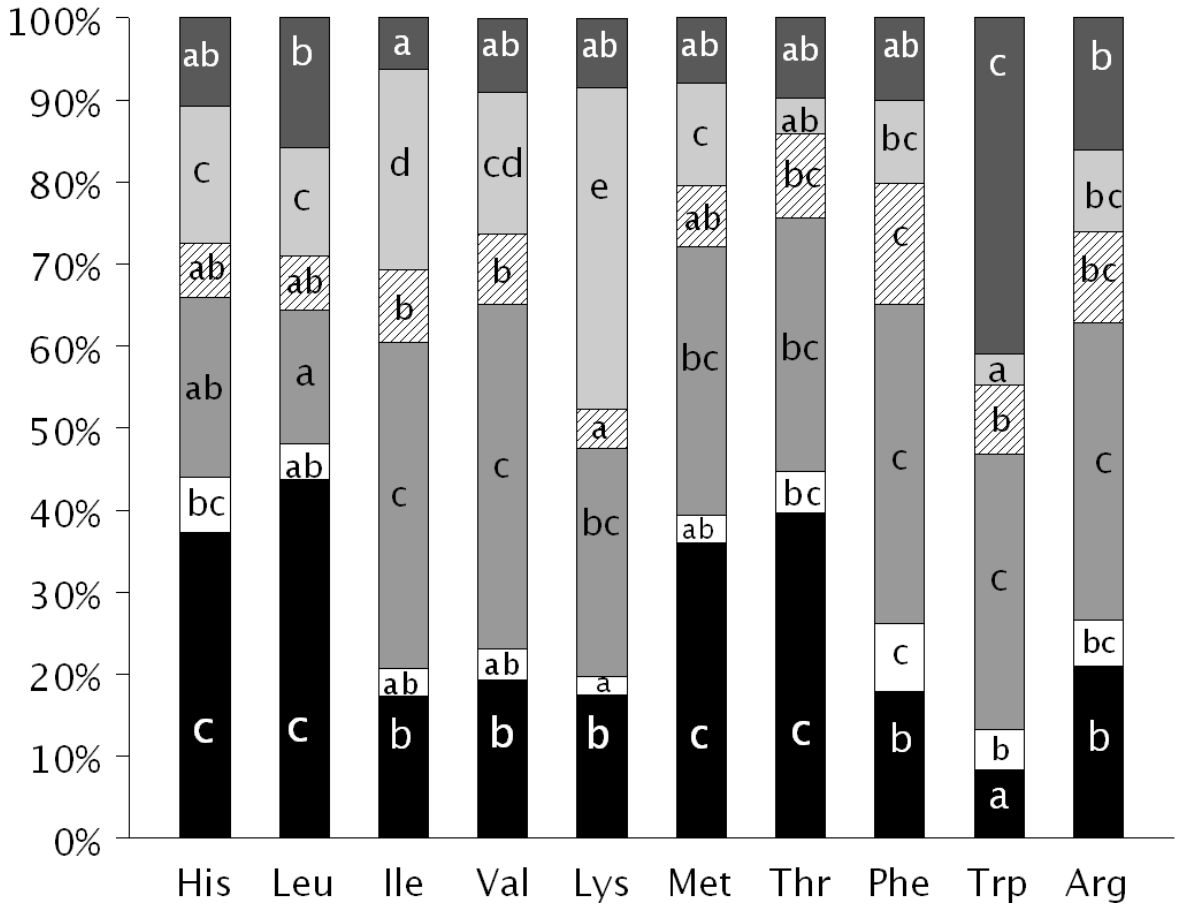

Figure 1. Metabolic budgets of IAA in Senegalese sole juveniles. Proportion (%) of the total tube-fed ^{14}C - labelled IAA that was retained in muscle (■), liver (□), other tissues (▩) and gut (▨), oxidised (▩) or evacuated (■). Mean values and standard deviation (n = 6 for His, Leu, Ile, Val, Met, Thr, Phe and Arg; n = 9 for Lys and Trp). Mean values within the same compartment with unlike superscript letters were significantly different ($P < 0.05$).

4.3.2. Metabolic budgets of indispensable amino acids

Some variation in retentions for liver and gut fractions were observed among the IAA tube-fed (Figure 1), with significantly higher retentions for phenylalanine (8.39 ± 2.3 and 14.81 ± 3.1 % in liver and gut, respectively) and lower retentions for lysine (2.26 ± 0.7 and 4.72 ± 1.8 % in liver and gut, respectively). Histidine, leucine, methionine and threonine were the most retained IAA in muscle (37.25 ± 12.6, 43.69 ± 7.7, 35.96 ± 5.1 and 39.73 ± 3.3 %, respectively), while isoleucine, valine, lysine, phenylalanine and arginine showed lower retentions (17.30 ± 2.8, 19.28 ± 1.2, 17.49 ± 4.1, 17.83 ± 4.9 and 21.02 ± 7.4 %, respectively). Tryptophan was the IAA with lowest (8.28 ± 3.5 %) retention in muscle (Figure 1). Furthermore, total retention (whole body) was

slightly different for the ten IAA tube-fed when compared to retention in muscle. For instance, threonine was the most retained IAA in the whole body while lysine and tryptophan showed the lowest values (Table 2). In addition, AA catabolism significantly varied among the different IAA. Lysine was the most catabolised IAA (39.2 %) followed by isoleucine, valine, histidine, leucine, methionine, phenylalanine, and arginine with 24.4, 17.2, 16.7, 13.1, 12.4, 10.1, and 10.0 % of IAA oxidised, respectively (Figure 1). Moreover, tryptophan was significantly less absorbed than the other IAA ingested, while histidine, leucine, isoleucine, valine, lysine, methionine, threonine, phenylalanine and arginine showed similar values for absorbed IAA (Table 2).

Table 2. Metabolic budgets for Senegalese sole juveniles tube-fed with ^{14}C - labelled IAA.

	Water	CO_2 trap	Retained*	Digested†
His	10.8 ± 1.1 ^{ab}	16.7 ± 4.3 ^{d}	72.6 ± 4.7 ^{b}	89.2 ± 1.1 ^{a}
Leu	15.9 ± 8.4 ^{b}	13.1 ± 3.0 ^{c}	71.0 ± 8.0 ^{b}	84.1 ± 8.4 ^{a}
Ile	6.3 ± 6.9 ^{a}	24.4 ± 5.9 ^{d}	69.3 ± 3.2 ^{b}	93.7 ± 6.9 ^{a}
Val	9.1 ± 1.3 ^{ab}	17.2 ± 1.4 ^{cd}	77.3 ± 2.0 ^{bc}	90.9 ± 1.3 ^{a}
Lys	8.5 ± 1.7 ^{ab}	39.2 ± 3.2 ^{e}	52.9 ± 2.3 ^{a}	91.8 ± 0.9 ^{a}
Met	7.9 ± 1.5 ^{ab}	12.4 ± 3.0 ^{c}	79.6 ± 3.5 ^{bc}	92.1 ± 1.5 ^{a}
Thr	9.7 ± 4.6 ^{ab}	4.4 ± 1.3 ^{ab}	85.8 ± 4.2 ^{c}	90.3 ± 4.7 ^{a}
Phe	10.1 ± 1.9 ^{ab}	10.1 ± 0.9 ^{bc}	79.9 ± 1.2 ^{bc}	90.0 ± 1.9 ^{a}
Trp	41.0 ± 5.8 ^{c}	3.7 ± 3.0 ^{a}	55.3 ± 7.5 ^{a}	59.0 ± 5.8 ^{b}
Arg	16.1 ± 7.9 ^{b}	10.0 ± 7.2 ^{bc}	73.9 ± 14.2 ^{bc}	84.0 ± 7.9 ^{a}

Data are presented as means ± SD (n = 6-9), expressed as a percentage (%) of the total ^{14}C - labelled IAA counted. * Retained fraction was calculated by the sum of liver, gut, muscle and other tissues fractions for each IAA tube-fed. † Digestibility was obtained by the sum of retained and oxidised (CO_2 trap) fractions for each IAA tube-fed. Mean values within a column with unlike superscript letters were significantly different ($P<0.05$).

4.3.3. Relative bioavailability and ideal profile of indispensable amino acids

Relative bioavailability results show higher values for histidine, leucine, isoleucine, valine, methionine, threonine, phenylalanine and arginine compared to lysine. Histidine presents the highest value among all IAA. On the other hand, tryptophan seems to present lower relative bioavailability when compared to

lysine (Table 3). In addition, the ideal IAA profile shows some changes compared to the sole carcass IAA profile (Table 3). For instance, histidine, methionine, threonine, phenylalanine and arginine seem to present lower values whereas lysine and tryptophan show higher levels.

Table 3. Bioavailability, other related estimates, and ideal IAA profile for Senegalese sole juveniles.

	rIAA (%)*	Apparent retention (%)†	Retention (%)‡	Relative bioavailability§	Apparent IAA profile (g 100 g-1 IAA) ‖	Ideal IAA profile (g 100 g-1 IAA)¶
His	25.89	72.58	98.46	1.65	5.64	4.14
Leu	-5.76	71.01	71.01	1.19	16.13	16.41
Ile	-9.31	69.34	69.34	1.17	8.38	8.73
Val	-18.37	73.72	73.72	1.24	10.29	10.08
Lys	7.27	52.24	59.51	1.00	16.91	20.52
Met	-15.34	79.62	79.62	1.34	6.26	5.68
Thr	-8.62	85.83	85.83	1.44	9.63	8.10
Phe	-9.19	79.89	79.89	1.34	9.03	8.16
Trp	-39.92	55.28	55.28	0.93	2.63	3.44
Arg	-3.91	73.92	73.92	1.24	15.09	14.74

* Relative differences between IAA from the diet and carcass (rIAA, %) = ($_{diet}$pIAA$_i$ - $_{carcass}$pIAA$_i$) x 100/($_{carcass}$pIAA$_i$), where $_{diet}$pIAA and $_{carcass}$pIAA are the contributions of a given IAA to the diet and carcass IAA profiles, respectively.

† Apparent retention = sum of liver, gut, muscle and other tissues fractions for each IAA tube-fed.

‡ Retention, calculation depends if rIAA is positive (= rIAA + apparent retention, to take into account unavoidable AA losses due to dietary imbalances) or negative (=rIAA).

§ Relative bioavailability = retained IAA$_i$/retained lysine.

|| Sole Carcass IAA profile (Data from Silva et al., 2010).

¶ Corrected Ideal IAA profile, calculated as IAA$_i$ in Senegalese sole carcass IAA profile/IAA$_i$ relative bioavailability, and back-calculated to 100%.

4.4. Discussion

4.4.1. Methodology

The present tube-feeding methodology permits unabsorbed labelled nutrients emptied from the gut, to be distinguished from labelled molecules originating from catabolism of the absorbed IAA in juvenile fish. Since endogenous nitrogen is reabsorbed in final parts of fish digestive tract (Dabrowski and Dabrowska, 1981) probably as a result of enzyme reabsorption

(Hofer and Schiemer, 1981), eventual AA losses due to endogenous nitrogen secretion are expected to be negligible. Moreover, the present methodology can also identify where a particular nutrient is being retained and at which proportion. Although the analysis of isotopically labelled CO_2 is a well-established method for the study of AA catabolism in adult fish (Walton, 1985) as in other animals (e.g., Schreurs, et al., 1992; Wolfe, 1992), it is important to emphasize that this method differentiates between absorption (digestibility), catabolism (oxidation) and retention of a given IAA ingested.

It was previously suggested that unavoidable effects of the stress imposed when using this technique in fish larvae may affect fish metabolism (Rust et al., 1993). Previous studies (Rønnestad et al., 2000, 2001a; Aragão et al., 2004b) have shown that Senegalese sole post-larvae have low sensitivity to handling during this type of experimentation, resulting in 100 % survival after the tube-feeding experiments. In addition, Senegalese sole juveniles tube-fed in the present study were also alive and with a normal appearance and ventilation rate (after the first hour in the chambers) throughout the trials and at the end of the incubation period. Furthermore, at the end of the incubation time (24 hours), there was no evidence of remaining faeces after visual inspection in dissected fish, suggesting an accomplished gut clearance. Which is in line with that observed by Dias et al (2010) for total egestion of a single meal. In order to evaluate whether the physiological status of sole juveniles was being affected by the methodology setup, results showed comparable plasma cortisol levels between fish that had or had not been tube-fed before and at the end of the incubation period (Conceição et al., unpublished results). Plasma cortisol is commonly used to assess stress in fish due to its responsiveness to acute stressors and its functional significance in physiological processes (Barton and Iwama, 1991). Conceição et al. (unpublished results) observed that the handling procedure from this experimental setup, but not the tube-feeding itself, appears to induce a mild accute stress response in sole. Moreover, fish seem to recover normally from handling procedure, with cortisol levels returning to basal levels at the end of the 24h incubation period (Conceição et al., unpublished results). This is in line with what could be expected. Another study performed with Senegalese sole showed that cortisol levels peak at 1 hour, dropping quickly after that and returning to basal levels within 4 hours, after an acute stress (Costas et al., 2011). In addition to that, AA oxidation only starts 6 hours after tube-feeding

using the same experimental setup (Conceição et al., unpublished results). This suggests that the acute stress suffered was not sufficient to elicit an AA catabolic response driven by cortisol as previously observed in other species (Mommsen et al., 1999), and that handling procedures from this methodology do not seem to have a significant effect on Senegalese sole metabolism. This idea is further supported by quick adaptation of Senegalese sole juveniles to confinement in small respirometers, very similar in size to the present setup, as shown by oxygen consumption rates showing a moderate increase and then dropping to resting metabolism values after 2-3 hours in the respirometers (Castanheira et al., 2011).

Another possible concern with the present methodology is the calculation of the fraction "other tissues" and the muscle estimates by sampling. However, the recovery test (Table 2) shows that the estimated values for retention of each IAA in other tissues (the blood, skin, skeleton, fins, head, brain, gills, kidney and mucus fractions) and in muscle (retention in dorsal and ventral muscle fractions) are relatively fixed proportions. Calculations for quantifying IAA retention in muscle and other tissues fractions from the experimental setup are thus supported and validated. Moreover, the amount of unrecovered IAA was very small and was considered to be negligible.

Taken together, the results obtained in this study supports that the tube-feeding technique for Senegalese sole juveniles is a valid tool for *in vivo* tracer studies on AA metabolism.

4.4.2. Indispensable amino acids metabolism

Results from the present study point to various differences in absorption (digestibility) capacity for different IAA in Senegalese sole juveniles, as well as their relative utilization for protein deposition or energy production. A striking observation was the low digestibility of tryptophan (59 %). Available data of tryptophan digestibility in fish is scarce since it is destroyed by acid hydrolysis during routine AA analyzes. Still, juvenile rainbow trout (*Oncorhynchus mykiss*) fed a fish meal based diet showed lower apparent digestibility coefficients of tryptophan in several parts of the digestive tract when compared with all other IAA (Dabrowski and Dabrowska, 1981). In addition, a low digestibility

(approximately 70%) of tryptophan compared to other IAA was also observed in white seabream (*Diplodus sargus*) larvae (Saavedra et al., 2008). For the IAA studied in the present study, at least two carrier mediated transport systems specific to basic (histidine, lysine and arginine) and neutral (leucine, isoleucine, valine, methionine, threonine, phenylalanine and tryptophan) free AA (FAA) are involved in the transport across the brush-border membrane (Collie and Ferraris, 1995). However, carrier-mediated transporters often have overlapping specificities and interactions between FAA seem to interfere with the uptake of a specific AA (Storelli et al., 1989; Vilella et al., 1990). For instance, lysine appears to have both stimulatory and inhibitory effects on the uptake of arginine depending on their relative concentrations (Berge et al., 1999). Therefore, an abundance of IAA deriving from protein digestion could have competed or at least interfered with the transport of tryptophan, leading to a low relative absorption of this particular IAA. On the other hand, tryptophan usage by gut microbiota may also be the cause for the increased tracer found in water. In fact, anaerobic fermentation of tryptophan by colonic bacteria gives rise to indoles, which are eventually excreted in the urine in humans (Blaut and Clavel, 2007). Some of these bacteria belong to the genus *Bacillus* (Macfarlane et al., 1986), which has been also found in Senegalese sole intestinal microbiota (Martin-Antonio et al., 2007). Therefore, tryptophan may have been utilized by these particular bacteria before being absorbed in the intestinal tract, and thus releasing ^{14}C-labelled metabolites into the incubation water. In any case, the low tryptophan digestibility should be taken into account when formulating sole feeds, and it is important to verify if this poor digestibility for tryptophan also occurs in other fish species.

The high digestibilities observed for all the IAA studied but one (tryptophan) are in agreement with previous studies of AA digestibility in fish (Dabrowski, 1983; Kaushik et al., 1994). For instance, most IAA showed AA availabilities higher than 78 % in Siberian sturgeon (*Acipenser baeri*) fed either a commercial diet or two experimental isoproteic and isoenergetic diets (Kaushik et al., 1994). Unfortunately, data regarding tryptophan digestibility was not available. On the other hand, since histidine, leucine, isoleucine, valine, lysine, methionine, threonine, phenylalanine and arginine from the present study were similarly absorved/digested, the higher arginine, threonine and phenylalanine retentions in the gut fraction may be indicative of gut proteins synthesis and/or

other molecules rich in these particular IAA. For instance, threonine is the major component of intestinal mucin in animals, thus regulating intestinal barrier integrity and function (Li et al., 2009).

The present study shows higher histidine, leucine, methionine and threonine retentions in muscle, suggesting a sparing of these particular IAA for muscle growth in Senegalese sole juveniles. Methionine and leucine were also highly retained (around 80 and 85 %, respectively) in Senegalese sole larvae (Rønnestad et al., 2000, Aragão et al., 2004b), while a 60 % retention was observed for methionine in white seabream larvae (Saavedra et al., 2008). However, these values indicate whole body retentions. Since total retentions from the current study were also calculated (80 and 71 % for methionine and leucine, respectively), these results are comparable and suggest that differences in body retention for methionine and leucine may be species and/or developmental stage dependent. Conceição et al. (1998) had already stated that the AA profile of larval fish seems to be more variable than larger fish, resulting from allometric growth of the larvae. For instance, when comparing the IAA profiles of larval (Conceição et al., 1998) and juvenile (Hoffman et al., 1995) African catfish (*Clarias gariepinus*), it can be observed that the percentage contribution of arginine and threonine to total IAA contents tend to increase, while valine and isoleucine decrease. On the other hand, Kaushik and Luquet (1977) also observed high contents of histidine in muscle of rainbow trout. Still, a lower availability of histidine in Siberian sturgeon (*Acipenser baeri*) juveniles when compared to other IAA was reported (Kaushik et al., 1994), probably indicating a decreased availability for tissue maintenance and growth. However, the present study shows that dietary histidine is highly available in sole juveniles, suggesting again species differences in IAA biovailability.

In the present study, results from methionine, leucine, arginine and phenylalanine catabolism appear to be comparable to the observed in other studies with tube-fed Senegalese sole post-larvae (around 15 % for methionine, 14.5 % for leucine, 9.2 % for arginine, and 10 % for phenylalanine; Rønnestad et al., 2000; Aragão et al., 2004b; Pinto et al., 2009) and plaice (*Pleuronectes platessa*) juveniles fed a diet with 50 % crude protein level (around 14.6 % for leucine and 9.2 % for phenylalanine; Cowey, 1975). However, a preferential utilization of lysine as an energy substrate was observed in this study, indicating

a selective catabolism of this individual IAA. This observation was surprising since other studies with fish larvae (Rønnestad et al., 2001a; Conceição et al., 2002; Applebaum and Rønnestad, 2004) and mammals (Chavez and Bayley, 1976; Newport et al., 1976) did not show this preferential usage of lysine for energy production. In fact, the lysine trial was repeated (total fish used n = 9 instead of n = 6, as for other IAA) and the amount of tracer oxidised was always consistent among replicates. Even if lysine was in slight apparent excess in the diet (Table 3), this does not seem to explain its high utilization for energy production. It would be interesting to verify to what extent this preference of lysine as an energy substrate also occurs in other telesot species. In fact, different amounts of oxidised lysine were observed in unfed Senegalese sole post-larvae and herring (*Clupea harengus*) larvae (around 11.5 and 23 %, respectively), suggesting a species specific usage of lysine for energy production.

4.4.3. Bioavailability and ideal profile of indispensable amino acids

In the present study, histidine, leucine, isoleucine, valine, methionine, threonine, phenylalanine and arginine appear to have higher relative bioavailabilities compared to lysine, suggesting a preferential use of lysine for energy production, once absorption efficiencies of other IAA (except tryptophan) were similar to lysine (Table 2). On the contrary, tryptophan showed a lower relative bioavailability compared to lysine, which is explained by with the lower absorption efficiency observed in the present study. In addition, histidine and threonine showed the highest relative bioavailability values.

Correction of the IAA profile obtained by HPLC with the bioavailability data, indicate some important changes in the qualitative IAA requirements of Senegalese sole juveniles when compared to IAA profile used alone. Histidine, methionine, threonine and phenylalanine requirements appear to decrease, while lysine and tryptophan requirements augment due to the increased catabolism and the lower absorption efficiency observed, respectively. Therefore, optimized diet formulations must take into account biovialability of individual IAA. For sole, the tryptophan and lysine requirements seem to deserve particular attention. IAA requirements for a given species are often estimated based on a dose response for lysine requirements, and then applying the ideal protein concept (ARC, 1981; Boisen et al., 2000) to estimate the requirements of other IAA based on the

carcass IAA profile (e.g., Small and Soares Jr., 2000; Wilson, 2002; Peres and Oliva-Teles, 2008). If IAA requirements for sole are based on such a simplified method, an overestimation of the requirements for most IAA will occur, once lysine has been shown here to have a low bioavailability relative to all other IAA except tryptophan.

4.5. Conclusions

The experimental setup used in this study may be a valuable tool for improving knowledge on fish AA utilization and requirements. The relative absorption (digestibility) capacity for different AA as well as their relative utilization for energy production can be assessed by this methodology. Results from the present study point to lower absorption (digestibility) capacity for tryptophan compared to other IAA in Senegalese sole juveniles, as well as several differences in relative utilization of individual IAA for energy production. In particular, lysine seems to have an important role as an energy substrate in Senegalese sole juveniles. In the present study, histidine, leucine, methionine and threonine showed a higher retention in muscle than the other IAA, suggesting a sparing for muscle growth. The present study also estimated the relative bioavailability of individual IAA in Senegalese sole, which together with the carcass IAA profile allowed defining the ideal dietary IAA profile for this species.

4.5. Acknowledgements

This study was supported by the IDEIA project “Optidietas” (Agência de Inovação, Portugal, with the support of the European fund FEDER). B. C. was supported by Fundação para a Ciência e Tecnologia, Portugal (SFRH/BD/38697/2007).

B. C. carried out the main experimental work and wrote the manuscript under the supervision of L. E. C. C., who was also responsible for the experimental design; M. F. C. assisted with experimental work and radioactivity counting. The authors also acknowledge Jorge Dias (CIMAR/CCMAR) for critically reviewing the manuscript.

There are no conflicts of interest in connection with the present study.

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Chapter 5

Effects of dietary amino acids and repeated handling on stress response and brain monoaminergic neurotransmitters in Senegalese sole (*Solea senegalensis* Kaup, 1858) juveniles

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Costas, B., Aragão, C., Soengas, J.L., Míguez, J.M., Rema, P., Dias, J., Afonso, A., Conceição, L.E.C., 2011. Effects of dietary amino acids and repeated handling on stress response and brain monoaminergic neurotransmitters in Senegalese sole (*Solea senegalensis* Kaup, 1858) juveniles. Comparative Biochemistry and Physiology, Part A, *in press*. DOI: 10.1016/j.cbpa.2011.08.014.

Effects of dietary amino acids and repeated handling on stress response and brain monoaminergic neurotransmitters in Senegalese sole (*Solea senegalensis* Kaup, 1858) juveniles

Abstract

The present study aimed to assess the effects of increased availability of dietary amino acids (AA) on brain monoamine neurotransmitters and the metabolic processes resulting from stressful situations in fish. Senegalese sole (*Solea senegalensis*) juveniles (24.2 ± 0.4 g wet weight) were weekly subjected to an acute handling stressor (HDLG) or remained undisturbed (CTL). Additionally, both treatments were fed a control or a high protein (HP) diet (CTL, CTL HP, HDLG and HDLG HP). The HP diet had a slightly increase in the levels of digestible indispensable AA, together with tyrosine and cysteine. Repeated handling induced a stress response after 14 and 28 days in fish held at both HDLG and HDLG HP treatments. While dietary treatment and handling stress activated the serotonergic system at 14 days, these effects were not observed after 28 days. In addition, the HP diet minimized the decrease in plasma indispensable AA due to repeated handling stress after 28 days. It was concluded that HP diet decreased post-stress plasma glucose and lactate levels in HDLG HP specimens only at 14 days of treatment. Moreover, dietary treatment was also effective in stimulating DA synthesis and release, thus dietary phenylalanine supplementation can increase DA biosynthesis in fish.

Keywords: cortisol; dopamine; lysozyme activity; serotonin; tryptophan; tyrosine

5.1. Introduction

Senegalese sole (*Solea senegalensis*) is high-value flatfish that presents a great potential for future farming at commercial scale. However, growth and survival from juvenile to market-size fish is not fully controlled with regard to rearing technology and husbandry conditions, feeding behavior and nutritional requirements (Imsland et al., 2003). Among the different factors that may induce high mortality during the juvenile stage, stress might be one of the key issues. The few existing studies focusing on the stress response of this species relate to chronic stressors, pointing to an elevation of plasma cortisol values in fish

subjected to chronic handling (Aragão et al., 2008), high stocking densities (Costas et al., 2008; Salas-Leiton et al., 2010) or osmotic challenge (Arjona et al., 2007, 2009; Aragão et al., 2010).

Several brain monoamine neurotransmitters, including dopamine (DA) and serotonin (5-hydroxytryptamine, 5-HT), are believed to be involved in the control of behavioral and physiological stress responses in teleost fish (Øverli et al., 1999, 2001). For instance, handling and predator exposure can increase brain levels of the major serotonin metabolite, 5-hydroxyindole-3-acetic-acid (5-HIAA), and/or brain 5-HIAA/5-HT ratios (Winberg and Nilsson, 1993). In addition, several studies have suggested that brain 5-HT may have a stimulatory action on the HPI axis (Winberg and Lepage, 1998; Øverli et al., 1999; Höglund et al., 2000). Brain tryptophan (5-HT precursor) and tyrosine (DA precursor) availabilities are major determinants of the 5-HT and DA synthesis rates, and elevated dietary intake of these amino acids (AA) increases brain tryptophan and tyrosine levels, which in turn augment the rate of brain 5-HT and DA biosynthesis in mammals, respectively (Fernstrom, 1983, 1990; Boadle-Biber, 1993). Furthermore, this enhanced biosynthesis of brain 5-HT due to increased dietary intake of tryptophan has been also confirmed in fish (Johnston et al., 1990; Aldegunde et al., 1998, 2000; Winberg et al., 2001; Lepage et al., 2002), although it was not investigated for tyrosine yet. In fact, research on supplementing tyrosine and/or phenylalanine (tyrosine precursor) to experimental diets and its potential influence on fish physiology is currently limited (Li et al., 2009). Moreover, the major factor regulating tyrosine and tryptophan uptake into the mammalian brain is a transport carrier located at the blood-brain barrier, which also transports other large neutral AA (LNAA; i.e. phenylalanine, leucine, isoleucine and valine). Therefore, tyrosine and tryptophan will compete for the same carrier as the other LNAA (Boadle-Biber, 1993; Aldegunde et al., 1998, 2000; Fernstrom and Fernstrom, 2007). However, when feeding mammals a diet rich in carbohydrates and poor in protein, the ratio of plasma tryptophan/LNAA increases and gives tryptophan the advantage in the competition for access to the brain (Fernstrom et al., 1973; Fernstrom, 1983). In addition, increased plasma tryptophan levels and plasma tryptophan/LNAA ratios were observed in fish fed tryptophan supplemented diets, when the levels of the other LNAA in those diets were constant (Lepage et al., 2002).

Stress conditions that induced high plasma cortisol levels also modified fish AA metabolism in several teleost species (Milligan, 1997; Pinto et al., 2007; Aragão et al., 2008, 2010; Costas et al., 2008, 2011). Changes in plasma free AA levels may be indicative of AA requirements in fish (Wilson, 2002). In fact, it has been suggested that fish under stressful conditions present additional AA requirements, due to either increased energetic demands or for the synthesis of stress-related proteins and other compounds related with the stress response (Aragão et al., 2008, 2010; Costas et al., 2008). The role of specific AA and their metabolites on key metabolic pathways that are necessary for growth, immunity or resistance to environmental stressors and pathogens have been recently reviewed in mammals and fish (Li et al., 2007; Li et al., 2009). Emerging evidence shows that dietary tryptophan supplementation can inhibit aggression or reduce cannibalism and stress-induced anorexia and cortisol rise in several teleosts (Hseu et al., 2003; Lepage et al., 2003; Höglund et al., 2007). Furthermore, a positive effect of an arginine-enriched diet on the resistance of channel catfish (*Ictalurus punctatus*) to infection with *Edwardsiella ictaluri* has been demonstrated (Buentello and Gatlin, 2001).

The main objective of this study is to assess whether increased availability of dietary AA can mitigate the metabolic processes impinging on AA requirements of Senegalese sole when it is exposed to stressful situations. This gains particular importance in this species since it has been reported to be highly susceptible to opportunistic pathogens under stressful conditions (Costas et al., 2008). In addition, since changes in brain monoamine neurotransmitters and plasma lysozyme activity due to handling stress are well documented, it will be verified to what extent dietary treatment can influence these parameters.

5.2. Materials and Methods

5.2.1. Fish

Senegalese sole (*S. senegalensis*) juveniles (24.2 ± 0.4 g wet weight) originated from the natural spawning of wild broodstock and were reared according to standard larval and juvenile rearing protocols (Dinis et al., 1999).

Before the experiment, fish were acclimated for 15 days using a flow-through seawater system (temperature: 20 ± 1 ºC; salinity: 36 g L^{-1}; dissolved oxygen: above 90% saturation level), comprised by a flat-bottomed fiberglass tank.

This study was directed by trained scientists (following FELASA category C recommendations) and conducted according to the guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE.

5.2.2. Experimental design

At the beginning of the experiment, fish were fasted for 24 h, anaesthetised with 2-phenoxyethanol (500 ppm, Sigma-Aldrich, Germany) and individually measured, weighed, and marked with water ink (Acualux Titan, Spain). Fish were distributed in eight flat-bottomed fibreglass tanks (70 cm length x 30 cm width x 20 cm depth, volume 20 L, water flow rate 54 L h^{-1}), using a partial-recirculated seawater system (temperature: 20 ± 1 ºC; salinity: 36 g L^{-1}; dissolved oxygen: 90 % above saturation level). Each tank contained ten fish. Fish were kept at a photoperiod of 12 h light:12 h dark and the light intensity at water surface was 200 lux. Initial density was around 1.25 kg m^{-2}. Fish were fed daily by automatic feeders, over a 24-h period.

The experimental period lasted for 28 days and comprised four treatments randomly assigned to duplicate tanks: control fish fed the Control diet (CTL), control fish fed the HP diet (CTL HP), handling fish fed the Control diet (HDLG) and handling fish fed the HP diet (HDLG HP). Fish in HDLG groups were weekly chased with a net in order to capture all fish inside each tank and thereafter the net was held in the air during 3 min. Immediately after air exposure the fish were returned to the experimental tanks. Fish in CTL groups were reared without any disturbance, except from daily tank cleaning procedures. Two sampling periods were established: intermediate and final (after 14 and 28 days of experiment, respectively). Experimental sampling procedures were identical during both periods.

5.2.3. Sampling

Fish were fasted 24 h prior to sampling in order to avoid any influence of feeding on cortisol and glucose levels (Arends et al., 1999). Each tank was individually sampled; thus, five fish were quickly taken out from each tank at a time and anaesthetized with 2-phenoxyethanol (1000 ppm, Sigma-Aldrich). Blood was withdrawn from the caudal vein of each fish using heparinised syringes. After each sampling, blood was centrifuged at 1500 × *g* during 2 min at room temperature. The collected plasma was frozen in liquid nitrogen and stored at -80 ºC for further analysis of cortisol, glucose, lactate, lysozyme activity and free AA. After blood sampling, all fish from each tank were weighed and measured. Thereafter, fish were sacrificed by decapitation, and the brain was quickly removed, weighed, frozen in liquid nitrogen, and kept at -80 ºC for further analysis of monoamine neurotransmitters. Moreover, liver form each fish was dissected and weighed for hepatosomatic index calculation.

5.2.4. Experimental diets

Two diets were formulated, a low protein reference diet (Control) containing 39 % fish meal as the main protein source and a high protein diet (HP) where fish meal was increased up to 44.5 %. In addition, digestible protein from Control and HP diets was calculated according to Dias et al. (2010), being 46.9 and 51.2 %, respectively. Therefore, HP diet had a slightly increase in the levels of digestible indispensable AA, together with tyrosine and cysteine, when compared to the Control diet. In addition, L-tryptophan [0.5 % on a dry matter (DM) basis] was added to the HP diet. Wheat gluten and corn gluten were chosen as complementary protein sources due to their high protein content and potential high digestibility in fish, while soybean meal is known to have both high crude protein content (44 % DM) and a reasonably balanced AA profile (Gatlin et al., 2007). In the absence of specific data on vitamin, mineral and trace element requirements of Senegalese sole, requirement data for other species were applied (NRC, 1993; Kaushik, 1998). With the aim of increasing the feed acceptance, 5 % squid meal was added to both Control and HP diets. All dietary ingredients were supplied by Sorgal S.A. (Ovar, Portugal) and were finely ground, mixed and dry pelleted through a 3.2 mm die at 50 ºC (CPM, C-300 model, San Francisco, CA,

USA). The diets were dried at 37 ºC for 24-h and stored in a refrigerator (4 ± 1 ºC) until use. Formulation and proximate composition of the experimental diets are presented in Table 1 and the corresponding AA profile in Table 2. The AA patterns (g 100 g^{-1} AA) presented differences between experimental diets, with arginine, methionine, lysine and phenylalanine being significantly higher in the HP diet (Table 2).

Table 1. Ingredients and proximal composition of experimental diets.

	Experimental diets	
	Control	HP
Ingredients (%)		
Fish meal Herring	39.0	44.5
CPSP G	2.5	2.5
Squid meal	5.0	5.0
Soybean meal 48	15.0	15.2
Corn gluten	8.0	9.0
Wheat meal	22.3	15.5
Gelatin	1.0	1.0
Fish oil	6.9	6.5
¸-Tryptophan	-	0.5
Choline chloride	0.1	0.1
Mineral mix*	0.3	0.3
Vitamin mix†	0.3	0.3
Proximate composition		
Dry matter (% DM)	89.7	89.7
Crude protein (% DM)	52.1	56.3
Crude fat (% DM)	12.3	12.3
Ash (% DM)	8.2	9.3
Gross Energy (kJ g^{-1} DM)	21.9	22.0
NFE	24.4	23.3

*Minerals (g or mg kg^{-1} diet): Mn (manganese oxyde), 20 mg; I (potassium iodide), 1.5 mg; Cu (copper sulphate), 5 mg; Co (cobalt sulphate), 0.1 mg; Mg (magnesium sulphate), 500 mg; Zn (zinc oxide), 30 mg; Se (sodium selenite), 0.3 mg; Fe (iron sulphate), 60 mg; Ca (calcium carbonate), 2.15 g; dibasic calcium phosphate, 5 g; KCl (potassium chloride), 1 g; NaCl (sodium chloride), 0.4 g.

†Vitamins (mg or IU kg^{-1} diet): vitamin A (retinyl acetate), 8000 IU; vitamin D3 (DL-cholecalciferol), 1700 IU; vitamin K3 (menadione sodium bisulfite), 10 mg; vitamin B12 (cyanocobalamin), 0.02 mg; vitamin B1 (thiamine hydrochloride), 8 mg; vitamin B2 (riboflavin), 20 mg; vitamin B6 (pyridoxine hydrochloride), 10 mg; folic acid, 6 mg; biotin, 0.7 mg; inositol, 300 mg; nicotinic acid, 70 mg; pantothenic acid, 30 mg; vitamin E (Lutavit E50), 300 mg; vitamin C (Lutavit C35), 500 mg; betaine (Betafin S1), 500 mg.

HP, high protein; CPSP G, fish soluble protein concentrate (hydrolysed white fish meal); fish oil extracted from sardine; DM, dry matter; NFE, nitrogen free extracts = 100 – (CP + CL + CA).

Table 2. Amino acid composition (g 100 g^{-1} AA) of experimental diets.

	Experimental diets	
	Control	HP
IAA		
His	3.2 ± 0.1	3.5 ± 0.1
Arg	6.9 ± 0.0	7.1 ± 0.1 *
Ile	4.9 ± 0.2	5.2 ± 0.1
Leu	8.9 ± 0.1	8.9 ± 0.2
Val	4.4 ± 0.0	4.3 ± 0.3
Thr	4.7 ± 0.1	4.8 ± 0.1
Met	2.2 ± 0.0	2.6 ± 0.1 *
Lys	7.6 ± 0.0	8.4 ± 0.2 *
Phe	5.0 ± 0.0	5.7 ± 0.1 *
DAA		
Asx	9.8 ± 0.1	9.5 ± 0.9
Glx	16.6 ± 0.2	16.1 ± 0.3
Ser	4.9 ± 0.1	4.5 ± 0.3
Gly	5.1 ± 0.0	4.9 ± 0.5
Ala	6.1 ± 0.1	6.0 ± 0.2
Pro	5.4 ± 0.1	5.1 ± 0.2
Tyr	4.4 ± 0.1	4.2 ± 0.2

Trp and Cys were not analysed. Values are mean ± SEM (n = 3). Row means followed by asterisk indicate significant differences between experimental diets (Student's *t*-test; $P < 0.05$). HP, high protein.

5.2.5. Analytical procedures

Plasma cortisol levels were determined by radioimmunoassay (RIA) as described by Rotllant et al. (2006). Briefly, 50 µL of plasma samples were diluted in 950 µL phosphate buffer containing 1 g L^{-1} gelatin, pH 7.6, and denatured at 80 ºC for 1 h. Duplicate aliquots (100 µL) of diluted denatured plasma were then used in the assay. Due to technical constrains, only plasma cortisol levels from final sampling were analysed. Glucose and lactate analysis were performed on plasma samples using commercially available Spinreact kits (Glucose HK Ref. 1001200; Lactate Ref. 1001330), adapted for 96-well microplates.

Diets and plasma were analysed for total and free AA contents, respectively. Diet samples were hydrolyzed in 6M HCl at 106 °C over 24 h in nitrogen-flushed glass vials. Plasma samples were deproteinised by centrifugal ultrafiltration (10 kDa cut-off, 2500 × *g*, 20 min, 4 ºC). After deproteinisation,

samples were pre-column derivatized with phenylisothiocyanate (PITC; Pierce), using the PicoTag method (Waters, USA) according to Cohen et al. (1989). External standards were prepared along with the samples, using physiological amino acid standard solutions (acid/neutral and basics from Sigma) and a glutamine solution. Norleucine was used as an internal standard. Samples and standards were analysed by high performance liquid chromatography (HPLC) in a Waters Reversed-Phase Amino Acid Analysis System equipped with a PicoTag column (3.9 x 300 mm), using the conditions described by Cohen et al. (1989). Resulting peaks were analysed with the Breeze software (Waters). During acid hydrolysis of dietary samples, asparagine is converted to aspartate and glutamine to glutamate, so the reported values for these amino acids (Asx and Glx) represent the sum of the respective amine and acid. Moreover, tryptophan was not determined since it is destroyed by acid hydrolysis. Due to technical constraints, cysteine in the samples was not quantified and only plasma samples from fish held at HDLG HP treatment were analysed at intermediate sampling.

The brain contents of DA, 3,4-dihydroxyphenylacetic acid (DOPAC, a major DA metabolite), 5-HT and 5-HIAA were analysed by high performance liquid chromatography with electrochemical detection (HPLC-EC) as previously described by Gesto et al. (2006). Briefly, tissues were homogenized by ultrasonic disruption in 0.5 mL of mobile phase with the following composition: 85 mM NaH_2PO_4, 0.72 mM octanosulfonic acid, 18 % methanol, and adjusted to pH 3.0. Homogenates were centrifuged (16000 × *g* during 10 min at room temperature) and supernatants were diluted (1:10 supernatant:mobile phase) prior to analysis. A 20 µl aliquot of each sample was injected into the HPLC system consisting on a Jasco PU2080 pump equipped with a Rheodyne 7725i injection valve, and an ESA Coulochem detector (Bedford, USA). The detection system consisted on a double analytical M5011 ESA cell with electrode potentials set at +20 mV and +300 mV respectively. All separations were performed at room temperature at a flow rate of 0.8 mL min^{-1}. Acquisition and integration of chromatograms were performed by using the BiocromXP software (Micron analitica, Madrid, Spain).

Lysozyme activity was measured using a turbidimetric assay based on the method described by Ellis (1990) with some modifications (Wu et al., 2007). Briefly, a solution of *Micrococcus lysodeikticus* (0.5 mg mL^{-1} 0.05 M sodium phosphate buffer; pH 6.2) was prepared. In a microplate, 15 µL of plasma and

250 µL of the above suspension were added. The reaction was carried out at 25 ºC and the absorbance at 450 nm was measured after 0.5 and 4.5 min. Lyophilized hen egg white lysozyme (Sigma) was serially diluted in sodium phosphate buffer (0.05 M; pH 6.2) and used to develop a standard curve. The amount of lysozyme in the sample was calculated using the formula of the standard curve.

5.2.6. Data analysis

Data on proximate composition of diets and initial weight, final weight, total length and liver weight were used to calculate nitrogen free extracts (NFE), relative growth rate (RGR), weight gain, condition factor (K), voluntary feed intake (VFI), and hepatosomatic index (HSI) as follows:

NFE = 100 – (Crude protein + Crude lipids + Crude ash);
RGR (% day^{-1}) = (e^{g} – 1) x 100, with g = [ln(W_2) – ln(W_1)] $days^{-1}$, where W_1 and W_2 are the initial and final wet weights, respectively;
Weight gain (%) = [(Final wet weight) – (Initial wet weight) × (initial wet weight)$^{-1}$] × 100;
K (g cm^{-3}) = (wet weight) × (total length)$^{-3}$ × 100;
VFI = Crude feed intake/average body weight ((initial body weight + final body weight)/2)/days;
HSI (%) = (liver weight) × (final wet weight)$^{-1}$ × 100.

5.2.7. Statistics

All results are expressed as means ± standard error of the mean (SEM). Data were analysed for normality (Kolomogorov-Smirnov test) and homoscedasticity of variance (Levene's test) and, when necessary, these requisites were achieved by log-transformation. Data from experimental diets were analysed by *t*-test, while data from fish were analysed by two-way analysis of variance (ANOVA) with stress and dietary treatment as main factors. When significant differences were obtained from the ANOVA, Student's *t*-tests were carried out for paired-comparisons to analyse the effect of handling stress and dietary treatment. The level of significance used was $P \leq 0.05$ for all statistical tests.

5.3. Results

5.3.1. Diets and growth performance

Experimental diets were well accepted and survival was 100 % for all treatments at the end of the experimental period. Weekly handling or diet did not affect intermediate or final RGR, weight gain, K, VFI and HSI in Senegalese sole juveniles (Table 3).

Table 3. Relative growth rate (RGR), weight gain, condition factor (K) and hepatosomatic index (HSI) in *S. senegalensis* after 14 (Intermediate sampling) or 28 (Final sampling) days held at different treatments.

Parameters	Treatments			
	CTL	CTL HP	HDLG	HDLG HP
Intermediate sampling				
RGR (% day^{-1})	0.6 ± 0.2	0.9 ± 0.2	0.4 ± 0.2	0.7 ± 0.3
Weight gain (%)	9.4 ± 2.6	13.4 ± 2.8	6.5 ± 2.1	10.1 ± 3.9
K (g cm^{-3})	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
HSI (%)	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.2
Final sampling				
RGR (% day^{-1})	0.6 ± 0.2	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.2
Weight gain (%)	17.9 ± 5.3	18.2 ± 4.7	15.7 ± 3.2	19.5 ± 5.5
K (g cm^{-3})	1.2 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	1.1 ± 0.1
HSI (%)	0.9 ± 0.1	1.1 ± 0.2	1.1 ± 0.1	1.2 ± 0.2

Values are means ± SEM (n = 10). Differences were not significant among groups (two-way ANOVA; $P > 0.05$).

5.3.2. Stress indicators

Plasma cortisol levels were significantly higher in both HDLG groups when compared to CTL groups at the end of the experimental period with no significant dietary effects (Figure 1). Plasma glucose and lactate levels showed a similar pattern, being significantly lower in HDLG HP fish when compared to HDLG specimens at intermediate sampling. In addition, lactate levels were significantly

higher in fish held at HDLG treatment than unstressed specimens at the same sampling time. Similar to cortisol values, glucose and lactate levels were significantly higher in both HDLG groups when compared to CTL groups at final sampling with no significant effect of dietary treatment (Table 4).

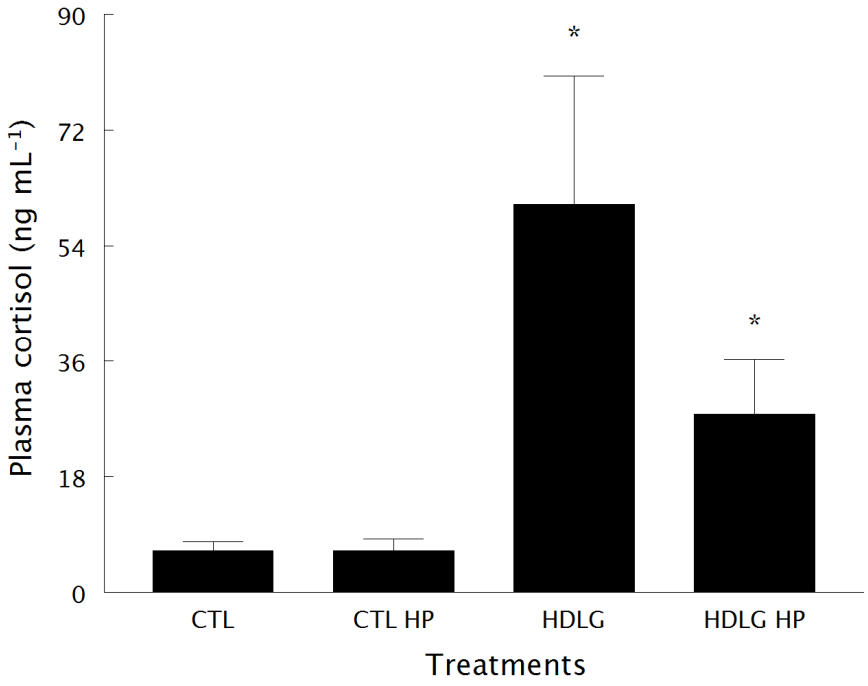


Figure 1. Plasma cortisol levels in *S. senegalensis* after 28 days held at different treatments. Values are means ± SEM (n = 10). * stands for significant differences attributed to handling stress (Student's *t*-test; $P < 0.05$).

5.3.3. Plasma free amino acid levels

Plasma free AA levels from fish held at HDLG HP treatment showed similar values for total AA, and the sum of either dispensable or indispensable AA at both intermediate (results not shown) and final sampling times (Table 4). Moreover, total plasma free AA levels were not significantly affected by either repeated handling stress or dietary treatments at the end of the experimental period (Table 4), and the same outcome was observed for the sum of dispensable or indispensable AA in the plasma (results not shown). However, some individual AA showed different patterns. Regarding individual dispensable AA, glutamate, cysteine and ornithine levels were significantly lower in fish held at HDLG treatment than in fish held at both CTL and CTL HP treatments. Moreover, HP diet

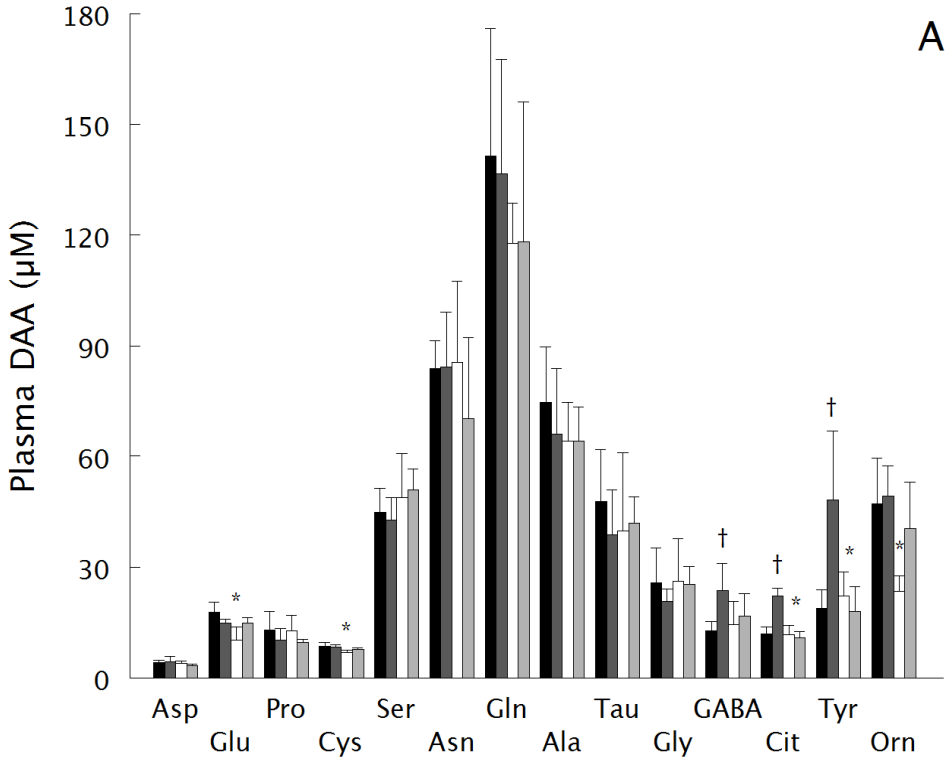
Table 4. Plasma glucose, lactate and total free amino acid (AA) levels in *S. senegalensis* after 14 (Intermediate sampling) or 28 (Final sampling) days held at different treatments.

Plasma parameters	Treatments				P-value		
	CTL	CTL HP	HDLG	HDLG HP	Diet	Stress	Diet x Stress
Intermediate sampling							
Glucose (mM)	2.0 ± 0.1	2.0 ± 0.2	2.7 ± 0.2	1.8 ± 0.1†	0.006	NS	0.006
Lactate (mM)	0.7 ± 0.1	0.4 ± 0.1	1.8 ± 0.2 *	0.5 ± 0.1 †	<0.001	<0.001	<0.001
Total free AA (µM)	ND	ND	ND	1404.4 ± 178.1	-	-	-
Final sampling							
Glucose (mM)	1.9 ± 0.1	1.7 ± 0.1	2.5 ± 0.3 *	2.3 ± 0.4 *	NS	0.034	NS
Lactate (mM)	0.5 ± 0.1	0.6 ± 0.2	1.6 ± 0.3 *	1.1 ± 0.2 *	NS	0.002	NS
Total free AA (µM)	1486.2 ± 170.9	1358.6 ± 73.3	1325.8 ± 201.1	1290.0 ± 85.8	NS	NS	NS

Values are means ± SEM (n = 10). *P* values from two-way ANOVA. * indicates significant differences attributed to repeated handling (Student's *t*-test; $P < 0.05$). † means significantly different from HDLG treatment (Student's *t*-test; $P < 0.05$). ND, not determined. NS, not significant.

significantly increased γ-aminobutyric acid (GABA), citruline and tyrosine levels in unstressed sole (Figure 2A). Regarding individual indispensable AA, arginine, lysine, isoleucine and leucine levels decreased significantly in fish held at HDLG treatment compared to fish from both CTL and CTL HP groups. Moreover, lysine, isoleucine and leucine levels were significantly higher in fish held at HDLG HP treatment than those from fish held at HDLG treatment. In addition, fish held at CTL HP treatment presented significantly higher phenylalanine levels than fish held at CTL treatment (Figure 2B).

Plasma tryptophan and LNAA levels, and the tyrosine/LNAA ratio were not significantly different among treatments. However, a significant decrease in the tryptophan/LNAA ratio was observed in fish held at HDLG HP treatment (Table 5).



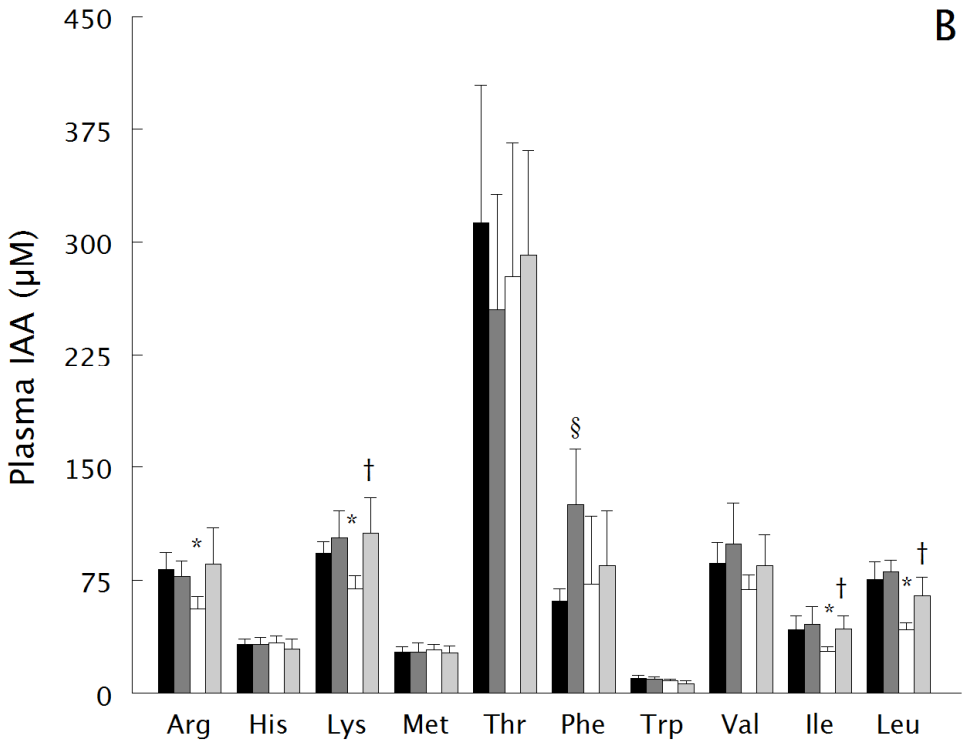


Figure 2. Dispensable (A) and indispensable (B) plasma free amino acid levels in *S. senegalensis* after 28 days held at different treatments: CTL (■), CTL HP (■), HDLG (□) or HDLG HP (□). Values are means ± SEM (n = 6). * and § mean significantly different from CTL treatment due to stress or HP diet, respectively (Student's *t*-test; $P < 0.05$). † stands for significant differences attributed to HP diet (Student's *t*-test; $P < 0.05$).

5.3.4. Brain monoamine levels

Regarding results from intermediary sampling, fish held at CTL HP treatment showed significantly higher brain DA, 5-HIAA and 5-HT levels than fish from CTL treatment, while 5-HIAA and 5-HT values increased significantly in fish held at HDLG and HDLG HP treatments when compared to fish from CTL treatment. Moreover, 5-HIAA/5-HT ratio was significantly higher in fish held at HDLG treatment than in fish held at CTL treatment (Table 6).

Results from final sampling did not show such drastic changes. Only fish held at HDLG HP treatment presented significantly higher brain DOPAC and DA levels than fish from CTL treatment (Table 6).

Table 5. Plasma tryptophan and large neutral amino acids (LNAA; tyrosine, phenylalanine, leucine, isoleucine and valine) levels, and tryptophan/LNAA ratio in *S. senegalensis* after 28 days (Final sampling) held at different treatments.

Plasma parameters	Treatments				P-value		
	CTL	CTL HP	HDLG	HDLG HP	Diet	Stress	Diet x Stress
Tryptophan (μM)	9.8 ± 1.0	9.1 ± 0.8	8.4 ± 0.4	6.4 ± 0.8	NS	NS	NS
LNAA (μM)	297.9 ± 15.1	357.0 ± 16.3	247.6 ± 12.1	301.6 ± 14.4	NS	NS	NS
Tyrosine/LNAA ratio	0.06 ± 0.01	0.11 ± 0.01	0.12 ± 0.01	0.11 ± 0.02	NS	NS	NS
Tryptophan/LNAA ratio	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.02 ± 0.00†	0.002	NS	NS

Values are means ± SEM (n = 6). NS not significant. † stands for significant differences attributed to supplemented diet (Student's *t*-test; $P < 0.05$).

Table 6. Brain concentrations (ng mg^{-1} of tissue) of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA), and DOPAC/DA and 5-HIAA/5-HT ratios in *S. senegalensis* after 14 (Intermediate sampling) or 28 (Final sampling) days held at different treatments.

Brain neurotransmitters	Treatments				*P*-value		
	CTL	CTL HP	HDLG	HDLG HP	Diet	Stress	Diet x Stress
Intermediate sampling							
DOPAC	29.8 ± 1.7	33.9 ± 3.1	33.7 ± 2.2	33.8 ± 3.6	NS	NS	NS
DA	235.6 ± 14.5	301.0 ± 15.8*	252.0 ± 19.6	298.7 ± 16.6	0.003	NS	NS
DOPAC/DA ratio	0.12 ± 0.01	0.12 ± 0.01	0.15 ± 0.01	0.12 ± 0.01	NS	NS	NS
5-HIAA	41.1 ± 6.3	70.6 ± 9.1*	69.4 ± 4.3*	76.0 ± 4.1*	0.011	0.017	NS
5-HT	676.3 ± 54.3	1146.6 ± 50.2*	956.2 ± 61.0*	1130.7 ± 71.0*	<0.001	0.037	0.020
5-HIAA/5-HT ratio	0.05 ± 0.01	0.07 ± 0.01	0.08 ± 0.01*	0.06 ± 0.01	NS	0.017	0.004
Final sampling							
DOPAC	23.2 ± 1.6	27.6 ± 1.4	26.0 ± 1.3	32.7 ± 2.8*	NS	0.004	NS
DA	193.0 ± 26.6	248.1 ± 33.9	199.5 ± 52.6	262.5 ± 28.8*	0.033	NS	NS
DOPAC/DA ratio	0.12 ± 0.01	0.12 ± 0.01	0.14 ± 0.01	0.13 ± 0.01	NS	NS	NS
5-HIAA	50.0 ± 4.8	60.6 ± 7.6	58.5 ± 3.2	59.7 ± 4.7	NS	NS	NS
5-HT	841.3 ± 27.2	924.3 ± 49.4	908.7 ± 43.1	945.9 ± 45.0	NS	NS	NS
5-HIAA/5-HT ratio	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	NS	NS	NS

Values are means ± SEM (n = 10). Two-way ANOVA; $P < 0.05$. NS not significant.

5.3.5. Lysozyme activity

Regarding plasma lysozyme activity, fish from CTL HP, HDLG and HDLG HP treatments presented significantly higher values than fish from CTL treatment at intermediate sampling, while those levels also increased in specimens from CTL HP and HDLG HP treatments when compared to values in fish from CTL and HDLG treatments, respectively (Figure 3A). Moreover, both HDLG groups showed significantly higher lysozyme activity than unstressed specimens at final sampling, with no effect of dietary treatment (Figure 3B).

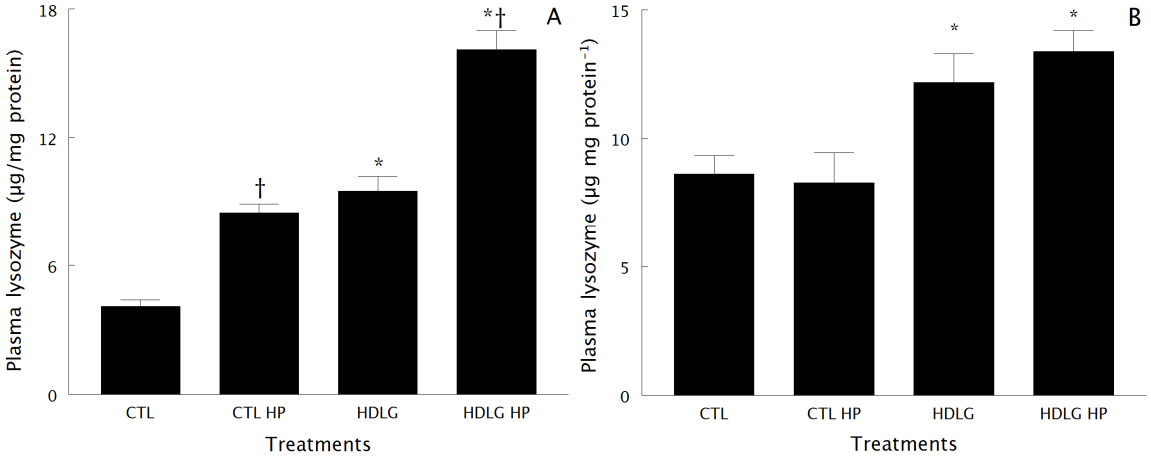


Figure 3. Plasma lysozyme activity in *S. senegalensis* after 14 (A) and 28 (B) days held at different treatments. Values are means ± SEM (n = 10). * stands for significant differences attributed to handling stress, and † for significant differences attributed to HP diet (Student's *t*-test; $P < 0.05$).

5.4. Discussion

The major conclusions drawn from this study were threefold. First, feeding sole a diet with a slightly increase in all indispensable AA, together with tyrosine and cysteine, with respect to a reference diet decreases post-stress plasma glucose and lactate levels after 14 days of treatment, whereas no dietary effect was observed after 28 days. Second, dietary treatment and handling stress

activated the serotonergic system at intermediate sampling, while these effects were not observed at final sampling. Third, dietary treatment was also effective in stimulating DA synthesis and release, thus supporting the hypothesis that DA biosynthesis can be increased through dietary phenylalanine (tyrosine) supplementation in fish.

The stress response in fish from intermediate sampling will be discussed based on brain monoamines and plasma glucose and lactate values since plasma cortisol was not analysed at this particular sampling time. Secondary stress responses include, among others, measurable changes in blood glucose and lactate (Barton, 2002). Therefore, measurement of plasma glucose and lactate levels provides an effective method to monitor the secondary stress response in fish. Furthermore, plasma glucose and lactate levels usually increase following stressful situations such as hypoxia, handling, crowding or repeated weekly crowding (Arends et al., 1999; Gesto et al., 2008; Basrur et al., 2010; Conde-Sieira et al., 2010). Moreover, brain 5-HIAA/5-HT ratios have been found to correlate positively with plasma cortisol levels (Winberg and Lepage, 1998), and increased 5-HIAA/5-HT ratios have been observed in fish after social subordination and stress challenge (Winberg and Nilsson, 1993; Höglund et al., 2000; Gesto et al., 2008). In the current study, increased plasma lactate levels and brain 5-HIAA/5-HT ratio from fish held at HDLG treatment appear to be indicative of a stressful status in these specimens. The gradual decline of the magnitude of the physiological response with repeated exposures to stressors/stimuli is well known (Koolhaas et al., 2011). In fish, plasma cortisol levels rise at the beginning of the stressful condition and decrease to initial values in a few days (Tort et al., 1996), showing adaptation to the new situation. Since plasma cortisol levels from the current study were significantly higher at final sampling, it is tempting to speculate that HDLG specimens could have had similar cortisol values at intermediate sampling. Interestingly, plasma glucose and lactate levels as well as brain 5-HIAA/5-HT ratio were significantly lower in specimens held at HDLG HP treatment than in fish from HDLG group at intermediary sampling, while plasma glucose, lactate and cortisol levels increased in both HDLG and HDLG HP treatments at the end of the experimental period. Therefore, dietary treatment appears to influence the Senegalese sole secondary stress response by minimizing the subsequent mobilization of energy substrates, at least after 14 days of feeding and under this particular stressor. However, HP

diet seems to loose these effects after 28 days of feeding. Similarly, rainbow trout (*Oncorhynchus mykiss*) fed diets supplemented with tryptophan (4 and 8 times the tryptophan content of the control diets) for 7 days, and thereafter subjected to an acute stress, showed significantly lower cortisol levels than stressed fish fed the control diets (Lepage et al., 2002, 2003), while a similar increase in plasma cortisol levels was observed among treatments after 28 days of feeding (Lepage et al., 2003).

Growth and survival were not affected by repeated handling stress in agreement with that observed in previous studies with this species under chronic stressful conditions (Aragão et al., 2008; Costas et al., 2008; Salas-Leiton et al., 2010). In addition, wedge sole (*Dicologoglossa cuneata*) and gilthead seabream (*Sparus aurata*) chronically submitted to high stocking densities did not show significant differences in feed intake and growth performance when compared to control fish (Montero et al., 1999; Herrera et al., 2009). Although it is widely accepted that stressful conditions decrease growth in fish (Pottinger, 2008), contradictory results in different studies are probably related to duration, intensity and type of stressors employed. In addition, species-specific strategies to cope with stress can be also one of the reasons. In fact, Salas-Leiton et al. (2010) observed that Senegalese sole subjected to high stocking density decreased growth rate after 40 days of experiment, developing thereafter a compensatory growth strategy resulting in no differences in growth after 60 days with respect to specimens reared at low density.

To our knowledge, this is the first study in flatfish showing that brain monoamine neurotransmitters are significantly affected by both repeated handling stress and dietary treatments in which the most significant changes were observed after 14 days of treatment. Modulation of the brain serotonergic system by stress appears to be in accordance with that previously reported in rainbow trout subjected to acute stress and social subordination (Winberg and Nilsson, 1993; Lepage et al., 2002, 2003; Gesto et al., 2008). Moreover, increased 5-HIAA and 5-HT levels observed in fish held at HDLG HP treatment are probably due to both HP diet and handling stress. Rainbow trout fed diets supplemented with tryptophan also showed higher 5-HIAA levels than fish fed control diets in hypothalamus and brain stem after stress challenge (Winberg et al., 2001; Lepage et al., 2002). Unfortunately, since there are no data available regarding plasma

free AA levels at intermediary sampling, comparison between plasma tryptophan and brain 5-HT as well as plasma tyrosine and brain DA levels is not possible at this time. Still, HP diet effectively stimulated the synthesis and release of both DA and 5-HT levels in the brain of Senegalese sole held at CTL HP treatment when compared to specimens from CTL treatment, and the same outcome was observed for brain 5-HIAA and 5-HT levels in fish from HDLG HP treatment. The first and rate-limiting step in the biosynthesis of 5-HT is the hydroxylation of tryptophan to 5-hydroxytryptophan, a reaction catalysed by the enzyme tryptophan hydroxylase, which does not appear to be subjected to any inhibition by 5-HT. Consequently, an elevation of brain tryptophan levels results in an increase in the rate of 5-HT synthesis both in mammals (Boadle-Biber, 1993) and in fish (Aldegunde et al., 1998, 2000; Winberg et al., 2001). Similarly, the first and rate-limiting step in catecholamine synthesis is the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine by tyrosine hydroxylase, being subsequently decarboxylated to form DA in catecholaminergic neurons (Winberg and Nilsson, 1993). Therefore, it is likely that the observed increase in brain monoamine levels in this study are due to increased availability of tryptophan and tyrosine in those neurons. Furthermore, increased phenylalanine levels from the HP diet resulted in a higher synthesis and release of brain DA in fish from CTL HP treatment after both 14 and 28 days of treatment, as well as increased plasma phenylalanine and tyrosine levels at final sampling. In addition, while dietary tryptophan did not have any apparent effect on brain 5-HT biosynthesis at the end of the experimental period, phenylalanine still increases brain DA synthesis and release in fish from both CTL HP and HDLG HP treatments. Although plasma phenylalanine, tyrosine and tryptophan levels in fish from HDLG group were similar to those from fish held at HDLG HP treatment, the increased brain DOPAC and DA levels in HDLG HP specimens could be related to differences in tryptophan/LNAA and tyrosine/LNAA ratios. In fact, tryptophan/LNAA ratio was significantly lower in fish held at HDLG HP treatment whereas tyrosine/LNAA ratio did not show significant diferences. Therefore, tryptophan could give some advantage to tyrosine and other LNAA in the competition for access to the brain. Therefore, those data point to differential changes in brain indoleamines and catecholamines in relation with the stress response in sole, which appear to be modulated by increased dietary AA availability.

Plasma total AA levels from the present study were not different among treatments at the end of the experimental period, being in agreement with that previously observed by Aragão et al. (2008) in Senegalese sole subjected to the same stressor for 60 days. However, since increased post-stress plasma cortisol, glucose and lactate levels were observed in fish from this study, changes on AA metabolism are expected (see Introduction). In fact, a significant decrease in plasma arginine, lysine, leucine, isoleucine, glutamate, cysteine and ornithine were observed in fish held at HDLG treatment with respect to unstressed specimens. This suggests that these particular AA are involved in metabolic pathways with an important role during the stress response. For instance, arginine serves as the precursor for the synthesis of nitric oxide (NO) in terrestrial animals (Wu and Morris, 1998), and physiological levels of NO stimulate glucose uptake and oxidation in liver and muscle in mammals (Jobgen et al., 2006). Lysine is one of the substrates for the synthesis of L-carnitine, which in fish is required for the transport of long-chain fatty acids to mitochondria, were they are oxidised (Harpaz, 2005). Leucine and isoleucine can be easily used as energetic substrates in skeletal muscle, which is the major site of branched-chain AA oxidation (Van Waarde, 1988). Glutamate and cysteine are used for the synthesis of glutathione (Wu et al., 2004), which have been observed to effectively minimize oxidative stress following reoxygenation in goldfish (*Carassius auratus*) previously submitted to 8 hours of anoxia (Lushchak et al., 2001). Interestingly, despite the fact that plasma stress-related parameters from fish held at HDLG HP treatment remained significantly higher than those from unstressed specimens, HDLG HP specimens did not show the altered plasma indispensable AA profile observed in fish from HDLG treatment. Therefore, dietary treatment appears to be also effective in minimizing cortisol-induced changes on AA metabolism.

Interestingly, plasma tryptophan levels from the present study were not significantly affected either by dietary treatment or repeated handling stress. Increased plasma tryptophan levels would be expected at least in fish held at CTL HP treatment. However, this result is probably linked to the low level of tryptophan supplementation employed. Similarly, rainbow trout fed a diet supplemented with either 2 or 4 times the tryptophan level of the control diet did not show significant differences in plasma tryptophan levels, which only increased with respect to control fish when the level of supplementation reached was 8 times the tryptophan content of the control diet (Lepage et al., 2002).

Moreover, Senegalese sole subjected to various chronic stressors showed a significant decrease in plasma tryptophan levels (Pinto et al., 2007; Aragão et al., 2008; Costas et al., 2008). In the current study, unaltered plasma tryptophan levels from fish held at HDLG treatment with respect to unstressed fish could be related to the duration of the stress imposed. In fact, a tendency to decrease in plasma tryptophan levels was observed in stressed specimens at final sampling (28 days of treatment), in line with the results from the previous (longer duration) studies with chronically stressed Senegalese sole (Pinto et al., 2007; Aragão et al., 2008; Costas et al., 2008).

Plasma lysozyme activity was differentially affected by experimental protocol used in the current study. While HP diet significantly increased plasma lysozyme activity only at intermediate sampling, handling stress induced a similar response at both sampling times. Therefore, it appears that this particular stressor enhances plasma lysozyme activity regardless of time employed, while dietary treatment was only effective after 14 days of feeding. This non-specific humoral immune response appears to depend on the species and type and duration of the stress imposed. For instance, changes on lysozyme activity in response to a stressor present contradictory results in different studies. In some cases, lysozyme activity decreases or no consistent effects are observed (Olsen et al., 1993; Cnaani and McLean, 2009). However, in other studies this parameter significantly increased in stressed specimens (Demers and Bayne, 1997; Rotllant et al., 1997; Caipang et al., 2009). Moreover, information regarding influence of AA on innate humoral immunity is scarce. However, Costas et al. (unpublished results) have recently observed that Senegalese sole fed a diet with significantly higher arginine, isoleucine, leucine, threonine, valine and methionine levels than the control diet increased plasma lysozyme, complement and peroxidase activities after 12 weeks of feeding. More innate immune parameters must be assessed, including bacterial challenge tests, in order to evaluate the Senegalese sole immune status after stress challenge and dietary treatment.

In conclusion, Senegalese sole appears to show no adaptation or predictability to repeated weekly handling for 14 and 28 days, as indicated by energy metabolites and 5-HIAA/5-HT ratio. Moreover, HP diet appears to decrease some of the negative effects attributed to cortisol release after HPI axis activation. Following 28 days of treatment, there was no effect of the increased

dietary intake of AA on either plasma cortisol, glucose or lactate levels. In addition, dietary treatment was also effective in activating dopaminergic and serotonergic systems at intermediate sampling, whereas only DA synthesis and release was observed at final sampling. In addition, the decrease in plasma individual indispensable AA due to repeated handling stress was minimized by HP diet after 28 days. Development of diets supplemented with some key AA, for instance arginine, phenylalanine and tryptophan, would be instrumental for decreasing mobilization of energy substrates during periods of fish handling (i.e. grading) in the aquaculture industry.

5.5. Acknowledgements

This study was supported by project STRESSAA – POCTI/CVT/49324/2002 (FCT, Portugal and FEDER). Benjamín Costas and Cláudia Aragão were supported by Fundação para a Ciência e a Tecnologia, Portugal (SFRH/BD/38697/2007 and SFRH/BPD/37197/2007, respectively).

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Chapter 6

Different inflammatory responses of Senegalese sole (*Solea senegalensis* Kaup, 1858) following challenge with two *Photobacterium damselae* subsp. *piscicida* strains from different geographical origins

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Costas, B., Rêgo, P.C.N.P., Marques, J.F., Castro-Cunha, M., Afonso, A., 2011. Different inflammatory responses of Senegalese sole (*Solea senegalensis* Kaup, 1858) following challenge with two *Photobacterium damselae* subsp. *piscicida* strains from different geographical origins. Submitted.

Different inflammatory responses of Senegalese sole (*Solea senegalensis* Kaup, 1858) following challenge with two *Photobacterium damselae* subsp. *piscicida* strains from different geographical origins

Abstract

The present study aimed to investigate leucocyte responses to inflammation as well as some innate immune parameters of Senegalese sole (*Solea senegalensis*) following challenge with two strains of *Photobacterium damselae* subsp. *piscicida* belonging to the European and Japanese clones described for this bacterium. Pathogenicity assays were performed in order to assess the virulence of each *Photobacterium damselae* subsp. *piscicida* strain for sole. Afterwards, fish were intraperitoneally injected with PBS (control) or two concentrations (2×10^2 and 2×10^6 CFU mL^{-1}) of each bacterial strain and sampled after 6 and 24 h. Results showed that the European isolate induces a higher degree of response than the Japanese strain. Most significant changes were observed after 24 h. While blood neutrophilia and monocytosis correlated well with the increase in neutrophil and macrophage numbers in the peritoneal cavity, fish infected with the European isolate presented higher peritoneal cell numbers than fish challenged with the Japanese strain. In addition, alternative complement pathway activity and respiratory burst of head-kidney leucocytes increased significantly in fish infected with the European isolate. This study shows for the first time leucocyte responses to inflammation in both peripheral blood and peritoneal cavity of flatfish, presenting Senegalese sole, an inflammatory response similar to that observed for other teleosts. Furthermore, the increase in blood leucocytes was congruent with the augmented peritoneal leucocyte numbers, thus corroborating the hypothesis of cell migration to the inflammation focus in fish. The enhanced innate immune machinery displayed by Senegalese sole challenged with the European isolate is probably due to the higher degree of virulence presented by this *Photobacterium damselae* subsp. *piscicida* strain.

Keywords: alternative complement pathway; European clone lineage; macrophage; neutrophil; reactive oxygen species; Senegalese sole.

6.1. Introduction

Senegalese sole (*Solea senegalensis*) is a high-value flatfish with a great potential for future farming. It is commonly raised in semi-extensive earthen ponds and land-based facilities along the southern coast of the Iberian Peninsula (Dinis et al. 1999). However, some factors are not fully controlled and present some constrains for accomplishing a commercial-scale production of this species. Disease outbreaks appear to be the most significant limitation on the development of the industry, being from bacteria the infections more frequently diagnosed (Imsland et al. 2003).

Fish have many innate and acquired humoral and cell-mediated mechanisms to resist bacterial diseases (Ellis 1999). However, the innate immune system is of primary importance in combating infections due to the evolutionary status and poikilothermic nature of fish (Magnadotir 2006). When superficial barriers are breached by microorganisms, an inflammatory response is induced with the ultimate influx of phagocytes which have potent bactericidal properties (Ellis 2001). The involvement of neutrophils and macrophages in phagocytosis, killing and degradation of invading microoganisms, as well as different patterns of localization and mobilization into the infected areas are well documented in mammals (Lehrer et al. 1988, Haslett 1989, Silva et al. 1989, van Furth 1992, Densen et 1995) and fish (Steinhagen and Jendrysek 1994; Afonso et al. 1998a; do Vale et al. 2002). In fact, both macrophages and neutrophils can produce bactericidal reactive oxygen species during the respiratory burst on contact with bacteria (Lamas and Ellis 1994; Campos-Pérez et al. 1997). Furthermore, there are a number of soluble (complement, transferrins, anti-proteases, lysozyme, lectins, C-reactive protein) defenses that also increase in response to invading bacteria, being the complement system considered the most important defense factor (Ellis 2001). The functions of complement are numerous but it is most well known for its capacity to kill pathogens and participation in inflammatory reactions by attracting phagocytic cells to the site of injury (Holland and Lambris 2002).

The halophilic bacterium *Photobacterium damselae* subsp. *piscicida* (*Phdp*) is the aetiological agent of pasteurellosis or photobacteriosis in marine fish (Romalde 2002). It has become a serious problem for wild and cultured marine

fish due to its capacity for inducing massive mortality, ubiquitous geographical distribution, widespread antibiotic resistance and lack of efficient vaccines (Barnes et al. 2005), affecting more than 20 species worldwide, including Senegalese sole (Magariños et al. 1996, 2003; Zorrilla et al. 1999; Romalde 2002). *Phdp* represents a highly homogeneous group regarding lipopolysaccharides and protein profiles, and also from a serological standpoint (Romalde 2002; Magariños et al. 2003). However, several studies based on genetic techniques showed the existence of two clonal lineages (European and Japanese isolates) within *Phdp*, being highly correlated with the geographic origin of the strains (Magariños et al. 2000; Thyssen et al. 2000; Kvitt et al. 2002). In addition, both European and Japanese *Phdp* isolates do not possess strict host specificity, showing different degrees of virulence depending on the strain (Magariños et al. 1992; do Vale et al. 2003). However, quantitative data regarding host immune responses against *Phdp* isolates from both clonal lineages is not available. Although recent studies have contributed to give some insights regarding the methods of invasion and survival of this particular pathogen inside the host (do vale et al. 2005, 2007; Silva et al. 2010), little is known regarding Senegalese sole ability to cope with *Phdp*. Therefore, the aim of the present study was to contribute to this endeavour by investigating the response of both peripheral blood and peritoneal leucocytes to inflammation as well as some innate immune parameters following challenge with either European or Japanese *Phdp* isolates.

6.2. Materials and Methods

6.2.1. Experimental fish

Healthy Senegalese sole were obtained from a commercial fish farm, located in North-West Portugal, with no history of pasteurellosis. Fish weighing 96.5 ± 8.4 g were used for analysis of respiratory burst activity because they allowed the collection of a larger number of head-kidney cells, while fish weighing 36.5 ± 10.9 g were used for all the other experiments. Fish were maintained in recirculating aerated seawater, at 21–22 ºC. Water quality was maintained with mechanical and biological filtration and fish were fed to apparent satiety with commercial pellets. Ammonia and nitrite levels in the water were

measured twice a week using commercial kits and never exceeded 0.025 and 0.3 mg l^{-1}, respectively. Only healthy fish, as indicated by their activity and exterior appearance, were used in the experiments.

6.2.2. Bacterial strains

Phdp strains used in the present study were kindly provided by Dr. Ana do Vale (Institute for Molecular and Cell Biology, University of Porto, Portugal). Strain PC566.1 was isolated from Senegalese sole (Spain) by Professor Alicia E. Toranzo (Departamento de Microbiología y Parasitología, Facultad de Biología, University of Santiago de Compostela, Spain) and served as representative for the European lineage, while strain PP3 was isolated from yellowtail (*Seriola quinqueradiata*; Japan) by Dr. Andrew C. Barnes (Marine Laboratory, Aberdeen, UK) and served as representative for the Japanese lineage.

Bacteria were routinely cultured at 22 ºC in tryptic soy broth (TSB) or tryptic soy agar (TSA) (both from Difco Laboratories, Detroit, MI, USA) supplemented with NaCl to a final concentration of 1 % (w/v) (TSB-1 and TSA-1, respectively) and were stored frozen at -70 ºC in TSB-1 supplemented with 15% (v/v) glycerol.

To prepare the inocula for injection into the fish peritoneal cavities, stocked bacteria were cultured for 48 h at 22 ºC on TSA-1 and then inoculated into TSB-1, and cultured overnight at the same temperature, with continuous shaking (100 rpm). Exponentially growing bacteria were collected by centrifugation at 3500 × *g* for 30 min, resuspended in sterile phosphate buffered saline (PBS) and adjusted to 10-fold dilutions ranging from 2×10^2 to 2×10^6 CFU ml^{-1}. Plating serial dilutions of the suspensions onto TSA-1 plates and counting the number of CFU following incubation at 22 ºC confirmed bacterial concentrations of the inocula.

6.2.3. Virulence assays

Infectivity trials were conducted to determine the pathogenicity and host specificity of PC566.1 and PP3 strains, representative for the two clonal lineages described for *Phdp*. In parallel, the virulence of each strain was assessed and

defined according to previous studies (Magariños et al. 1992). Therefore, groups of 12 fish were randomly distributed in 8 L tanks with aerated seawater, and left to acclimate for 48 h prior to bacterial challenge. Afterwards, fish were anesthetised by immersion in ethylene glycol monophenyl ether (0.2 mL L^{-1}; Merck, Darmstadt, Germany) and inoculated by intraperitoneal injection (i.p.) with 100 µL of 10-fold dilutions ranging from 2×10^2 to 2×10^6 CFU mL^{-1}. Another group with fish i.p. injected with 100 µL sterile PBS was included as control for each experiment. Both trials were maintained in closed systems with filtered seawater for up to 21 days, at 21-22 ºC. Fish were fed daily at a ration of 1 % of total fish biomass. In addition, ammonia and nitrite levels were assessed daily and kept below 0.025 and 0.3 mg L^{-1}, respectively. The lethal dose (LD_{50}) was calculated for each strain by the method of Reed and Müench (1956). Dead fish were removed from each tank daily and analyzed to reisolate the inoculated strain.

6.2.4. Peritoneal leucocytes response to inflammation

Five groups of 12 fish were i.p. infected to assess eventual differences in innate immune responses of sole against PC566.1 and PP3. Fish were maintained under the same culture conditions and challenged as described above. Two bacterial concentrations were used (2×10^2 and 2×10^6 CFU mL^{-1}) for each strain. In addition, one group was i.p. injected with sterile PBS and served as control. After 6 and 24 hours, 6 fish per group were sacrificed by immersion in 1 mL L^{-1} of ethylene glycol monophenyl ether (Merck), weighed and sampled, as follows.

6.2.5. Blood and peritoneal leucocytes collection

Approximately 0.5 mL of peripheral blood per fish was collected in non-heparinised syringes following venipuncture of the caudal vein at the indicated times. While one drop was used for blood smears preparations, the remaining blood was allowed to clot for 3 h at room temperature, and thereafter centrifuged at 5000 × *g* for 5 min to obtain the serum. Individual samples were stored at -70 ºC until required.

The peritoneal cells were collected by a procedure described elsewhere (Afonso et al. 1997). Briefly, after sacrificed by an overexposure to anesthetic (see

above) and peripheral blood removed, the abdominal side of the fish was then cleaned with ethanol. Sterile PBS with osmotic strength adjusted to 310-340 mOsm L^{-1} and supplemented with 20 U heparin mL^{-1} was injected into the peritoneal cavity (1 mL per fish). Afterwards, PBS containing the peritoneal cells was collected and placed on ice until processed, as follows.

6.2.6. Light microscopy

Blood smears were prepared from fresh blood, air dried, and stained with Wright's stain (Haemacolor, Merck) after fixation for 1 min with formol-ethanol (10 % of 37 % formaldehyde in absolute ethanol). Detection of peroxidase activity to label neutrophils was done by a procedure described elsewhere (Afonso et al. 1998a). The slides were examined under oil immersion (1000×) and at least 200 leucocytes were counted and classified as thrombocytes, lymphocytes, monocytes and neutrophils. The relative percent of each leucocyte type was calculated.

Total cell counts from peritoneal exudates were performed with a hemocytometer. Cytospin preparations were made with a THARMAC Cellspin apparatus and stained as indicated above for blood smears. The lymphocytes, macrophages and neutrophils in the peritoneal exudates were differentially counted and the percentage of those cell types established after counting a minimum of 300 cells per slide.

6.2.7. *In vitro* respiratory burst activity

6.2.7.1. Head-kidney leucocytes isolation

Head-kidney cells were collected, isolated and maintained essentially as described by Secombes (1990). Briefly, the head-kidney was removed under aseptic conditions, pushed through a 100 µm nylon mesh and suspended in Leibovitz L-15 medium (L-15: Gibco, Scotland, UK) supplemented with 2% foetal bovine serum (FBS; Gibco), penicillin (100 IU mL^{-1}; P, Gibco), streptomycin (100 µg mL^{-1}; S, Gibco) and heparin (20 units mL^{-1}; Sigma). The suspensions were then loaded onto a 34:51 % Percoll (Sigma) density gradient and centrifuged at 400 × *g* and 4 ºC for 40 min. The band of cells laying at the interface of the Percoll gradient was collected and washed three times at 400 × *g* and 4 ºC for 5 min in

L-15, 0.1 % FBS, P/S and heparin. The viable cell concentration was determined by the Trypan blue exclusion test. Cells were counted in a haemocytometer and adjusted to 2×10^7 cells mL^{-1} in L-15, 0.1 % FBS, P/S and heparin. Afterwards, cells were plated in 96 well plates at 100 µL per well. After overnight incubation at 18 ºC, the non-adherent cells were washed off and the monolayers were maintained with L-15 supplemented with 5% FBS, until the respiratory burst was conducted after 24 h of incubation at 18 ºC.

6.2.7.2. Analysis of respiratory burst activity

Respiratory burst activity of head-kidney leucocytes was based on the reduction of ferricytochrome C method for the detection of O_2^- (Secombes, 1990). Briefly, the leucocytes monolayers were washed twice with phenol red-free Hank's balanced salt solution (HBSS) and 100 µL suspension of ferricytochrome C solution (2 mg ferricytochrome C mL^{-1} diluted in phenol red-free HBSS) containing 2×10^2 or 2×10^6 CFU *Phdp* mL^{-1} were added. Ferricytochrome C solution containing 10 µg mL^{-1} phorbol myristate acetate (PMA, Sigma) was added as a soluble stimulant of the respiratory burst and served as a positive control. Ferricytochrome C with PMA and 0.725 mg mL^{-1} superoxide dismutase (SOD, Sigma) was used to confirm the specificity of the reaction. For each parameter 3 or more wells of leucocytes per fish and bacterial strain were assayed. Plates were read 30 min after addition of reagents to the leucocytes and readings were then taken on a Power-Wave™ microplate spectrophotometer (BioTek) at 550 nm. Optical densities were converted to nmol of O_2^- produced by multiplying by 15.87 as described by Pick (1986), and the mean ± SD of each treatment was calculated.

6.2.8. Innate humoral parameters

Alternative complement pathway (ACP) activity was estimated as described by Sunyer and Tort (1995). The following buffers were used: GVB (Isotonic veronal buffered saline), pH 7.3, containing 0.1% gelatin; EDTA-GVB, as previous one but containing 20 mM EDTA; and Mg-EGTA-GVB, which is GVB with 10 mM Mg^{+2} and 10 mM EGTA. Rabbit red blood cells (RaRBC; Probiologica Lda, Portugal) were used for ACP determination. RaRBC were washed four times in GVB and resuspended in GVB to a concentration of 2.5×10^8 cells mL^{-1}. Twenty-five µL of RaRBC suspension were then added to 100 µL of serially diluted serum in Mg-

EGTA-GVB buffer. Samples were incubated at room temperature for 100 min with regular shaking. The reaction was stopped by adding 100 µL of cold EDTA-GVB. Samples were then centrifuged and the extent of hemolysis was estimated by measuring the optical density of the supernatant at 414 nm. The ACH50 units were defined as the concentration of serum giving 50% haemolysis of RaRBC. All analysis were conducted by triplicates.

Total peroxidase activity in serum was measured following the procedure described by Quade and Roth (1997). Briefly, 15 µL of serum were diluted with 135 µL of HBSS without Ca^{+2} and Mg^{+2} in flat-bottomed 96-well plates. Then, 50 µL of 20 mM 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB; Sigma) and 50 µL of 5 mM H_2O_2 were added. The colour-change reaction was stopped after 2 min by adding 50 µL of 2M sulphuric acid and the optical density was read at 450 nm in a Powerwave™ microplate spectrophotometer. The wells without serum were used as blanks. The peroxidase activity (units mL^{-1} serum) was determined defining one unit of peroxidase as that which produces an absorbance change of 1 OD.

Lysozyme activity was measured using a turbidimetric assay based on the method described by Ellis (1990) with some modifications (Wu et al. 2007). Briefly, a solution of *Micrococcus lysodeikticus* (0.5 mg mL^{-1} 0.05 M sodium phosphate buffer; pH 6.2) was prepared. To a microplate, 15 µL of serum and 250 µl of the above suspension were added to give a final volume of 265 µL. The reaction was carried out at 25 ºC and the absorbance (450 nm) is measured after 0.5 and 4.5 min. Lyophilized hen egg white lysozyme (Sigma) was serially diluted in sodium phosphate buffer (0.05 M; pH 6.2) and used to develop a standard curve. The amount of lysozyme in the sample was calculated using the formula of the standard curve.

6.2.9. Statistical analyses

All results are expressed as means ± standard deviation (SD). Data were analysed for normality (Kolomogorov-Smirnov test) and homoscedasticity of variance (Levene's test) and, when necessary, they were log-transformed before being treated statistically. Data were analysed by one-way ANOVA followed by Tukey's post hoc tests to identify significantly different groups. When statistical

assumptions of the ANOVA were not accomplished, data were analysed by *t*-test. All statistical analyses were performed using the computer package SPSS 15.0 for WINDOWS. The level of significance used was $P < 0.05$ for all statistical tests. All results expressed as a percentage were previously arcsine transformed (Zar 1999).

6.3. Results

6.3.1. Pathogenicity assays

Fish i.p. injected with strain PC566.1 presented higher mortality (LD_{50} of 5×10^3) than fish challenged with strain PP3 (LD_{50} of 1.2×10^4). In addition, while all fish i.p. injected with the highest concentration of the strain PC566.1 died, fish i.p. injected with 2×10^6 CFU mL^{-1} of the strain PP3 presented 91.7 % mortality (results not shown).

6.3.2. Cellular responses to infection

The percentage in the cellular type of the peripheral white blood cells from fish i.p. injected with strains PC566.1 and PP3 changed significantly after at both sampling times. After 6 h, a significant decrease in the percentage of lymphocytes was observed in fish i.p. injected with the high concentration of both *Phdp* strains, whereas in the percentage of neutrophils increase significantly in those specimens (Table 1). After 24 h, all fish challenged with both *Phdp* strains presented lymphopenia, with lowest values in fish i.p. injected with the high concentration of both PC566.1 and PP3. In addition, the percentage of monocytes and neutrophils increased significantly in all fish challenged with both *Phdp* strains with respect to control fish (Table 2).

Table 1. Proportion of peripheral leucocytes of Senegalese sole 6 h after injection of bacteria.

Leucocyte type	Inocula				
	PBS	PP3 (10^2)	PP3 (10^6)	PC566.1 (10^2)	PC566.1 (10^6)
Thrombocytes	37.9 ± 3.5	32.3 ± 7.9	37.8 ± 9.1	39.8 ± 4.4	35.5 ± 4.1
Lymphocytes	51.3 ± 3.8 ^{b}	55.7 ± 7.2 ^{b}	30.3 ± 9.4 ^{a}	47.3 ± 4.1 ^{b}	35.2 ± 8.6 ^{a}
Monocytes	5.6 ± 1.2	4.3 ± 0.5	6.1 ± 0.7	5.5 ± 1.0	8.2 ± 2.8
Neutrophils	5.2 ± 1.4 ^{a}	7.8 ± 3.7 ^{a}	25.8 ± 13.8 ^{b}	7.4 ± 2.5 ^{a}	21.1 ± 6.4 ^{b}

Values (%) are expressed as means ± SD (n = 6). Different letters mean significant differences among inocula for the same leucocyte type (One-way ANOVA; $P < 0.05$).

Table 2. Proportion of peripheral leucocytes of Senegalese sole 24 h after injection of bacteria.

Leucocyte type	Inocula				
	PBS	PP3 (10^2)	PP3 (10^6)	PC566.1 (10^2)	PC566.1 (10^6)
Thrombocytes	41.2 ± 2.9	38.6 ± 5.3	40.6 ± 4.7	39.1 ± 4.3	40.0 ± 3.9
Lymphocytes	48.3 ± 3.9 ^{c}	37.5 ± 3.4 ^{b}	24.3 ± 2.6 ^{a}	36.3 ± 5.9 ^{b}	22.5 ± 3.7 ^{a}
Monocytes	5.7 ± 1.9 ^{a}	10.6 ± 2.9 ^{b}	12.1 ± 4.7 ^{b}	10.0 ± 2.4 ^{b}	14.3 ± 5.4 ^{b}
Neutrophils	4.8 ± 1.2 ^{a}	13.3 ± 4.1 ^{b}	23.0 ± 7.2 ^{b}	14.6 ± 4.4 ^{b}	23.2 ± 9.0 ^{b}

Values (%) are expressed as means ± SD (n = 6). Different letters mean significant differences among inocula for the same leucocyte type (One-way ANOVA; $P < 0.05$).

Regarding peritoneal leucocytes response to infection, macrophages, neutrophils, eosinophilic granular cells (EGCs), lymphocytes and thrombocytes were observed in the peritoneal exudates of control and i.p. infected fish. However, thrombocytes and EGCs were only considered for total cell counts due to their very rare appearance, and thus not included within the differential counts. Total cells collected after 6 hours only increased significantly in fish i.p. injected with the high dose of the strain PC566.1 (Fig. 1). However, differential counting showed an increase in both the number and percentage of neutrophils in fish i.p. injected with 2×10^6 CFU mL^{-1} of both *Phdp* strains, with no changes in the values of lymphocytes and macrophages among treatments (Table 3). More drastic changes were observed after 24 hours. The number of total peritoneal leucocytes increased significantly in fish i.p. injected with both *Phdp* strains regardless of bacterial concentration with respect to control fish. In addition, fish i.p. injected with strain PC566.1 presented significantly higher values than fish from both PP3 groups (Fig. 1). On the other hand, differential counting showed and increase in the number of lymphocytes in fish challenged with both bacterial strains with respect to control fish, with highest values in fish i.p. injected with strain PC566.1. Moreover, while macrophages presented a similar pattern than that observed for lymphocytes, neutrophils increased parallel to bacterial concentration and strain (sole isolate), being the highest values in fish i.p. injected with strain PC566.1 (Table 4).

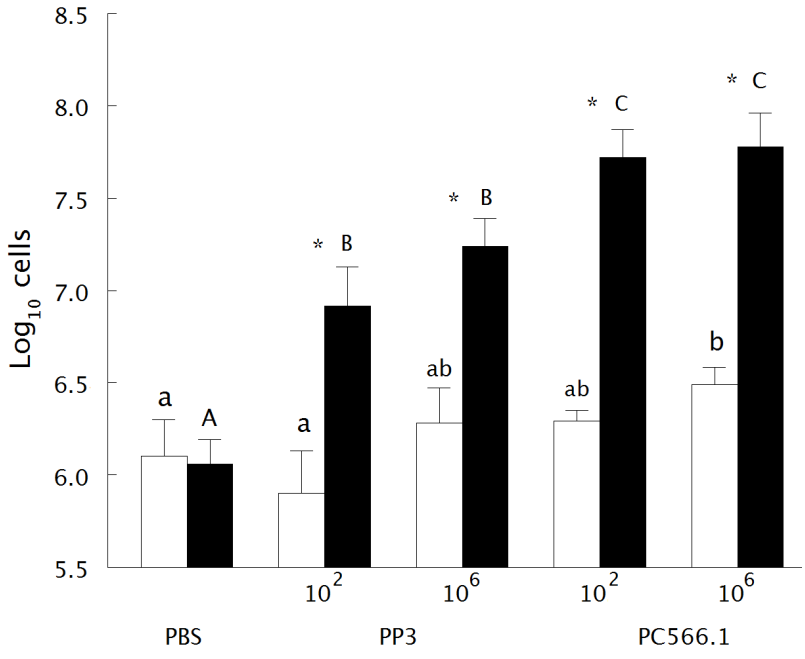

Figure 1. Leucocytes response in the peritoneal cavity of Senegalese sole at 6 (□) and 24 (■) h following intraperitoneal injection either with PBS (Control group) or PP3 and PC566.1 strains of *Phdp*, both at 10^2 or 10^6 CFU mL^{-1}. Values are expressed as means ± SD (n = 6). Different letters mean significant differences among inocula for the same time (One-way ANOVA; $P < 0.05$). Asterisk means significant differences in time for the same inoculum (*t*-test; $P < 0.05$).

Table 3. Leucocyte numbers and percentage in the inflamed peritoneal cavity of Senegalese sole 6 h after injection of bacteria.

Leucocyte type	Inocula				
	PBS	PP3 (10^2)	PP3 (10^6)	PC566.1 (10^2)	PC566.1 (10^6)
Lymphocytes	25.7 ± 15.6 (17.9)	35.0 ± 23.5 (37.8)	26.2 ± 17.1 (10.0)	44.8 ± 8.6 (21.2)	59.6 ± 52.1 (12.8)
Macrophages	98.9 ± 43.3 (72.2)	54.6 ± 46.5 (53.2)	72.5 ± 37.3 (28.3)	131.0 ± 28.9 (69.9)	157.0 ± 108.0 (40.8)
Neutrophils	14.4 ± 8.8 [a] (9.9)	10.8 ± 15.1 [a] (9.0)	125.3 ± 51.2 [b] (61.7)	18.7 ± 5.8 [a] (8.9)	193.2 ± 160.0 [b] (46.4)

Values ($\times 10^4$) are expressed as means ± SD (n = 6) of total leucocytes per peritoneal cavity. In brackets are percentage of the different leucocyte types. Different letters mean significant differences among inocula for the same leucocyte type (One-way ANOVA; $P < 0.05$).

Table 4. Leucocyte numbers and percentage in the inflamed peritoneal cavity of Senegalese sole 24 h after injection of bacteria.

Leucocyte type	Inocula				
	PBS	PP3 (10^2)	PP3 (10^6)	PC566.1 (10^2)	PC566.1 (10^6)
Lymphocytes	25.8 ± 4.5 [a]	68.4 ± 22.6 [b]	130.2 ± 38.7 [c]	259.6 ± 116.1 [c]	252.9 ± 127.0 [c]
	(21.8)	(6.2)	(7.2)	(12.4)	(4.2)
Macrophages	83.1 ± 23.0 [a]	341.5 ± 161.5 [b]	359.6 ± 157.6 [b]	1599.6 ± 547.1 [c]	1333.9 ± 277.2 [c]
	(70.1)	(36.2)	(19.5)	(38.7)	(15.4)
Neutrophils	9.5 ± 2.6 [a]	501.6 ± 222.0 [b]	1313.5 ± 426.8 [c]	2657.3 ± 725.4 [d]	4760.6 ± 793.6 [e]
	(8.1)	(57.6)	(73.3)	(48.9)	(80.4)

Values ($\times 10^4$) are expressed as means ± SD (n = 6) of total leucocytes per peritoneal cavity. In brackets are percentage of the different leucocyte types. Different letters mean significant differences among inocula for the same leucocyte type (One-way ANOVA; $P < 0.05$).

6.3.3. Innate immune responses to infection

The respiratory burst of head-kidney cells increased significantly following challenge with the high dose of both bacterial strains. Moreover, while leucocytes stimulated with the high concentration of strain PC566.1 presented increased values than leucocytes challenged with the same concentration of strain PP3, no differences were observed when leucocytes were stimulated with the low concentration of both bacterial strains (Figure 2).

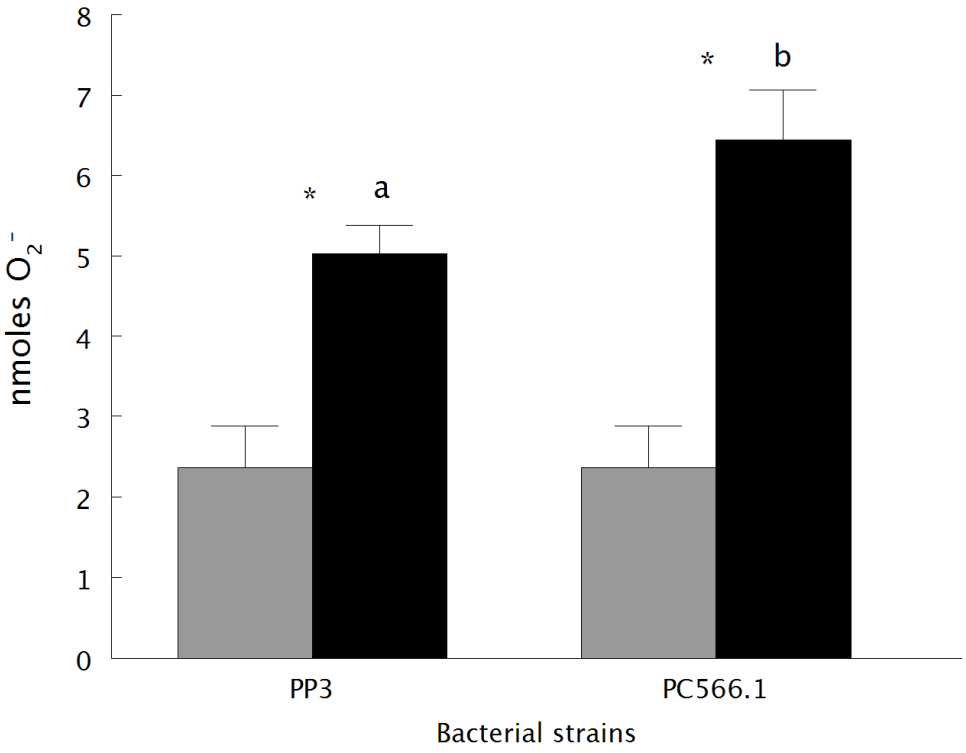

Figure 2. Respiratory burst activity of Senegalese sole head-kidney leucocytes following stimulation with different concentrations (10^2 [■] or 10^6 [■] CFU mL^{-1}) and strains of live *Phdp*. Data are expressed as means ± SD (n = 6). Different letters mean significant differences between bacterial strains for the same concentration (*t*-test; $P < 0.05$). Asterisk means significant differences between bacterial concentrations for the same strain (*t*-test; $P < 0.05$).

Innate humoral parameters also increased following challenge with both bacterial strains. The ACP activity increased significantly in fish i.p. injected with the high concentration of strains PC566.1 and PP3 after 6 and 24 h, being the highest values from fish challenged with strain PC566.1 at 24 h (Figure 3). Lysozyme activity increased significantly in fish i.p. injected with both bacterial strains and concentrations after 6 and 24 h (Figure 4). Peroxidase activity

increased significantly in fish i.p. injected with the high concentration of both bacterial strains at 6 h, while those values augmented in all infected groups at 24 h (Figure 5).

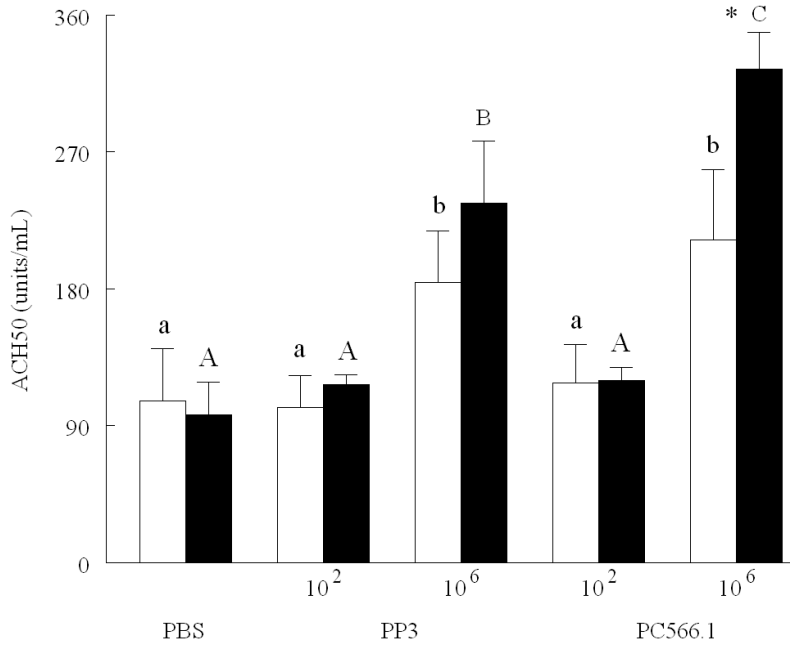

Figure 3. Plasma alternative pathway activity in Senegalese sole at 6 (□) and 24 (■) h following intraperitoneal injection either with PBS (Control group) or PP3 and PC566.1 strains of *Phdp*, both at 10^2 or 10^6 CFU mL^{-1}. Values are presented as ACH50 values, expressed as means ± SD (n = 6). Different letters mean significant differences among inocula for the same time (One-way ANOVA; $P < 0.05$). Asterisk means significant differences in time for the same inoculum (*t*-test; $P < 0.05$).

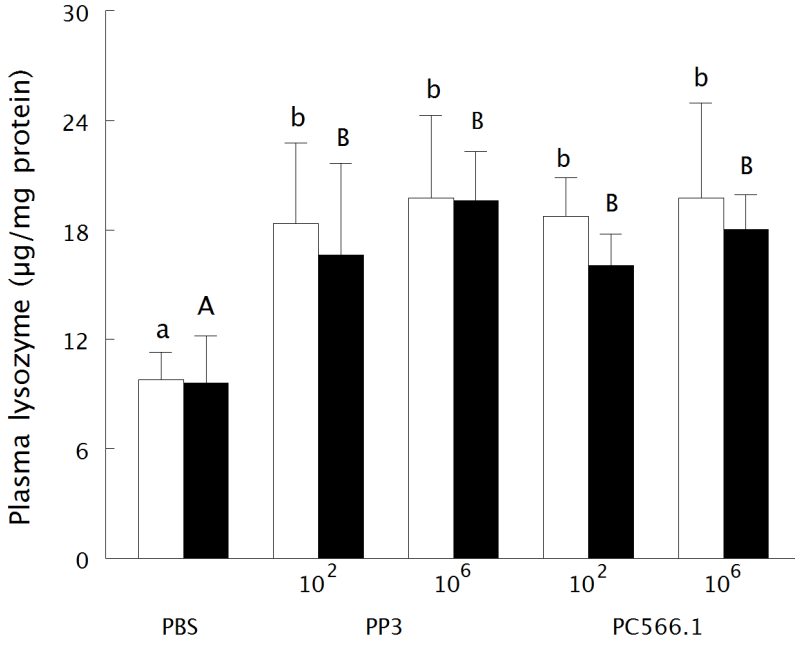

Figure 4. Plasma lysozyme activity in Senegalese sole at 6 (□) and 24 (■) h following intraperitoneal injection either with PBS (Control group) or PP3 and PC566.1 strains of *Phdp*, both at 10^2 or 10^6 CFU mL^{-1}. Values are expressed as means ± SD (n = 6). Different letters mean significant differences among inocula for the same time (One-way ANOVA; $P < 0.05$).

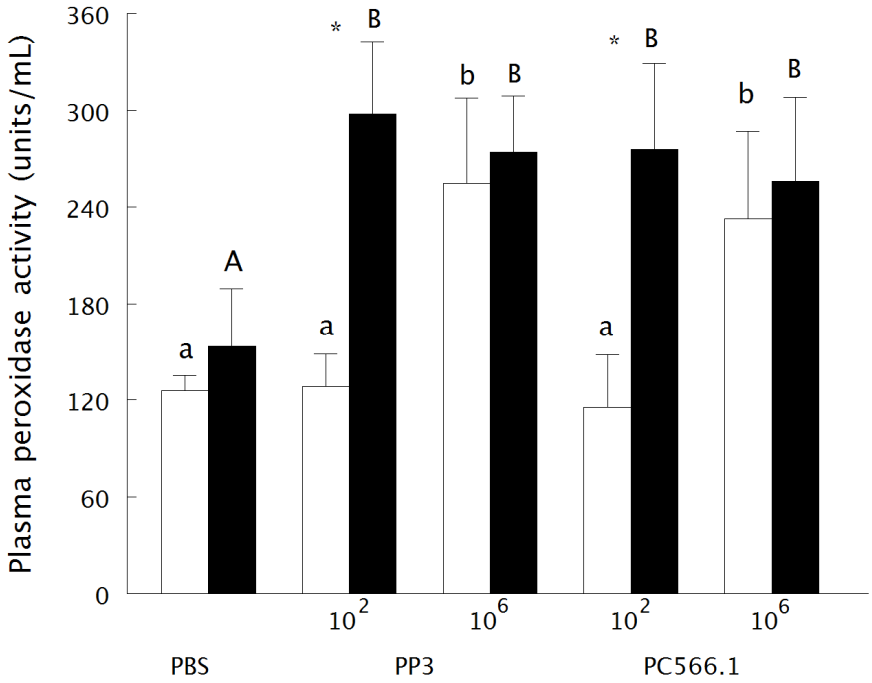

Figure 5. Plasma peroxidase activity in Senegalese sole at 6 (□) and 24 (■) hours following intraperitoneal injection either with PBS (Control group) or PP3 and PC566.1 strains of *Phdp*, both at 10^2 or 10^6 CFU mL^{-1}. Values are expressed as means ± SD (n = 6). Different letters mean significant differences among inocula for the same time (One-way ANOVA; $P < 0.05$). Asterisk means significant differences in time for the same inoculum (*t*-test; $P < 0.05$).

6.4. Discussion

For detailed and accurate studies to analyze the peritoneal phagocyte response to the presence of infectious agents, unequivocal differentiation between macrophages and neutrophils must be achieved. Sometimes, these two phagocytic cells can be difficult to distinguish by conventional light microscopic preparations, since the nuclear shape of some neutrophils is not as multilobular as in mammalian neutrophils. However, the cytochemical technique used in the present study allowed for the clear distinction between Senegalese sole macrophages and neutrophils, and therefore it was possible to study the appearance of each professional phagocyte to the site of inflammation. This technique has been validated and used for extensive studies of the inflammatory response in the peritoneal cavity of several teleosts following i.p. injection of bacteria (Afonso *et al.* 1998a; Afonso, Silva, Lousada, Ellis & Silva 1998b; Afonso, Oliveira, Ellis & Silva 1999; do Vale *et al.* 2002, 2003). The lack of EGCs in the Senegalese sole peritoneal exudates is in agreement to that observed by Afonso

et al. (1998a) and do Vale *et al.* (2002) and could be due to degranulation of these cells following injection of bacteria. Degranulation of EGCs at the injected site after inoculation of several degranulating agents, including bacteria, is well documented (Ellis 1985; Reite 1997; Matsuyama & Iida 1999). Furthermore, Senegalese sole macrophages are typically peroxidase-negative. However, some peroxidase-positive macrophages were observed, congruent with that described for rainbow trout (*Oncorhynchus mykiss*) and seabass (*Dicentrarchus labrax*) (Afonso *et al.* 1998a; do Vale *et al.* 2002). The identification of peroxidase-positive macrophages by light microscopy was achieved because the patterns of peroxidase staining in light microscopy preparations are different for neutrophils and macrophages. Peroxidase-positive macrophages were only observed in exudates collected after i.p. injection of bacteria, which is in agreement with that observed by Afonso *et al.* (1998a) and contrary to what was reported by do Vale *et al.* (2002), where peroxidase-stained macrophages were occasionally observed in exudates from non-infected seabass. It has been reported that the presence of peroxidase-positive material inside inflammatory peritoneal macrophages in fish may result from the uptake of neutrophils and neutrophilic components by macrophages (Afonso *et al.* 1998b; do Vale *et al.* 2002). In the present study, the observed increase in the number of peroxidase-positive macrophages after infection, correlated with the significant increase in the level of neutrophils, makes it tempting to speculate that the same mechanism also occurs in Senegalese sole.

To our knowledge, this is the first study in Senegalese sole showing leucocyte responses to inflammation in both peripheral blood and peritoneal cavity (inflammatory focus). Neutrophils from the reserve pools are quickly attracted to infectious foci by microbial products and chemotactic substances released by host cells, including macrophages (Neumann et al. 2001; do Vale et al. 2002). In the present study, the increase in the relative levels of neutrophils from peripheral blood in fish infected with the high concentration of both *Phdp* strains translated in a significant increase in the peritoneal neutrophil population already at 6 h after challenge. In addition, those levels augmented in all infected specimens after 24 h regardless of bacterial concentration. This blood neutrophilia was congruent with the increased plasma peroxidase acitvity. Moreover, the surge of neutrophil migration is commonly followed by monocyte recruitment in mammals and several fish species (Silva et al., 1989; Neumann et

al. 2001), including Senegelse sole (present study). Cases of blood neutrophilia and monocytosis were also observed in several fish species following challenge with bacteria (Bruno and Munro, 1986; Lamas et al., 1994). In this study, the observed monocytosis at 24 h was consistent with the increase in the peritoneal macrophage population at that time. Moreover, both neutrophils and macrophages from normal and infected sole showed similar patterns of mobilization and localization to that described previously for rainbow trout and seabass (Afonso et al. 1998a; do Vale et al. 2002). Interestingly, the percentage of blood lymphocytes from the current study decreased in fish infected with the high concentration of both *Phdp* strains already at 6 h after challenge. However, the peritoneal lymphocyte population only increased significantly after 24 h. In fact, those fish presented a higher degree of lymphopenia than the same specimens at 6 h. A reduction in the number of blood lymphocytes has been reported in fish after bacterial insults (Lamas et al. 1994; Balfry et al. 1997; Garcia et al. 2007), suggesting the migration of these cells to the site of inflammation. Therefore, results from the present study give further insights to the hypothesis of cell migration to inflammation foci in fish. In addition, the cytological technique used in the present study, applied to samples from both peripheral blood and exudates from the inflammatory focus, could be instrumental for studies of defense mechanisms against infection.

The peritoneal leucocytes response observed at 24 h following infection appears to be strain specific. While the percentage of neutrophils and macrophages were similar among fish i.p. injected with the same concentration of both *Phdp* isolates, specimens infected with PC566.1 showed an increase (around 4-5 fold) in the population of both phagocytes with respect to fish challenged with strain PP3. It is likely that this higher increase in peritoneal phagocyte numbers is related to the higher degree of virulence from the European isolate. Extensive phagocytosis of virulent *Phdp* by peritoneal macrophages and neutrophils as well as a significant increase in their population has been observed in seabass (do Vale et al. 2002). However, information on the interactions between *Phdp* pathogenesis and host phagocytes is still scarce. The susceptibility of *Phdp* to the phagocyte killing activity was observed in several fish species (Skarmeta et al. 1995; Barnes et al. 1999), including Senegalese sole (this study), suggesting that this bacterium is not well equipped for the protection against reactive oxygen species (ROS) produced by macrophages and neutrophils.

Interestingly, this study also showed that head-kidney cells challenged with strain PC566.1 displayed a higher ROS production than phagocytes exposed to strain PP3. Therefore, it is tempting to speculate that pathogen recognition receptors from sole phagocytes may have detected pathogen-associated molecular patterns associated to DNA structures unique for each isolate. In fact, the existence of genetic heterogeneity within the European and Japanese clonal lineages has been reported (Magariños et al. 2000). In the current study, even though Senegalese sole showed higher ROS production (*in vitro*) and phagocyte response to inflammation (*in vivo*) when challenged with 2×10^6 CFU mL^{-1} of the European isolate, these specimens presented a higher degree of mortality than fish challenged with the same concentration of the Japanese isolate. *Phdp* strains from different origin and degrees of virulence induced extensive apoptosis of peritoneal macrophages and neutrophils when around 10^6 CFU were i.p. injected in seabass, however, quantitative differences in apoptogenic activity among virulent isolates were not reported (do Vale et al. 2003). Recent studies showed that AIP56, a protein exotoxin abundantly secreted by virulent *Phdp*, is the factor responsible for the reported apoptogenic activity of this bacterium (do Vale et al. 2005), and also suggested that the AIP56-dependent pathogenicity mechanism appears to be decisive for the rapid course and high mortality of both experimental and natural acute fish pasteurellosis (do Vale et al. 2007). However, differential degrees of virulence of this AIP56-dependent pathogenicity mechanism related to *Phdp* origin have not been studied yet.

In the present study, plasma lysozyme and peroxidase contents correlate well with the increase in peripheral neutrophils. These phagocytes are thought to be the source of plasma lysozyme (Murray and Fletcher 1976) and peroxidase (Ellis 1999), and increases in lysozyme and peroxidase levels have been associated to increases in neutrophil numbers (Muona and Soivio 1992; Cerezuela et al. 2009). However, among the humoral factors assessed in the present study, ACP appears to be the mechanism of greater importance against *Phdp*. ACH50 values increased in fish i.p. injected with 2×10^6 CFU mL^{-1} of both bacterial strains after 6 and 24 h. Considering the role of both macrophages and neutrophils on phagocytosing this bacterium (do Vale et al. 2002), together with the significant increase in both phagocytes in infected fish (do Vale et al. 2002, 2003; this study), it is likely that the complement system may have contributed to stimulate this mechanism. By opsonising pathogens, complement proteins can

stimulate phagocytosis, a process that is mediated by complement receptors on the surface of phagocytic cells (Holland and Lambris 2002). Moreover, gilthead seabream (*Sparus aurata*) and seabass increased both the level of expression of C3 mRNA and ACH50 values at 24 h following challenge with *Phdp* (Mauri et al., 2011). Interestingly, Senegalese sole i.p. injected with the European isolate showed the highest ACH50 values at 24 h, which correlate well with the highest levels of peritoneal neutrophils at that same time. These data gives further support to the idea that Senegalese sole presents an increased immune response against the European isolate when compared to fish infected with the Japanese strain, probably due to its higher degree of virulence.

In conclusion, a number of changes were observed when Senegalese sole is challenged with *Phdp* strains from different origins (European and Japanese isolates) and concentrations. This study showed for the first time leucocyte responses to inflammation in both peripheral blood and peritoneal cavity of Senegalese sole, being consistent with that observed for other teleosts.. The percentage of peripheral blood leucocytes correlated well with the increase in peritoneal leucocytes numbers, thus corroborating the hypothesis of cell migration to the inflammatory focus in fish. Furthermore, Senegalese sole challenged with the European isolate displayed a higher innate immune response, showing increased levels of both peritoneal leucocytes and ACP activity *in vivo* and ROS production *in vitro* than specimens infected with the Japanese isolate. This augmented innate machinery is probably due to the higher degree of virulence presented by the European strain.

6.5. Acknowledgements

The authors are grateful to Ana do Vale for providing the bacterial strains used in this study. This work was developed under the project OPTISOLE – QREN IDT Co-Promoção Nº 1605, which was co-funded by the National Strategic Reference Framework – *QREN* under the Regional Operational Programme North – ON2, with 343751.84 € originating from the European Regional Development Fund – *FEDER*. Benjamín Costas and Paulo César N.P. Rêgo benefited from grants by Fundação para a Ciência e Tecnologia, Portugal (SFRH/BD/38697/2007 and SFRH/BD/30907/2006, respectively).

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Chapter 7

Dietary arginine and repeated handling increase disease resistance and modulate innate immune mechanisms of Senegalese sole (*Solea senegalensis* Kaup, 1858)

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Costas, B., Conceição, L.E.C., Dias, J., Novoa, B., Figueras, A., Afonso, A., 2011. Dietary arginine and repeated handling increase disease resistance and modulate innate immune mechanisms of Senegalese sole (*Solea senegalensis* Kaup, 1858). Fish & Shellfish Immunology 31, 838 – 847.

Dietary arginine and repeated handling increase disease resistance and modulate innate immune mechanisms of Senegalese sole (*Solea senegalensis* Kaup, 1858)

Abstract

Stress is known to impair immune function and disease resistance in fish. In the present study, repeated handling was employed as a chronic stressor in order to verify whether its attributed immunosuppressive effects could be minimized by dietary arginine supplementation. Therefore, Senegalese sole (*Solea senegalensis*) were air exposed daily for 3 min during 14 days (handling) or left undisturbed (control). In addition, both control and handled specimens were fed 3 diets with graded levels of arginine (Arg 4.4, Arg 5.7 and Arg 6.9 g 16 g^{-1} N). Following the 14 days stress challenge and feeding on those diets, fish were infected with *Photobacterium damselae* subsp. *piscicida* (strain PC566.1; LD_{50} 5 × 10^3 cfu mL^{-1}) and fed the same experimental diets. Respiratory burst activity and nitric oxide production of head-kidney leucocytes increased parallel to dietary arginine supplementation. HIF-1, HAMP-1, MIP1-alpha and gLYS expression values and some humoral parameters augmented in control specimens fed the Arg 5.7 and Arg 6.9 diets. Interestingly, repeated acute stress increased both disease resistance and some innate immune mechanisms in handled fish. The role of dietary arginine and repeated handling on Senegalese sole innate immunity and disease resistance are discussed.

Keywords: bacterial challenge; immune-related genes; nitric oxide; respiratory burst activity; stress response

7.1. Introduction

Senegalese sole (*Solea senegalensis*) is a high-value flatfish that presents a great potential for future farming at commercial scale. However, growth and survival from juvenile to market-size fish is not fully controlled with regard to rearing technology and husbandry conditions, feeding behavior and nutritional requirements (Imsland et al., 2003). Among the different factors that may induce high mortality during the juvenile stage, stress might be one of the key issues. Osmoregulatory and metabolic changes associated to stress responses have been assessed previously in this species (Arjona et al., 2009; Aragão et al., 2008,

2010; Costas et al., 2008, 2011a). However, little attention has been paid to study alterations in innate immune mechanisms after a stress challenge (Salas-Leiton et al., 2010; Costas et al., 2011b).

Confinement, high density, handling and transport are stress inducers which are highly relevant to aquaculture and have received considerable attention (Saeij et al., 2003; Binuramesh et al., 2005; Costas et al., 2008, 2011; Fast et al., 2008). The effect of stress on the immune system has been widely investigated and, although acute stress can have beneficial effects, chronic stress was found to inhibit an optimal immune response in both mammals and teleost fish (Sunyer et al., 1995; Sapolsky et al., 2000; Weyts et al., 1998a; Fast et al., 2008), leading to increased susceptibility to pathogens (Wendelaar Bonga, 1997). Corticosteroids can influence multiple aspects of the innate immune defense mechanisms in fish (Verburg-van Kemenade et al., 2009). Cortisol usually down-regulates the production of pro-inflammatory cytokines and nitric oxide (NO) to prevent damage due to an excessive inflammatory response (Saeij et al., 2003a). Moreover, phagocytosis of peripheral blood leucocytes from common carp (*Cyprinus carpio*) and hybrid tilapia (*Oreochromis niloticus* × *O. aureus*) was significantly depressed after *in vitro* cortisol administration (Law et al., 2001). Additionally, mRNA levels of g-type lysozyme and hepcidin antimicrobial peptide-1 genes decreased in liver and kidney of Senegalese sole submitted to high stocking density (Salas-Leiton, et al., 2010). Furthermore, high plasma cortisol levels resulted in reduction of leucocyte proliferation, numbers of antibody producing cells, and levels of virus-neutralising antibodies in fish (Verburg-van Kemenade et al., 2009).

Stress conditions that induced high plasma cortisol levels also modified amino acid (AA) metabolism in Senegalese sole (Pinto et al., 2007; Aragão et al., 2008, 2010; Costas et al., 2008, 2011a,b). Moreover, changes in plasma free AA levels may be indicative of AA requirements in fish (Wilson, 2002). In fact, it has been suggested that fish under stressful conditions present additional AA requirements, due to either increased energetic demands or for the synthesis of stress-related proteins and other compounds related with the stress response (Aragão et al., 2008, 2010; Costas et al., 2008). The role of specific AA and their metabolites on key metabolic pathways that are necessary for growth, immunity or resistance to environmental stressors and pathogens in fish have been recently

reviewed (Li et al., 2009). Thus, AA not only serve as constituents of proteins and energy sources, but are also converted into important biochemically active substances *in vivo*. Particularly, arginine is the precursor for the synthesis of nitric oxide (NO) and polyamines (Satriano et al., 1999). NO causes vasodilatation and stimulates microcirculation, improving thereby cardiac, pulmonary and cerebral functions in humans (Roth, 2007). In fish, macrophage NO production plays an important role in the cellular defense mechanisms and has been demonstrated in stimulated macrophages in several fish species (Neumann et al., 1995; Buentello and Gatlin, 1999; Tafalla and Novoa, 2000). Moreover, different studies revealed that L-arginine administration improved wound and bone healing in mammals (Roth, 2007). In fish, a positive effect of an arginine-enriched diet on the resistance of channel catfish (*Ictalurus punctatus*) to infection with *Edwardsiella ictaluri* has been also demonstrated (Buentello and Gatlin, 2001).

Therefore, the present study aimed to assess whether dietary arginine supplementation can minimize the immunosuppressive effects attributed to chronic stress in fish. Repeated handling stress will be used in the current study since it has been already established as a chronic stressor for this species. Similarly, it is also intended to verify to what extent dietary treatment can influence disease resistance and some aspects of the innate immune system in Senegalese sole.

7.2. Materials and Methods

7.2.1. Formulation and analytical procedures with experimental diets

Three practical diets were formulated to be isonitrogenous, isolipidic and isoenergetic (54 % protein, 8 % lipids, 21 kJ g^{-1} energy on a dry-matter (DM) basis). Marine-derived ingredients represented only 15 % of the formula. The rest of the protein fraction was achieved by means of a variable blend of soybean meal, soy protein concentrate, wheat meal, corn and wheat gluten, and wheat DDGS (dried distillers grains with solubles), whereas fish oil was the main fat source. Thus, the plant-protein fraction in these experimental diets was around 75 %. Moreover, L-arginine (0.8 and 1.5 %) was added to two of the diets to obtain graded levels of arginine (4.5, 6 and 7.5 % of crude protein) at the expenses of wheat gluten. Following analytical procedures final values were 4.4, 5.7 and 6.9 g

16 g^{-1} N, respectively. Therefore, experimental diets are referred from now on as Arg 4.4, Arg 5.7 and Arg 6.9. The Arg 4.4 diet was formulated according to known nutritional requirements of Senegalese sole and served as control (Dias et al., 2010). These plant protein-rich diets were further supplemented with lysine and mono-calcium phosphate to avoid imbalances. In the absence of specific data on vitamin, mineral and trace element requirements of Senegalese sole, requirement data for other species were applied (NRC, 1993; Kaushik, 1998). Experimental diets were manufactured by SPAROS Lda. (Faro, Portugal). Main ingredients were grinded (below 250 micron) in a Micropulverizer hammer mill (Hosokawa, model #1). Powder ingredients were then mixed accordingly to the target formulation in a double-helix mixer (TGC, model 500L). All diets were manufactured by extrusion (pellet size 3 mm), by means of a pilot-scale twin-screw extruder (CLEXTRAL BC45) with a screw diameter of 55.5 mm and temperature ranging 105-110 ºC. Upon extrusion, extruded feeds were dried in a convection oven (LTE OP 750-UF) for 2 hours at 60 ºC. Following drying, pellets were allowed to cool at room temperature, and subsequently the supplemental $_{L}$-arginine and fish oil were added under vacuum coating conditions in a DINNISEN Pegasus vacuum mixer (PG-10VCLAB). Formulation and proximate composition of experimental diets are presented in Table 1.

Diets were analysed for total amino acids content. Diet samples were hydrolyzed in 6M HCl at 106 °C over 24 h in nitrogen-flushed glass vials. Afterwards, samples were pre-column derivatized with phenylisothiocyanate (PITC; Pierce), using the PicoTag method (Waters, USA) described by Cohen et al. (1989). External standards were prepared along with the samples, using physiological amino acid standard solutions (acid/neutral and basics from Sigma) and a glutamine solution. Norleucine was used as an internal standard. Samples and standards were analysed by high performance liquid chromatography (HPLC) in a Waters Reversed-Phase Amino Acid Analysis System equipped with a PicoTag column (3.9 x 300 mm), using the conditions described by Cohen et al. (1989). Resulting peaks were analysed with the Breeze software (Waters). During analytical procedures, asparagine is converted to aspartate and glutamine to glutamate during acid hydrolysis, so the reported values for these amino acids (Asx and Glx) represent the sum of the respective amine and acid. Moreover, tryptophan was not determined since it is destroyed by acid hydrolysis. Amino acid profile of experimental diets is presented in Table 2.

Table 1. Ingredients and proximal composition of experimental diets.

	Experimental diets		
	Arg 4.4	Arg 5.7	Arg 6.9
Ingredients (%)			
Fishmeal 70 LT	5.0	5.0	5.0
CPSP	5.0	5.0	5.0
Squid meal	5.0	5.0	5.0
Soybean meal	17.0	17.0	17.0
Soycomil (PC)	3.2	3.2	3.2
Wheat gluten	13.9	13.0	12.1
Wheat meal	17.2	17.3	17.5
Wheat DDGS	10.0	10.0	10.0
Corn gluten	15.0	15.0	15.0
Fish oil	4.0	4.0	4.0
Vit & Min Premix*	1.0	1.0	1.0
DCP	2.7	2.7	2.7
L-Lysine	1.0	1.0	1.0
L-Arginine	-	0.8	1.5
Proximate composition			
Dry matter (% DM)	90.7	92.3	92.5
Crude protein (% DM)	53.9	54.1	56.4
Crude fat (% DM)	7.4	8.1	8.1
Ash (% DM)	6.6	6.6	6.8
Gross Energy (kJ g^{-1} DM)	21.7	21.3	21.1
NFE	31.1	31.2	28.7

CPSP, fish soluble protein concentrate (hydrolysed white fish meal); DCP, dibasic calcium phosphate; DDGS, dried distillers grains with solubles; DM, dry matter; NFE, nitrogen free extracts = 100 – (CP + CL + CA).

*Minerals (g or mg kg^{-1} diet): Mn (manganese oxyde), 20 mg; I (potassium iodide), 1.5 mg; Cu (copper sulphate), 5 mg; Co (cobalt sulphate), 0.1 mg; Mg (magnesium sulphate), 300 mg; Zn (zinc oxide), 30 mg; Se (sodium selenite), 0.3 mg; Fe (iron sulphate), 56 mg; Ca (calcium carbonate), 80 mg; KCl (potassium chloride), 750 mg; NaCl (sodium chloride), 0.4 g. Vitamins (mg kg^{-1} diet): vitamin A (retinyl acetate), 2.75 mg; vitamin D3 (DL-cholecalciferol), 0.04 mg; vitamin K3 (menadione sodium bisulfite), 10 mg; vitamin B12 (cyanocobalamin), 0.02 mg; vitamin B1 (thiamine hydrochloride), 8 mg; vitamin B2 (riboflavin), 20 mg; vitamin B6 (pyridoxine hydrochloride), 10 mg; folic acid, 6 mg; biotin, 0.7 mg; inositol, 300 mg; nicotinic acid, 70 mg; pantothenic acid, 30 mg; vitamin E (Lutavit E50), 300 mg; vitamin C (Lutavit C35), 500 mg; betaine (Betafin S1), 500 mg.

Table 2. Amino acid composition (g 16 g^{-1} N) of experimental diets.

	Experimental diets		
	Arg 4.4	Arg 5.7	Arg 6.9
IAA			
His	2.0 ± 0.1	2.2 ± 0.1	2.1 ± 0.1
Arg	4.4 ± 0.3 [a]	5.7 ± 0.5 [b]	6.9 ± 0.2 [c]
Ile	2.5 ± 0.3	2.7 ± 0.1	2.6 ± 0.1
Leu	7.4 ± 0.1	7.5 ± 0.4	7.3 ± 0.2
Val	3.2 ± 0.1	3.4 ± 0.1	3.2 ± 0.1
Thr	3.0 ± 0.1	3.1 ± 0.1	2.9 ± 0.1
Met	2.0 ± 0.2	2.1 ± 0.1	2.0 ± 0.0
Lys	4.3 ± 0.1	4.4 ± 0.1	4.2 ± 0.1
Phe	4.1 ± 0.1	4.2 ± 0.1	4.2 ± 0.0
DAA			
Asx	5.7 ± 0.2	5.8 ± 0.2	5.3 ± 0.3
Glx	16.8 ± 0.7	17.7 ± 0.4	16.6 ± 0.6
Ser	4.2 ± 0.2	4.5 ± 0.2	4.2 ± 0.3
Gly	3.0 ± 0.1	3.3 ± 0.3	3.2 ± 0.3
Ala	4.0 ± 0.3	4.3 ± 0.3	4.1 ± 0.2
Pro	6.0 ± 0.3	6.4 ± 0.5	6.3 ± 0.2
Tau	0.6 ± 0.0	0.7 ± 0.1	0.6 ± 0.0
Tyr	3.5 ± 0.1	3.6 ± 0.1	3.6 ± 0.0
Cys	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0

Trp was not analysed. Values are mean of duplicates ± SD. Row means followed by different letters indicate significant differences among dietary treatments (One-way ANOVA; $P < 0.05$).

7.2.2. Fish

Specimens originated from the natural spawning of wild broodstock and were reared according to standard larval and juvenile rearing protocols (Dinis et al., 1999) at the CCMAR (University of Algarve, Faro, Portugal) facilities. Prior to the experiment, fish were transported to the CIIMAR (University of Porto, Porto, Portugal) facilities and maintained in two separate recirculated seawater systems (temperature: 21-22 ºC; salinity: 34 ‰; natural photoperiod (April 2010); dissolved oxygen: above 90 % saturation level; water flow rate: 114 L h^{-1}; n = 25

fish/tank; density = 1.5 Kg m^{-2}) comprised by three flat-bottomed round tanks (volume: 300 L) for 14 days. During this acclimation period, fish were fed daily to apparent satiety (based on the assessment of feed remaining in the tanks) with the Arg 4.4 diet.

7.2.3. Experimental design

Experiments were directed by trained scientists (following FELASA category C recommendations) and were conducted according to the guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE. Senegalese sole (90.7 ± 35.7 g wet weight) were maintained in two separate recirculated seawater systems as indicated above. In one of the systems, fish were reared in triplicate tanks (n = 25) without any disturbance, except from daily tank cleaning procedures, and served as control. In the other system, handled fish were daily chased with a net in order to capture all fish inside each tank (triplicates; n = 25) and thereafter the net was held in the air during 3 min. Immediately after air exposure the fish were returned to the experimental tanks. Both control and handled fish were fed the Arg 4.4, Arg 5.7 and Arg 6.9 diets. Tanks were daily cleaned and temperature, salinity, dissolved oxygen and ammonia and nitrite levels in the water were daily measured. Fish were fed by hand four times a day at a daily feeding rate of 1.5 % biomass.

Experimental sampling was performed after 14 days. Eight fish were quickly taken out from each tank at a time and anaesthetized with ethyl 3-aminobenzoate methanesulfonate (MS-222, 200 mg L^{-1}; Sigma – Aldrich, Germany). Blood was withdrawn from the caudal vein of each fish using heparinised syringes, centrifuged (10000 × *g* for 10 min at 4 ºC) and the resulting plasma was stored at -80 ºC for further analysis. After blood collection, fish were individually weighed and the head-kidney subsequently dissected over an ice bed. Head-kidneys from 4 fish per tank were removed under aseptic conditions, placed in RNA*later* solution (Ambion) and stored at 4 ºC for 24 h followed by long term storage at -80 ºC prior to RNA extraction. In addition, head-kidneys from the remaining 4 fish per tank were processed as follows.

7.2.4. Functional immune assays

7.2.4.1. Cellular innate immune parameters

Leucocytes from head-kidney were isolated and maintained essentially as described by Secombes (1990). Briefly, the head-kidney was removed under aseptic conditions, pushed through a 100 µm nylon mesh and suspended in Leibovitz L-15 medium (L-15: Gibco, Scotland, UK) supplemented with 2% foetal bovine serum (FBS; Gibco), penicillin (100 IU mL^{-1}; P, Gibco), streptomycin (100 µg mL^{-1}; S, Gibco) and heparin (20 units mL^{-1}; Sigma). The suspensions were then loaded onto a 34:51 % Percoll (Sigma) density gradient and centrifuged at 400 × *g* and 4 ºC for 40 min. The band of cells laying at the interface of the Percoll gradient was collected and washed three times at 400 × *g* and 4 ºC for 5 min in L-15, 0.1 % FBS, P/S and heparin. The viable cell concentration was determined by the Trypan blue exclusion test. Cells were counted in a haemocytometer and adjusted to 1 x 10^7 cells mL^{-1} in L-15, 0.1 % FBS, P/S and heparin. Afterwards, cells were plated in 96-well plates at 100 µL per well. After overnight incubation at 18 ºC, the non-adherent cells were washed off and the monolayers were maintained with L-15 supplemented with 5% FBS, until the respiratory burst and NO assays were conducted after 24 and 72 h of incubation at 18 ºC, respectively.

Respiratory burst activity of head-kidney leucocytes was based on the reduction of ferricytochrome C method for the detection of O_2^- (Secombes, 1990). Briefly, the leucocytes monolayers were washed twice with phenol red-free Hank's balanced salt solution (HBSS) and 100 µL suspension of ferricytochrome C solution (2 mg ferricytochrome C mL^{-1} diluted in phenol red-free HBSS) containing 2 × 10^6 colony-forming units (cfu) *Photobacterium damselae* subsp. *piscicida* mL^{-1} were added. Ferricytochrome C solution containing 10 µg mL^{-1} phorbol myristate acetate (PMA, Sigma) was added as a soluble stimulant of the respiratory burst and served as a positive control. Ferricytochrome C with PMA and 0.725 mg mL^{-1} superoxide dismutase (SOD, Sigma) was used to confirm the specificity of the reaction. For each ferricytochrome C solution (i.e., bacteria, PMA and PMA+SOD) 3 or more wells of leucocytes per fish were assayed. Plates were read 30 min after addition of reagents to the leucocytes on a Power-Wave™ microplate spectrophotometer (BioTek) at 550 nm. Optical densities (OD) were converted to nmol of O_2^- produced by multiplying by 15.87 as described by Pick (1986), and

the mean ± SD of each treatment was calculated. Total cell counts were determined after respiratory burst assay by removing the medium and adding 100 µL of lysis buffer (0.1 M citric acid, 1 % Tween 20 and 0.05 % crystal violet). After 2 min the nuclei were counted in a haemocytometer. The mean of four wells was used as the total cell count per well for each fish. All results were expressed per 10^5 cells.

NO production of head-kidney leucocytes was assayed by the method described by Neumann et al. (1995) and modified by Tafalla and Novoa (2000). This method is based on the Griess reaction (Green et al., 1982) that quantifies the nitrite content of the leucocytes supernatants, since NO is an unstable molecule and degrades to nitrite and nitrate. To stimulate head-kidney leucocytes for NO production, cells monolayers were exposed to *Escherichia coli* serotype 0111: B4 LPS (Sigma) at a concentration of 10 µg mL^{-1} diluted in L-15 5% FBS. NG-Methyl-L-arginine (Sigma) at 1 mM and LPS were also added to some wells to confirm the specificity of the reaction. After 72 h incubation of leucocytes at 18 ºC, 50 µL of the leucocytes supernatants were removed from individual wells and placed in a separate 96-well plate. Afterwards, 100 µL of 1 % sulfanilamide (Sigma) in 2.5 % phosphoric acid was added to each well, followed by 100 µL of 0.1 % N-naphthyl-ethylene-diamine (Sigma) in 2.5 % phosphoric acid. OD was determined at 540 nm. The molar concentration of nitrite in the sample was determined from standard curves generated using known concentrations of sodium nitrite.

7.2.4.2. Humoral innate immune parameters

Lysozyme activity was measured using a turbidimetric assay based on the method described by Ellis (1990) with some modifications (Wu et al., 2007). Briefly, a solution of *Micrococcus lysodeikticus* (0.5 mg mL^{-1} 0.05 M sodium phosphate buffer; pH 6.2) was prepared. In a microplate, 15 µL of plasma and 250 µL of the above suspension were added. The reaction was carried out at 25 ºC and the absorbance at 450 nm was measured after 0.5 and 4.5 min. Lyophilized hen egg white lysozyme (Sigma) was serially diluted in sodium phosphate buffer (0.05 M; pH 6.2) and used to develop a standard curve. The amount of lysozyme in the sample was calculated using the formula of the standard curve. All analyses were conducted in triplicates.

Alternative complement pathway (ACP) activity was estimated as described by Sunyer and Tort (1995). The following buffers were used: GVB (Isotonic veronal buffered saline), pH 7.3, containing 0.1 % gelatin; EDTA-GVB, as previous one but containing 20 mM EDTA; and Mg-EGTA-GVB, which is GVB with 10 mM Mg^{+2} and 10 mM EGTA. Rabbit red blood cells (RaRBC; Probiologica Lda., Portugal) were used for ACP determination. RaRBC were washed four times in GVB and resuspended in GVB to a concentration of 2.5×10^8 cells mL^{-1}. Then, 10 µL of RaRBC suspension were added to 100 µL of serially diluted plasma in Mg-EGTA-GVB buffer. Samples were incubated at room temperature for 100 min with occasional shaking. The reaction was stopped by adding 100 µL of cold EDTA-GVB. Samples were then centrifuged and the extent of haemolysis was estimated by measuring the OD of the supernatant at 414 nm. The ACH50 units were defined as the concentration of plasma giving 50% haemolysis of RaRBC. All analyses were conducted in triplicates.

Total peroxidase activity in plasma was measured following the procedure described by Quade and Roth (1997). Briefly, 15 µL of plasma were diluted with 135 µL of HBSS without Ca^{+2} and Mg^{+2} in flat-bottomed 96-well plates. Then, 50 µL of 20 mM 3,3',5,5'-tetramethylbenzidine hydrochloride (Sigma) and 50 µL of 5 mM H_2O_2 were added. The colour-change reaction was stopped after 2 min by adding 50 µL of 2 M sulphuric acid and the OD was read at 450 nm. The wells without plasma were used as blanks. The peroxidase activity (units mL^{-1} plasma) was determined by defining that one unit of peroxidase produces an absorbance change of 1 OD.

7.2.5. Gene expression analysis

Total RNA isolation was conducted with TRIzol Reagent (Invitrogen) following manufacturer's specifications in combination with the RNeasy mini kit (Quiagen) for RNA purification. Moreover, RNA (5 µg per sample) was treated with DNase I (Ambion) to remove contaminating DNA, and first-strand cDNA was synthesized with SuperScript™ II Reverse Transcriptase (Invitrogen) following the manufacturer's instructions. Quantitative PCR assays were performed with the 7300 Real Time PCR System (Applied Biosystems) using 1 µL cDNA mixed with 12.5 µL of SYBR green PCR master mix (Applied Biosystems) and 0.5 µL (10 µM) of each specific primer in a final volume of 25 µL. cDNA amplification was carried out with specific primers (Table 3), which were designed using Primer 3 software

according to known qPCR restrictions (amplicon size, Tm difference between primers, GC content and self-dimer or cross-dimer formation). The alignments of Senegalese sole HIF-1 and MIP1-alpha genes sequences showed the highest identity with *Dicentrarchus labrax* (86 % homology; e-value of $3e^{-133}$) and *Paralichthys olivaceus* (71 % homology; e-value of $1e^{-23}$), respectively. The efficiency of the primer pairs was analyzed in serial five-fold dilutions of cDNA by calculating the slope of the regression line of the cycle thresholds (Ct) versus the relative concentration of cDNA (Livak and Schmittgen, 2001). Efficiency values for g-type lysozyme (gLYS), hypoxia-inducible factor (HIF-1), hepcidin antimicrobial peptide 1 (HAMP-1) and macrophage inflammatory protein 1α (MIP1-alpha) were −3.03, −3.41, −3.49 and −2.79, respectively. Melting curve analysis was also performed to verify that no primer dimers were amplified. The standard cycling conditions were 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min. All reactions were carried out as technical triplicates. The Ct values were compared through the comparative Ct method to the geometric mean derived from the expression of Senegalese sole 18S rRNA (Livak and Schmittgen, 2001). The expression of the candidate genes was normalized using the RNA 18S ribosomal gene of Senegalese sole as a housekeeping gene, as it showed a constant expression level and was constitutively expressed independently of treatments. Fold change units were calculated by dividing the normalized expression values of tissues from different treatments by the normalized expression values of the control (unstressed fish fed Arg 4.4).

Table 3. Primers sequences of the genes analysed by real time PCR.

Gene		Primer sequence 5' – 3'	Accession no.	Product size
g-type lysozyme (gLYS)	Fwd:	CTCATTGCTGGCATCATCTC	AB428773	89 bp
	Rev:	TACGCTCCTCTGCTTGGATT		
Hipoxia inducible factor (HIF-1)	Fwd:	TGCCACGCAGTAAAAATCAG	FF288855.1	140 bp
	Rev:	CTTCACCGCTTTGACTCGTA		
Hepcidin antimicrobial peptide 1 (HAMP-1)	Fwd:	AAACTGAGCAGCGTCTGACA	AB455099	109 bp
	Rev:	TGAATGCCTTCATCTTCACG		
Macrophage inflammatory protein 1α (MIP1-alpha)	Fwd:	TTGCAGAGCACAGAGGAGAG	FF282572.1	105 bp
	Rev:	TCGTCAATCGTCTTCCACAG		
rRNA 18s Senegalese sole	Fwd:	GCCGTTCTTAGTTGGTGGAG	AM882675	144 bp
	Rev:	CTCAATCTCGTGTGGCTGAA		

7.2.6. Bacterial challenge

The strain PC566.1 of *Photobacterium damselae* subsp. *piscicida* (*Phdp*) used in this study was kindly provided by Dr. Ana do Vale (Institute for Molecular and Cell Biology, University of Porto, Portugal) and isolated from infected Senegalese sole at a fish farm in Spain by Professor Alicia E. Toranzo (Departamento de Microbiología y Parasitología, Facultad de Biología, University of Santiago de Compostela, Spain) (Magariños et al., 2003). The virulence of the pathogen and challenge dose used for this study were determined from a preliminary LD_{50} experiment (Costas et al., unpublished results). Bacteria were routinely cultured at 22 ºC in tryptic soy broth (TSB) or tryptic soy agar (TSA) (both from Difco Laboratories, Detroit, MI, USA) supplemented with NaCl to a final concentration of 1 % (w/v) (TSB-1 and TSA-1, respectively) and were stored frozen at -70 ºC in TSB-1 supplemented with 15% (v/v) glycerol. To prepare the inocula for injection into the fish peritoneal cavities, stocked bacteria were cultured for 48 h at 22 ºC on TSA-1 and then inoculated into TSB-1, and cultured overnight at the same temperature, with continuous shaking (100 rpm). Exponentially growing bacteria were collected by centrifugation at 3500 × *g* for 30 min, resuspended in sterile phosphate buffered saline (PBS) and adjusted to 5 × 10^3 cfu mL^{-1}. Plating serial dilutions of the suspensions onto TSA-1 plates and counting the number of cfu following incubation at 22 ºC confirmed bacterial concentration of the inocula.

At the end of the 14 days experimental period, 17 fish from each tank were anaesthetised (MS-222, 200 mg L^{-1}) and injected intraperitoneally (i.p.) with either 100 µL PBS (controls; n = 8) or an equivalent volume of PBS containing 5 × 10^3 cfu mL^{-1} (n = 9) of *Phdp*. Fish i.p. injected with PBS were maintained in separate tanks separated from fish i.p. with *Phdp*. Tanks were daily cleaned and temperature, salinity, dissolved oxygen and ammonia and nitrite levels in the water were daily measured. Dead fish were removed from each tank daily for 21 days and analyzed to reisolate the inoculated pathogen. During this period, fish continued to be fed on the same experimental diets as before.

7.2.7. Statistical analysis

All results are expressed as means ± standard deviation (SD). Data were analysed for normality (Kolomogorov-Smirnov test) and homogeneity of variance (Levene's test) and, when necessary, they were log-transformed before being treated statistically. Data from experimental diets were analysed by one-way analysis of variance (ANOVA), while data from fish were analysed by two-way ANOVA with chronic stress and dietary treatment as dependent variables. When significant differences were obtained from the ANOVA, Tukey's post hoc tests were carried out to identify significantly different groups fed the experimental diets. In addition, Student's *t*-tests were carried out for paired-comparisons to analyse the effect of chronic stress. All statistical analyses were performed using the computer package SPSS 15.0 for WINDOWS. The level of significance used was $P \leq 0.05$ for all statistical tests.

7.3. Results

7.3.1. Diets

Feed ingredients and the analysed feed composition are given in Table 1. Protein, lipids, DM, ash and energy levels were similar in all diets, whereas the AA profiles (g 16 g^{-1} N) presented significant differences among dietary treatments. As expected, arginine levels increased directly proportional to the level of supplementation (Table 2). Tryptophan could not be analysed by the method used, and therefore is not part of the evaluation of the AA profile.

7.3.2. Innate immune parameters

7.3.2.1. Cellular responses

Respiratory burst activity increased parallel to arginine supplementation in head-kidney leucocytes from control specimens ($P < 0.001$). In addition, head-kidney cells from handled fish presented a decreased respiratory burst when compared to control fish fed the same amount of arginine. Leucocytes from handled specimens fed the Arg 6.9 diet showed an increased respiratory burst

compared to that of specimens fed the Arg 4.4 and Arg 5.7 diets (Figure 1). No significant interactions between chronic stress and dietary treatments were observed (P = 0.187). Nitric oxide production increased parallel to arginine supplementation in head-kidney leucocytes from both control and handled specimens (P < 0.001). Moreover, head-kidney cells from stressed fish fed the Arg 5.7 and Arg 6.9 diets showed an increased NO production (P < 0.001) compared to leucocytes from control specimens fed the same experimental diets (Figure 2), which seems to depend on both the stressor and arginine as shown by the significant interaction between chronic stress and dietary treatments (P < 0.001).

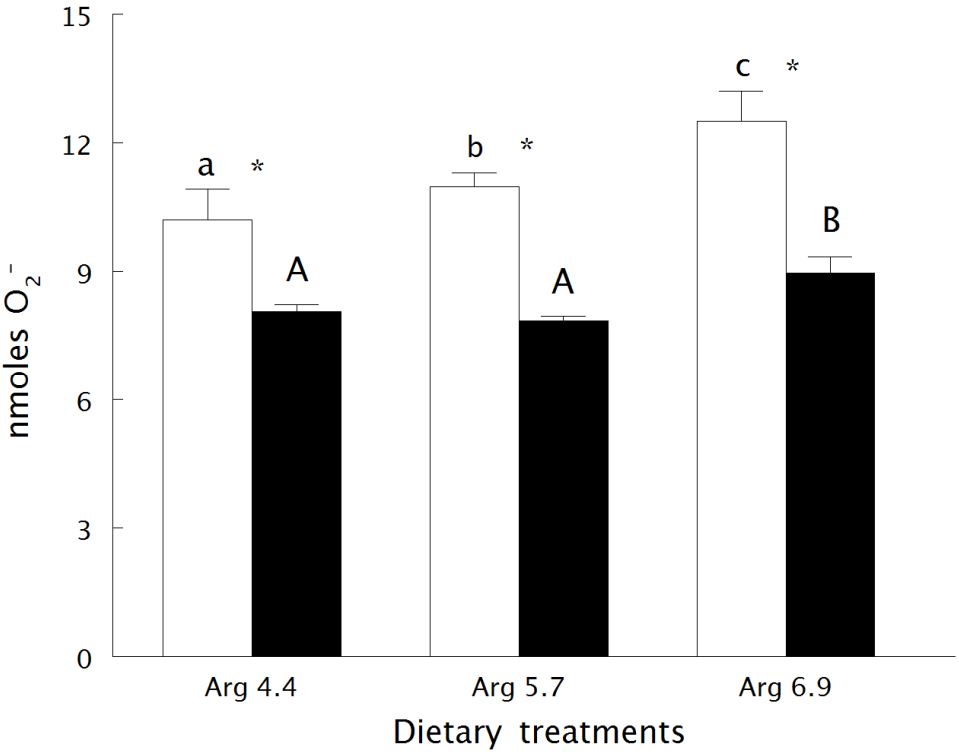

Figure 1. Respiratory burst activity of Senegalese sole head-kidney leucocytes after 14 days held at different treatments. Control (□) or air exposed (■) fish. Data are expressed as means ± SD (n = 4). Different letters stand for significant differences within control or handled fish (two-way ANOVA; P < 0.05), and * for significant differences attributed to air exposure (t-Test; P < 0.05).

7.3.2.2. Humoral responses

Dietary arginine supplementation and handling treatments induced similar responses in some humoral parameters of the innate immune system. While

plasma lysozyme increased in control fish fed the Arg 5.7 diet compared to fish fed the Arg 4.4 diet (P = 0.005), those levels were significantly higher (P = 0.002) in handled fish compared to control fish fed the same amount of arginine (Table 4). No significant interactions between chronic stress and dietary treatments were observed (P = 0.243). Similar to that observed for lysozyme, ACP activity increased in control fish fed the Arg 5.7 and Arg 6.9 diets (P < 0.001), while those levels were significantly higher in handled specimens when compared to control fish. In addition, handled specimens fed the Arg 6.9 diet showed significantly higher levels (P = 0.003) than handled fish fed the Arg 4.4 diet (Table 4). No significant interactions between chronic stress and dietary treatments were observed (P = 0.505). Plasma peroxidase activity was significantly higher (P < 0.001) in control fish fed the Arg 6.9 diet than in unstressed specimens fed the Arg 4.4 and Arg 5.7 diets, with no significant changes among handled fish (Table 4). No significant interactions between chronic stress and dietary treatments were observed (P = 0.433).

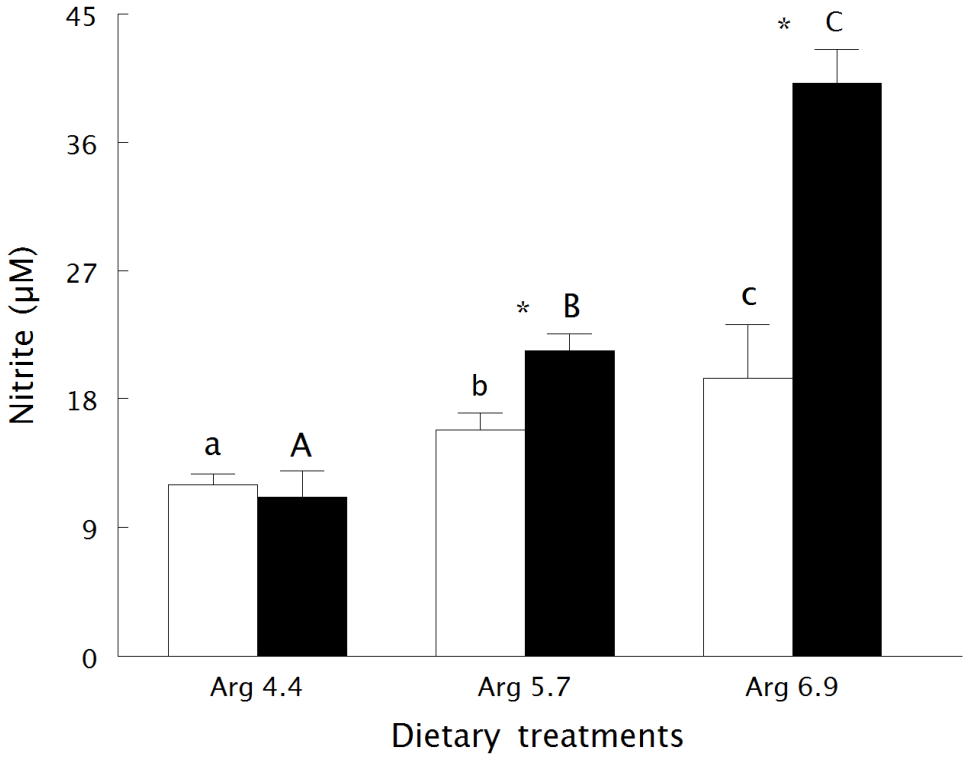

Figure 2. Nitric oxide production of Senegalese sole head-kidney leucocytes after 14 days held at different treatments. Control (□) or air exposed (■) fish. Data are expressed as mean concentration of nitrites (µM) ± SD (n = 4). Different letters stand for significant differences within control or handled fish (two-way ANOVA; P < 0.05), and * for significant differences attributed to air exposure (t-Test; P < 0.05).

Table 4. Effects of different treatments on humoral non-specific immune parameters of Senegalese sole after 14 days of feeding.

Humoral parameters	Treatments					
	Control			*Handling*		
	Arg 4.4	Arg 5.7	Arg 6.9	Arg 4.4	Arg 5.7	Arg 6.9
Lysozyme (μg mg protein^{-1})	14.0 ± 2.1 ^{a}	20.5 ± 2.0 ^{b}	17.1 ± 2.7 ^{ab}	26.9 ± 7.5 *	23.3 ± 7.8 *	27.8 ± 8.1 *
ACH50 (units mL^{-1})	123.9 ± 33.8 ^{a}	223.9 ± 54.8 ^{b}	262.9 ± 44.1 ^{b}	221.9 ± 46.5 ^{A*}	260.3 ± 47.5 ^{AB*}	348.6 ± 74.8 ^{B*}
Peroxidase (units mL^{-1})	115.1 ± 24.6 ^{a}	287.1 ± 146.7 ^{a}	610.1 ± 203.4 ^{b}	107.5 ± 34.5	116.3 ± 37.1	106.2 ± 13.0

Values are means ± SD (n = 8). Different letters stand for significant differences within control or handling fish (two-way ANOVA; $P < 0.05$), and * for significant differences relative to fish held at control Arg 4.4 (*t*-Test; $P < 0.05$).

7.3.3. Gene expression

The level of expression of gLYS and HIF-1 increased significantly in handled specimens regardless of dietary treatment (P = 0.003 and < 0.001, respectively). Control fish fed the Arg 5.7 diet showed increased gLYS expression levels compared to control fish fed the Arg 4.4 diet (Fig. 3A; P = 0.031). In addition, the level of expression of HIF-1 increased in control fish fed the Arg 5.7 (P = 0.049) and Arg 6.9 (P = 0.036) diets when compared to control fish fed the Arg 4.4 diet (Fig. 3B). Furthermore, HAMP-1 expression values increased significantly (P = 0.047) in control fish fed the Arg 5.7 and Arg 6.9 diets compared to control fish fed the Arg 4.4 diet, with no significant changes among handled fish (Fig. 3C). No significant interactions between chronic stress and dietary treatments were observed for gLYS, HIF-1 and HAMP-1 (P = 0.808, 0.552 and 0.230, respectively). Similar to that observed for HIF-1 and HAMP-1 mRNA transcripts, control fish fed the Arg 5.7 and Arg 6.9 diets showed higher MIP1-alpha expression levels (P = 0.023 and 0.031, respectively) than control fish fed the Arg 4.4 diet. Moreover, handling treatment induced the highest level of expression (9.2 fold increase) of MIP1-alpha in fish fed the Arg 4.4 diet (P = 0.022), whereas handled specimens fed the Arg 5.7 diet showed higher MIP1-alpha expression values (2.5 fold increase; P = 0.044) than control fish fed the Arg 4.4 diet (Fig. 3D). In addition, mRNA transcripts of this particular gene seem to depend on both the stressor and experimental diets as shown by the significant interaction between chronic stress and dietary treatments (P = 0.002). In fact, diets Arg 5.7 and 6.9 reduced the effect of stress in MIP1-alpha expression values.

7.3.4. Bacterial challenge

Handled specimens presented an increased resistance to *Phdp* than unstressed fish regardless of dietary treatment, whereas control fish fed the Arg 6.9 diet were less susceptible to the pathogen than control fish fed the Arg 4.4 and 5.7 diets. Control Senegalese sole started to die at day 5 whereas first mortalities were only recorded at day 6 in handled fish (Fig. 4).

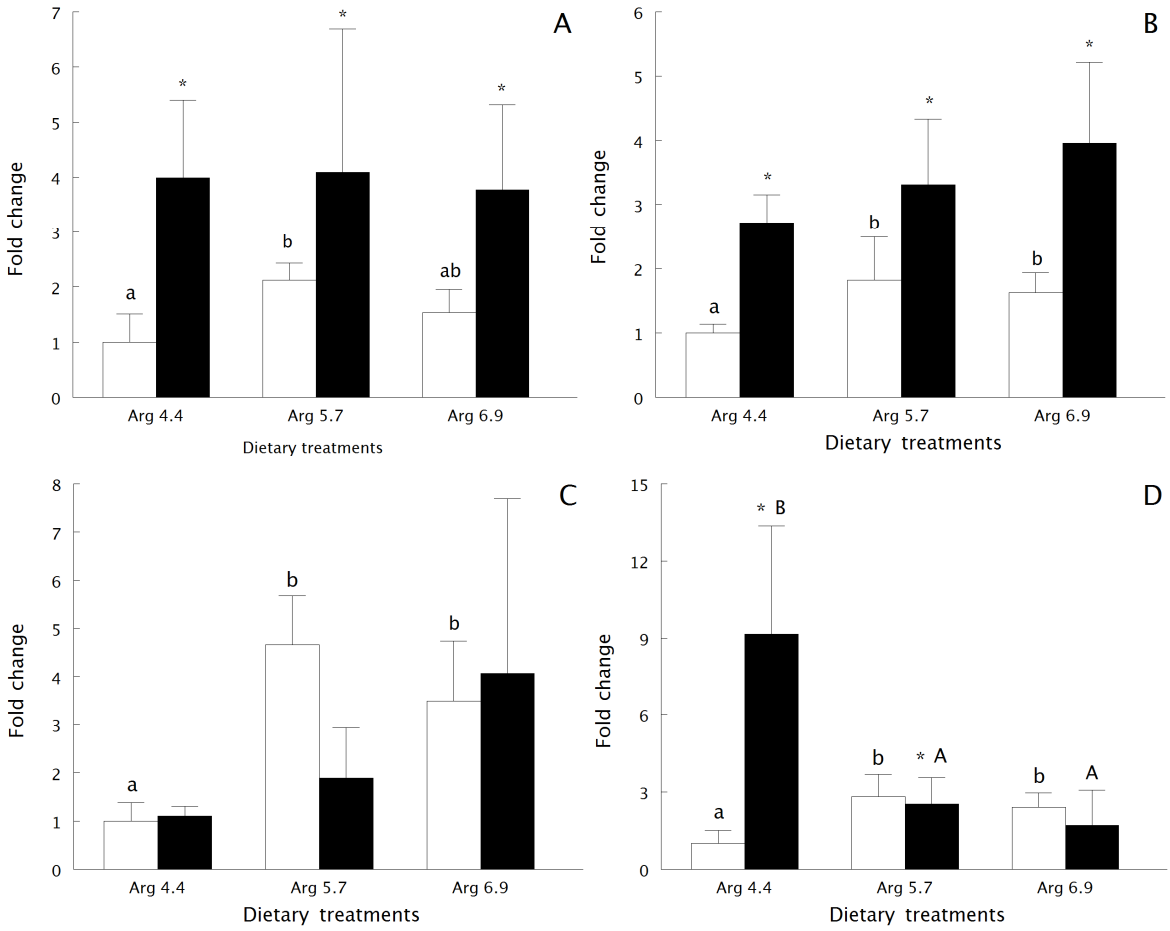

Figure 3. Quantitative expression of gLYS (A), HIF-1 (B), HAMP-1 (C) and MIP1-alpha (D) in the head-kidney of Senegalese sole after 14 days held at different treatments. Data are expressed as means ± SD (n = 4). Bars represent the fold increase in expression of each gene as compared to control specimens fed the Arg 4.4 diet, previously normalized to endogenous 18s expression levels. Different letters stand for significant differences within control or handled fish (two-way ANOVA; $P < 0.05$), and * for significant differences attributed to air exposure (*t*-Test; $P < 0.05$).

7.4. Discussion

The increased disease resistance presented by handled fish from this study was surprising since chronic stress is known to impair the immune system (Wendelaar Bonga, 1997). Previous studies have demonstrated that air exposure once a week represents a chronic stressful condition for Senegalese sole after 28 and 60 days (Aragão et al., 2008; Costas et al., unpublished results). Therefore, handling procedures from the present study are expected to induce a chronic

stress response in sole. However, exactly how the hormones involved in this response affect the immune system is still unclear. Although repeated acute stress is usually considered as a chronic stressful situation, it is not necessarily detrimental as long as the rapid physiological response mounted in the presence of the stressor returns to resting levels upon cessation of stressor, and as long as the frequency of repeated stress responses is not such that it results in a constant increased exposure to stress-related hormones (Dhabhar, 2009). It was previously reported that cortisol levels in air exposed Senegalese sole peak at 1 h, dropping quickly after that and returning to basal levels within 4 h (Costas et al., 2011b). Therefore, it is possible that the daily short-time stress exposure from this study was not enough to maintain chronically high cortisol levels, and may have activated some survival-promoting aspects, resulting in physiological conditions which may elucidate the increased disease resistance observed in handled fish regardless of dietary treatment.

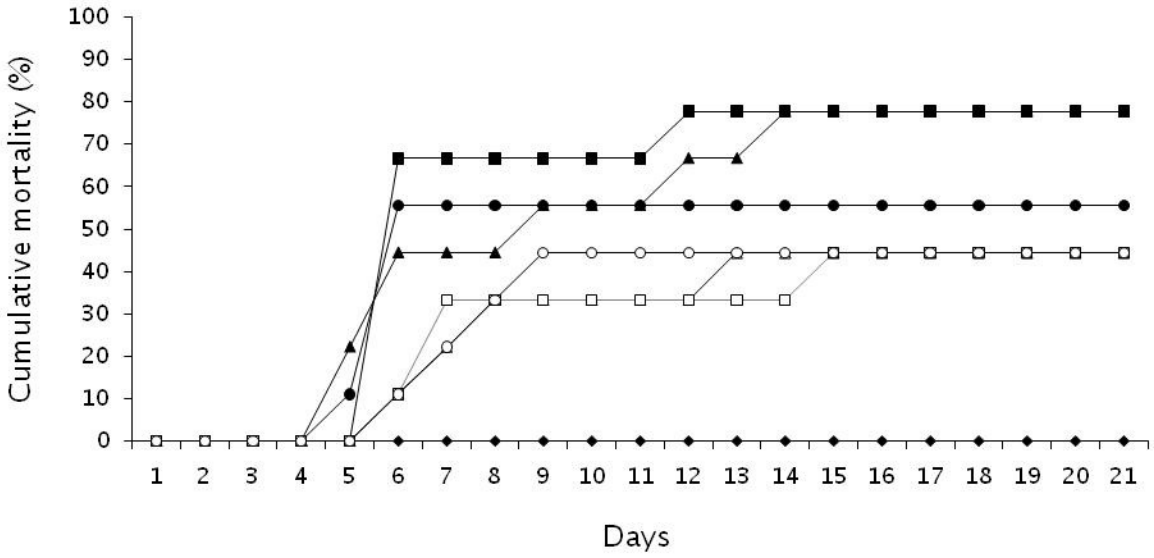

Figure 4. Cumulative mortality (%) of Senegalese sole held at different dietary treatments following chronic stress challenge and i.p. injection of *Photobacterium damseale* subsp. *piscicida* (strain PC566.1; LD_{50}, 5×10^3 cfu mL^{-1}). Control fish fed Arg 4.4 (▲), Arg 5.7 (■) or Arg 6.9 (●), and handled fish fed Arg 4.4 (Δ), Arg 5.7 (□) or Arg 6.9 (○) i.p. injected with *Photobacterium damselae* subsp. *piscicida* (n = 9 per treatment), or the same treatments (♦) i.p. injected with PBS (n = 8 per treatment).

Head-kidney leucocytes from handled specimens showed different responses for both cellular and humoral immune parameters. The general decrease in the respiratory burst of head-kidney cells from handled fish was consistent with the suppressive effects generally attributed to long term exposure to stressors. Similarly, Binuramesh et al. (2005) reported a decrease in the respiratory burst activity of peripheral blood leucocytes of Mozambique tilapia (*Oreochromis mossambicus*) submitted to confinement for 28 days. Moreover, the same outcome has also been observed in head-kidney leucocytes of rainbow trout (*Oncorhynchus mykiss*), dab (*Limanda limanda*), common carp (*Cyprinus carpio*) and gilthead seabream (*Sparus aurata*) following handling, anoxic shock, crowding and *in vitro* exposure to various cortisol concentrations (Angelidis et al., 1987; Pulsford et al., 1994; Yin et al., 1995; Esteban et al., 2004). Similarly, several authors have also demonstrated the inhibitory effect of cortisol on NO production by fish macrophages (Wang and Belosevic, 1995; Saeij et al., 2003a). However, when head-kidney leucocytes from carp were stimulated with high concentrations of LPS (25 and 50 μg mL^{-1}), cortisol increased NO production (Saeij et al., 2003a). Since head-kidney cells are a mixed population of macrophages, neutrophils and some lymphocytes, the ability of cortisol to increase NO production is most likely attributed to its inhibitory effect on the apoptosis of neutrophils (Weyts et al., 1998b). Therefore, the increased NO production of head-kidney cells from handled fish fed the Arg 5.7 and Arg 6.9 diets was most likely produced by the neutrophils population.

In this study, the stimulatory action of dietary arginine supplementation observed for NO production in both control and handled fish is consistent with previous findings (Buentello and Gatlin, 1999), and proved the efficiency of dietary treatments on influencing the innate immune system of sole. Furthermore, the lack of difference in NO production between control and handled fish fed the Arg 4.4 diet, together with increased NO values in fish fed higher arginine amounts or in stressed fish, suggests that dietary arginine supplementation may have a major role on NO production. To our knowledge, this is the first report in fish showing an increase in the respiratory burst of head-kidney cells after dietary arginine treatment. This finding was surprising since a dangerous outcome from the combination of increased NO formation and augmented generation of superoxide anion is the formation of peroxinitrite, which has a high cytotoxic potency (Roth, 2007). However, it has been reported

that carp head-kidney phagocytes (neutrophils and macrophages) are highly resistant to nitrosative stress by increasing both total intracellular glutathione (GSH) and the activity of γ-glutamylcysteine synthetase, an enzyme with the ability to synthesize new GSH (Saeij et al., 2003b). Therefore, a similar detoxifying mechanism may have been employed by head-kidney cells of undisturbed Senegalese sole, being congruent with the higher resistance to *Phdp* presented by fish fed the Arg 6.9 diet.

Handled specimens from the current study also showed an increase in plasma lysozyme and ACP activities as well as in the level of expression of gLYS. These innate humoral immune responses appear to depend on the species and type and duration of the stress imposed. In some cases, plasma lysozyme and ACP activities and gLYS expression values decrease or no changes are observed (Sunyer et al., 1995; Salas-Leiton et al., 2010; Costas et al., 2011b; Mauri et al., 2011), while in other studies plasma lysozyme activity significantly increased in stressed specimens (Demers and Bayne, 1997; Rotllant et al., 1997; Caipang et al., 2009). These differential effects may be achieved by differences in overall glucocorticoid sensitivity or receptivity of the immune response being affected (Dhabhar, 2009). Therefore, the effects of stress or cortisol on the fish immune system could be described to be regulatory and not necessarily inhibitory. For instance, stress hormones induced apoptosis in carp lymphocytes but inhibited apoptosis of neutrophils, indicating a selective regulation of innate and adaptive immune responses (Weyts et al., 1998a, 1998b). Moreover, a drop in ambient temperature reduced the number of circulating lymphocytes in carp, whereas the relative percentage of circulating granulocytes nearly doubled (Engelsma et al., 2003). Since high cortisol levels are expected in handled fish from the present study, an increase in peripheral neutrophil numbers is plausible and would correlate well with the augmented plasma lysozyme levels. These phagocytes are thought to be the source of plasma lysozyme (Murray and Fletcher, 1976), and increased lysozyme levels have been associated with enhanced neutrophil numbers (Muona and Soivio, 1992). Moreover, complement proteins can stimulate phagocytosis by opsonising pathogens, a process that is mediated by complement receptors on the surface of phagocytic cells (Holland and Lambris 2002). Increased plasma ACP activity has been associated with augmented neutrophil numbers in the Senegalese sole peritoneal cavity (Costas et al., unpublished results). Considering the role of both macrophages and neutrophils

on phagocytosing bacteria, together with the significant increase in expression values of gLYS and plasma lysozyme and ACH50 values in handled fish from the current study, it is likely that stressed specimens had a higher level of protection than control fish to resist *Phdp* at the moment of infection. The increased level of expression of HIF-1 observed in handled specimens would further support this hypothesis as suggested by the biological function of HIF-1 described by Cramer et al. (2003). These authors reported that HIF-1 is essential for the regulation of glycolytic capacity and energy metabolism in myeloid cells (granulocytes and monocytes/macrophages), and that in the absence of this gene the cellular ATP pool is drastically reduced, resulting in impairment of myeloid cell aggregation, motility, invasiveness, and bacterial killing.

The level of expression of MIP1-alpha increased drastically in handled fish fed the Arg 4.4 diet, being consistent with the higher disease resistance revealed by these specimens when compared to control fish. MIP1-alpha belongs to the pro-inflammatory CC chemokines, which are small proteins that control cellular migration (Laing and Secombes, 2004). Similarly, the level of expression of interleukin-1β (a pro-inflammatory cytokine) increased in Atlantic salmon (*Salmo salar*) after 7, 14 and 28 days submitted to daily handling (Fast et al., 2008). In contrast, MIP1-alpha expression values from the current study decreased parallel to arginine supplementation in handled specimens. This outcome could be related to the aforementioned expected increase in GSH levels due to nitrosative stress. In fact, NO and MIP1-alpha expression values from handled specimens showed an inverse linear relationship ($y = -2.0293\times + 31.48$; $R^2 = 0.4662$; $p = 0.021$). Interestingly, Barret et al. (1999) observed a decrease in the mRNA levels of MIP1-alpha in macrophages treated with extracellular GSH, suggesting that the regulation of macrophage pro-inflammatory genes is mediated in part by an oxidant-dependent mechanism. Based on the present study, it is tempting to speculate that the expected increase of intracellular GSH in leucocytes from handled specimens, acting as an antioxidant due to enhanced NO and respiratory burst levels, could be an important mediator of the stress-induced pro-inflammatory gene regulation in sole.

Dietary arginine supplementation clearly increased some aspects of the Senegalese sole innate immune response. While *in vitro* cellular responses increased parallel to dietary arginine supplementation, *in vivo* expression values

of most immune-related genes and humoral mechanisms augmented in control specimens fed the Arg 5.7 and Arg 6.9 diets. The up-regulation of HAMP-1 gene transcripts could be related to an increased availability of polyamines for leucocytes growth and differentiation. Arginine is a precursor substrate for polyamines biosynthesis and seabream leucocytes incubated with polyamines showed increased HAMP-1 expression values (Satriano et al., 1999; Reyes-Berrecil et al., 2011). Hepcidin gene expression has been demonstrated to be involved in both iron regulation and immunity (Rodrigues et al., 2006). Therefore, considering the role of HAMP-1, HIF-1, gLYS and MIP1-alpha genes on innate immunity, together with the increased cellular and humoral immune parameters observed in control fish fed arginine supplements, the importance of dietary arginine on the innate immune mechanisms of Senegalese sole is clearly demonstrated in the present study. Furthermore, these data are congruent with the increased disease resistance observed in control fish fed the Arg 6.9 diet. However, undisturbed fish fed the Arg 5.7 diet failed to resist *Phdp* in a similar way to control fish fed the Arg 6.9 diet. This outcome is difficult to explain according to results from innate immune parameters. It is tempting to speculate that Arg 6.9 diet may have stimulated polyamine synthesis, thus increasing their availability for neutrophils proliferation in the head-kidney. This hypothesis is further supported by the increased plasma peroxidase levels observed in undisturbed fish fed the Arg 6.9 diet. Enhanced peroxidase values have been associated with increases in neutrophil numbers in fish (Cerezuela et al., 2009).

In summary, the suppressive effect of stress on the innate immune system is highly disputable and does not necessarily translate in decrease resistance to infection. Depending on the duration and severity of the stressor, increased glucocorticoid levels may enhance innate and adaptive immune responses while similar stress level may also suppress immune function. In the current study, daily handling stress for 14 days induced a higher disease resistance in handled fish than in undisturbed specimens, together with increased cellular (NO production) and humoral immune responses (plasma lysozyme and ACP activities) as well as gLYS and HIF-1 expression values at the time of bacterial infection. However, the exact mechanisms by which stressed Senegalese sole resisted to *Phdp* infection remain to be elucidated, and future studies should address mechanisms of action and regulation of cytokines and other immune-related genes following bacterial challenge in previously stressed fish. Furthermore,

dietary arginine supplementation enhanced most aspects of the innate immune mechanisms assessed and increased disease resistance at the maximum level of supplementation. It is concluded that the Arg 6.9 diet could be used in practical aquaculture conditions, to assist Senegalese sole to resist bacterial infections.

7.5. Acknowledgements

This study was partially supported by project CSD2007-00002 Aquagenomics funded by the program Consolider-Ingenio 2010 from the Spanish Ministerio de Ciencia e Innovación. SPAROS participation in this study had the support of project ANYFISH, co-funded by PO Algarve 21, QREN and FEDER (European Union). Benjamín Costas benefited from grant by Fundação para a Ciência e a Tecnologia, Portugal (SFRH/BD/38697/2007). The authors are grateful to Dr. Ana do Vale (Institute for Molecular and Cell Biology, University of Porto, Portugal) for providing the bacterial strain used in this study.

7.6. References

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Chapter 8

Partial fish meal replacement by plant-protein sources and indispensable amino acids supplementation enhance some innate immune parameters of Senegalese sole (*Solea senegalensis* Kaup, 1858) juveniles

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Costas, B., Silva, J.M.G., Conceição, L.E.C., Dias, J., Valente, L.M.P., Afonso, A., 2011. Partial fish meal replacement by plant-protein sources and indispensable amino acids supplementation enhance some innate immune parameters of Senegalese sole (*Solea senegalensis* Kaup, 1858) juveniles. Submitted.

Partial fish meal replacement by plant-protein sources and indispensable amino acids supplementation enhance some innate immune parameters of Senegalese sole (*Solea senegalensis* Kaup, 1858) juveniles

Abstract

The effect of partial or total replacement of fish meal by plant-protein ingredients balanced with indispensable amino acids (IAA) on some innate immune parameters was assessed in Senegalese sole juveniles. A reference diet (F45) contained fish meal as the main protein source, while four plant-protein-based diets contained either 5 % (F15) or no fishmeal (F5). These diets also contained 10 or 5 % of protein from marine sources, respectively. The rest of the protein derived from a blend of soybean meal, corn and wheat gluten. Thus, the plant-protein fraction in these test diets ranged from 70–80 % of crude protein level. The plant-based diets were supplemented either with a mixture of IAA (F15 + IAA, F5 + IAA) or only with lysine (F15 + Lys, F5 + Lys). Fish around 9.5 g were fed the experimental diets by automatic feeders over 24 hours. After a period of 12 weeks, head-kidney leucocytes and serum were collected. Head-kidney leucocytes respiratory burst activity was significantly higher in fish fed the F15 + Lys diet. In addition, fish fed the F15 + IAA diet showed the highest serum lysozyme, alternative complement pathway and peroxidase activities. Results suggest that an enhancement of these innate immune parameters appears to be related both with IAA dietary balance and partial fish meal replacement by plant-protein sources. However, the effects of such long-term immunomodulatory pattern associated to changes in dietary protein quality and its effectiveness on promoting disease resistance require further assessments.

Keywords: Indispensable amino acids; Fish meal; Plant proteins; Humoral immune parameters; Respiratory burst; Senegalese sole.

8.1. Introduction

The increasing demand of fish meal-based diets in the aquaculture sector is not sustainable and has raised the need to search for alternative protein sources. Most research has been focused on fish meal replacement by plant-

protein ingredients, which are not well balanced in indispensable amino acids (IAA) according to the requirements of fish (Tacon and Jackson, 1985). Some studies have shown that a sensible blending of different plant-protein sources is more appropriate to obtain adequate amino acid (AA) profile compared to the incorporation of a single plant-protein source (Watanabe et al., 1995; Regost et al., 1999; Espe et al., 2006). In addition, some crystalline AA may have to be supplemented to fulfil the AA requirements of a given species (Espe et al., 2006). Furthermore, nutrients can have significant effects on disease resistance mechanisms, and the influence that dietary factors may have on disease outbreaks in cultured fish has been recognised for many years (Blazer, 1992). The suppression of the non-specific immune capacity by high concentrations of dietary soybean proteins has been reported in fish (Burrells et al., 1999). Moreover, fish fed diets with 100 % protein provided by different plant sources were more susceptible to bacterial infection compared with fish fed diets with animal protein sources (Neji et al., 1993). However, Sitjà-Bobadilla et al. (2005) observed that fish meal replacement by a mixture of plant-protein sources induced different innate immune responses in gilthead seabream (*Sparus aurata*), including an enhancement of the respiratory burst and a decrease in complement values at the 75 % level of replacement. On the other hand, the major fate of AA is towards protein synthesis, but emerging evidence shows that many AA regulate key metabolic pathways that are crucial to maintenance, growth, reproduction and immune responses (Li et al., 2009). A positive effect of an arginine-enriched diet on the resistance of channel catfish (*Ictalurus punctatus*) to infection with *Edwardsiella ictaluri* has been demonstrated (Buentello and Gatlin, 2001). In addition, several studies indicate that an inadequate intake of branched-chain amino acids (BCAA: leucine, isoleucine and valine) results in immune impairment (Li et al., 2007).

Senegalese sole (*Solea senegalensis* Kaup, 1858) constitutes a new high valuable option in Southern European aquaculture, mostly due to a wide market and good prices. Recently, Senegalese sole capacity to perform well with marine-derived protein replacement by plant-protein ingredients has been showed (Silva et al., 2010). The importance of a balanced AA profile, achieved through a supplementation of either a mixture of crystalline AA or only lysine, the putative first limiting amino acid, on the overall growth performance and nutrient utilization of juvenile sole was assessed by Silva et al. (2009). However, the effect

of fish meal replacement by plant-protein sources on innate immune parameters still needs to be assessed, and little is known about the effects of IAA balance on non-specific immune mechanisms in fish. Therefore, the aim of the present study was to contribute to this endeavour by assessing some innate immune parameters. This gains particular importance in a fish species such as Senegalese sole, which and has been demonstrated to be highly susceptible to opportunistic pathogens under stressful conditions (**Chapter 2**).

8.2. Material and methods

Five extruded isonitrogenous and isoenergetic diets (57 % crude protein, 23 kJ g^{-1} energy on a DM basis) were formulated: a reference diet contained fish meal as the main protein source (F45, containing 37 % fish meal and 45% of the dietary protein being from marine sources) and four plant-protein diets either containing 5 % (F15) or no fishmeal (F5). The F15 diets also contained 10 % of protein being from marine sources while the F5 diets contained only 5 %. The rest of the protein derived from a blend of soybean meal, corn and wheat gluten. Thus, the plant-protein fraction in these test diets ranged from approximately 70-80 % of crude protein level. These plant-based diets were supplemented with either a mixture of indispensable AA (arginine, histidine, isoleucine, leucine, lysine, threonine, tryptophan, valine and methionine) required to mimic the AA pattern of the reference diet (F15 + IAA, F5 + IAA) or only with lysine, which was added to all diets according to both the level present in the reference diet but also considering the requirement previously determined for Senegalese sole juveniles (Silva et al., 2008). Plant-protein rich diets were further supplemented with mono calcium phosphate to avoid any phosphorus deficiency. Upon ingredient grinding with a hammer mill (0.8 mm sieve) and its mixing in a paddle mixer, all diets were manufactured using a twin-screw extruder (2.0 mm pellet size). Subsequently oil was added under vacuum coating conditions in a DINNISEN Pegasus vacuum mixer (PG-10VCLAB). The diets were dried at 45 ºC for 12 h and stored at 4 ºC until use. Ingredients and amino acids composition of the experimental diets are presented in Table 1. For additional details on diet composition see Silva et al. (2009).

Table 1. Ingredients and indispensable amino acids composition of experimental diets.

	Experimental diets				
	F45	F15+IAA	F15+Lys	F5+IAA	F5+Lys
Ingredients (%)					
Marine fish protein sources	45.0	15.0	15.0	5.0	5.0
Plant protein sources	32.5	51.8	57.3	64.5	68.9
Wheat flour	18.0	18.1	16.8	14.6	13.7
Fish oil	4.0	5.8	5.8	6.6	6.6
Crystalline AA supplements	0.0	6.1	1.9	5.4	1.9
Vitamins and Minerals	0.5	0.5	0.5	0.5	0.5
Mono Ca Phosphate	0.0	2.7	2.7	3.4	3.4
Indispensable amino acids composition (g 16 g^{-1} N)					
Arginine	4.6^{b}	5.4^{a}	4.4^{b}	5.2^{a}	4.4^{b}
Histidine	2.0^{ab}	2.1^{a}	1.8^{b}	2.1^{ab}	1.9^{b}
Isoleucine	3.6^{b}	3.8^{a}	3.5^{b}	4.0^{a}	3.5^{b}
Leucine	7.5^{c}	8.0^{ab}	7.9^{ab}	8.2^{a}	7.9^{b}
Lysine	5.3	5.5	5.8	5.9	5.8
Threonine	3.3^{b}	3.6^{a}	3.0^{c}	3.6^{a}	3.0^{c}
Valine	4.0^{b}	4.7^{a}	3.8^{b}	4.9^{a}	3.8^{b}
Methionine	1.9^{b}	2.2^{a}	1.5^{c}	2.1^{a}	1.4^{c}
Phenylalanine	3.9^{b}	4.0^{ab}	4.4^{ab}	4.2^{ab}	4.5^{a}

For further details on ingredients and crystalline IAA supplements please refer to Silva et al. (2009). Tryptophan was not analysed. Different letters within the same row indicate significant differences among dietary treatments for a given IAA (One-way ANOVA; $p \leq 0.05$).

The study was carried out at the CIIMAR facilities (Porto, Portugal) with Senegalese sole (*Solea senegalensis*) juveniles. After arrival at the experimental facilities from a commercial fish farm (Stolt Sea Farm S.A., Spain), fish were acclimated for two weeks and fed a commercial sole diet (Skretting, 59 % DM crude protein and 16 % DM crude fat). For each treatment, 54 fish of 9.5 ± 0.1 g (average initial body weight ± SD) were distributed in triplicate tanks (18 fish per tank). Therefore, 15 white fibre glass tanks (50 x 35 cm) were set up in a seawater recirculation system (temperature: 20 ± 1 ºC; salinity: 30 ‰; photoperiod: 16 h light:8 h dark; water flow rate: 2 L min^{-1}). Temperature, dissolved O_2, salinity, pH and nitrogenous compounds were monitored during the entire trial and maintained at levels within limits recommended for this species. The experiment lasted for 12 weeks and fish were fed by automatic feeders over 24 hours. Every day the feed amount left uneaten was monitored and used to adjust daily ration when necessary as previously described elsewhere (Borges et al. 2009).

At the end of the feeding trial, fish were fasted for 24 hours and blood and head-kidney were collected from 8 fish per dietary treatment. Blood was withdrawn from the caudal vein using non-heparinised syringes and left to clot during 4 hours at room temperature. The resulting serum was then collected and centrifuged (10000 x *g* for 5 min at room temperature). Serum samples were kept at - 80 ºC until further analysis.

Head-kidney leucocytes were isolated and maintained essentially as described by Secombes (1990). Briefly, under aseptic conditions, the head-kidney was removed, pushed through a 100 µm nylon mesh and suspended in Leibovitz L-15 medium (L15, Gibco) supplemented with 2% foetal bovine serum (FBS, Gibco), penicillin (100 IU mL^{-1}; P, Gibco), streptomycin (100 µg mL^{-1}; S, Gibco) and 20 units heparin mL^{-1} (B. Braun Medical Lda). The suspensions were then loaded onto a 30 %:51 % Percoll density gradient and centrifuged at 400 *g* and 4 ºC for 40 min. The band of cells laying at the interface of the Percoll gradient was collected, counted in haemocytometer and adjusted to 1 x 10^7 cells mL^{-1} in L15, 0.1 % FBS, P/S, heparin. Afterwards, cells were plated in 96 well plates at 100 µL per well. After 24 h, non adherent cells were removed by washing the cultures three times with L15. The respiratory burst activity was based on the reduction of ferricytochrome C method for the detection of O_2^- (Secombes 1990). To the monolayers were added 100 µL of ferricytochrome C solution (2 mg ferricytochrome C mL^{-1} phenol red-free HBSS). Ferricytochrome C solution containing 10 µg mL^{-1} phorbol myristate acetate (PMA, Sigma) was added to 3 wells of leucocytes monolayers per fish as a soluble stimulant of the respiratory burst. Ferricytochrome C with PMA and 0.725 mg mL^{-1} superoxide dismutase (Sigma) was used to confirm the specificity of the reaction. Plates were read 60 min after addition of reagents to the leucocytes on a Powerwave™ microplate spectrophotometer (BioTek) at 550 nm. Optical densities were converted to nmol of O_2^- produced by multiplying by 15.87 as described by Pick (1986), and the mean ± SD of each treatment was calculated.

Lysozyme activity was measured using a turbidimetric assay based on the method described by Ellis (1990) with some modifications (Wu et al., 2007). Briefly, a solution of *Micrococcus lysodeikticus* (0.5 mg mL^{-1} 0.05 M sodium phosphate buffer; pH 6.2) was prepared. To a microplate, 15 µL of serum and

250 µL of the above suspension were added to give a final volume of 265 µL. The reaction was carried out at 25 ºC and the absorbance (450 nm) is measured after 0.5 and 4.5 min. Lyophilized hen egg white lysozyme (Sigma) was serially diluted in sodium phosphate buffer (0.05 M; pH 6.2) and used to develop a standard curve. The amount of lysozyme in the sample was calculated using the formula of the standard curve. All analysis were conducted by triplicates.

Alternative complement pathway (ACP) activity was estimated as described by Sunyer and Tort (1995). The following buffers were used: GVB (Isotonic veronal buffered saline), pH 7.3, containing 0.1% gelatin; EDTA-GVB, as previous one but containing 20 mM EDTA; and Mg-EGTA-GVB, which is GVB with 10 mM Mg^{+2} and 10 mM EGTA. Rabbit red blood cells (RaRBC; Probiologica Lda, Portugal) were used for ACP determination. RaRBC were washed four times in GVB and resuspended in GVB to a concentration of 2.5×10^8 cells mL^{-1}. Ten µL of RaRBC suspension were then added to 40 µL of serially diluted serum in Mg- EGTA-GVB buffer. Samples were incubated at room temperature for 100 min with regular shaking. The reaction was stopped by adding 100 µL of cold EDTA-GVB. Samples were then centrifuged and the extent of hemolysis was estimated by measuring the optical density of the supernatant at 414 nm. The ACH50 units were defined as the concentration of serum giving 50% haemolysis of RaRBC. All analysis were conducted by triplicates.

Total peroxidase activity in serum was measured following the procedure described by Quade and Roth (1997). Briefly, 15 µL of serum (triplicates per fish) were diluted with 135 µL of HBSS without Ca^{+2} and Mg^{+2} in flat-bottomed 96-well plates. Then, 50 µL of 20 mM 3,3',5,5'-tetramethylbenzidine hydrochloride (Sigma) and 50 µL of 5 mM H_2O_2 were added. The colour-change reaction was stopped after 2 min by adding 50 µL of 2M sulphuric acid and the optical density was read at 450 nm in a Powerwave™ microplate spectrophotometer. The wells without serum were used as blanks. The peroxidase activity (units mL^{-1} serum) was determined defining one unit of peroxidase as that which produces an absorbance change of 1 OD.

Statistical analysis was performed using the computer package SPSS 15.0 for WINDOWS®. All results are expressed as means ± standard deviation (SD). Data among treatments were analysed by one-way analysis of variance (ANOVA). When

significant differences were obtained from the ANOVA, multiple comparisons were carried out performing Tukey – HSD mean comparison test. The level of significance used was $P \leq 0.05$ for all statistical tests.

8.3. Results and discussion

Head-kidney leucocytes respiratory burst activity (O_2^- production) was significantly higher in fish fed the F15 + Lys diet, and no significant differences were found among the other experimental groups (Figure 1). The increased O_2^- production observed in leucocytes from fish fed the F15 + Lys diet could suggest an important role of Lys on oxidative radical production. However, this enhancement on O_2^- production cannot be related to dietary Lys alone since leucocytes from fish fed the F5 + Lys diet did not show any changes with respect to leucocytes from fish fed the other experimental diets. Moreover, both F15 + Lys and F5 + Lys diets presented similar contents in Lys and other IAA (Silva et al., 2009). It is likely that this enhanced O_2^- production observed in leucocytes from fish fed the F15 + Lys diet is also related to other elements associated to the partial fish meal replacement by plant-protein sources (e.g. presence of anti-nutritional factors, altered levels of dietary trace elements). Increased leucocyte killing activity, assessed by oxidative radical production, was previously observed in fish fed soybean protein or a mixture of plant-protein sources (Rumsey et al., 1994; Sitjà-Bobadilla et al., 2005). Still, in the present study this effect of partial fish meal replacement on O_2^- production seems to depend on the replacement level, as no effect was observed with the F5 + Lys or F5 + IAA. On the other hand, carrier-mediated transporters often have overlapping specificities and interactions between AA seem to interfere with the uptake of a specific AA (Collie and Ferraris, 1995). The fact that leucocytes from fish fed the F15 + IAA diet did not show a higher O_2^- production may be related to a competition among different free IAA for the same transporters and thus producing an inhibitory effect on the uptake of Lys. Therefore, leucocytes O_2^- production from Senegalese sole may be increased by feeding fish a diet with a moderate fish meal replacement by a mixture of plant-protein sources and Lys supplementation. However, since fish fed diets with a high protein proportion provided by different plant sources have been shown to be more susceptible to bacterial infection compared with fish fed diets with animal protein sources (Neji et al., 1993;

Burrells et al., 1999), fish fed these plant-protein based diets should be submitted to a bacterial challenge in order to verify its effects on disease resistance.

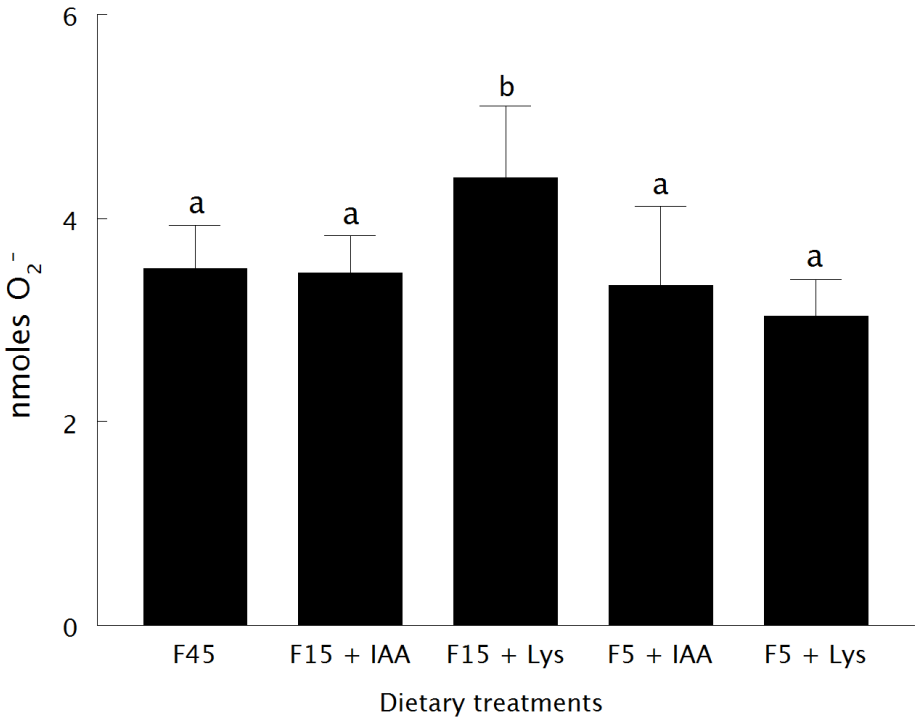

Figure 1. Respiratory burst activity from head-kidney leucocytes of Senegalese sole fed the experimental diets. Data are presented as nmol of O_2^- produced, expressed as means ± SD (n = 8). Different letters mean significant differences among groups (ANOVA; $P \leq 0.05$).

The humoral non-specific immune parameters assessed in the present study were also significantly affected by dietary treatments. While fish fed the F15 + IAA diet showed the significantly highest serum lysozyme and ACP activities, fish fed the F15 + Lys and F5 + IAA diets showed significantly higher values than fish fed the F5 + Lys diet for serum lysozyme activity (Figure 2), and than fish fed the F45 and F5 + Lys diets for ACP activity (Figure 3). Moreover, serum peroxidase activity was significantly higher in fish fed the F15 + IAA diet than fish fed the F15 + Lys, F5 + IAA and F5 + Lys diets (Figure 4). The enhanced lysozyme, ACP and peroxidase activities observed in fish fed the F15 + IAA diet may suggest that a moderate fish meal replacement (diet F15 + IAA) while keeping a well balanced dietary IAA profile may play an important role on the

Senegalese sole humoral non-specific immune mechanisms. In fact, arginine, isoleucine, threonine, valine and methionine were more abundant in diets F15 + IAA and F5 + IAA than in F45, F15 + Lys and F5 + Lys (Table 1). BCAA supplementation may increase liver-associated lymphocytes and NK cells, as well as lectin-dependent cellular cytotoxicity in mammals (Tsukishiro et al., 2000). Interestingly, the health-promoting effects of dietary arginine beyond meeting requirement for protein synthesis have been reported in fish (Buentello and Gatlin, 2001). In fact, arginine serves as the precursor for the synthesis of nitric oxide in terrestrial animals (Wu and Morris, 1998), a molecule with an important role in both innate and acquired immunity (Bogdan et al., 2000).

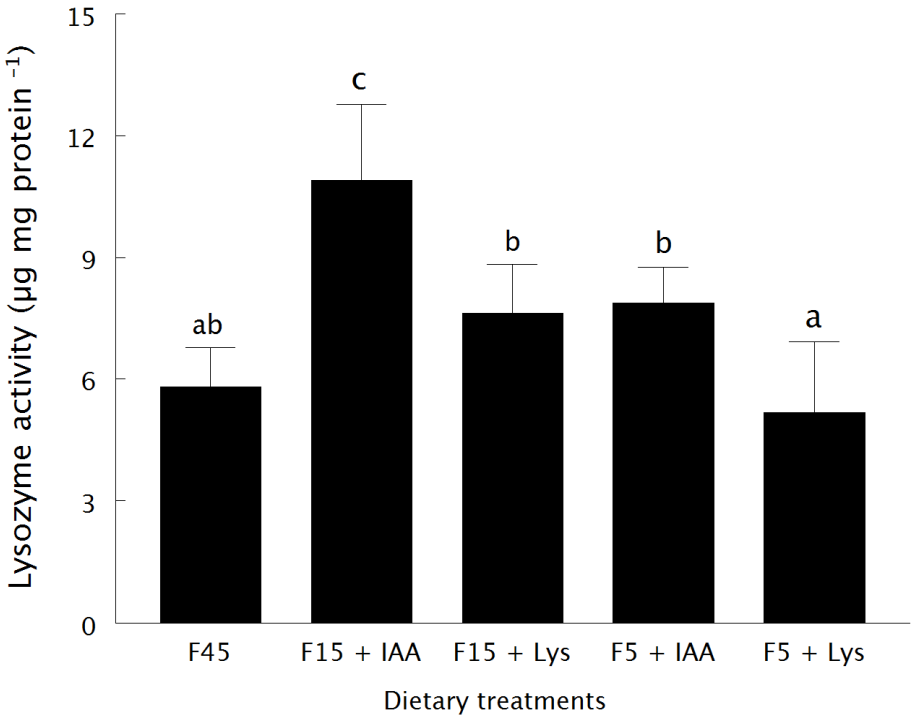

Figure 2. Serum lysozyme activity of Senegalese sole fed the experimental diets. Data are expressed as means ± SD (n = 8). Different letters mean significant differences among groups (One-way ANOVA; $P \leq 0.05$).

Moreover, the increased humoral non-specific immune parameters observed in the present study appear to be also related to the partial fish meal replacement by plant-protein sources, as already discussed above for head-kidney leucocytes O_2^- production, since IAA contents were similar between diets F15 + IAA and F5 + IAA. In fact, a high fish meal replacement (F5 + IAA) seems to

counteract the effects of the moderate fish meal replacement level (diet F15 + IAA). Lysozyme and ACP activities have been reported to increase in fish fed diets with partial replacement of fish meal by plant proteins (Krogdahl et al., 2000; Sitjà-Bobadilla et al. 2005) or different dietary treatments, for instance, increased dietary vitamin C (Ortuño et al., 1999), α-tocopherol acetate (Ortuño et al., 2000) and levamisole (Mulero et al., 1998).

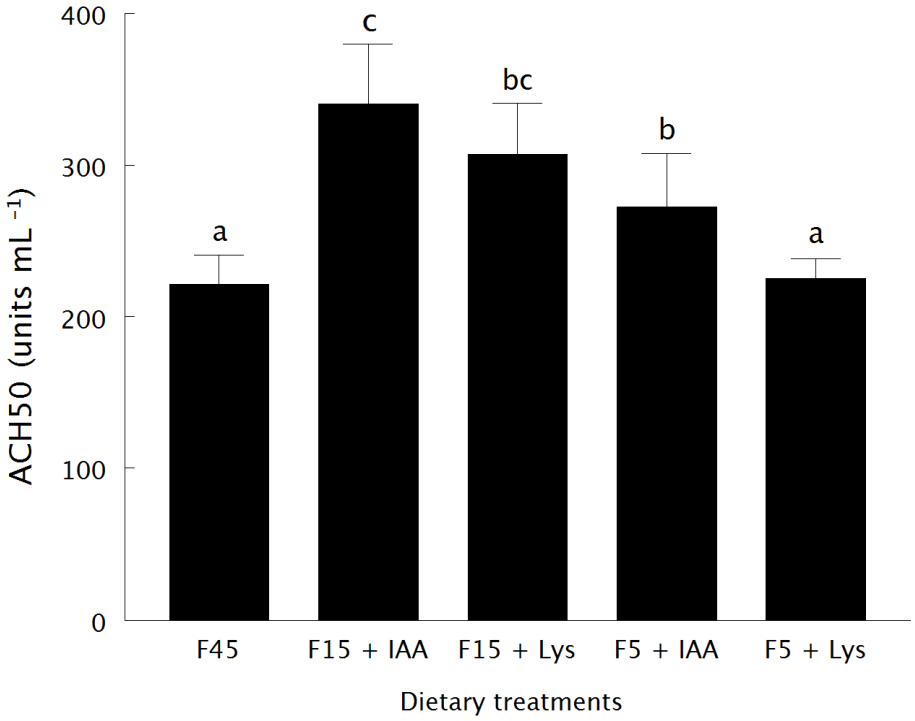

Figure 3. Serum alternative complement pathway activity (ACH50 units) of Senegalese sole fed the experimental diets. Data are expressed as means ± SD (n = 8). Different letters mean significant differences among groups (One-way ANOVA; $P \leq 0.05$).

The graded inclusion of different plant-protein sources in these diets implies unavoidable dietary differences other than the IAA and the interpretation of the present results becomes complex. For instance, the F45 diet is expected to have a higher iron concentration due to the higher fish meal proportion than the other experimental diets, and shifting of immunoregulatory balances by iron excess or deficiency may produce deleterious physiological effects (Maita, 2007). Different levels and combinations may also bring quantitative and qualitative differences in presence of anti-nutritional factors (Gatlin et al., 2007), dietary trace elements (Sugiura et al., 1999), complex carbohydrates and other

metabolism-modulating molecules. Therefore, nutritional factors other than IAA contents may affect somehow some of the results discussed. This is in line with the increased O_2^- production in fish fed the F15 + Lys diet, as well as the significantly lower final body weight observed in this treatment (Silva et al., 2009). Moreover, a persistent stimulation of non-specific immune parameters could be deviating energy for other metabolic processes, suggesting that an equilibrium remains to be found. For instance, Sitjà-Bobadilla et al. (2005) observed an increase in some metabolic and antioxidant factors when including plant-protein sources in the diet. Still, we believe the present results are relevant as fish meal replacement by vegetable ingredients is a reality in the aquafeed industry, and its consequences other than growth need to be better understood.

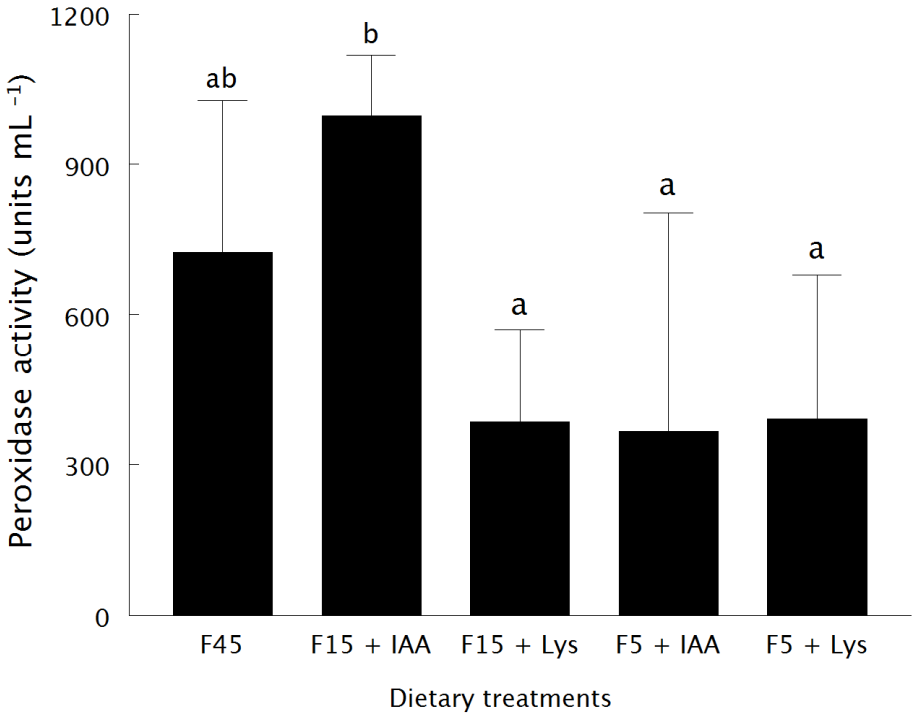

Figure 4. Serum peroxidase activity of Senegalese sole fed the experimental diets. Data are expressed as means ± SD (n = 8). Different letters mean significant differences among groups (One-way ANOVA; $P \leq 0.05$).

In conclusion, both fish meal replacement by vegetable ingredients and varying dietary IAA contents induced differential responses on cellular and humoral innate immune mechanisms. Provided that utmost care is taken to provide sole juveniles with a balanced dietary IAA supply, high replacement levels

of fish meal by a mixture of plant-protein sources seems to activate some non-specific immune parameters instead of being immune-compromising compared to the fish meal based (control) diet. However, this study focused only on a few selected immune parameters and a more complex response at the whole animal level is needed to confirm these conclusions. In addition, the long-term persistency of such an immunostimulated status may represent a physiological 'cost', which could affect growth rates and might not necessarily support an enhanced resistance to disease. This balance between the enhancement of non-specific immune parameters and growth performance, substantiated with bacterial challenge tests appears to be a matter of further research for this species.

8.4. Acknowledgements

This work was supported by the IDEIA project "Optidietas" (Agência de Inovação, Portugal, with the support of the European fund FEDER). Joana M.G. Silva and Benjamín Costas benefited from grants by Fundação para a Ciência e Tecnologia, Portugal (SFRH/BD/24857/2005 and SFRH/BD/38697/2007, respectively).

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Chapter 9

General discussion, conclusions and future research

9.1. General discussion

9.1.1. Stress duration and type of stressor employed affect stress response in sole

Acute and chronic stressful conditions are both characterised by the activation of the brain-sympathic-chromaffin cells (BSC) and hypothalamic-pituitary-interrenal (HPI) axes with the subsequent release of catecholamines and cortisol into circulation (Perry and Reid, 1993; Wendelaar Bonga, 1997). Senegalese sole primary and secondary stress responses to acute (**Chapter 3**) and chronic (**Chapters 2 and 5**) stressful events were in general similar to that observed in many other teleosts (Waring et al., 1996; Arends et al., 1999; Montero et al., 1999; Jentoft et al., 2005; Cnaani and McLean, 2009; Herrera et al., 2009; Trushenski et al., 2010). However, some particularities have been observed with sole.

Cortisol concentrations reported in **Chapters 2 and 5** are quantitatively similar to those reported previously for Senegalese sole reared under similar chronic stressors (Aragão et al., 2008; Salas-Leiton et al., 2010). Fold increase cortisol values (9-fold) from **Chapter 5** are similar to those (7-fold) reported by Aragão et al. (2008), while absolute cortisol values from **Chapter 2** (28.3 ng mL^{-1}) were close to those (~20 ng mL^{-1}) reported by Salas-Leiton et al. (2010). Interestingly, high stocking density from that study was 3 times higher than that employed in **Chapter 2**. According to that reported for seabass (Rotllant et al., 2003), the initial increase in plasma cortisol values due to crowding could have been higher when sole was submitted to a stocking density of 30 kg m^{-2} (Salas-Leiton et al., 2010) instead of 9 kg m^{-2} (**Chapter 2**). However, those values appear to decrease to similar values after two months, probably due to a negative feedback of cortisol at the level of the hypothalamus and pituitary, thus modulating adrenocorticotrophic hormone secretion and consequently cortisol production (Balm et al., 1994; Mommsen et al., 1999). On the other hand, Senegalese sole endocrine responses to acute stress are in line to that previously reported by Barton and Iwama (1991), being the highest increase in plasma cortisol within 30 min – 1 h after a stressful disturbance. However, cortisol concentrations showed in **Chapter 3** are much higher than those reported in several teleosts following air exposure (Waring et al., 1996; Arends et al., 1999;

Jentoft et al., 2005; Cnaani and McLean, 2009; Trushenski et al., 2010). In addition, characteristic cortisol elevations of fish in response to acute stressors tend to range within about 30 and 300 ng mL^{-1} (Barton, 2002). Still, there are notable exceptions. For instance, several fish species in the family Salmonidae showed cortisol values up to 900 ng mL^{-1}, being the highest plasma cortisol level recorded in striped bass (*Morone saxatilis*), with nearly 2000 ng mL^{-1} during recovery following 5 h of hauling (Barton and Iwama, 1991). In this Thesis, high cortisol values from **Chapter 3** may also be related to genetic factors. Within a single strain or population, variation in stress responses also has a genetic component and some fish may be predisposed to consistently exhibit high or low cortisol responses to stressors (Pottinger et al., 1992; Tort et al., 2001).

Netting and mild hypoxia for 3 min appears to induce an increase in plasma lactate levels immediately after air exposure (**Chapter 3**). Although these data are consistent with the known increase in plasma lactate levels following oxygen restriction (van Raaij et al., 1996) and after BSC axis activation in fish (Barton and Iwama, 1991), an increased anaerobic muscle activity associated with the observed avoidance behaviour (once inside the net) may play a role in the early lactate production. A similar observation was pointed out by Arends and colleagues (1999) in gilthead seabream air exposed for 3 min. Still, the rapid increase in plasma lactate levels observed in air exposed sole could also be attributed to a strategy representative of genetic adaptations that this species may have developed in its natural habitat. The distribution of fishes to diversified habitat makes them unique with regard to their physiologic and behavioural responses to stress (Peter, 2011). For instance, the observed increase in plasma free fatty acids instead of glucose and lactate in turbot after air exposure may also be considered as a genetic adaptation developed in its natural habitat (Waring et al., 1996). Furthermore, the sea raven (*Hemitripterus americanus*), a sedentary, benthic marine fish, took about 4 h to reach its peak cortisol level following an acute stressor, a strategy that may help conserve energy in a normally inactive species having a slow metabolic rate (Vijayan and Moon, 1994).

In summary, Senegalese sole appears to differ from other fish species in cortisol (high concentration at peak levels) and lactate (early production) responses after acute stress, whereas no major changes with respect to other teleosts were observed following chronic stress challenge. These differences in

Senegalese sole endocrine and metabolic responses may depend on duration and type of the stressor imposed, in line with that suggested for other teleosts (Wendelaar Bonga, 1997; Barton, 2002). This heterogeneity in the stress response of fish is in general attributed to different experimental conditions such as temperature, mineral and ionic composition of water, season, life stage, physiological condition, social factors, and inherited or acquired individual characteristics including nutritional status and energy reserves (Wendelaar Bonga, 1997; Barton, 2002). Therefore, stress responses to aquaculture-related stressors should be assessed for a given species in order to obtain a more accurate picture of the endocrine and metabolic responses that may have been adopted. However, genetic factors also seem to play an important role, and selection of phenotypic characters (e.g. high- or low-cortisol responders) could be considered in order to improve production.

9.1.2. Stress duration and type of stressor employed affect amino acid and glucose metabolism in sole

Length of time and severity of stressors are important factors that will likely influence how fish respond (Barton, 2002). In Senegalese sole, plasma free AA patterns appear to depend on both duration and type of stressor applied. Total plasma free AA increased significantly at 24 h in air exposed Senegalese sole (**Chapter 3**) whereas fish submitted to weekly handling for 28 days did not show significant changes with respect to undisturbed specimens (**Chapter 5**). Similarly, both weekly handling for 63 days and chronic exposure to high levels of total ammonia nitrogen for 52 days did not affect total plasma free AA levels in sole (Pinto et al., 2007; Aragão et al., 2008). In contrast, those levels decreased parallel to increasing stocking density after 63 days (**Chapter 2**). These differences in different studies may be indicative of diverse metabolic adjustments due to both intensity and duration of the stressor. Stress usually imposes a metabolic load on fish that consists of two components, an energy demand for activity required to cope with the disturbance, and an energy cost to correct the accompanying hydromineral imbalance (Barton and Iwama, 1991). Interestingly, plasma glucose levels increased slightly or no changes were observed in chronically stressed sole by handling procedures (Aragão et al., 2008; **Chapter 5**), in contrast to that observed following acute stress (**Chapter 3**).

Plasma glucose usually increases following the stress-induced release of catecholamines into circulation (Gamperl et al., 1994; Wendelaar Bonga, 1997). These adrenergic hormones, among other functions, serve to mobilize energy reserves through glycolysis as an adaptive response to meet increased metabolic demands necessary for enabling the fish to cope with the stress (Gamperl et al., 1994; Wendelaar Bonga, 1997). Therefore, mild increase or significant absence in the response of plasma glucose to repeated handling, which implies the involvement of the catecholamine response, suggests a general habituation to the repeated stressor. When an acute stressor is repeated over a period of time, some habituation may occur, which may result in reductions in post-stress cortisol and glucose levels (Jentoft et al., 2005).

A more detailed picture can be drawn when we look at the role of individual AA. Different stressful husbandry conditions seem to affect the metabolism of individual AA, even when those conditions did not affect total free AA levels (Pinto et al., 2007; Aragão et al., 2008). According to several authors, these changes may reflect the synthesis of proteins or other specific compounds related to the stress response or may be indicative of a higher usage of AA as energetic substrates (Milligan, 1997; Pinto et al., 2007; Aragão et al., 2008; **Chapters 2 and 3**). In some cases, it has been suggested that the changes in plasma levels of FAA in fish under stressful conditions may be indicative of specific AA requirements (Aragão et al., 2008). Recent studies using functional transcriptomics and comparative proteomics also evidence that stress affects intermediary metabolism, and in particular AA metabolism. An acute stressor has been shown to bring significant changes in transcriptome level of genes involved in gluconeogenesis, energy metabolism, protein degradation, as well as immune response in trout liver (Wiseman et al., 2007). An increased proteolytic potential mediated by glucocorticoid signalling in liver of stressed fish suggests that susceptible hepatic proteins may serve as transient sources of AA for hepatic metabolism, including gluconeogenesis. Interestingly, Senegalese sole submitted to a similar acute stressor appear to spare some indispensable AA for the synthesis of compounds related to the stress response or fatty acid transport, whereas dispensable AA were probably mainly used either as energy sources or in gluconeogenesis (**Chapter 3**). This preferential usage of dispensable AA as energy sources and/or in gluconeogenesis has also been observed in chronically stressed Senegalese sole (Aragão et al., 2010; Costas et al., 2010; **Chapter 2**).

Moreover, AA metabolism appears to depend on duration of the stressor applied when specific mechanisms are studied. For instance, Alves et al. (2010) identified glutamine synthetase (GSase) as a down-regulated protein in liver of gilthead seabream chronically exposed to two different stressors (repeated handling and high stocking density). This enzyme catalyzes glutamine formation from ammonia and glutamate, an ammonia detoxification mechanism in liver. The down-regulation of GSase expression in stressed fish was suggested to result from an early increase in GSase after stress exposure, followed by a feedback mechanism to reduce this accumulation after some time (Alves et al., 2010). This hypothesis is supported by the observed up-regulation of GSase in trout liver at 1h following acute stress (Wiseman et al., 2007). Similarly, GSase was up-regulated in rainbow trout at 1 and 6 h following acute stress whereas lasting the stressor up to 7 and 21 days down-regulated this gene (Cairns et al., 2008).

The methodology utilized in this Thesis for the study of digestibility and retention efficiency of individual indispensable AA is known to induce an acute stress response due to handling procedures (**Chapter 4**). However, the increased usage of lysine for energy production observed in sole from that study appears to be species specific rather than a catabolic effect of cortisol. It was observed that AA oxidation only starts 6 hours after tube-feeding using the same experimental setup (Conceição et al., unpublished results), and plasma cortisol levels are relatively low at this time (**Chapter 3**). On the other hand, Senegalese sole seems to poorly digest tryptophan. In **Chapter 4,** it was already discussed that some other fish species also seem to have some difficulties in absorbing this indispensable AA. This poor tryptophan digestibility presented by sole may be partially related to side effects of the stress response due to handling procedures. Moreover, Senegalese sole submitted to an acute stress presented an increased usage of methionine after 3 min and 1 h (**Chapter 3**). This is in line with that observed in chronically stressed chickens (*Gallus domesticus*) which presented a 50 % increase in the jejunal uptake of methionine (Mitchell and Carlisle, 1992). Since the transport of methionine and tryptophan across the brush-border membrane involves the same carrier mediated transport system (Collie and Ferraris, 1995), an eventual increase in the uptake of methionine due to increased metabolism following acute stress may have interfered to some extent on an early uptake o tryptophan. It would be interesting to compare these data with

individual AA digestibility following chronic stress challenge in sole as well as in other teleosts.

In summary, aquaculture-related stressful conditions induce a number of changes in the metabolism of individual AA which are needed in a range of physiological processes necessary for coping with an increased energy demand and higher synthesis of proteins or other specific compounds related to the stress response. The few existing studies regarding the effects of stressful rearing conditions on AA metabolism in fish other than sole, focused only on the effects of acute stress, thus restraining discussion on species specific differences. Changes in AA metabolism after acute stress challenge appear to be transient and some AA are differentially utilized in sole. For instance, plasma branched-chain AA decreased after 1 h in air exposed sole (**Chapter 3**), while those levels increased in rainbow trout at 2 and 4 h following severe exercise (Milligan, 1997), as well as in 24 h confined tilapia (Vijayan et al., 1997). On the other hand, chronic stress induces a number of changes that may influence AA requirements. Individual indispensable AA appear to be particularly important in sole during chronic stressful rearing conditions, being plasma arginine, branched-chain AA, lysine, methionine and tryptophan levels commonly decreased with respect to control specimens. Therefore, supplementation of those particular AA in the diet may represent a strategy to minimize AA mobilization and tissue proteolysis (mainly liver and muscle), which may translate in improvement of farming results through better welfare and ultimately increased disease resistance.

9.1.3. Stress response affects innate immunity and disease resistance in Senegalese sole

The suppressive effect of stress on the innate immune system is highly disputable and does not necessarily translate in decrease resistance to infection (Dhabhar, 2009; Verburg-van Kemenade et al., 2009). Depending on the duration and severity of the stressor, increased glucocorticoid levels may enhance innate and adaptive immune responses while similar hormone levels may suppress immune function. In the present Thesis, duration (acute or chronic) of handling procedures induced different responses in some innate immune parameters of Senegalese sole. While plasma lysozyme activity decreased at 4 h after air

exposure (**Chapter 3**), those levels increased in sole submitted to weekly handling for 14 and 28 days (**Chapter 5**) or daily handling for 14 days (**Chapter 7**). Similarly, complement activity (ACH50) followed an inverse linear relationship with respect to plasma cortisol levels after air exposure (**Chapter 3**), whereas daily stressed sole for 14 days presented higher ACH50 values than undisturbed specimens (**Chapter 7**). Interestingly, respiratory burst activity followed the opposite pattern, showing increased values at 2 and 4 h after air exposure (**Chapter 3**) while daily handling for 14 days significantly decreased this cellular response (**Chapter 7**). In addition, the augmented NO production as well as gLYS and HIF-1 expression values in chronically stressed sole translated in a higher resistance to *Phdp* (**Chapter 7**). Similarly, activation of innate immunity after stress challenge was observed in several teleosts. For instance, relevant genes associated with acute inflammation followed similar kinetics, showing interleukin (IL)-1β, inducible nitric oxide synthase (iNOS), α_2-macroglobulin and serum amyloid protein A increased expression values after acute stress (Huising et al., 2003). In addition, the level of expression of IL-1β increased in Atlantic salmon after 7, 14 and 28 days submitted to daily handling (Fast et al., 2008). Moreover, acute stress resulted in an increase of both phagocytosis of head-kidney leucocytes and circulating granulocytes in several fish species (Pulsford et al., 1994; Engelsma et al., 2003). In addition, cortisol and LPS synergistically stimulate the expression of IL-1β mRNA in head kidney phagocytes of common carp (Engelsma et al., 2003). In contrast, leucocytes respiratory burst activity decreased in specimens submitted to handling, anoxic shock or crowding (Angelidis et al., 1987; Pulsford et al., 1994; Yin et al., 1995; Ortuño et al., 2002). Similarly, several *in vitro* studies demonstrated that cortisol inhibits LPS-induced expression of pro-inflammatory cytokines IL-1β, IL-12p35, IL-11, tumor necrosis factor-alpha (TNF-α) and iNOS (Saeij et al., 2003; Huising et al., 2005; Fast et al., 2008; Stolte et al., 2008; Castillo et al., 2009). Moreover, cortisol can stimulate apoptosis of B-cells (Weyts et al., 1998a), and inhibit apoptosis of neutrophils (Weyts et al., 1998b), an adaptive response to prolong the life span of neutrophils that form the first-line of defense against pathogens. Therefore, stress can have opposite effects on innate immunity depending on duration and type of stressor applied, and also depending on the immune mechanisms being studied.

Indeed, the effects of stress on the immune system are difficult to interpret *in vivo*, since a number of different hormones are involved via the HPI axis. Thus,

it is likely that *in vivo* neuroendocrine-immune interactions are dependent on the actions of various hormones and cytokines, as well as on their interactions. In fact, *in vitro* adrenaline and cortisol administration, but not ACTH, reduced the LPS-stimulated expression of IL-1β, whereas ACTH in combination with LPS increased the level of expression of TNF-α in the head-kidney of gilthead seabream (Castillo et al., 2009). This finding could explain the increased immune function frequently observed in Senegalese sole submitted to repeated handling (**Chapters 5 and 7**). Other hormones released during BSC and HPI axes activation may have influenced innate immune mechanisms in a higher degree, decreasing the suppressive effects of cortisol. In contrast, a different situation probably occurs during constant chronic situations such as high stocking density, playing cortisol its attributed immunosuppressive role. This hypothesis is supported by the increased susceptibility to opportunistic pathogens observed in Senegalese sole reared at high stocking density (**Chapter 2**). Although immune function was not assessed in this study, Salas-Leiton et al. (2010) observed a significant decrease in HAMP1 and gLYS transcripts in the liver of Senegalese sole reared at high stocking density.

Numerous studies in mammals and fish have reported the adverse effects of stress on health (Barton and Iwama, 1991; Wendelaar Bonga, 1997; Calcagni and Elenkov, 2006; Magnadottir, 2010). During common aquaculture practices, long term exposure to stressors usually induces suppressive effects on the immune system and disease resistance in fish (Wendelaar Bonga, 1997; Magnadottir, 2010). However, the aforementioned actions of stress or cortisol on the fish immune system depend on both duration and intensity of the stressors applied. Although many studies have studied in detail the effects of either cortisol or stress on immune competence in *in vivo* or *in vitro* models, evidence of its consequent effects on disease resistance are scarce. In this Thesis, the exact mechanisms by which stressed Senegalese sole resisted better to *Phdp* infection remain to be elucidated. Future studies addressing mechanisms of action and regulation of cytokines and other immune-related genes following bacterial challenge in previously stressed fish deserve further attention.

9.1.4. Dietary amino acids reduce the metabolic changes associated to stress response in Senegalese sole and may improve welfare

Fish present additional AA requirements when submitted to stressful rearing conditions, due to either increased energy demands or for the synthesis of stress-related proteins and other compounds related with the stress response (Aragão et al., 2008, 2010; **Chapters 2 and 3**). Therefore, the present Thesis proposes the possibility of mitigating the negative metabolic effects of stress by altering dietary AA levels, based on the effects of cortisol on AA metabolism (see General Introduction) and on that suggested by Aragão et al. (2008). This hypothesis is supported in **Chapter 5** for chronically stressed sole. In this study, feeding sole a diet with a slightly increase in all indispensable AA, together with tyrosine and cysteine, with respect to a reference diet resulted in a decrease in post-stress plasma glucose and lactate levels after 14 days of treatment, thus minimizing the negative effects attributed to cortisol release after HPI axis activation. Although this study did not measure cortisol levels at 14 days, handling stress activated the serotonergic system and increased plasma glucose and lactate levels. Moreover, this is further supported by another study reporting high plasma cortisol levels in Senegalese sole submitted to air exposure once a week for 60 days (Aragão et al., 2008).

In general, plasma cortisol levels rise at the beginning of the stressful condition and decrease to initial values in a few days (Pickering and Stewart, 1984; Tort et al., 1996), showing adaptation of the fish to the new situation. Cortisol data presented in **Chapter 5** showed the incapacity of Senegalese sole to adapt to chronic handling procedures after 28 days of treatment, supported by increased plasma glucose and lactate levels. In addition, there was no evidence of an effect of dietary treatment on neither of those parameters. However, it was observed that a slight increase in the availability of some dietary AA may have a significant impact on AA metabolism, as indicated by changes in plasma AA levels when comparing both chronically stressed treatments. As already discussed in **Chapter 5**, some individual AA (i.e., arginine, lysine, leucine, isoleucine, glutamate, cysteine and ornithine) were probably used in different mechanisms of oxidative stress and energy metabolism in fish from handling treatment, while those changes were not observed in handling specimens fed the high protein diet (increased AA availability). Therefore, while dietary treatment does not seem to

minimize the increased energy needs due to chronic stress, some metabolic processes related with the stress response appear to be absent in handling groups fed the high protein diet. For instance, the lack of change in plasma levels of glutamate and cysteine may be indicative of a lower glutathione synthesis and, to some extent, may suggest a better welfare condition than fish from handling treatment fed the control diet.

Therefore, providing some key AA in the diet may represent a metabolic advantage during predictable stressful events (e.g. handling and crowding associated to grading procedures), which may have a significant effect on growth and welfare in the longer term. Those effects on metabolism appear to be stronger after 14 days compared to 28 days of feeding, as indicated by the reduction of plasma glucose and lactate levels. Still, 28 days of feeding appear to have some effect on other processes related to the stress response. It would be interesting to consider intracellular glutathione levels and nitric oxide production in future experiments assessing the effects of amino acid supplementation on the stress response of chronically stressed fish.

9.1.5. Dietary amino acid supplementation enhances innate immune parameters and disease resistance in sole

Recent evidence shows that some AA and their metabolites are important regulators of key metabolic pathways that are necessary for immune mechanisms and resistance to pathogens in various teleosts (Li et al, 2009). However, despite the clear theoretical importance of those AA in immune function, few studies have been performed in fish to explore the full effects of this important group of nutrients on immune function. In this Thesis, some innate immune parameters were enhanced due to either increased dietary AA (**Chapter 5**) or amino acid supplements, including a mixture of all indispensable AA (**Chapter 8**), lysine (**Chapter 8**) or arginine (**Chapter 7**) alone. The efficacy of dietary arginine on increasing innate immunity and disease resistance has been successfully documented for sole (**Chapter 7**), in line with that previously reported for mammals and fish (Wu and Morris, 1998; Roth, 2007; Buentello and Gatlin, 1999; 2001). On the other hand, results from **Chapter 5** are not conclusive since only one humoral immune parameter (lysozyme activity) was assessed. Still, data

obtained from lysozyme activity in **Chapters 5, 7** and **8** are consistent, and together with data from peroxidase activity in **Chapters 7** and **8**, makes tempting to speculate that AA supplements may have increased the numbers of circulating neutrophils. In this case, arginine would probably be the main AA responsible since it is the main precursor of polyamines, which in turn are required for cell growth and differentiation (Soda et al., 2005). Experimental diets from **Chapters 7** and **8** showed higher arginine, isoleucine, threonine, valine, lysine or methionine levels than that in control diets, suggesting that these particular AA and/or its derived compounds may play a role on innate immune mechanisms in sole. According to literature and data from **Chapter 7**, arginine plays a pivotal role between nutrition and immunity as an indispensable AA and the precursor for polyamines and NO synthesis. However, data from **Chapter 8** also point to other indispensable AA (BCAA, threonine, lysine or methionine) as potential nutrients for use in functional feeds or as nutraceutics[1].

Immunonutrition has been a subject of profound research in mammals during the last decades, with particular emphasis to be used in humans (e.g., Grimble and Grimble, 1998; Roth, 2007; Grimble, 2009). For instance, BCAA supplementation may increase liver-associated lymphocytes and natural killer cells, as well as lectin-dependent cellular cytotoxicity in mammals (Tsukishiro et al., 2000). Moreover, threonine inhibits apoptosis, stimulates lymphocyte proliferation and increases antibody production (Li et al., 2007). In addition, and similar to that observed in fish submitted to stressful conditions, severe trauma and infection in humans cause large decreases in plasma glycine, serine, and taurine levels, suggesting an increased utilization of these AA, together with methionine and cysteine (Grimble and Grimble, 1998). In fact, dietary methionine supplementation inhibits inflammation and enhances T cell function in humans (Grimble, 2009).

Therefore, the impact of dietary AA supplements on fish immune mechanisms has not been investigated in any depth, and its potential use as nutraceutics should be taken into account. In addition, this takes particular importance in situations of chronic stress where AA metabolism is affected and immune function is commonly impaired.

[1] These terms could be defined as feeds supplemented with specific ingredients or compounds to achieve desirable immunological or physiological benefit, other than a purely nutritional effect.

9.2. Conclusions

According to the results presented in this Thesis, the following conclusions can be highlighted:

- Senegalese sole presents stress (**Chapters 2 and 3**) and immune (**Chapter 6**) responses similar to that find in other teleosts.

- Senegalese sole presents a lower absorption (digestibility) capacity for tryptophan compared to other indispensable AA (**Chapter 4**). In addition, several differences in relative utilization of individual indispensable AA for energy production were observed. Particularly, lysine seems to have an important role as an energy substrate in sole juveniles.

- **Chapter 5** gives some insights on the possibility of mitigating the increased metabolic cost attributed to cortisol release, which during chronic stressful conditions are detrimental for the organism. In fact, dietary AA levels, in particular arginine and methionine, appear to modulate AA metabolism following chronic stress challenge.

- Dietary arginine supplementation enhanced some innate immune mechanisms and disease resistance in sole, increasing NO production and respiratory burst of head-kidney leucocytes in a dose dependent manner (**Chapter 7**).

- Interestingly, in **Chapter 7** daily handling stress for 14 days augmented expression levels of HIF-1 and gLYS, and increased disease resistance regardless of dietary treatment.

- Finally, **Chapter 8** contributes to the development of practical, environmentally sustainable and cost-effective plant protein based diets for Senegalese sole juveniles, with putative stimulatory effects on some innate immune parameters.

9.3. Further research

The present Thesis proposes the possibility of mitigating the negative effects of stress and disease susceptibility of fish through dietary AA supplements. Although this hypothesis is supported by **Chapters 5 and 7**, further studies are required for validating this nutritional strategy in order to improve welfare and survival in chronically stressed fish. It was observed that stress response and immune function vary with type of stressors and stress duration. Therefore, once an optimal level of AA supplementation is achieved for a given species, its beneficial effects should be validated during different stressful conditions commonly found in aquaculture.

A new field of research regarding functional feeds in aquaculture is arising. However, most studies are focusing on pre- and probiotics and immunostimulants. To our knowledge, this Thesis represents the first structured attempt to include AA supplements in functional feeds in order to improve welfare and survival in chronically stressed fish. In fact, **chapter 7** shows the beneficial effects that arginine supplementation presents for fish, and other indispensable AA appear to have a similar role (**Chapters 5 and 8**). Therefore, the potential use of specific AA as nutraceutics for fish should be taken into account in future studies.

In **Chapter 7** it was speculated that increased numbers of peripheral neutrophils in chronically stressed sole may have had a role on disease resistance. This hypothesis was based on reported data from many other studies where different stressors (e.g. transport, handling, injection, crowding) decreased numbers of circulating B-lymphocytes and increased numbers of circulating neutrophils (Angelidis et al., 1987; Pickering and Pottinger, 1987; Pulsford et al., 1994; Espelid et al., 1996). Therefore, stressful rearing conditions do not always suppress immune function and further studies are required in order to elucidate the mechanisms that stressed fish may have adopted during resistance to bacterial challenge. These studies should consider leucocyte trafficking and redistribution, cytokines kinetics and the release of hormones other than cortisol.

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