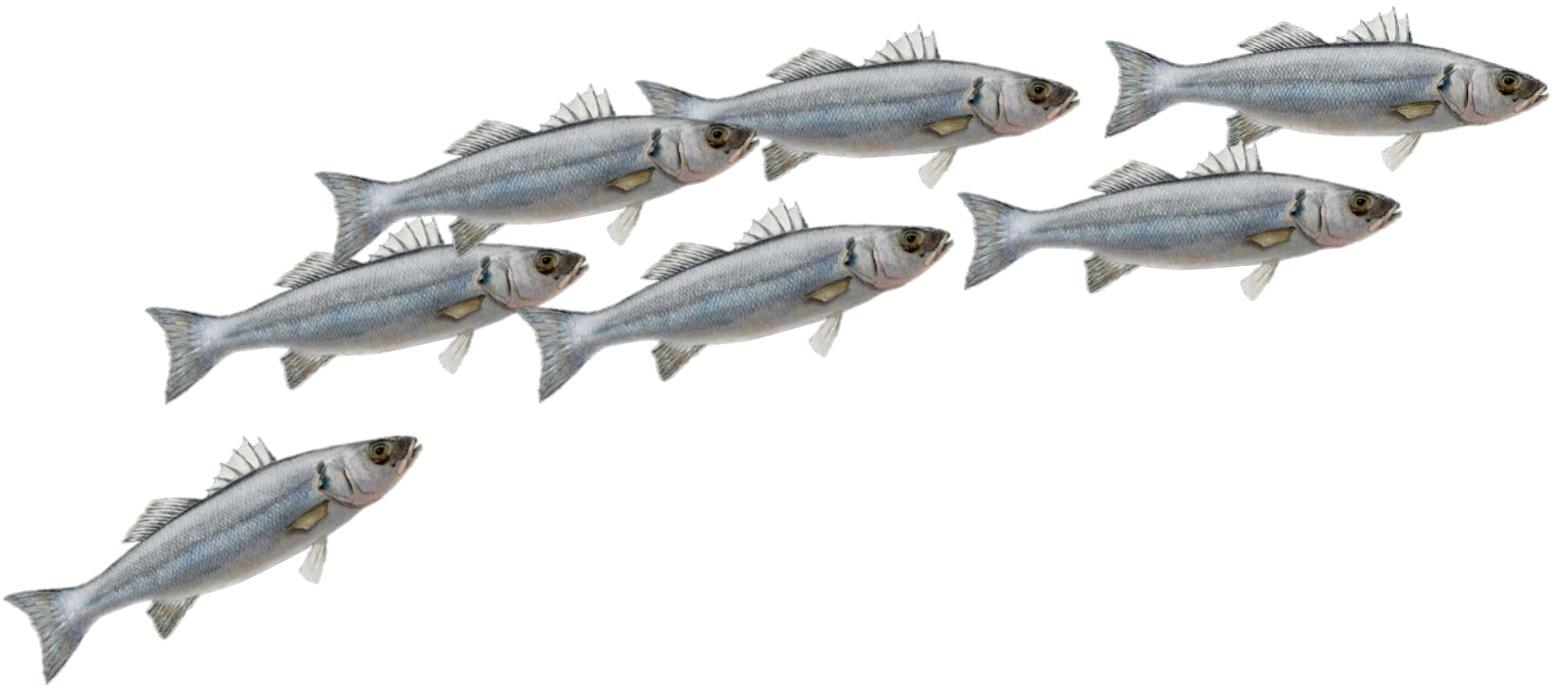

Amino acids as novel nutraceutics to modulate immune mechanisms and increase disease resistance in fish

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List of Papers

This thesis also includes three scientific papers published in international peer-review journals originating from part of the results obtained in the experimental work, referenced to as:

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Azeredo R., Machado M., Afonso A., Fierro-Castro C., Reyes-López F. E., Tort L., Gesto M., Conde-Sieira M., Míguez J.M., Soengas J.L., Kreuz E., Wuertz S., Peres H., Oliva-Teles A. and Costas B. 2017. Neuroendocrine and immune responses undertake different fates following tryptophan or methionine dietary treatment: tales from a teleost model. Front Immunol. 8, DOI: 10.3389/fimmu.2017.01226.

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Summary

Fish farming might take many different forms of husbandry that vary how fish are held, feeding routines and cleaning practices. Regardless of specific fish husbandry practices, there are inherent features to this activity that are inevitable and that may compromise fish health and welfare. Stocking density in rearing tanks, social dynamics among individuals and environmental factors such as water temperature and oxygenation, availability of hiding places, etc., might create stressful environments for fish, thereby compromising other physiological responses such as that of the immune system. Indeed, different and apparently unrelated physiological events are orchestrated by the same mediators, sharing resources and interacting to regulate the same response to an allostatic state scenario.

The use of functional diets to improve fish health is a passive strategy that can be applied alone or in combination with other prophylactic actions to promote their efficiency. In fish, several compounds have already been shown to exert immune-enhancing effects while amino acids have only recently started to be considered as fish functional ingredients in fish diets. Amino acids are well known for their versatility, as they are conspicuously involved in distinct regulatory mechanisms, signaling pathways and metabolic reactions.

The present study intends to evaluate amino acids immunomodulatory effects in a teleost species, the European seabass. Specifically, different amino acids (glutamine, arginine, citrulline, methionine and tryptophan) are tested regarding their ability to modulate innate immune mechanisms and consequently modulate fish immune status, inflammatory response and, ultimately, disease resistance. Thereby, this Thesis aims at building knowledge on the potential use of amino acids as a dietary strategy to promote health in farmed fish.

Considered one of the most versatile amino acids, arginine is involved in several immune mechanisms which makes this an interesting amino acid to be considered in immunomodulation strategies. Arginine immunomodulatory effects were evaluated *in vitro* in **Chapter 2** and *in vivo* in **Chapters 5** and **6**. While arginine supplementation to seabass head-kidney leucocyte (HKL) culture increased extracellular ATP levels, it did not induced nitric oxide (NO) production (**Chapter 2**). Moreover, fish fed arginine-supplemented diets had lower plasma NO level than those fed the control, non-supplemented diet (**Chapter 5**). Immune- and polyamine biosynthesis-related gene expression was generally down-regulated by arginine (**Chapters 2** and **5**), which suggests an impairment of both immune response and cell proliferation and

differentiation, as confirmed by lower cell counts in fish fed arginine-supplemented diets both before (immune status) and after bacterial challenge (inflammatory response, **Chapter 6**). In conclusion, arginine dietary administration for 2 and 4 weeks seems to compromise fish immune condition and response. Since fish immune status is already depressed before any immune stimulation, it is suggested that arginine-mediated suppressive effects might be related to toxicity of arginine metabolites such as NO. Further research is required to ascertain this hypothesis, which should include shorter administration periods and a time-course sampling.

As precursor of S-adenosylmethionine, methionine is directly involved in methylation pathways, polyamines and cysteine biosynthesis. In addition, because cysteine is one of glutathione compounds, increased methionine availability may also modulate the redox potential. Upon immune stimulation, seabass HKL cultured in methionine-supplemented media (M1 and M2) showed enhanced NO production and respiratory burst compared to cells incubated in non-supplemented medium (L-15, **Chapter 2**). In **Chapter 3**, fish fed methionine-supplemented diet (MET) and intra-peritoneally (i.p.) injected with UV-killed *Photobacterium damselae* subsp. *piscicida* (*Phdp*) showed a non-significant increasing trend regarding peripheral leucocytes respiratory burst compared to fish fed a control diet (CTRL). Compared to fish fed CTRL, the number of circulating and peritoneal leucocytes was higher in MET-fed fish, both before and after the immune challenge, suggesting that the involvement of methionine in immune mechanisms is that of promoting cell proliferation. Dietary methionine surplus also significantly modulated immune-related genes expression levels (**Chapters 2** and **3**), an effect that might be associated to altered methylation patterns. Furthermore, as no relevant immunomodulation was observed in peripheral lymphoid tissues (skin and posterior gut) of i.p. *Phdp*-injected, MET-fed fish (**Chapter 4**), the role methionine plays in immune mechanisms seems to be limited to the inflammatory focus and the associated systemic response.

Tryptophan is precursor of serotonin (5-HT), a monoamine and neurotransmitter involved in activating and inhibitory mechanisms related to the hypothalamus-pituitary-interrenal (HPI) axis, ultimately modulating cortisol production. On the other hand, tryptophan is catabolized by 2, 3-indoleamine dioxygenase (IDO) leading to the formation of metabolites that mediate immunosuppressive effects. Immune-related effects of tryptophan supplementation were tested under the same conditions used for methionine. The majority of tryptophan-mediated immunomodulatory effects that were observed were related to immunosuppression, as denoted by down-regulation of immune-related

gene expression and cellular-mediated immune defences (**Chapters 2 and 3**). Moreover, fish fed tryptophan-supplemented diet (TRP) also showed decreased abundance of neutrophils (**Chapter 3**). Since brain monoamine content and plasma cortisol levels increased in TRP-fed fish, the aforementioned immunosuppressive effects could be associated to an enhanced neuroendocrine response. Still, since similar impairing effects were observed *in vitro* (**Chapter 2**) in tryptophan-treated HKL cultures where no cortisol is produced, IDO-mediated tryptophan metabolism should also be accounted for the observed immune response impairment. On the contrary, several immune-related genes were up-regulated in the posterior gut of i.p. *Phdp*-injected, TRP-fed fish (**Chapter 4**). Altogether, the present results show clear and mainly inhibitory effects of tryptophan supplementation on the innate immune response of seabass. Nonetheless, further research is needed, especially that focused on IDO-mediated tryptophan metabolism in different lymphoid tissues.

Despite considered non-essential amino acids, glutamine and citrulline may become conditionally essential in certain situations. The immune response, especially leucocyte function, rely on high availability of both glutamine and citrulline given their importance for energy production and *de novo* synthesis of arginine, respectively. To test the effects of increased glutamine concentration on the immune response, primary cultures of European seabass HKL were incubated in glutamine-supplemented media at two doses (G1 and G2) and were subjected to immune stimulation (**Chapter 2**). In unstimulated, G1- and G2-treated HKL, extracellular ATP levels were higher than in L-15 cell cultures, which denotes the importance of this amino acid as an energy source for cells of the immune system. Concomitantly, compared to control cells (L-15), G1- and G2-treated cells presented a generally enhanced immune response, as observed by increased NO and immune-related gene expression.

To evaluate the involvement of citrulline on the immune status and inflammatory response, European seabass were fed a citrulline-supplemented diet (CIT) for 2 and 4 weeks and were then subjected to a bacterial challenge (**Chapter 6**). Results point at immunosuppressive effects, with lower leucocyte numbers and lysozyme activity in fish fed CIT compared to fish fed a basal diet. The similarity between citrulline- and arginine-mediated effects suggests that citrulline is indeed used by immune cells for *de novo* synthesis of arginine. Still, further research on enzymatic activity is needed to confirm the enhanced conversion of citrulline to arginine.

Keywords: innate immunity, inflammation, neuroendocrine response, functional ingredients, amino acids, leucocytes, cell proliferation, primary cell culture

Resumo

A produção de peixe em aquicultura assume diferentes formas quanto ao volume de produção, método de cultivo e padrões de alimentação. Independentemente da estratégia adotada, qualquer unidade de produção tem associadas determinadas características que podem constituir riscos que podem comprometer a saúde e o bem-estar animal. As altas densidades e a própria dinâmica social, aliadas a condições ambientais tais como a temperatura e oxigenação da água e disponibilidade de abrigos constituem fatores de stress para a maior parte das espécies cultivadas. Deste modo, e eficiência de respostas fisiológicas que de algum modo estão associadas à ativação da resposta neuro-endócrina, tais como a resposta imunitária, poderá ser menor.

A administração de dietas funcionais é frequentemente usada como estratégia para promover a resposta imunitária em humanos e em outros mamíferos. Estudos nutricionais em peixes têm demonstrado o potencial de diversos compostos como promotores da capacidade imunitária. Contudo, a investigação de aminoácidos e do envolvimento dos mesmos em mecanismos de imunidade inata é um tema ainda recente em peixes. Os aminoácidos são considerados, na sua maioria, moléculas particularmente versáteis que, além de serem as unidades estruturais proteicas, estão envolvidas em diversas vias de sinalização, ações reguladoras bem como outras vias metabólicas.

A presente Tese tem como principal objetivo avaliar o papel modulador de aminoácidos na resposta imunitária de uma espécie de peixe teleósteo. Em particular, os efeitos da glutamina, arginina, citrulina, triptofano e metionina são averiguados a nível da condição imunitária, da resposta inflamatória bem como da resistência a doença em robalo Europeu. Deste modo, os estudos aqui apresentados contribuem para a caracterização mais aprofundada do potencial destes aminoácidos como ingredientes funcionais em dietas de aquicultura e assim otimizar estratégias que assegurem o bem-estar e a saúde de peixes em cultivo.

A participação da arginina em diversos mecanismos de defesa, não só em mamíferos mas também em peixes, aponta para a sua importância na resposta imunitária. O efeito da sua suplementação nas defesas imunitárias foi testado *in vitro* no **Capítulo 2** e *in vivo* nos **Capítulos 5** e **6**. No **Capítulo 2**, a adição de arginina em duas doses diferentes a meios de cultura primária de leucócitos (meios A1 e A2) do rim anterior de robalo Europeu levou a um aumento da concentração de ATP no meio de cultura de células estimuladas com lipopolissacarídeo (LPS) relativamente a células cultivadas em meio sem suplementação de arginina (L-15). Contudo, não se observou

um aumento na produção de óxido nítrico (NO) em células incubadas em A1 e A2, como seria esperado dada a presença de maior concentração de precursor. A suplementação nutricional de arginina levou à diminuição da produção de NO (**Capítulo 5**), inibição da expressão de genes associados à resposta imunitária e à síntese de poliaminas (**Capítulos 2** e **5**) e a uma menor concentração de leucócitos periféricos (**Capítulo 6**), comparativamente a peixes alimentados com uma dieta basal, não suplementada. Esta supressão geral da condição e resposta imunitária aumentou a suscetibilidade dos peixes à infeção por *Photobacterium damselae* subsp. *piscicida* (*Phdp*), levando a maiores mortalidades em peixes alimentados com dieta suplementada com arginina do que em peixes alimentados com a dieta basal (**Capítulo 5**). Estas observações apontam para um potencial efeito tóxico de metabolitos da arginina que, quando em níveis superiores aos valores fisiológicos, inibem a resposta imunitária. Assim, níveis de arginina acima do requisito do robalo parecem ser prejudiciais, nas condições testadas, e não benéficos para a sua condição imunitária. Contudo, são necessárias diferentes abordagens e estudos para confirmar a hipótese da toxicidade destes metabolitos, incluindo análises tais como atividade enzimática do metabolismo da arginina e variação no tempo da concentração de NO.

A metionina, além de ser um abundante aminoácido na constituição de proteínas, é também precursora de S-adenosilmetionina, estando assim consequentemente envolvida em vias de metilação, síntese de poliaminas e cisteína. É também precursora da glutationa, um importante antioxidante, e por isso um aumento da sua concentração poderá influenciar o potencial redox. No **Capítulo 2**, leucócitos mantidos em meio suplementado com duas doses de metionina (M1 ou M2) e estimulados com LPS e/ou *Vibrio anguillarum* demonstraram produzir mais NO e anião superóxido (O_2^-) do que células mantidas em L-15. Da mesma forma, no **Capítulo 3**, peixes alimentados com uma dieta suplementada com metionina (MET) e injetados com *Phdp* inativada por UV apresentaram maior concentração de O_2^- comparativamente ao grupo alimentado com uma dieta basal (CTRL), apesar da ausência de significância estatística nestes resultados. O número de leucócitos no sangue bem como no peritoneu foi igualmente superior nos peixes alimentados com MET relativamente àqueles alimentados com CTRL, tanto antes como depois do estímulo imunitário. Este aumento da resposta celular sugere que a relação da metionina com a resposta imunitária poderá ser ao nível da proliferação celular, provavelmente via síntese de poliaminas. Relativamente ao papel deste aminoácido a nível de expressão genética, os resultados dos **Capítulos 2** e **3** demonstram o efeito modulador da suplementação da metionina em genes associados à resposta imunitária. Uma vez que a S-adenosilmetionina é um importante

dador de grupos metilo, a suplementação de metionina poderá afetar padrões de metilação e deste modo modular a expressão genética. Em contrapartida, as respostas imunitárias do muco da pele bem como do intestino posterior em peixes alimentados com MET e injetados com *Phdp* não foi significativamente afetada (**Capítulo 4**), sugerindo que o papel da metionina em mecanismos de defesa é direcionado para o foco inflamatório e para a resposta sistémica a ele associada.

A degradação de triptofano via enzima triptofano hidroxilase resulta na síntese de serotonina (5-HT) que é uma monoamina envolvida em mecanismos de ativação/inibição do eixo hipotálamo-hipófise-interrenal (HPI) nos peixes. Por outro lado, o triptofano é também metabolizado pela enzima 2, 3 – indolamina dioxigenase (IDO), através da qual se formam metabolitos envolvidos em mecanismos imunossupressores. Os efeitos da suplementação de triptofano nas defesas imunitárias do robalo foram avaliados em condições semelhantes àquelas escolhidas para a metionina, nos **Capítulos 2**, **3** e **4**. Em geral, as alterações induzidas pelo aumento da concentração de triptofano, tanto em culturas de leucócitos *in vitro* (**Capítulo 2**) como em dietas experimentais *in vivo* (**Capítulo 3**), demonstraram padrões de inibição da resposta imunitária em termos de expressão genética e da produção de mediadores da resposta inflamatória. No **Capítulo 3**, o número de neutrófilos diminui no sangue de peixes alimentados com uma dieta suplementada com triptofano (TRP) comparativamente aos peixes alimentados com CTRL, depois de serem injetados com *Phdp*. Além da observada supressão da resposta inflamatória, a suplementação de triptofano aumentou a concentração de monoaminas no cérebro dos robalos, incluindo os níveis de 5-HT, bem como a concentração de cortisol no plasma de peixes alimentados com TRP e injetados com *Phdp*. Tendo em conta que os sistemas imunitário e neuro-endócrino comunicam entre si e regulam-se reciprocamente, a inibição da resposta inflamatória observada em peixes alimentados com TRP poderá estar associada à ativação do eixo HPI, comprovada pelo aumento da produção de cortisol nestes peixes. Contudo, e uma vez que no **Capítulo 2** se observaram efeitos semelhantes a nível da resposta imunitária onde não é possível a interação com cortisol, as consequências da suplementação de triptofano poderão estar associadas à atividade da IDO e dos metabolitos por ela formados. No **Capítulo 4**, a resposta imunitária na superfície da pele de peixes alimentados com TRP demonstrou ser menos intensa do que a observada em peixes alimentados com CTRL, ao mesmo que a expressão de genes associados à defesa imunitária no intestino posterior foi intensificada. Assim, o envolvimento do triptofano no sistema imunitário parece estar dependente das condições fisiológicas do peixe e do tecido em causa. Deste modo, são necessários mais estudos para esclarecer e melhor

caracterizar a via de metabolização do triptofano durante a resposta inflamatória atendendo a cada contexto específico.

Apesar de serem considerados não-essenciais, a glutamina e a citrulina podem tornar-se aminoácidos indispensáveis em determinadas ocasiões. Durante a resposta imunitária os leucócitos utilizam a glutamina como fonte de energia e a citrulina como precursora de arginina para produção de NO. O efeito da glutamina na imunidade inata foi testado em culturas primárias de leucócitos de robalo no **Capítulo 2**, através da suplementação de glutamina em duas concentrações (G1 e G2). No que diz respeito aos níveis de ATP, estes foram mais altos nos meios de cultura de leucócitos não-estimulados e tratados com G1 e G2 do que em culturas sem suplementação de glutamina. Ao mesmo tempo, a produção de NO e os níveis de expressão de genes associados à resposta imunitária aumentaram em leucócitos de rim tratados com G1 e G2, demonstrando a importância da glutamina para a eficiência da resposta celular e apontando para a capacidade estimulante deste aminoácido durante a inflamação.

No **Capítulo 6,** os efeitos da suplementação nutricional da citrulina no sistema imunitário do robalo foram testados ao fim de 2 e 4 semanas de alimentação, bem como após a injeção i.p. de *Phdp*. Em paralelo com a arginina, a maior disponibilidade de citrulina teve efeitos supressores sobre mecanismos de defesa tais como o número de leucócitos no sangue e a atividade da lisozima no plasma, comparativamente a peixes alimentados com uma dieta basal. Esta semelhança entre os resultados obtidos em ensaios com arginina e citrulina sugere que os peixes teleósteos utilizam a citrulina como substrato para a síntese *de novo* de arginina e assim, indiretamente, a sua resposta imunitária é modulada da mesma forma que perante o suplemento direto de arginina. Contudo, são necessários estudos de atividade enzimática para confirmar esta hipótese, tendo como foco as enzimas argininosuccinato sintetase e argininosuccinato liase.

Palavras-chave: imunidade inata, inflamação, resposta neuro-endócrina, ingredientes funcionais, aminoácidos, leucócitos, proliferação celular, cultura celular primária

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List of Abbreviations

5-HIAA	5-hydroxyindoleacetic acid
5-HT	Serotonin
AA	Amino acids
ACTH	Adrenocorticotropin hormone
BBB	Blood-brain-barrier
BSA	Bovine serum albumin
CFU	Colony forming units
CRH	Corticotropin-releasing hormone
CV	Coefficients of variation
DA	Dopamine
DOPAC	3, 4-dihydroxyphenylacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCR	Feed conversion rate
FCS	Foetal calf serum
GALT	Gut-associated lymphoid tissue
GR	Glucocorticoid receptor
HBSS	Hank's balanced salt solution
Hg	Haemoglobin
HK	Head-kidney
HKL	Head-kidney leucocyte
HPA	Hypothalamus-pituitary-adrenal
HPI	Hypothalamus-pituitary-interrenal
HPLC-EC	high performance liquid chromatography with electrochemical detection
Ht	Haematocrit
i.p.	intra-peritoneally
IDO	2, 3-indoleamine dioxygenase
IFNγ	interferon γ
IgM	Immunoglobulin M
iNOS	Inducible nitric oxide synthase
KP	Kynurenine pathway
LPS	Lipopolyssacharide
M1	Classically activated macrophage
M2	Alternatively activated macrophage
MALT	Mucosa-associated lymphoid tissue
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume

MSC	Myeloid suppressor cells
NA	Noradrenaline
NAD^+	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
O_2^-	Superoxide anion
OD	Optic density
PAMP	pathogen-associated molecular pattern
PBS	Phosphate buffer saline
Phdp	*Photobacterium damselae* subsp. *piscicida*
PMA	Phorbol myristate acetate
RBC	Red blood cells
RLU	Relative light units
ROI	Reactive oxygen intermediates
SALT	Skin-associated lymphoid tissue
SAM	S-adenosylmethionine
SGR	Specific growth rate
TAC	Total antioxidant capacity
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TCBS	Thiosulfate citrate bile salts sucrose
TDO	Tryptophan 2, 3-dioxygenase
T_H1	T-helper 1 cell
T_H2	T-helper 2 cell
TMB	Tetramethylbenzidine hydrochloride
TNFα	Tumour necrosis factorα
TSA	Tryptic soy agar
TSA-1	Tryptic soy broth with 1 % NaCl
TSA-2	Tryptic soy broth with 2 % NaCl
TSB	Tryptic soy broth
TSB-1	Tryptic soy agar with 1 % NaCl
TSB-2	Tryptic soy agar with 2 % NaCl
UPLC	Ultra-high-performance liquid chromatography
vaLPS	*Vibrio anguillarum* lipopolysaccharide
Vang	*Vibrio anguillarum*
VSI	Viscerosomatic index
WBC	White blood cells

Chapter 1

General Introduction

1.1 Functional diets in aquaculture

Aquaculture now provides half of all fish for human consumption, which has now reached 20 kg per capita [1]. To respond to the needs of an increasing world population, aquaculture emerged as a fast growing food sector that involves the inherent dangers of intensive animal farming regarding environment and local communities, farmed fish health and of course, final consumers. Maintenance routines, rearing conditions or even seasonal climatic variations were, and still are on the base of several constraints and challenges in an aquaculture farm. Problems would multiply due to poor regulatory control and lack of standardized strategies. Such was the case of the use of antimicrobial drugs or other chemicals, which in early days of fish farming was poorly legislated and disproportionately used, most of the times adopted as a prophylactic strategy [2]. Residues of antibiotic in fish as well as in the surrounding environment and the alarming increase in antibiotic resistance – which is then transferred to the consumer – began to be the focus of many studies [3-5]. Fortunately, knowledge on the risks of such conducts now reinforces regulatory changes and the need for alternative solutions that must start with good husbandry practices and immunization programmes, thereby reducing antibiotics use to therapeutic purposes alone.

A correct and adapted nutrition provides the nutrients and energy required for growth, reproduction and general homeostasis and may therefore be regarded as an important tool of health management that potentiates efficiency of immunization. Indeed, healthy individuals are most likely to efficiently manage vaccination. Fish feeds are known to represent the highest production costs. Hence, optimizing fish feeds formulation to meet not only their nutritional requirements but an additional health benefit or improved product quality has been regarded as a promising approach to improve welfare and consequently farm's profitability. These feeds, generally known as functional diets, are usually supplemented with specific ingredients known to directly or indirectly increase the efficiency of a given biological mechanism [6]. They should be both economically attractive and environmentally friendly, and thus animal raw materials should represent a low fraction in the formulated feed [7]. Probiotics [8,9], prebiotics [10,11], nucleotides [12-14], pigments [15,16], vitamins [17,18] and amino acids (AA) [19] are some good examples of successful feed additives supplemented to fish diets at different concentrations to achieve different goals.

Functional AA are those involved in other cellular processes beyond protein building [19] which imply that feed formulation must account for these specific requirements. Indeed, drastic decreases in plasma pool of certain AA have been denoted by fish upon disturbance of homeostasis [20-23]. AA are versatile molecules, involved in multiple

processes such as metabolic regulation, antioxidant protection, stress response, osmoregulation, reproduction and behaviour among others [6]. AA display key features of great relevance when supplemented in aquafeeds: i) AA are innocuous for fish, the environment and its inhabitants at reasonable concentrations; ii) some are already commercialized as feed-grade and iii) periods of administration of an AA-supplemented diet are not necessarily long. Relevant effects have been obtained in many and diversified areas with AA dietary treatments ranging from 1 week [24,25] to 8 weeks [26,27]. Furthermore, Pohlenz and co-workers [28] demonstrated synergistic interactions between AA which co-supplementation in one diet increased vaccination efficiency in channel catfish, *Ictalurus punctactus*.

A great number of studies have been focused on AA supplementation to animal diets but the majority used avian or mammalian models. AA research in fish has been receiving more attention, and though results are still far from being conclusive, recent evidences suggest those are also worthy of further investigation.

1.2 Innate immune response in teleosts

Fish are the first phylogenetic group exhibiting a fully developed adaptive immune system, similar to that of mammals, therefore representing a unique link in the study of the immune system. Although teleosts present a rather limited adaptive immunity in comparison to mammalian specific immune response, innate immune defences are remarkably developed and efficient [29-31]. As aquatic animals, fish are in close and constant contact with microorganisms, whether being on their skin/mucus surface or other mucosal organs such as gills and gut. Therefore, in distinguishing commensals from pathogens, as well as in mounting the corresponding tolerance-leading or inflammatory responses, fish highly rely on powerful innate immune mechanisms. Mediating these mechanisms is an array of effector players classically categorized as cellular and humoral components of the innate immune response: natural killer cells, phagocytes and thrombocytes, hormones, cytokines, protease inhibitors, lysins, precipitins and agglutinins. All these mediators communicate and regulate the actions of one another, creating a complex and very effective network of pro- and anti-inflammatory actions [32-36]. Such richness and complexity, together with different pathogen recognition receptors, grants fish immune defences that, though unspecific, are able to readily recognize and even discriminate from different types of pathogenic microorganism, e.g. viruses, gram-negative or gram-positive bacteria. Moreover, innate does not necessarily mean constitutive, as different cells and molecules are quite dynamic in terms of

activation state and readiness of response, which consequently guarantees strength in each immune response [29,37].

Fish mucosal surfaces are the interface between pathogens and the host millieu and together they constitute the mucosal-associated lymphoid tissue (MALT) [29,38]. Skin (mucus), gills and gut are the first physical barriers, and are provided with diffuse resident cells, enzymes and antimicrobial peptides that readily respond to invading microorganisms, preventing their entrance to the host. A simultaneous systemic response takes place and is characterized by increased blood flow and vessels permeability, migration and accumulation of phagocytes and activation of lytic mediators (e.g. proteases, anti-proteases and lysozyme, among others) [30,39].

Inflammatory processes are orchestrated by pro- and anti-inflammatory mediators that, at different but gradual stages, act to a successful pathogen clearance and resolution of inflammation without or with limited self-damage. In case inflammatory response is not successful at pathogen clearance or in scenarios of a prolonged, unrestrained response, it may developed into a systemic situation typically associated with leucocytosis, activation of complement cascades, decreased plasmatic iron and amino acid mobilization from muscle to liver for intense protein synthesis and secretion [37].

The so-called macrophage polarization is a key feature in determining these timeframes. Macrophage polarization gives respect to type of activation and consequent differentiation of myeloid cells such as macrophages, which determines their nature and activity [40,41]. Macrophage polarization has also been studied in teleosts [42,43] and it seems that the acquired phenotypes share similarities with those observed in mammals. Macrophages are often categorized in 3 phenotypes, acquired upon activation by distinct immune stimuli [43]: i) innate-activated macrophages respond to a microbial stimulus alone (for instance, bacterial lipopolysaccharide, LPS); ii) classically activated macrophages (M1) are induced by LPS and T helper type 1 cells (T_H1)-produced interferon γ (IFNγ); and iii) alternatively activated macrophages (M2), stimulated by T_H2-syntethized interleukin 4 and interleukin 13. M2 can still be functionally sub-classified in three types which is also dependent on the trigger [41]. M1 macrophages present a generally stronger response than innate-activated macrophages, mainly in terms of respiratory burst activity, immune-related enzymes expression and antigen presentation [41], but both phenotypes denote pro-inflammatory activity. On the other hand, M2 macrophages mostly mediate regulatory functions, refraining those of their M1 counterparts from being self-destructive, and at the same time contributing to the resolution of inflammation and tissue repair. A very interesting review, which authors wisely entitled “The ‘macrophages first’ point of view”, calls attention to the fact of a general assumption that macrophages

polarization is dependent on a specific T-cell-driven environment, hence, in scenarios of on-going specific immune processes [43], despite macrophages themselves being key players of innate immune responses. Authors then address other reviews/studies suggesting that macrophages differentiate simply by sensing microbial/parasite infection and subsequently direct T lymphocytes to produce cytokines that would, respectively, extend M1/M2 polarization and not initiate it [44,45]. It is therefore important to state that it is the adaptive immunity which needs and relies on innate immune mechanisms to initiate and direct specific responses and not the inverse situation [45].

1.3 Neuroendocrine-immune interactions

In order to efficiently respond and ultimately survive pathogen invasion and killing, animals (including fish) have developed two physiological responses: the immune response and the stress response. The two share common pathways and effector organs, a fact that necessarily involves a highly-regulated, bi-directional network of signalling molecules [46]. Indeed, interrenal and haematopoietic tissues are entwined in the teleosts head-kidney. Thus, chromaffin, interrenal and immune cells are in close contact and communicate between each other. Moreover, lymphoid tissue is innervated with both sympathetic and parasympathetic nerves that are associated both to inhibition and to stimulation of immune functions [47]. The head-kidney is therefore a central organ, particularly under stressful conditions, with endocrine, nervous and immune tissues being in close contact and orchestrated by local and shared regulatory mechanisms [46].

Analogous to the hypothalamus-pituitary-adrenal (HPA) axis from mammals, fish neuroendocrine response occurs in a cascade of events referred to as the hypothalamus-pituitary-interrenal (HPI) axis (Fig. 1). Activation of the HPI axis involves production of hormones and neurotransmitters both in the hypothalamus and in the pituitary, which will in turn be sent, across the blood-brain barrier (BBB) and transported to peripheral organs. Adrenocorticotropin hormone (ACTH), synthetized in the pituitary, stimulates interrenal cells to produce cortisol (Fig. 1) [48]. Cortisol is the principal corticosteroid in fish and thus is frequently used as stress biomarker [49]. Besides its essential role in the stress response, cortisol is also implicated in osmoregulation, growth and reproduction.

Leucocytes are sensitive to a wide repertoire of neuroendocrine responses. For instance, glucocorticoids influence the balanced successive secretion of pro- and anti-inflammatory cytokines and, in particular, cortisol can have profound and differential effects on the fish immune system [50]. The type of stressor is a crucial determinant on cortisol immunomodulatory effects. Chronic stress is associated to a prolonged demand

of additional energy to sustain the stress response, an allostatic state that deviates energy from other physiological processes. Together with continuous cortisol production, these conditions compromise the immune status, inhibiting defences and consequently, the efficiency of an immune response. On the other hand, an acute-stressor, which typically characterizes a fight-or-flight reaction, may actually stimulate fish immune system. It occurs in a short period of time with high intensity, and there is often no serious effects on immune mechanisms [48], but it may actually prime immune cells which will eventually more rapidly respond to an immune challenge [51].

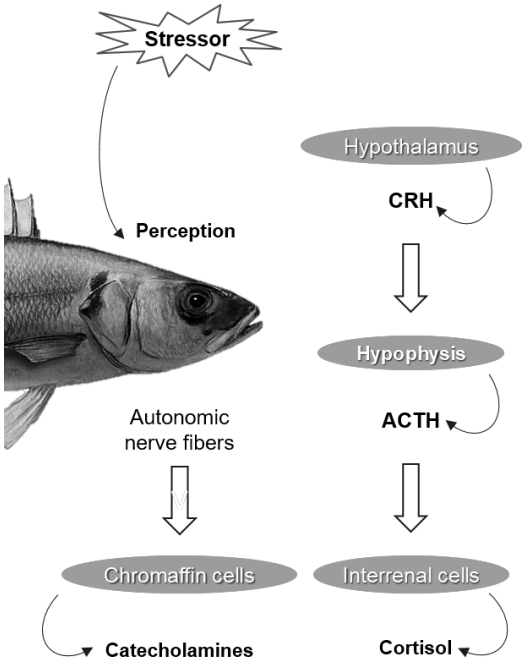

Fig. 1 Hypothalamus-pituitary-interrenal axis of teleosts. Main events following brain perception of stress. CRH: corticotropin-releasing hormone; ACTH: adrecorticotropin hormone.

An important evidence of neuroendocrine and immune systems interaction is the presence of glucocorticoid, adrenergic, cholinergic and opioid receptors in leucocytes, making them sensitive to a wide repertoire of neuroendocrine responses. On the other hand, endocrine cells present cytokine and toll-like receptors [50,52].

Most studied fish species demonstrated the presence of nuclear glucocorticoid receptors (GR) in immune cells [53-55], through which cortisol mediates its suppressive effects. Two GR isoforms are present in the majority of these species, as a result of genome duplication, and their cortisol sensitivity is quite different [50]. While GR2 appears to be more sensitive to physiological levels of circulating cortisol (present in non-stressed fish), GR1 responds only to higher levels, as observed in stressed animals. Nonetheless, immuno-modulation is mediated by both GR isoforms, which individual expression is solely dependent on cortisol concentration [53,56]. GR-mediated inhibitory effects of cortisol

are directly triggered by binding of the glucocorticoid/receptor complex to DNA or transcription modulators (such as nuclear factor - kappa B) [57]. Interestingly, *in vitro* and *in vivo* evidences of down-regulation of GR binding sites in leucocytes have been reported upon cortisol treatment [58] and different hypothesis were formulated. It has been suggested that immune cells bearing such receptors might have acquired the ability to counteract cortisol effects by down-regulating GR gene expression in a negative feedback mechanism. Such regulatory mechanism would enable the immune system to avoid, or at least reduce, the inhibitory effects of a prolonged stress response. Lower GR numbers can also be attributed to the receptor translocation to the nucleus [53], where it has transactivation activity. Moreover, Freeman et al. [59] addresses the fact that GR gene expression in several cells and tissues of adult humans is regulated around a "set-point" by glucocorticoid itself, which is another possible explanation for down-regulation of GR expression in the presence of higher circulating cortisol levels.

Although cortisol is associated to immunity suppression events, it is important to acknowledge that glucocorticoids (cortisol) are part of an integrated response to stress/immune challenge aiming to achieve homeostasis and balance between pro-inflammatory and anti-inflammatory responses, whilst minimizing damage to the host. Cortisol synthesis as well as GR expression are therefore vital and their presence/activity should not be targeted to be completely supressed.

Just as neuroendocrine mechanisms affect immune responses, peripheral immune signals also modulate central, neuroendocrine events. Besides their classical roles as regulators of the systemic and inflammatory responses, cytokines induce other physiological processes such as fever, loss of appetite and fatigue, among others [50]. Fever might not seem to be applicable in fish, which are ectothermic animals, but their temperature has indeed been shown to increase in particular situations. In goldfish, *Carassius auratus*, for instance, temperature rose upon LPS or interleukin 2 injections [60]. Nonetheless, exactly how these peripheral signals are effectively perceived by the central nervous system is a matter of discussion, but some hypothesis have been postulated. Prostaglandins and the enzyme cyclooxygenase-2 are well-known for their role in mammalian fever induction and regulation [61-63]. It is possible that these molecules, known to increase in inflammatory conditions [64], mediate communication between peripheral immune signals and the brain, since prostaglandins are able to cross the BBB [50]. A second hypothesis is that of pathogen-associated molecular patterns (PAMP's) triggering macrophages or endothelial cells present in areas of the brain that are BBB-deficient [65]. These cells will then produce and release cytokines that, in turn, activate receptors present in these brain regions. Finally, besides leucocytes, astrocytes and glia cells are also immunocompetent cells (i.e. following proper stimulation) and evidences

point at the presence of cytokines gene expression in teleosts brain [66-68]. Altogether, these observations demonstrate that inflammatory processes can also modulate central, neuroendocrine responses by directly or indirectly trigging hormone- or neurotransmitter-producing cells. As observed in rainbow trout, cortisol levels increased following HPI axis activation by IL1β injection [69]. HPI axis activation implies the synthesis of corticotropin-releasing hormone (CRH) which then induces pituitary-production of ACTH. CRH increased in juvenile tilapia, *Oreochromis mossambicus,* in response to a 10-days exposure to LPS without elevation of cortisol production [70], suggesting that immunomodulation is performed at the upmost point of the HPI axis.

Data on teleost modulation of the neuroendocrine response by immune signalling pathways is still scarce. However, despite the obvious distance between mammals and lower vertebrates, neuroendocrine and immune processes seem to be evolutionarily conserved among groups, allowing the establishment of parallelisms that are at least useful as starting points.

1.4 Amino acids in immune mechanisms

The assembling of any physiological response requires energy and precursors for anabolic processes which means that requirements of certain nutrients might occasionally increase. Not differently from other nutrients, AA requirements are traditionally evaluated according to growth efficiency and body composition [71]. Hence, and especially for the so-called conditionally essential AA, dietary provision might not be sufficient to fulfil the needs of a particularly critical situation. Meeting these higher AA requirements may also be compromised by the currently used fish feeds formulation, since fish meal is often replaced by vegetable ingredients and final AA balance might not be adequate. These higher requirements are easily observed in stressful situations upon which certain AA plasma levels are drastically reduced [20]. Such changes are of course dependent on each AA and the needs of each particular situation.

The immune response is an especially demanding process marked by intense gene expression and synthesis of immune-related proteins as well as cellular proliferation, migration and trafficking. It engages tight regulatory mechanisms and tissue repair actions. Naturally, the efficiency as well as the resolution of these processes rely on an adequate input of precursor molecules, and among them are specific AA.

This thesis is focused on four AA: glutamine, arginine, tryptophan and methionine. Therefore, a brief review on each one's main roles during the innate immune response is presented separately.

Glutamine

Together with glutamate, glutamine represents ≈20 % of protein constitution and its plasma and muscle abundance is one of the highest in teleost fish [6]. Glutamine and glutamate are in constant interconversion and are both involved in a wide range of metabolic pathways. Glutamate is a neurotransmitter and may be converted to α-ketoglutarate that is an intervenient of the Krebs cycle (Fig. 2).

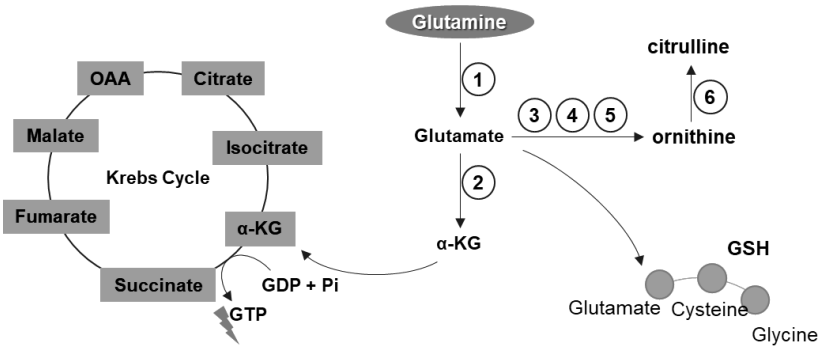

Fig. 2 Glutamine metabolic pathways with implications in the immune response. Glutamate can be incorporated in glutathione and thereby promote the antioxidant capacity; it can also be converted into ornithine and citrulline, hence, indirectly provide substrate for nitric oxide synthesis; glutamate is a preferred source of energy in cells of the immune system, by being converted to α-ketoglutarate that fuels the Krebs cycle. **α-KG**: α-ketoglutarate; **GSH**: glutathione; **OAA**: oxaloacetate; **1**: glutaminase; **2**: glutamate-dehydrogenase; **3**: glutamate-5-kinase; **4**: glutamate-5-semialdehyde dehydrogenase; **5**: ornithine aminotransferase; **6**: ornithine transcarbamylase

Glutamine is considered a non-essential AA. Its availability may be limiting during immune responses, as it is of critical importance to leucocytes as a major energy source, with utilization rates higher than that of glucose [72]. It is also a promoter of cell division and of phagocytosis [73]. Though there is a vast research on glutamine utilization by mammalian macrophages, studies in teleost are scarce and glutamine involvement in the immune system has only been superficially addressed. Buentello and Gatlin [74] observed a synergistic effect of glutamine and arginine on nitric oxide (NO) production in channel catfish macrophages incubated in culture media supplemented with both AA. It was suggested that glutamine was used as fuel in these cells resulting in improved NO production rates from its precursor, arginine.

Innate immune responses typically involve an expressive increase in cellular oxidative stress, as result of activation of phagocytes killing mechanisms such as respiratory burst and NO production [75]. Glutathione is one among many antioxidants which scavenges free radicals to prevent the host from self-damage. Glutathione is composed by glutamate, cysteine and glycine (Fig. 2). Therefore, an adequate provision of glutamine may contribute to the maintenance of the oxidative status.

Arginine and citrulline

Arginine requirements are often high in fish and the almost inexistent endogenous synthesis and its high contribution to protein synthesis makes arginine an essential AA [6]. Arginine is a precursor for the synthesis of nitric oxide, polyamines, proline, glutamate, creatine, and agmatine which grants it a metabolic versatility that might be of interest for many applications.

The importance of arginine as immune modulator is related to two important metabolic pathways. Under inflammatory conditions, fish produce NO and ornithine from arginine, via the inducible nitric oxide synthase (iNOS) and arginase, respectively [74,76]. The two pathways lead to opposite directions regarding inflammatory process and help determining macrophage polarization (Fig. 3). Triggered by LPS and IFNγ, macrophages express iNOS and present an intense NO production against infectious agents [74]. In contrast, arginase activity is observed in macrophages that differentiated in response to anti-inflammatory T-cell produced cytokines. Arginase converts arginine to ornithine, which is the precursor of proline and polyamines [77]. Arginase-expressing macrophages, frequently referred to as alternatively activated macrophages, prevail in a later phase of inflammation, suppressing the activity of other immune cells, thereby leading to resolution of the immune response (Fig. 3).

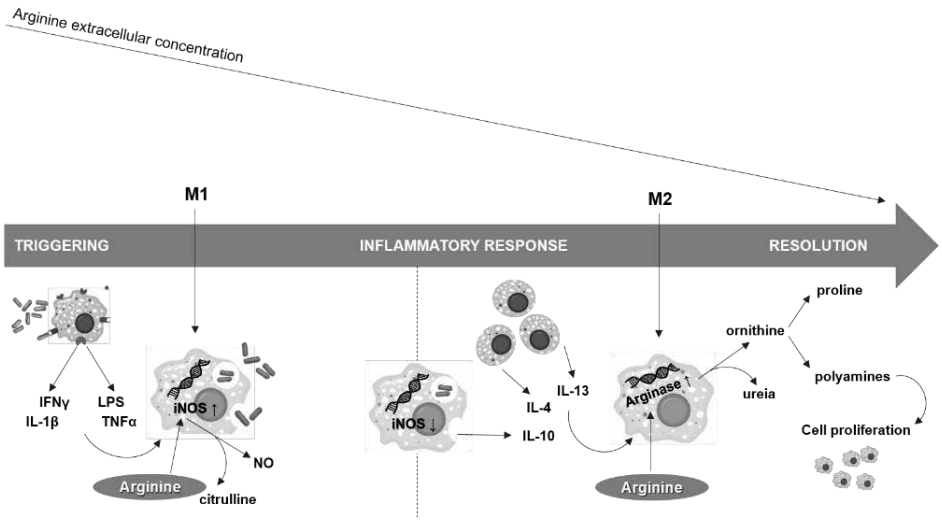

Fig. 3 Macrophage activation, phenotype determination and arginine metabolism during the inflammatory response. **M1**: classically activated macrophage; **M2**: alternatively activated macrophage; **IFNγ**: interferon γ; **LPS**: lipopolysaccharide; **IL-1β**: interleukin-1β; **TNFα**: tumor necrosis factor-α; **iNOS**: inducible nitric oxide synthase; **NO**: nitric oxide; **IL-10**: interleukin-10; **IL-4**: interleukin-4; **IL-13**: interleukin-13

Adding arginine to fish diets or to cell culture media induced [25,78] and impaired different aspects of the immune response [79]. Context seems to be determinant for arginine-mediated effects. Briefly, arginine is metabolized by macrophages throughout the entire inflammatory process and time, together with response progression, dictate

environments chemical composition and cells phenotype, hence, arginine metabolic fate [80-83].

Arginine is gradually becoming an attractive research line given its multitude of possible applications. But particularly in immunology, it is the paradox in resultant effects that mostly entices the will for exploring arginine potential.

Citrulline is both metabolite and precursor of arginine. It is formed via iNOS activity, along with NO, and it has also been shown to influence macrophage NO synthesis upon immune stimulation, which points at its importance as arginine precursor [84]. In channel catfish, dietary administration of gabaculine to inhibit ornithine aminotransferase lowered plasma citrulline and arginine concentration [85], indicating that the physiological needs of this fish species for arginine might be met by both dietary and endogenous *de novo* synthesis sources.

Tryptophan

Similar to mammals, tryptophan is an essential AA for fish, not only for protein synthesis, but also as precursor of relevant functional molecules such as serotonin, melatonin and nicotinic acid [86]. Excluding protein synthesis, most of tryptophan is totally degraded along the kynurenine pathway (KP), yielding nicotinamide adenine dinucleotide (NAD^+) (Fig. 4). Complete tryptophan degradation mostly occurs in the liver and it starts with an oxidation step mediated by tryptophan 2, 3-dioxygenase (TDO), also known as tryptophan pyrrolase. NAD^+ synthesis depends on the organism uptake of dietary niacin. In fish, tryptophan conversion to niacin is limited and, as observed by Ng and co-workers [87], supplementing channel catfish diets devoid of niacin with graded levels of tryptophan did not enhance this vitamin endogenous synthesis.

While most of the KP steps are well-established in mammals, only part of the enzymes have been identified so far in teleost fish [88,89]. Therefore, whether the exact sequence of events is present and which regulatory mechanisms determine enzyme activities in lower vertebrates, is a matter of further investigation.

The same degradation pathway is present in macrophages, though in these cells TDO is replaced by indoleamine 2, 3-dioxygenase (IDO) (Fig. 4). This enzyme has higher affinity to tryptophan than TDO does, and its activity suppresses TDO's [90]. IDO is induced by inflammatory stimuli such as LPS or IFN-γ and it has been observed not only in mammals but also in fish [88].

The role that tryptophan plays in IDO-expressing cells is mediated by bioactive compounds produced further down the cascade. Besides IDO-mediated tryptophan depletion from extracellular environment, which prevents microorganisms from utilizing it, tryptophan metabolties such as 3-hydroxyanthranilic acid and quinolinic acid are able

to regulate T cell function and to modulate the oxidative status (Fig. 3) [91]. Thus, tryptophan-IDO-mediated effects are mostly regulatory effects, often leading to immune tolerance scenarios.

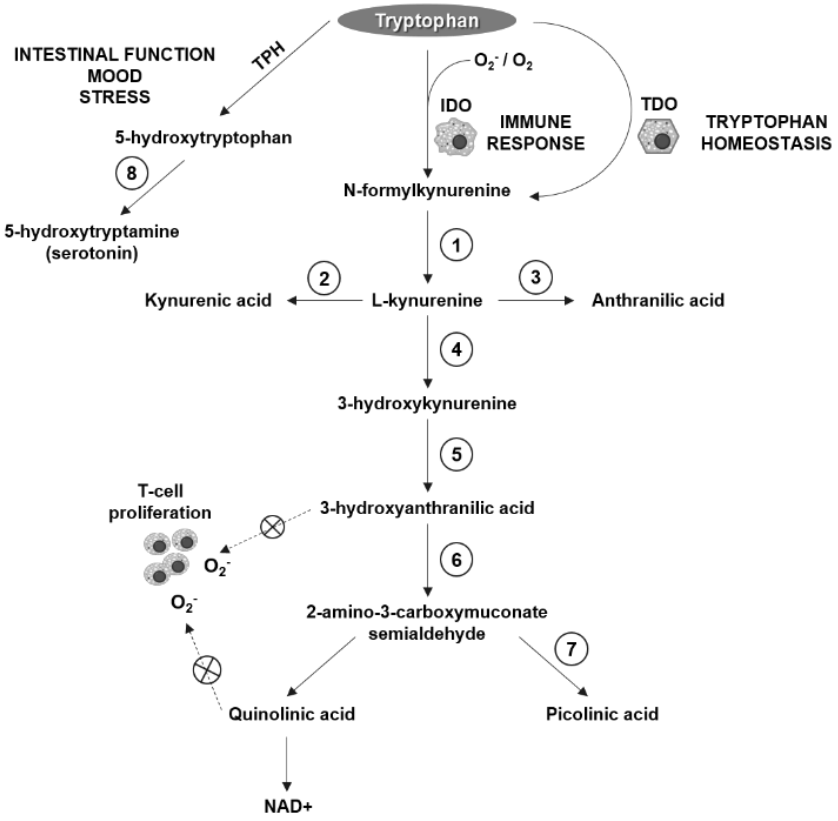

Fig. 4 Tryptophan metabolism in mammals and its implication in the immune response. **TPH**: tryptophan hydroxylase; **IDO**: 2, 3-indoleamine dioxygenase; **TDO**: tryptophan 2, 3-dioxygenase; **NAD+**: nicotinamide adenosine dinucleotide; **1**: arylformamidase; **2**: kynurenine amino-transferase; **3**: kynureninase; **4**: kynurenine 3-hydroxylase; 5: kynureninase; **6**: 3-hydroxyanthranilic acid oxygenase; **7**: picolinate carboxylase. Adapted from Le Floc'h (2011).

Tryptophan might also indirectly modulate the immune response by means of a neuroendocrine pathway. Serotonin (5-HT) is produced from tryptophan in a reaction involving tryptophan hydroxylase (Fig. 4), which is the rate-limiting enzyme in this pathway. 5-HT is commonly associated to animal behaviour, appetite and aggression [92]. 5-HT is a monoamine which is simultaneously a neurotransmitter in the central nervous system and a paracrine or endocrine signal in the gut and blood [93]. Interestingly, 5-HT might either induce or inhibit ACTH production in the pituitary, and consequently increase or decrease interrenal cortisol synthesis [94]. Because tryptophan hydroxylase is not saturated at tryptophan physiological concentrations, 5-HT production can be promoted with tryptophan supplementation, a fact already observed in rainbow trout [94,95]. While this negative feedback mechanism seems to be dose-dependent in rainbow trout, the same might not be true for other fish species. Indeed, Senegalese sole, *Solea senegalensis*,

fed tryptophan-supplemented diets at 2 × the requirement level presented increased disease resistance and humoral defences than fish fed a control diet, in both resting and stressful conditions [96]. However, when fed higher tryptophan amounts (4 × the requirement level), disease resistance was only higher in stressed fish, whereas higher mortalities were observed in undisturbed ones.

Methionine

S-adenosylmethionine (SAM) is a major methyl donor and is produced mostly in the liver during the methylation cycle by methionine adenosyltransferase [97].

Methionine metabolism can be directed to three pathways with implications in the immune response (Fig. 5): i) it provides SAM that is then decarboxylated and turned into an aminopropane donor that fuels polyamine turnover [98]; ii) SAM is directly involved in methylation of several cell constituents such as DNA, adrenergic, dopaminergic and serotonergic molecules [6]; iii) it leads to the transsulfuration pathway that ends up in the formation of glutathione from homocysteine [99]. Cysteine synthesis through transsulfuration, as well as transmethylation and remethylation are well-established for mammalian liver, and in spite differences may exist among species, these reactions are also likely to be present in teleosts [6].

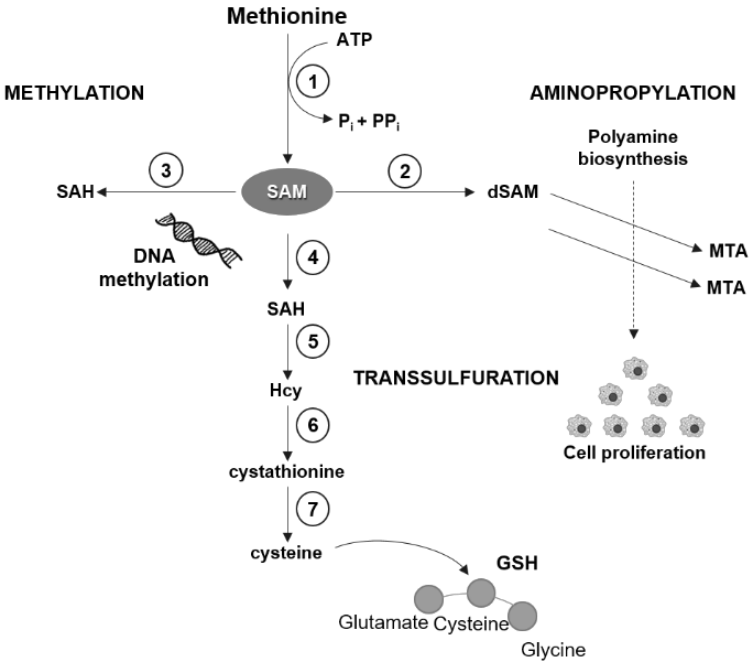

Fig. 5 Methylation, aminopropylation and transsulphuration pathways of methionine metabolism. **SAM:** S-adenosylmethionine**; dSAM:** decarboxylated S-adenosylmethionine; **SAH:** S-adenosylhomocysteine**; Hcy:** homocysteine**; GSH:** glutathione**; MTA:** methylthioadenosine; **1**: methionine adenosyltransferase; **2**: S-adenosylmethionine decarboxylase; **3**: DNA methyltransferase; **4**: S-adenosylmethionine methyltransferase; **5**: S-adenosylhomocysteine hydrolase; **6**: cystathione β-synthase; **7**: γ-cystathionase

As a biological response that critically relies on a solid cellular reaction, immune response is associated to intensive DNA replication and cell division. Polyamines ensure stability and fidelity of replication, and studies in fish demonstrate increased levels of polyamines in so demanding circumstances [100-102]. High methionine intake has shown to increase hepatic SAM content [103]. Thus, it seems logical to assume that promotion of polyamine biosynthesis through higher methionine input might enhance immune cellular response. Methionine dietary supplementation for 14 days improved innate humoral and cellular responses of European seabass intra-peritoneally injected with UV-killed *Photobacterium damselae* subsp. *piscicida* [104].

As precursor of cysteine, methionine may also play a role in increasing glutathione synthesis and thus improve the oxidative status during inflammatory response (Fig. 4). Head-kidney and spleen glutathione as well as other free radical scavengers augmented in juvenile Jian carp, *Cyprinus carpio* var. *Jian*, fed graded levels of methionine hydroxy analogue [105]. On the other hand, deficiency of methionine or of its metabolites may compromise the oxidative status and consequently affect normal inflammatory processes [106,107].

Due to high inclusion of plant-derived ingredients in current fish feeds, methionine is often considered the first-limiting AA in diets formulation. It is also among other essential AA which plasma pool sharply decreases during stressful situations [108].

1.5 Thesis main objectives

As a recent and fast growing animal production sector, fish farming has its own bottlenecks typically associated to sub-optimum rearing conditions and lack of scientific knowledge. As lower vertebrates, fish share most of the metabolic pathways and intermediate messengers with other vertebrate species such as mammals. Yet, some differences exist that need to be acknowledged in order to optimize husbandry procedures such as nutrition and health-management routines.

Given their deep involvement in immune-related pathways, and in light of what is already established for humans and other mammalian models, AA stand out as good candidates to be used as nutraceutics, not only to promote fish immune status and disease resistance but also to increase the efficiency of other immunization strategies such as vaccination.

This thesis is an approach to advance understanding on the potential of glutamine, arginine, citrulline, tryptophan and methionine in modulating innate immunity given their specific roles in different immune mechanisms. It also evaluates whether such effects ultimately change disease resistance of European seabass. It thereby intends to gather

new data on promising feed additives that might represent healthier and more sustainable strategies to improve farmed fish welfare.

Aquaculture functional diets and fish innate immune response are generally addressed in Chapter 1 of this thesis, together with a brief explanation on the importance of neuroendocrine-immune interactions and of each AA specific immune roles. An *in vitro* approach to AA immunomodulatory effects is presented in Chapter 2, where primary head-kidney leucocyte cultures treated with different AA supplementations accordingly develop different innate immune responses against bacterial antigens. Methionine and tryptophan immune-related actions are then tested *in vivo* in Chapter 3, where both the immune status and inflammatory response of seabass are evaluated after 15 days being fed diets supplemented with 2 × the requirement level of each AA. Furthermore, neuroendocrine-immune interactions are assessed and AA effects on the HPI axis are estimated. Chapter 4 further explores the contribution of the same AA in immunity, this time describing the development of peripheral immune processes in response to a peritoneal insult, analyzing gut and skin mucus responses. Arginine in particular is addressed in Chapters 5 and 6, where experimental feeding trials were set up to determine whether arginine improves, impairs or does not change seabass immune condition, response and disease resistance after bacterial infection. Finally, an integrated discussion is presented in Chapter 7, where results regarding each AA from different experiments are linked and the potential as feed additives is commented. Loose ends are exposed and debated followed by proposals that might enlighten them.

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Chapter 2

Amino acids as modulators of the European seabass, *Dicentrarchus labrax*, innate immune response: an *in vitro* approach

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Amino acids as modulators of the European seabass, *Dicentrarchus labrax*, innate immune response: an *in vitro* approach

Abstract

Teleost innate immune system is a developed and powerful defence system in fish. Conditions in aquaculture farms are particularly prone to disease. Thus, health and welfare ensuring strategies are an urgent call to which nutrition is gradually becoming a most regarded achievement tool. This study intended to evaluate the role of different amino acids on immune-related signalling pathways as well as their potential as enhancers of European seabass, *Dicentrarchus labrax*, leucocyte function. To achieve these goals, primary cultures of head-kidney leucocytes were established and kept in amino acid (glutamine, arginine, tryptophan or methionine) supplemented culture media in two doses. The effects of amino acids treatments were then evaluated after stimulation with either *Vibrio anguillarum* or *Vibrio anguillarum* lipopolysaccharides by measuring nitric oxide production, extracellular respiratory burst, ATP and arginase activities, and expression of immune-related genes. Glutamine, arginine and tryptophan showed to be particularly relevant regarding cell energy dynamics; arginine and tryptophan supplementation also resulted in down-regulation of important immune-related genes. Immune responses in cells treated with methionine were generally enhanced but further studies, particularly those of enzymes activity, are essential to complement gene expression results and to better understand this nutrients' immune role in fish.

2.1 Introduction

In spite of being among the oldest vertebrates, teleost are able to mount both innate and adaptive immune responses. While the latter is known to be less sophisticated than responses seen in higher vertebrates, the innate immune response is remarkably developed and powerful [1], and fish highly rely on it [1,2]. Regardless of organism phylogeny, innate immune mechanisms are orchestrated by a great variety of cells and effector intermediates that are absent in specific immune responses [3]. Such feature grants the host with innate immune defences that, though unspecific, are able to react against a wide range of pathogens [4]. Although pathogens are considered part of normal biological interactions of fish, they may become a serious problem in aquaculture. Hence, as fish farming is a fast growing industry there is an urgent need to investigate and control fish diseases, as well as deeply understanding fish immunity.

It is now widely accepted that nutritional approaches are essential to alleviate diseases among farmed aquatic animals [5]. In particular, immune-nutritional strategies have been studied in order to highlight the importance of individual amino acids (AA) as nutraceutics for farmed fish. Glutamine, for instance, is a key source of energy for leucocytes [6]. *In vivo* and *in vitro* experiments with arginine demonstrated its involvement on immune regulation, cell proliferation and neuro-endocrine mechanisms, but its practical health implications in farmed fish are still controversial [7-10]. Tryptophan is involved in immune tolerance mechanisms mediated by indoleamine 2, 3-dioxygenase (IDO), which metabolizes tryptophan in leucocytes [11,12]. The importance of tryptophan during dendritic cells activation has also been demonstrated [13]. Methionine supplementation of feeds affected fish immune status, as observed by increased leucocyte numbers [14] and enhanced humoral immune response [14,15]. Nonetheless, few studies were centred on the exact pathways through which AA mediate their immune effects.

One of the main players of innate response, particularly in inflammatory processes, is the phagocyte. Phagocytes are differentiated leucocytes (mostly neutrophils and macrophages) able to engulf and digest other cells (pathogens or self-apoptotic or necrotic cells). Phagocytes are directly involved in both induction and resolution of inflammation via paracrine and autocrine signalling [16] by producing pro- and anti-inflammatory mediators. However, particularly in macrophages, such response only happens upon their activation/differentiation into mature cells that phenotypically present the required machinery. Based on stimuli that triggers different activation states, macrophages are classified in four groups (as recently reviewed by Forlenza and colleagues [17]): innate activated macrophages (triggered by microbial stimulus alone);

classically activated macrophages (M1, resulting from synergistic effect of both microbial stimulus and interferon γ (IFNγ); alternatively activated macrophages (M2, induced by interleukin 4 and interleukin 13); and regulatory macrophages (activated by microbial stimulus, immune complexes, and interleukin 10). Other than macrophages it has been shown that other immune cells such as phagocytic lymphocytes are present in fish, a feature that is not found in higher vertebrates, except in very low proportions [18].

This study will focus only on innate activated macrophages, which are a valuable instrument in *in vitro* studies focused on the innate immune response [19-22]. Moreover, primary leucocyte cultures are useful tools in functional studies as they allow to evaluate responses under well controlled conditions and without the interference of external and unpredictable factors. In addition, it allows the evaluation of important cellular responses to bacteria or bacterial antigens and the assessment of signalling pathways eventually affected by treatments.

Antigens, such as lipopolysaccharides (LPS) from *E. coli,* are currently the most used immune response-triggering stimuli due to feasibility and commercial availability. However, though LPS easily elicits innate activation of mammal macrophages [23-25] this is not the case in fish. Fish immune cells seem to be considerably less susceptible to LPS and much higher concentrations have to be administered (i.e. μg ml^{-1}) for a response. Presence of residual peptidoglycans or bacterial DNA in impure LPS preparations are believed to be the structures recognized by fish leucocytes, therefore responsible for inducing an immune response [26].

We hereby present an *in vitro* study on the innate immune response of head-kidney leucocytes (HKL) against *Vibrio anguillarum* (*Vang*) or *Vibrio anguillarum* LPS (vaLPS), where we evaluated the modulatory effects of AA on immune-related signalling pathways. We also aimed to evaluate whether each AA surplus is able to improve the immune response in innate activated macrophages.

2.2 Material and Methods

2.2.1 *Vibrio anguillarum* cell inactivation

The addition of viable bacteria to eukaryotic cell cultures necessarily involves nutrients consumption. Consequently, not only nutrients availability is compromised and AA treatments jeopardized, but cell medium pH is altered. Therefore, stimulating HKL with live bacteria would probably lead to a noxious *in vitro* environment that do not serve present study main goals. Hence, bacteria cultures were inactivated by UV-exposure. Since UV exposure inactivates bacteria by disrupting nucleic acids, integrity of the main structures is assured thus allowing normal antigen recognition by eukaryotic cells. *Vang*

was kindly provided by Professor Alicia E. Toranzo (Departamento de Microbiología y Parasitología, Facultad de Biología, University of Santiago de Compostela, Spain) and previously isolated from gilthead seabream, *Sparus aurata*. Bacteria were first cultured for 24 h at 22 °C in selective medium thiosulfate citrate bile salts sucrose (TCBS) agar (VWR, Prolabo). Colonies were then inoculated into tryptic soy broth (TSB) supplemented with NaCl to a final concentration of 1 % (w/v) and incubated overnight at 22 °C. Bacterial solution was inactivated by exposure to UV-light for 2 h preceded by alternating UV-light exposure for 10 min (2 min UV-light with 1 min interval), which proved to increase the efficiency of bacterial inactivation. Bacterial growth was not observed when UV-killed bacteria were plated in tryptic soy agar. Bacteria were then recovered by centrifugation at 3,500 rpm for 30 min and the pellet was then re-suspended in sterile phenol-red free Hank's Balanced Salt Solution (HBSS) at final concentration of 1×10^7 colony forming units (CFU) ml^{-1}.

2.2.2 *Vibrio anguillarum* lipopolysaccharides extraction and purification

V. anguillarum LPS was extracted by hot phenol-water according to the method described by Rezania et al. [27] with slight modifications. One hundred ml of bacterial suspension was centrifuged at 10,000 × *g* for 5 min and washed twice with 0.15 M phosphate buffer saline (PBS) (pH=7.2) containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$. Pellets were re-suspended in 10 ml PBS and sonicated on ice for 10 min. To eliminate protein and nucleic acid contaminants, the samples were treated with proteinase K (100 µg ml^{-1}; Roche, Mannheim, Germany) for one hour at 65 °C, followed by overnight incubation at 37 °C with RNase (40 µg ml^{-1}; Roche) and DNase (20 µg ml^{-1}; Roche) in presence of 1 µl ml^{-1} 20 % $MgSO_4$ and 4 µl ml^{-1} chloroform. Next, an equal volume of hot (65-70 °C) 90 % phenol was added to the mixture followed by vigorous shaking at 65-70 °C for 30 min. After being cooled on ice, the mixture was transferred to 50 ml polypropylene tubes and centrifuged at 3,500 × *g* for 30 min. The supernatants were recovered and extra phenol phases were removed using 20 ml hot (65-70 °C) distilled water. Sodium acetate at 0.5 M final concentration and 10 volumes of 95 % ethanol were added to the extracts to precipitate vaLPS by incubating overnight at -20 °C. Tubes were then centrifuged at 3,500 × *g,* 4 °C for 30 min and pellets were re-suspended in 1 ml distilled water, followed by extensive dialysis (SnakeSkin dialysis tubing of 10 K MWCO, Thermo Fischer Scientific) against distilled water at 4 °C. Purified vaLPS, without any residual phenol was lyophilized, re-suspended in PBS to a final concentration of 2 mg ml^{-1}, and kept at -20 °C until use. Visualization was achieved by SDS-PAGE (12 %)

electrophoretic resolution of 20 µg purified vaLPS and consequent staining following the improved silver stain protocol described by Zhu et al. [28].

2.2.3 Fish and establishment of HKL primary cell cultures

European seabass (*Dicentrarchus labrax*) of 200 ± 50 g were obtained from a commercial fish farm. The animals were acclimatized to laboratory conditions for 3 weeks in a recirculation water system maintained at 18 °C and daily fed a commercial diet (Skretting, Spain). No clinical signs of disease or illness were observed in the animals.

HKL were isolated and maintained following Secombes [29] methodology with some modifications. Briefly, European seabass head-kidney was aseptically removed and pushed through a 100 µm nylon mesh in Leibovitz L-15 medium (Gibco, Scotland, UK) supplemented with 2 % foetal calf serum (FCS, Gibco), penicillin (Gibco; 100 IU ml^{-1}), streptomycin (Gibco; 100 µg ml^{-1}), gentamicin (200 µg ml^{-1}) and heparin (Braun, 30 U ml^{-1}). The cell suspension was then layered over a 31:45 % Percoll column (Sigma) and centrifuged at 400 × *g* and 4 °C for 40 minutes. Cells were recovered from the Percoll gradient interface, loaded onto a new Percoll gradient and centrifuged again in the same conditions. Then, the cell band was washed three times in L-15 medium 2 % FCS, heparin and antibiotics at 600 × *g* and 4 °C for 10 minutes. Cells were re-suspended in L-15 medium, 0.1 % FCS and antibiotics, and both leucocyte viability and concentration were determined by the trypan blue exclusion method, using a Neubauer chamber. Cell suspension was then adjusted to 1×10^7 cells ml^{-1} and plated in 96-well plates at 100 µl per well (for respiratory burst, nitric oxide (NO), ATP assay and arginase activity measurements) or 24-well plates at 500 µl per well (for gene expression). Plates were incubated for 2 hours at 18 °C and non-adherent cells were then washed off with HBSS.

The experiments were approved by the Animal Welfare Committee of the Interdisciplinary Centre of Marine and Environmental Research and carried out in a registered installation (N16091.UDER) and were performed by trained scientists in full compliance with national rules and following the European Directive 2010/63/EU of the European Parliament and the European Union Council on the protection of animals used for scientific purposes.

2.2.4 Experimental conditions

L-15 is a standard eukaryotic cell culture media which composition contains the amino acids considered in this study. Monolayers were kept for 24 h at 18 °C in L-15 10 % FCS supplemented with each AA at 1 × or 1.5 × basal concentration to the following final concentrations: $_L$-glutamine (G1, 4.1 mM or G2, 5.1 mM), $_L$-arginine (A1, 5.7 mM or

A2, 7.2 mM), L-tryptophan (T1, 0.2 mM or T2, 0.25 mM) and L-methionine (M1, 1 mM or M2, 1.25 mM). A control group without addition of AA, henceforth referred as L-15 group, was also included. HKL cultures were visually inspected and photographed (Olympus IX71).

Functional immune responses were evaluated following stimulation with either *Vang* (added at a final concentration of 1×10^6 CFU ml^{-1}) or vaLPS (10 µg ml^{-1}) according to preliminary studies for each analysis. NO production was tested using both stimuli, as preliminary tests showed relevant results. A control group with no stimulus (CTRL) was always included. For gene expression studies cell monolayers were only stimulated with vaLPS to avoid bacterial RNA contamination. Every treatment analyses were carried out in triplicate wells, and a total of six biological replicates were used.

2.2.5 Nitric oxide

NO production was measured indirectly based on the Griess reaction previously described by [30] and modified according to [31]. As NO is a very unstable molecule and is promptly converted into nitrite and further into nitrate, this method quantifies nitrites content in the supernatant of macrophage primary cell cultures. Following 24 h incubation with each AA treatment or L-15 alone, cell culture media was replaced by fresh solution containing either *Vang* or vaLPS. Then, plates were incubated at 18 °C for 72 or 96 h. At the end of each incubation time, 50 µl of leucocytes supernatant were transferred into a new 96-well plate to which 100 µl of sulfanilamide (Sigma, 1 % in phosphoric acid at 2.5 %) and 100 µl N-naphthylethylene-diamine (Sigma, 0.1 % in phosphoric acid at 2.5 %) were added. Absorbance was read at 540 nm (Synergy HT, Biotek) and nitrites molar concentration was obtained against a sodium nitrite standard curve.

2.2.6 Respiratory Burst

Prior to respiratory burst assessment, primary cell cultures were stimulated for 4 or 24 hours at 18 °C with *Vang* solution containing each AA treatment or L-15 alone. A control group with no stimulus was also included. Respiratory burst activity of HKL was measured based on the reduction of ferricytochrome C method for detection of extracellular O_2^- [28]. After each incubation time, leucocyte monolayers were firstly washed twice with HBSS. Then, 100 µl of ferricytochrome C solution (Sigma, 2 mg ml^{-1} in HBSS) with phorbol myristate acetate (PMA, Sigma, 10 µg ml^{-1}) was added to the wells. To confirm the reaction specificity, wells containing ferricytochrome C, PMA and superoxide dismutase (SOD, Sigma, 300 uni ml^{-1}) were included for each assay. Plates were

incubated for 30 min at 18 °C, and the absorbance was measured at 550 nm (Synergy HT, Biotek). Optic density (OD) units were transformed into nmols of O_2^- by multiplying by the conversion factor 15.87, as described by [32].

2.2.7 Arginase activity

Arginase activity was only measured in the lysate of HKL cultures supplemented with A1, A2 or in control conditions (L-15). Cells were incubated for 4 or 24 h at 18 °C with *Vang*. Then, cell monolayers were removed from each well (≈ 10^6 cells ml^{-1}) with cold HBSS without Ca^{2+} or Mg^{2+}, and the cell suspension was centrifuged for 10 min at 1,000 × *g* and 4 °C. HKL were then lysed for 10 minutes with 100 µl Tris-HCl solution (pH 7.4, 1 mM pepstatin A, 1 mM leupeptin, and 0.4 % (w/v) Triton X-100). Afterwards, cell lysates were centrifuged at 13,000 × *g* and 4 °C, and the supernatant recovered. Arginase activity was then performed with the Arginase Activity Assay Kit (Sigma) according to manufacturer's instructions. Briefly, 40 µl of supernatant was added to duplicate wells. Fifty µl of 1 mM urea standard solution and 50 µl water were also added to separate wells. Then, 10 µl of arginine substrate buffer was added to one of the sample wells and plates were incubated for 2 h at 37 °C. Afterwards, 200 µl of urea reagent was added to all wells to stop the reaction followed by the addition of 10 µl of arginine substrate buffer to the other sample well. Plates were incubated again for 60 min at room temperature and absorbance was measured at 430 nm (Synergy HT, Biotek). OD units were converted to units ml^{-1} according to manufacturer's instructions.

2.2.8 ATP assay

Stimulation of cells for the ATP production analysis was achieved by incubating cell monolayers with vaLPS for 4 or 24 h at 18 °C, with prior 24 h incubation with each AA treatment or L-15 alone. The ATP concentration in HKL cultures was measured with the ATP Determination Kit (Molecular Probes) following manufacturer's indications. In summary, 10 µl of each well supernatant was transferred to a new plate containing 90 µl of standard reaction solution comprising dithiothreitol, D-luciferin and luciferase, and luminescence was read (Synergy HT, Biotek). Background luminescence values were subtracted and ATP concentration was calculated from a previously prepared ATP standard curve.

2.2.9 Gene expression

Total RNA was extracted with NZYol reagent (NZYTech, Portugal) following manufacturer's instructions, and resuspended in free nuclease water (NZYTech). RNA

was quantified using Take 3 Microvolume Plate (Biotek) and samples were then treated with DNase using RQ1 RNase-free DNase kit (Promega) following manufacturer's indications. The integrity of total RNA was assessed on denaturing agarose gels. Total RNA (600 ng) per sample was used for cDNA synthesis, which was performed using NZY First-Strand cDNA Synthesis Kit (NZYTech) according to manufacturer's instructions.

A set of primers was chosen to evaluate immune-relevant gene expression profile. The chosen genes were: interleukin-1β (*il1β*), cyclooxygenase 2 (*cox2*), macrophage migration inhibitory factor (*mif*), and transforming growth factor β (*tgfβ*). To estimate polyamine synthesis modulation, three genes involved in this pathway as well as in AA metabolism were selected: s-adenosylmethionine decarboxylase 1 (*amd*), ornithine decarboxylase (*odc*) and diamine acetyltransferase 1 (*sat*). Primer sequences are listed in Table 1. Efficiency of each primer pair was determined by real-time PCR according to Pfaffl [33]. Quantitative PCR reactions were carried out in an Eppendorf Mastercycle ep realplex. Each reaction contained 1 µl of diluted cDNA (1:5 dilution) mixed with 10 µl of NZYSpeedy qPCR Master Mix and 0.4 µl (10 mM) of each specific primer, in a final volume of 20 µl. The thermal conditions used were 10 min at 95 °C of pre-incubation, followed by 40 cycles at 95 °C for 15 s and annealing temperature for 1 min. Melting curve analysis was performed to verify that no primer dimers were amplified. The expression of target genes was normalized using European seabass ubiquitin (*ubqt*) gene as housekeeping gene, since it was constitutively expressed independently of treatments. Fold change units were calculated by dividing the normalized expression values from different treatments by the normalized expression values of the respective controls.

Table 1. Forward and reverse primers for real-time PCR.

Gene name	Abbr	GenBank	*Eff*[1]	AT[2]	nt[3]	Primer sequence (5'- 3')
Interleukin 1 β	*Il1β*	AJ269472.1	97.7	57	105	F AGCGACATGGTGCGATTTCT R CTCCTCTGCTGTGCTGATGT
Cyclooxygenase 2	*cox2*	AJ630649.1	81.3	60	160	F CATTCTTTGCCCAGCACTTCACC R AGCTTGCCATCCTTGAAGAGTC
Macrophage migration inhibitory factor	*mif*	FN582353	89.3	60	76	F GCTCCCTCCACAGTATTGGCAAGAT R TTGAGCAGTCCACACAGGAGTTTAGAGT
Transforming growth factor β	*tgfβ*	AM421619.1	105.4	55	143	F ACCTACATCTGGAACGCTGA R TGTTGCCTGCCCACATAGTAG
S-adenosylmethionine decarboxylase	*amd*	KM225770	99.3	57	63	F CTGACGGAACTTACTGGACCATC R CGAAGCTGACGTAGGAGAACT C
Ornithine decarboxylase	*odc*	KM225771	99.9	60	69	F GGGCTGTAGTTATGACACTGGCATCC R GCTGAATCTCCATCTTGCTTGCACAGT
Diamine acetyltransferase 1	*sat*	KM225772	91.6	63	55	F GCATCATCGCTGAAATCCAAGGAGAGAACA R CCAACCACCTTCAGGCCGTCACT
Ubiquitin	*ubqt*	FN565665	92.7	55	79	F TGCTCCCAATCCAGATGATCC R TGTCTCGATGGCGTTGCTT

[1] Efficiency of PCR reactions were calculated from serial dilutions of tissue RT reactions in the validation procedure.
[2] Annealing temperature (°C)
[3] Amplicon size in nucleotides

2.2.10 Statistical Analysis

Statistical analyses were performed with STATISTICA (StatSoft, Inc. 2013, version 12) for WINDOWS. Results are expressed as means ± standard deviation. Homogeneity (Levene's test) and normality were checked and, when necessary, outliers were removed and data were log-transformed before analysis. Data were analysed by multifactorial analysis of variance (ANOVA) with time, AA and stimulus as factors, and with $p \leq 0.05$ as significance level chosen for rejection of the null hypothesis. A multiple-comparison Tukey HSD test was performed to identify differences between groups.

2.3 Results

HKL were exposed to AA treatments by supplementing Leibovitz L-15 culture medium with each AA to the following final concentrations: $_{L}$-glutamine (G1, 4.1 mM or G2, 5.1 mM), $_{L}$-arginine (A1, 5.7 mM or A2, 7.2 mM), $_{L}$-tryptophan (T1, 0.2 mM or T2, 0.25 mM) and $_{L}$-methionine (M1, 1 mM or M2, 1.25 mM). A control group was included without addition of AA, henceforth referred as L-15 group.

2.3.1 Nitric oxide

A complete description of results can be found as Appendix I (Table 1), and Figure 1 provides a selected subset of most relevant results, regarding the effects of AA surplus on NO production. For clarity, results for each AA are described separately below.

Glutamine

At 72 h of incubation, G1-treated cells showed higher NO production than L-15-treated HKL (Appendix I, Table 1C). A time-dependent effect was denoted in cells treated with G2, in which NO was higher at 96 h than at 72 h (Appendix I, Table 1C). When incubated with *Vang*, G1- and G2-treated cells produced higher amounts of NO than their CTRL and vaLPS-incubated counterparts (Fig. 1). Moreover, NO levels in G1-treated cells were higher than in every other AA treatment when held under CTRL conditions (i.e. no stimuli) (Fig. 1).

Arginine

NO production increased with time only in A2-treated cells (Appendix I, Table 1C), but no differences were observed in NO production of A1- and A2- treated cells relatively to L-15-treated cells (Appendix I, Table 1B). Compared to CTRL or vaLPS-stimulated cells, incubation with *Vang* augmented NO production in both A1 and A2 groups (Fig. 1).

Tryptophan

NO production increased with time in both tryptophan supplementations (Appendix I, Table 1C), but was not higher than in the L-15-treated cells (Appendix I, Table 1B). Furthermore, T1- and T2- treated cells incubated with bacteria synthetized more NO than the respective CTRL and vaLPS groups (Fig. 1).

Methionine

The highest NO levels were measured in M2-treated cells, which were higher than in L-15, A1, A2 and T1 groups at 96 h of incubation (Appendix I, Table 1C). NO levels increased in time in both methionine-supplemented groups (Appendix I, Table 1C). An interactive effect was observed between vaLPS and methionine, as vaLPS-stimulated M1 and M2 cells presented higher NO production than vaLPS-stimulated L-15 HKL, as well as their methionine CTRL counterparts, respectively (Fig. 1). Moreover, regarding M2-treated cells, these levels were also higher than those measured in every other AA treatments. Finally, both M1 and M2 *Vang*-stimulated cells presented higher NO production than their CTRL counterparts (Fig. 1).

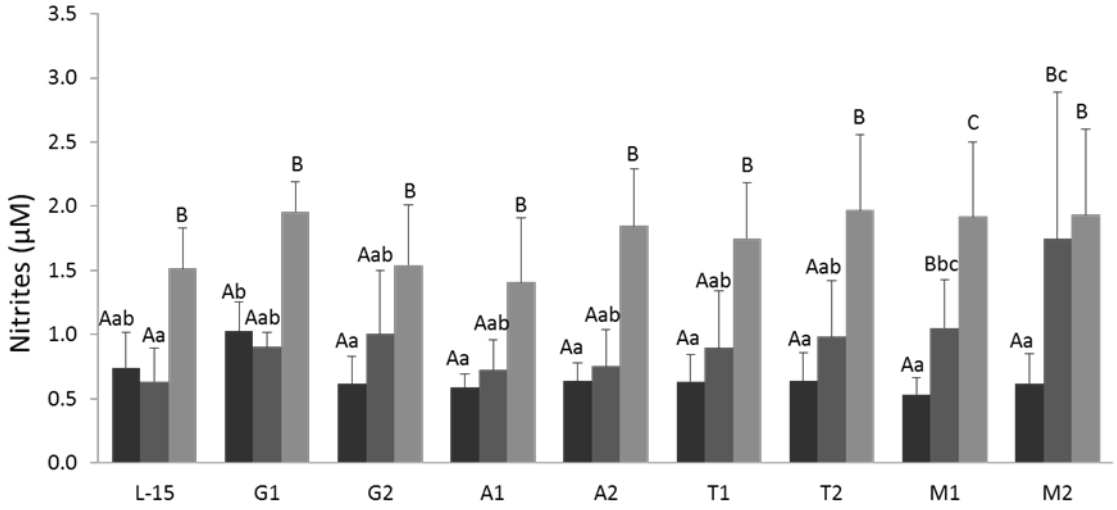

Fig. 1 Nitric oxide content measured as total nitrites in the supernatant of HKL subjected to AA treatments under CTRL (■), vaLPS (■) or *Vang* (■) stimulation conditions, regardless time of incubation. The AA treatments depicted in the X-axis are as follows: L-15, control; G1, 4.1 mM $_{L}$-glutamine; G2, 5.1 mM $_{L}$-glutamine; A1, 5.7 mM $_{L}$-arginine; A2, 7.2 mM $_{L}$-arginine; T1, 0.2 mM $_{L}$-tryptophan; T2, 0.25 mM $_{L}$-tryptophan; M1, 1 mM $_{L}$-methionine; M2, 1.25 mM $_{L}$-methionine. Values represent means ± SD (n = 6 biological replicates). Different capital letters stand for statistically significant differences between stimuli within the same AA treatment, regardless time of incubation. Different low case letters denote statistically significant differences between AA treatments, within the same stimulus (Multifactorial ANOVA; Tukey post-hoc test; $P \leq 0.05$).

2.3.2 Respiratory Burst

A complete description of results on respiratory burst can be found as Appendix I, Table 2.

Synthesis of O_2^- presented the highest level at 4 h, regardless of stimuli or AA treatment (Appendix I, Table 2B). Likewise, O_2^- was higher in the CTRL group than in *Vang*-stimulated cells (Appendix I, Table 2). Figure 2 provides a selected subset of the most relevant results, regarding the effect of AA treatments, regardless of stimulation or incubation time.

Cells treated with T2, M1 or M2 increased the production of O_2^- compared to L-15 and G1 groups (Fig. 2). In M1-treated cells supernatant, O_2^- levels were also higher than those observed in G2 and A1, while O_2^- produced by M2-treated cells were increased compared to those of A1 group (Fig. 2).

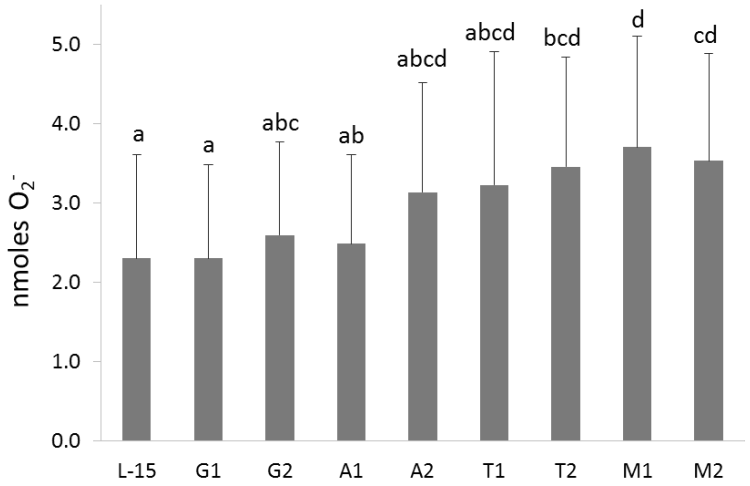

Fig. 2 Extracellular superoxide anion (O_2^-) production in HKL subjected to the experimental treatments. The AA treatments depicted in the X-axis are as follows: L-15, control; G1, 4.1 mM $_L$-glutamine; G2, 5.1 mM $_L$-glutamine; A1, 5.7 mM $_L$-arginine; A2, 7.2 mM $_L$-arginine; T1, 0.2 mM $_L$-tryptophan; T2, 0.25 mM $_L$-tryptophan; M1, 1 mM $_L$-methionine; M2, 1.25 mM $_L$-methionine. Values represent means ± SD (n = 6 biological replicates). Different low case letters denote statistically significant differences between AA treatments, regardless stimulation or incubation time (Multifactorial ANOVA; Tukey post-hoc test; $P \leq 0.05$).

2.3.3 ATP

A complete description of results on extracellular ATP concentration in the supernatant of cells subjected to the different treatments can be found as Appendix I, Table 3.

Figure 3 provides a selected subset of the most relevant results, regarding extracellular ATP concentration in the supernatant of HKL incubated with AA treatments and stimulated with vaLPS for 4 or 24 h. For clarity, results for each AA are described separately below.

Glutamine

The highest ATP levels were produced by G2-treated cells incubated for 24 h in CTRL conditions which were higher than those of L-15, A1, A2, T1 and M2 groups, under the same conditions (Fig. 3B). An increase in ATP production with time was observed both in G1- and G2 groups under control conditions, but not in stimulated cells.

Arginine

ATP levels in stimulated A2-treated cells, but not in A1-treated cells, were higher than in their CTRL counterparts incubated for 4 h, as well as in stimulated L-15, G1, G2 and M2 cells in the same conditions (Fig. 3A). Moreover, ATP levels increased with time in the supernatant of stimulated A1-treated monolayers (Fig. 3).

Tryptophan

Both tryptophan supplementation levels improved ATP production particularly in CTRL HKL incubated for 4 h, compared to the L-15 group (Fig. 3A). At 4 h of incubation, ATP in non-stimulated T1-treated cells supernatant was also higher than all the other AA treatments under the same conditions (Fig. 3A). Moreover, T1 treatment led to augmented ATP production in cells stimulated with vaLPS for 4 h, compared to L-15-treated cells (Fig. 3A). In non-stimulated T2-treated cells, ATP production was higher than that produced by L-15 cells incubated for 24 h (Fig. 3B).

Methionine

ATP production in M1-treated cells was higher than in L-15 group when incubated in CTRL conditions for 24 h (Fig. 3B).

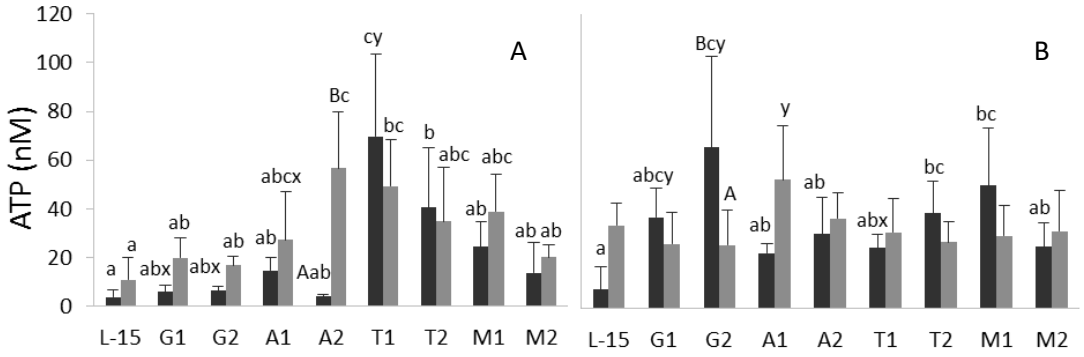

Fig. 3 Extracellular ATP concentration in the supernatant of HKL subjected to the experimental treatments for 4 (A) or 24 h (B) under CTRL (■) or vaLPS (■) conditions. The AA treatments depicted in the X-axis are as follows: L-15, control; G1, 4.1 mM L-glutamine; G2, 5.1 mM L-glutamine; A1, 5.7 mM L-arginine; A2, 7.2 mM L-arginine; T1, 0.2 mM L-tryptophan; T2, 0.25 mM L-tryptophan; M1, 1 mM L-methionine; M2, 1.25 mM L-methionine. Values represent means ± SD (n = 6 biological replicates). A and B stand for statistically significant differences between stimuli within the same AA treatment and time of incubation. Different low case letters denote statistically significant differences between AA treatments within the same stimuli and incubation time. x and y stand for statistically significant differences between incubation time, within the same AA treatment and stimuli (Multifactorial ANOVA; Tukey post-hoc test; $P \leq 0.05$).

2.3.4 Arginase Activity

The main purpose of measuring arginase activity was to understand the metabolic fate of arginine in innate-activated HKL, in parallel observation of NO synthesis. We also seek to find out whether it is dose-dependent. A complete description of results can be found as Appendix I, Table 4.

Arginase activity was not affected by any arginine treatment, though a general increase with time was denoted (Appendix I, Table 4B).

2.3.5 Gene expression

A complete description of results on gene expression can be found as Appendix I, Table S5 online.

Gene expression of *cox2* was down-regulated with time, regardless of cells being stimulated or not and independent of AA treatments, whereas it increased with the addition of vaLPS to cell medium (Appendix I, Table 5B).

Glutamine

G2-treated cells led to up-regulation of *cox2* compared to L-15, A1, A2, T2 and M2 groups, regardless of time or stimulation, while in G1-treated cell it was only higher than A1, A2, T2 and M2 groups (Appendix I, Table 5B). Though not statistically significant, *mif* mRNA levels were the highest in both glutamine treatments particularly when stimulated with vaLPS for 4 h. *tgfβ* was up-regulated in G1-treated cells compared to A1, A2, T2, M1 and M2 groups but only when incubated for 4 h (Appendix I, Table 5B). On the other hand, comparatively to L-15-treated cells, *amd* and *odc* expression was inhibited in G1- and in G2-treated cells incubated for 24 h (Appendix I, Table 5B). Also compared to L-15-treated cells, *odc* expression was inhibited in both glutamine supplementation levels both in CTRL and vaLPS-stimulated groups (Appendix I, Table 5C). *sat* gene expression increased in time in both glutamine supplementations but no further effects were observed.

Arginine

Relatively to L-15-treated cells, expression of *il1β* was inhibited in unstimulated A2-treated HKL but was higher in both vaLPS-stimulated arginine supplementations than in their CTRL counterparts (Appendix I, Table 5C). *cox2* gene expression was inhibited in A2-treated cells, regardless time of incubation or stimulation (Appendix I, Table 5B). A decreasing trend was observed in both *mif* and *tgfβ* mRNA levels but it was devoid of statistical significance. *amd* and *odc* expression were down-regulated only at 24h, in A1 treated-cells and in both A1- and A2-treated cells, respectively (Appendix I, Table 5B). While *odc* was down-regulated in both unstimulated arginine supplementations, it was not significantly altered upon vaLPS stimulation (Appendix I, Table 5C). In contrast, when incubated with vaLPS for 24 h, *sat* gene expression increased in A1- and A2- stimulated cells compared to their CTRL groups, an effect not observed in the other AA treatments or L-15-treated cells (Appendix I, Table 5A).

Tryptophan

Lower expression of *odc* was observed at 24 h of incubation in T1-treated cells (Appendix I, Table S5 B), as well as in both tryptophan supplementations treatments in CTRL conditions (Appendix I, Table 5C). However, in stimulated cells, *odc* down-regulation was only detected in T1-treated (Appendix I, Table 5C). A non-significant decreasing trend was denoted in gene expression patterns of *il1β*, *cox2*, *tgfβ*, and *amd* in both tryptophan treatments (Appendix I, Table 5B).

Methionine

Both *il1β* and *cox2* were down-regulated in M2-treated cells, though *il1β* inhibition occurred solely in CTRL conditions (Appendix I, Table 5B and 5C, respectively). *mif* gene expression was up-regulated from 4 to 24 h in vaLPS-stimulated M2-treated cells (Appendix I, Table 5A) whereas *odc* expression was down-regulated by both methionine treatments in cells incubated for 24 h (Appendix I, Table 5B). Such inhibitory effect was observed in both CTRL and vaLPS-stimulated conditions. *amd* gene expression was also inhibited in M1-treated cells but only when incubated for 24 hours while M2-treated cells showed higher *amd* mRNA levels at 24 h than at 4 h of incubation (Appendix I, Table 5B). *sat* was up-regulated at 4 h in vaLPS-stimulated M1-treated cells but only relatively to T2-treated HKL under the same conditions (Appendix I, Table 5A).

2.4 Discussion

An *in vitro* approach was carried out to evaluate possible effects of AA supplementation and the response of HKL upon immune stimulation over time. The expression of important immune-related genes was measured and indicators of cellular immune response were quantified.

The panel of genes selected to unveil possible AA modulatory effects was carefully assembled so that it would illustrate both innate immune mechanisms and polyamine biosynthesis mediated by innate activated macrophages. Being key players of several immune pathways, the impact of AA deficiency or excess might go as far as shifting original metabolism direction already at transcriptional levels. Unfortunately, variability of gene expression data was very high, not allowing to clearly unravelling presumed effects, but just trends without statistical significance.

The present study showed that cell stimulation triggers the innate immune response of European seabass HKL, either cultured in L-15 alone or L-15 supplemented with individual AA. A general increase of NO parallel to upregulation of *cox2*, *il1β* and *tgfβ* genes point at modulatory effects that go beyond gene expression level. Immune cells

activation is necessarily accompanied by increased NO production, as observed in the present study and several other studies in teleost [17,30,34]. Moreover, each immune mechanism that is triggered implies an increased demand for energy to sustain the immune response. Extracellular ATP is believed to be a crucial mechanism of cell to cell communication during immune responses and extremely elevated concentrations (> 100 µM) are indicative of cell death [35]. During inflammatory conditions, extracellular increasing levels of ATP are perceived by the cells through purinergic receptors promoting chemotaxis and interleukin 1 production in myeloid cells [36,37]. Accordingly, in the present study higher ATP production was observed to increase when cells were stimulated, though such increasing trend was more evident at 4 h of incubation. The observed decrease in superoxide anion production in stimulated cells compared to CTRL groups was probably related to exhaustion of respiratory burst, as already reported by Hardie et al. [38].

Up-regulation of inflammatory genes is to be expected shortly after an immune response is triggered [7,39,40]. As mediator of prostaglandins biosynthesis, *cox2* is considered a key inflammatory signal [9,41], together with other pro-inflammatory mediators such as *il1β* and tumour necrosis factor alpha (TNFα), and their expression generally increase upon immune stimulation [42,43]. Accordingly, both *il1β* and *cox2* were modulated in this study, their gene expression increasing in vaLPS-stimulated cells and decreasing over time irrespective of AA treatments. In contrast, while similarly higher in vaLPS-stimulated cells, *tgfβ* mRNA expression increased in time. *tgfβ* is a potent negative regulator of haematopoiesis, modulating proliferation, differentiation, and function of several cell types [44]. Hence, it can be considered an anti-inflammatory intermediate that counteracts other earlier cytokines actions as the immune response develops, thus controlling the inflammatory process in a later stage. *mif* has been previously been characterized as a constitutively expressed gene in immune cells, being stored as preformed protein and promptly released upon immune stimulation with LPS [45]. This fact might help explain the absence of a significant up-regulation in stimulated cells. Still, recent findings demonstrated the responsiveness of this gene transcription to microbial products, thereby promoting inflammatory responses [46]. Differently from pro-inflammatory cytokines, highest *mif* expression levels were not observed at 4 h of vaLPS incubation, but this may be explained by the immediate translated protein availability.

HKL incubated in glutamine-supplemented media (i.e. G1 and G2) enhanced NO and energy production. Long incubation periods are necessary for the assessment of reactive nitrogen species as their concentrations are on the µM range. In this study, NO concentration was particularly low, but this was to be expected, as intensity of NO production seems to be species specific [19,31,47]. Nevertheless, G1-treated cells showed

an augmented response at 72 h regardless being activated or not, with NO production as high as that measured later at 96 h, and also the highest amongst all treatments in CTRL conditions. NO is exclusively synthetized from arginine, but glutamine can be recycled to arginine via citrulline during the urea cycle in mammals [48]. Hence, besides being a by-product of arginine upon NO synthesis, citrulline is also the primary endogenous source of arginine and evidences have showed the same to occur in lower vertebrates such as teleost fish, though at much lower rates [49,50]. In channel catfish, *Ictalurus punctatus,* activated macrophages, medium citrulline concentration decreased below detection levels while NO production was improved when arginine and glutamine were simultaneously added [51]. Hence, one could attribute an increased NO production to glutamine-associated nitrogen pool as it has been done in mammals [52].

In immune cells such as lymphocytes or macrophages, glutamine utilization rates are even higher than those of glucose [53]. Indeed, these cells take advantage of glutamine availability when energy demands increase due to immune stimulation [52,54]. Being so, glutamine studies have been focused on its role as energy source [55,56]. Glutamine addition to HKL monolayers might have benefited these cells in terms of energy balance, as supported by the enhanced ATP production, promoting the synthesis of proinflammatory signals (*cox2*) and increasing the efficiency of immune mechanisms such as NO production. It would also be expected that glutamine treatment resulted in increased respiratory burst, as previously denoted by Cheng and co-workers [57,58]. Differently, superoxide anion production was not affected by glutamine supplementation to the media. A possible explanation would be that glutamine, being a glutathione precursor, could have increased glutathione pool[59-61], which in turn would contribute to reduce levels of free radicals such as superoxide anion. Nonetheless, this hypothesis would need to be confirmed by glutathione quantification analysis, and this should be taken into account in future studies concerning glutamine and the immune response.

Arginine, besides its obvious and direct role in innate immune response as NO sole precursor, is also determinant in designating T cell function in differentiated myeloid cells [62,63]. In this work, however, arginine supplementation to HKL monolayers did not affect NO synthesis nor arginase activity. Both inducible nitric oxide synthase (iNOS) and arginase activities are cytokine-induced but their expression occurs in differently activated myeloid cells [17]. While iNOS is typically expressed by innate- or classically-activated macrophages, arginase activity is a trait of alternatively-activated macrophages [64]. Because these two macrophage pathways have opposite and competing actions, i.e. iNOS is pro-inflammatory and arginase down-regulates inflammation, expression patterns should be opposite and consequently so should NO production and arginase activity. In accordance, and in spite of absence of an AA effect, arginase activity was

higher at 24 h compared to levels at 4 h which, supported by *tgfβ* transcript data, seems to indicate that anti-inflammatory processes start to develop later on in the immune response.

Although NO concentrations did not show significant changes in the supernatants of both A1- and A2-treated cells, higher NO levels appear to occur in stimulated HKL with the highest arginine surplus. Such absence of significant effects could be related to the incubation time, since there is a clear time effect. In channel catfish, peritoneal macrophages incubated in an arginine-supplemented medium produced much higher NO amounts than control cells in response to 96 h of LPS incubation [51]. In the present experiment, arginine concentration was much higher than that used in the aforementioned study. Being kept in such conditions for 120 h, arginine-treated macrophages might have suffered from earlier toxic effects of NO that impaired cell function, as suggested by Mills [65], hence no further increased was observed at the end of LPS incubation period. Still, additional and earlier measuring points are required to confirm this hypothesis.

In spite of *amd* expression patterns were lower in A1-treated HKL (an observation transversal to most AA treatments), such inhibition was not present in cells incubated with A2. Moreover, differently from other groups, arginine supplementation did not inhibited *odc* in stimulated cells. This, together with levels of *sat* mRNA in 24 h-vaLPS-stimulated cells being highest upon arginine surplus, may suggest that activation of these cells might have promoted polyamine turnover in a dose- and time-dependent manner. Andersen et al. [66] tested the effect of doubling arginine concentration on Atlantic salmon, *Salmo salar*, hepatocytes primary cell cultures and observed no alteration on *sat* mRNA levels. However, the abundance of the translated enzyme was highest in cells of individuals fed arginine-supplemented diets. Our results under CTRL conditions are in accordance with those of Andersen and colleagues, but our data on the presence of an immune stimulus suggests the potential of using arginine to boost polyamine turnover. Naturally, gene expression analysis per se is not determinant for the evaluation of final protein abundance, but differences observed between arginine group and L-15 or other AA treatments, at least points out the most likely pathway through which these macrophages are metabolizing arginine. The almost unaltered NO production and respiratory burst response, which were the lowest responses among all AA treatments, might further support that polyamine biosynthesis is the preferred metabolic pathway of macrophages treated with arginine. Accordingly, both pro-inflammatory genes *il1β* and *cox2* were down-regulated in cells incubated with the highest arginine surplus. Despite of conflicting information, previous *in vivo* and *in vitro* studies on arginine immune

modulation describe similar inhibitory effects that generally impair several immune mechanisms [7,8,67].

In the present study, HKL stimulated with vaLPS in combination with arginine treatment raised ATP synthesis, in contrast to any other treatment which reduced ATP production upon immune stimulation. This suggests that arginine could also have been used for energy production besides NO production. Cheng and co-workers [68] recently reviewed the importance of the cross-talk between immune response and metabolism, not only at the organism level, but also at individual cells level. In fish, high levels of ATP can be generated during partial AA catabolism and, particularly, high ATP yields in the conversion of arginine to alanine have been observed [69]. Alanine would further be advantageous since it forms a useful carrier of AA carbon to further metabolism elsewhere [70]. It is thus tempting to speculate that arginine catabolism was used to support an increased energy demand upon immune stimulation, supported by low superoxide anion and NO production in combination with poor arginase activation.

Indeed, energy demand must be compensated by intense ATP production, which in turn rely on adequate NAD+ supply. In the present study, at early phase of incubation and regardless of the presence of a stimulus, the highest ATP levels were observed in T1- and T2-treated HKL. In mammals, in the absence of niacin, tryptophan is the sole source of substrate for NAD+ production [12], via the kynurenine pathway. Although kynurenine pathway is far from being completely established if fish, evidences of presence of key enzymes have been reported in fish head-kidney cells [71]. In our *in vitro* model, cells were incubated with Leibovitz's L-15 medium, which already contains niacin, so the base level of this compound was the same for all AA treatments. So, comparatively to other AA treatments, the two tryptophan groups are expected to be benefited.

The focus of tryptophan involvement on the immune response has mostly been on the enzyme IDO which seems to be associated to regulation of T-cell function and thereby to immunosuppressive effects [12,72,73]. In accordance, inflammatory mediators mRNA levels were repeatedly (though not significantly) lower in T2-treated cells, suggesting that an inflammatory signal might have been impaired. However, while tryptophan failed to enhance NO production, extracellular superoxide anion levels were enhanced, which could be linked to improved cellular performance ensured by higher ATP production. Immunosuppression is mediated by 3-hydroxykynurenine, 3-hydroxyanthranilic acid and quinolinic acid, which are major downstream breakdown products of tryptophan catabolism known to inhibit immune cells proliferation and function [12]. *amd* is indirectly involved in polyamine biosynthesis, hence in cell proliferation, and was lower in both tryptophan supplementation levels. Altogether, observations seem to point out an attenuated inflammatory response caused by higher

tryptophan availability. Once the nucleotide sequence of the indoleamine 2, 3 dioxygenase is available for European seabass, future work on tryptophan and fish immune response should include the evaluation of its expression patterns coupled with those of dendritic and T-cell markers.

In this study, the most expressed modulatory effects were those induced by methionine supplementation, that boosted innate immune defences such as NO and superoxide anion production and enhanced ATP yields. One of the most remarkable effects of methionine immune modulation was the increase HKL reactivity to vaLPS. While M1-treated cells already showed higher vaLPS-induced NO response than that of L-15-treated cells, the highest methionine supplementation elevated LPS-induced NO to concentrations as high as those seen against *Vang* itself. The low sensitivity of fish to LPS is associated to the pathogen recognition receptor toll-like receptor 4, which is known to be activated by LPS in mammals but, when present, is believed to have different functions in teleosts [74,75]. Although methionine improves HKL immune response to vaLPS it does not necessarily mean that it modulates immune cells activation pathways, but it highlights the potential of methionine to strengthen cells immune response. This hypothesis is also supported by the improved ATP production and respiratory burst in these cells. Methionine was the most powerful AA at improving leucocytes oxygen radicals (both NO and superoxide anion production). As precursor of cysteine, a constituent of glutathione, methionine is expected to regulate cellular redox potential and, ultimately, the amount of free radicals such as superoxide anion. However, the ability of this AA to increase other non-directly related mechanisms (NO, ATP, gene expression) suggests that methionine might also indirectly modulate immune defences such as the respiratory burst, via methylation or polyamines biosynthesis pathway. As observed by Kuang et al. [15], the activity of superoxide dismutase and other antioxidant enzymes in the head-kidney of juvenile Jian carp, *Cyprinus carpio var. Jian*, were reduced upon addition of graded levels of dietary methionine hydroxyl-analogue.

Unexpectedly, methionine down-regulated the expression of two important inflammatory genes, *il1β* and *cox2*. Such impairment was stronger in unstimulated HKL treated with the higher methionine level. *cox2* mRNA levels seemed to respond to methionine on a dose-response basis, as values were higher in M1-treated cells compared to L-15 cells but lower in M2-treated cells, especially upon vaLPS stimulation, although the difference was not statistically significant. Though much higher than non-treated cells, a similar behaviour was observed with superoxide and ATP production. Such dose-effect suggests that methionine might be beneficial within a certain range of concentrations, an issue in need of further research.

Polyamine turnover is fuelled by the transferring of aminopropyl groups, which enables polyamines interconversion [76]. Decarboxylated s-adenosylmethionine is the donor molecule for this interconversion and this is its sole role, with *amd* being enzyme mediating the decarboxylation. Previous data on methionine dietary supplementation clearly demonstrated the stimulatory effect of this AA on peripheral leucocyte proliferation and, consequently, on the improvement of innate humoral response [14]. In this study, methionine supplementation increased *amd* gene expression from 4 to 24 h of incubation, which was not observed in any other treatment. These results suggest that methionine immune modulation might be mediated by an improvement of polyamine biosynthesis, which in turn increases immune cell proliferation. However, as *odc* was conversely inhibited in both methionine-treated groups, this hypothesis requires additional research.

In summary, the present *in vitro* study highlighted relevant immunomodulatory effects of different AA on seabass HKL. HKL took advantage of glutamine, arginine, and tryptophan as energy sources that, yielding higher ATP amounts, seem to enhance these cells immune performance. Arginine seemed to potentiate macrophages acquisition of an immune suppressive phenotype, as supported by arginase activity increase over time and enhanced *sat* gene expression, which might indicate improved polyamine turnover. Tryptophan enhanced respiratory burst, but it also down-regulated inflammatory-related and polyamine-related gene expression which seems to point at the presence of immune-suppressive (IDO-mediated) mechanisms. Differently, methionine treatment seemed to improve cellular innate immune defences and it may also play a role on cell proliferation. Altogether, these results signal clear immunomodulatory effects of AA, which highlights the potential for the establishment of immune-nutritional strategies in aquaculture that need to be further investigated.

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Chapter 3

Neuroendocrine and immune responses undertake different fates following tryptophan or methionine dietary treatment: tales from a teleost model

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Neuroendocrine and immune responses undertake different fates following tryptophan or methionine dietary treatment: tales from a teleost model

Abstract

Methionine and tryptophan appear to be fundamental in specific cellular pathways involved in the immune response mechanisms, including stimulation of T-regulatory cells by tryptophan metabolites or pro-inflammatory effects upon methionine supplementation. Thus, the aim of this study was to evaluate the immunomodulatory effect of these amino acids on the inflammatory and neuroendocrine responses in juveniles of European seabass, Dicentrarchus labrax. To achieve this, goal fish were fed for 14 days methionine and tryptophan-supplemented diets (MET and TRP, respectively, 2× dietary requirement level) or a control diet meeting the amino acids requirement levels (CTRL). Fish were sampled for immune status assessment and the remaining fish were challenged with intraperitoneally injected inactivated Photobacterium damselae subsp. piscicida and sampled either 4 or 24 h post-injection. Respiratory burst activity, brain monoamines, plasma cortisol, and immune-related gene expression showed distinct and sometimes opposite patterns regarding the effects of dietary amino acids. While neuroendocrine intermediates were not affected by any dietary treatment at the end of the feeding trial, both supplemented diets led to increased levels of plasma cortisol after the inflammatory insult, while brain monoamine content was higher in TRP-fed fish. Peripheral blood respiratory burst was higher in TRP-fed fish injected with the bacteria inoculum but only compared to those fed MET. However, no changes were detected in total antioxidant capacity. Complement factor 3 was upregulated in MET-fed fish but methionine seemed to poorly affect other genes expression patterns. In contrast, fish fed MET showed increased immune cells numbers both before and after immune challenge, suggesting a strong enhancing effect of methionine on immune cells proliferation. Differently, tryptophan effects on inflammatory transcripts suggested an inhibitory mode of action. This, together with a high production of brain monoamine and cortisol levels, suggests that tryptophan might mediate regulatory mechanisms of neuroendocrine and immune systems cooperation. Overall, more studies are needed to ascertain the role of methionine and tryptophan in modulating (stimulate or regulate) fish immune and neuroendocrine responses.

Key-words: feed additives; innate immunity; inflammation; amino acids; aquaculture; functional diets

3.1 Introduction

The neuroendocrine–immune interaction is an evolutionary conserved phenomenon among vertebrates. Two physiological responses evolved to help animals survive pathogen invasion and overcome situations of distress: the immune response and the stress response. In mammals, it has been recognized that both responses share some pathways [1]. The importance of neuroendocrine–immune interactions in the physiological regulation of immune and brain functions is evident because vertebrate lymphoid tissue is innervated by parasympathetic and sympathetic nerve fibres which are implicated both in stimulation and inhibition of immune functions [2]. Different hormones and cytokines, as well as their interactions, are involved in the same neuroendocrine-immune mechanisms [3]. These interactions are especially evident in lower vertebrates such as fish in which, for instance, cytokines and neuropeptides are performing roles in both neuroendocrine and immune systems [4]. Scientific evidence also shows that leucocytes are sensitive to a wide repertoire of neuroendocrine mediators. For instance, glucocorticoids influence the balanced successive secretion of pro- and anti-inflammatory cytokines and, in particular, cortisol can have profound and differential effects on the fish immune function [4].

Immune responses and stressful conditions can also affect amino acid requirements and metabolism [5]. In fact, amino acid requirements may increase as direct consequence of metabolic changes associated with inflammation and infection [6]. In fish, as in mammals, amino acids are versatile molecules displaying several functions besides protein constitution. For instance, amino acids have been shown to play important parts in immune mechanisms [7-10]. Methionine has several features that make it a relevant amino acid during innate immune responsive mechanisms. Methionine appears to have clear pro-inflammatory effects on juvenile carp, *Cyprinus carpio* [11,12]. S-adenosyl-methionine directly participates in polyamine biosynthesis by successively adding aminopropane to the forming polyamines, important elements to cell proliferation [13]. In addition, free radical scavengers, such as melatonin or glutathione, control the increased oxidative damage caused by the production of reactive oxygen or nitrogen species. Methionine is precursor of cysteine, which is one of the three glutathione constituents, and thus an essential element for its production.

Understanding the regulatory mechanisms of the immune system is necessary upon the analysis of a particular immune response. According to Frumento et al. [14], Grohmann et al. [15] and Le Floc'h et al. [16], the role of tryptophan during the immune response is mainly related to regulatory processes. As substrate of 2, 3-indoleamine dioxygenase (IDO), tryptophan metabolic cascades lead to anti-inflammatory signalling molecules, the main effectors being regulatory T-cells. Considering this, an increase in the tryptophan

availability in the organism may be regarded as a strategy to counteract the deleterious aspects of an eventual exacerbated innate immune response. However, considering the existing link between the immune and the neuroendocrine systems mentioned above, tryptophan effects must also be evaluated from an alternative perspective. Serotonin (5-HT) is a monoamine neurotransmitter that is produced from tryptophan in the central nervous system and its synthesis is known to control the adrenocorticotropic hormone release, which in turn directly regulates cortisol production [17]. The few available studies in fish have shown that the teleost immune response, like that of mammals, is sensitive to 5-HT-induced immunomodulation [18]. Lepage et al. [19] observed that tryptophan supplementation in juvenile rainbow trout, *Oncorhynchus mykiss*, had opposed effects on cortisol levels, which were slightly increased in non-stressed fish, while were reduced in stressed-fish.

Increasing evidence shows that dietary supplementation of specific amino acids to animals and humans with infectious diseases enhances the immune status, thereby reducing morbidity and mortality [7]. In fish, the immunomodulatory role of amino acids has only recently started to be studied, and the underlying cellular and molecular mechanisms still need to be unfold. Therefore, the main goal of this study was to gather evidence on the specific fate of amino acids arbitrating the elaborate neuroendocrine-immune network. The present study focused on several neuroendocrine and immunological aspects of sham or antigen-stimulated animals, using the European seabass, *Dicentrarchus labrax*, as model species. Particular emphasis was given to the study of monoamine responses in specific brain regions, the expression patterns of a panel of immune-related genes in both the head-kidney (HK) and blood, as well as peripheral blood cell dynamics in which the respiratory burst was determined.

3.2 Material and Methods

3.2.1 Formulation and proximal composition of experimental diets

Three isonitrogenous (44.9 % crude protein) and isolipidic (14.9 % crude fat) diets were formulated. Fish meal and a blend of plant feedstuffs were used as protein sources, whereas fish oil was the main fat source. The plant-protein fraction represented almost 50 % of the total feed composition. In two of these diets, $_{L}$-tryptophan or $_{L}$-methionine was added at the expenses of fish meal to a final concentration of 2 × the requirement level determined for seabass. A non-supplemented diet was used as a control diet (CTRL) meeting the amino acid requirement of European seabass [20]. The two supplemented diets were considered dietary treatments and will be referred to as TRP (tryptophan-supplemented diet) and MET (methionine-supplemented diet).

More detailed information on diets composition and proximate analysis is given in Table 1. All ingredients were ground, mixed together and dry-pelleted in a laboratory pellet mill (CPM, California Pellet Mill, Crawfordsville, IN, USA). Proximate analysis of the diets was performed according to the Association of Official Analytical Chemists methods [21] and amino acids analysis was carried out according to Banuelos-Vargas et al. [22] (Table 2).

Table 1. Ingredients and proximate composition of experimental diets as percentage of dry matter (% DM)

	Experimental diets		
	CTRL	**TRP**	**MET**
Ingredients (%)			
Fish meal[1]	34.1	33.5	33.2
Soybean meal[2]	15.0	15.0	15.0
Corn gluten[3]	10.0	10.0	10.0
Wheat gluten[4]	5.0	5.0	5.0
Wheat meal[5]	16.7	16.6	16.2
Fish oil	13.9	13.4	14.0
Vitamin premix[6]	1.0	1.0	1.0
Choline chloride (50 %)	0.5	0.5	0.5
Mineral premix[7]	1.0	1.0	1.0
Binder[8]	1.0	1.0	1.0
Agar	1.0	1.0	1.0
Dibasic calcium phosphate	0.84	0.91	0.96
L-Methionine[9]	—	—	1.16
L-Tryptophan[9]	—	0.52	—
Proximate analyses (% dry weight)			
Dry matter (%)	95.2	94.9	94.3
Crude protein	44.9	45.2	45.0
Crude lipid	15.5	16.5	16.9
Ash	10.5	10.4	10.5

[1]Pesquera Centinela, Steam Dried LT, Chile (CP: 71.4%; CL 9.3%). Sorgal, S.A. Ovar, Portugal

[2]Soybean meal (CP: 54.9%; CL:2.1%), Sorgal, S.A. Ovar, Portugal

[3]Corn gluten (CP: 72.2%; CL: 2.0%), Sorgal, S.A. Ovar, Portugal

[4]Wheat gluten (CP: 84.4%; CL: 2.1%), Sorgal, S.A. Ovar, Portugal

[5]Wheat meal (CP: 13.9%; CL: 1.8%), Sorgal, S.A. Ovar, Portugal

[6]Vitamins (mg kg^{-1} diet): retinol, 18000 (IU kg^{-1} diet); calciferol, 2000 (IU kg^{-1} diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.

[7]Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dicalcium phosphate, 8.02 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.4 (g kg^{-1} diet).

[8]Aquacube. Agil, UK.

[9]Feed grade amino acids, Sorgal, SA. Ovar, Portugal

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

	Experimental Diet		
	CTRL	**TRP**	**MET**
Arginine	7.74	7.11	7.02
Histidine	3.78	3.63	4.12
Isoleucine	5.05	4.64	4.71
Leucine	9.73	9.71	9.49
Lysine	6.66	6.96	6.68
Methionine	2.57	2.42	4.95
Phenylalanine	5.39	5.16	5.16
Tyrosine	4.04	3.96	3.98
Threonine	4.68	4.38	4.51
Tryptophan	1.12	2.24	1.10
Valine	5.38	5.10	5.12
AsparticAcid	8.20	7.54	7.60
Glutamic Acid	16.39	16.14	16.09
Serine	4.15	4.42	4.29
Glycine	3.98	4.18	4.20
Alanine	4.89	4.92	4.97
Proline	4.99	5.50	4.84

3.2.2 Bacteria inoculum preparation

Photobacterium damselae subsp. *piscicida*, strain PP3 (*Phdp*) isolated from Japanese amberjack (*Seriola quinqueradiata*, Japan) by Dr. Andrew C. Barnes (Marine Laboratory, Aberdeen, UK) and kindly provided by Dr. Ana do Vale (Institute for Molecular and Cell Biology, University of Porto, Portugal). Bacteria were first cultured for 48 h at 22 °C in tryptic soy agar (Difco Laboratories) supplemented with 1 % NaCl (w/v) (TSA-1). Colonies were then inoculated into tryptic soy broth equally supplemented with NaCl (TSB-1) and incubated overnight at 22 °C. Bacteria under exponentially growth were centrifuged at 3,500 x *g* for 30 min, resuspended in TSB-1 with glycerol at a final concentration of 15 % (v/v) and stored at -80 °C as a stock solution. *Phdp* inoculum was obtained by culturing bacteria from the stock solution as previously described and by suspending in sterile Hank's Balanced Salt Solution (HBSS) at a final concentration of 1 × 10^6 colony forming units (CFU) ml^{-1}, according to Costas et al. [23]. Bacteria were killed by 2 h UV-light exposure. No bacterial growth was observed when UV-killed bacteria were plated on TSA-1.

3.2.3 Fish and experimental design

This study was carried out at the Marine Zoological Station, Porto, Portugal. After two weeks of acclimatization, juvenile European seabass (274.7 ± 20.4 g) fed a commercial diet were randomly distributed in six fibreglass tanks (300 l; n=15) in a seawater recirculation system (temperature: 25 ± 1 °C; salinity: 35 ppt; natural light-dark cycle). Dietary treatments were randomly assigned to duplicate tanks and fish were fed twice a day until apparent satiety. The feeding trial lasted for 14 days and O_2, salinity, pH, temperature and nitrogenous compounds were monitored daily. At the end of the feeding trial, 3 fish per tank (6 fish per dietary treatment, n=6) were euthanized by immersion in 2-phenoxyethanol (1,500 ppm; Sigma) and sampled. Blood was collected from the caudal vessel with heparinized syringes and kept in heparinized tubes at 4 °C until analysed. The HK was removed and kept in RNAlater® solution (Ambion Inc., Austin, USA) at 4 °C for 24 h and then stored at -80 °C until assayed. Liver and brain were collected was well and brain was dissected in the following regions: hypothalamus, optic tectum and telencephalon (including olfactory bulb). Both liver and brain samples were immediately frozen in dry ice and later stored at -80 °C.

Thereafter, the remaining fish (12 fish per tank) were intraperitoneally (i.p.)-injected with either 100 µl of UV-killed *Phdp* or HBSS (sham group), according to an inflammatory model previously established [24] and redistributed into new tanks according to dietary treatment and stimuli. Afterwards, fish were euthanized and sampled (six fish per dietary treatment, per sampling time, n=6) either 4 or 24 h post-inoculation as mentioned above.

The experiments were approved by the Animal Welfare Committee of the Interdisciplinary Centre of Marine and Environmental Research and carried out in a registered installation (N16091.UDER) and were performed by trained scientists in full compliance with national rules and following the European Directive 2010/63/EU of the European Parliament and the European Union Council on the protection of animals used for scientific purposes.

3.2.4 Analytical procedures with blood and peritoneal leucocytes

An aliquot of gently homogenised blood was used to perform total white blood cells (WBC) counts. The remaining blood was centrifuged at 10,000 × *g* for 10 min at 4 °C and plasma stored at -80 °C until assayed. Blood smears were air dried and stained with Wright's stain (Haemacolor; Merck) after fixation with formol-ethanol (10 % of 37 % formaldehyde in absolute ethanol). The slides were examined (1,000×). At least 200 leucocytes were counted per smear and classified as thrombocytes, lymphocytes, monocytes and neutrophils. Detection of peroxidase activity was carried out as described

by [24] in order to facilitate identification of neutrophils. The relative percentage and absolute value ($\times 10^4$ ml^{-1}) of each cell type was subsequently calculated.

i) Respiratory burst in blood leucocytes

The respiratory burst in peripheral leucocytes was evaluated according to Nikoskelainen et al. [25] with some modifications. Briefly, 4 µl of blood were added to 96 µl of HBSS in a 96-well flat bottom, white polystyrene plate. Afterwards, 100 µl of freshly prepared luminol solution (2 mM luminol in 0.2 M borate buffer pH 9.0, with 2 µg ml^{-1} phorbol 12-myristate 13-acetate) were added to each well. Luminol-amplified chemiluminescence was measured every 3 min for 2 h in a luminescence reader (BioTek Synergy HT microplate reader) for generation of kinetic curves. Each sample was run in triplicate and controls contained no blood. The integral luminescence in relative light units (RLU) was calculated.

ii) Peritoneal leucocytes

The peritoneal cells were collected according to a procedure initially described for mice [26] and posteriorly adapted for fish [27]. Briefly, following anaesthesia and blood collection, 5ml of cold HBSS supplemented with 30 units ml^{-1} heparin were injected into the peritoneal cavity. Afterwards, the peritoneal area was slightly massaged in order to disperse the peritoneal cells in the injected HBSS. The HBSS containing the suspended cells was then collected. Total peritoneal cell counts were performed with a haemocytometer. Cytospin preparations were then made with a THARMAC Cellspin apparatus and stained as described above for blood smears. The lymphocytes, macrophages and neutrophils in the peritoneal exudates were counted and the percentage of each cell type was established after counting a minimum of 200 cells per slide. The concentration ($\times 10^4$ ml^{-1}) of each leucocyte type was also calculated.

3.2.5 Liver total antioxidant capacity

Liver samples were freeze-dried for 48 h (Alpha 1-4 LOC-1M, Christ) and homogenized with a TissueLyser (Qiagen). Tris-HCl buffer [50 mM Tris-HCl pH 7.5, 4 mM EDTA, 50 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 250 mM sucrose] was added to powdered frozen liver at a ratio of 5:1 v/w. After vortexing, samples were centrifuged at 20,000 × g for 30 min, at 4 °C, and the supernatant was transferred to a new vial.

Total protein content was determined in duplicate according to Bradford [28] using the Roti®-Quant reagent and a bovine serum albumin (BSA) dilution series [29]. Briefly, 50 µl

of supernatant were diluted with water (1:300). Then, 200 µl of Roti®-Quant reagent (1×) were added to each well and the plate was incubated for 10 min at room temperature, protected from light. Absorbance was read at 595 nm with a microplate Infinite 200 reader (TECAN).

Total antioxidant capacity (TAC) was analysed to quantify the activity of both antioxidant proteins and smaller molecules using a TAC assay kit (Sigma) as described before. In brief, supernatant was diluted with phosphate buffer (1:250) and 100 µl of the diluted sample with 100 µl of a Cu^{2+} working solution, mixed and incubated for 90 min at room temperature. The absorbance was read at 570 nm. TAC was determined in duplicate with a Trolox standard curve and concentration calculated based on the protein content.

3.2.6 Brain monoamine content

The brain content of noradrenaline (NA), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC, a major DA metabolite), 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) were analysed by high performance liquid chromatography with electrochemical detection (HPLC-EC) as previously described by Gesto et al. [30]. Briefly, tissues were homogenized by ultrasonic disruption in 0.5 ml of mobile phase with the following composition: 85 mM sodium hydrogen phosphate, 0.72 mM octanosulfonic acid, 18 % methanol, and adjusted to pH 3.0. Homogenates were centrifuged (16,000 x *g* for 10 minutes at room temperature) and prior to analysis supernatants were diluted 1:1 (supernatant/mobile phase) for optic tectum and 1:2 for telencephalon and hypothalamus. A 20 µl aliquot of each sample was injected into the HPLC system consisting of a Jasco PU2080 pump equipped with a Jasco AS-2057 autosampler, and an ESA Coulochem II detector (Bedford, USA). The detection system included a M5011 ESA analytical 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 ChromNAV version 1.12 software (Jasco Corp.).

3.2.7 Plasma cortisol

Cortisol was extracted from 20 µl of plasma in 180 µl of diethyl ether (Sigma). Cortisol levels in plasma were determined using a commercial ELISA kit (RE52061, IBL International GMBH, Germany), with a sensitivity of 0.05 ng/ml and intra- and inter-assay coefficients of variation (CV) of 2.98 and 3.51 %, respectively. This kit was previously validated for teleosts [31] and to validate this test for European seabass plasma samples

two different tests were performed: dilution parallelism and recovery. The dilution parallelism test consisted of 4 consecutive dilutions of a European seabass plasma sample with high and known concentration of cortisol, which was then compared with the standard curve. The curve obtained from seabass plasma was parallel to the standard curve, thus validating the test for this species (results not shown). The recovery test consisted of adding increasing known amounts of cortisol to a European seabass plasma sample, using kit standards (standards D, E, and F). A recovery value of 84.5 ± 11.9 % was obtained, reinforcing the validity of the kit for European seabass. Main cross-reactivities (>1 %; given by the supplier) were 30 % for prednisolone, 11 % for 11-Desoxy-Cortisol, 4.2 % for cortisone, 2.5 % for prednisone, and 1.4 % for corticosterone. Since cortisol is the main steroid produced by fish interrenal tissue, cross-reactivity with other steroids was assumed negligible.

3.2.8 Immune-related gene expression in HK

For HK immune-related gene expression, RNA was extracted with Tri Reagent (Sigma) following the manufacturer's instructions, and resuspended in free nuclease water (Invitrogen). RNA was quantified using a NanoDrop-1000 spectrophotometer (Thermo Scientific). Total RNA per sample (3 µg) was used for cDNA synthesis, which was performed using a SuperScript® III Reverse Transcriptase kit (Invitrogen) and Oligo-dT primer (Promega), according to manufacturer's instructions.

A set of different primers was designed to evaluate immune-relevant gene expression profiles. The chosen genes were: interleukin-1β (*il1β*), matrix-metalloproteinase 9 (*mmp9*), glutathione peroxidase (*gpx*), complement factor 3 (*c3*), hepcidin (*hamp*), glucocorticoid receptor (*gr*) and melanocortin 2 receptor (*mc2r*). Primer sequences were designed with Primer-Blast software (NCBI) and the structural primer analysis was performed with Oligoanalyzer (IDT®). Primer sequences are listed in Table 3.

To set up the real-time PCR, the optimal amplification condition of each primer pair was standardized by a temperature annealing gradient in conventional PCR. Efficiency of each primer pair was determined by real-time PCR according to Pfaffl [32]. Quantitative PCR reactions were carried out in a CFX384 Touch™ Real-Time PCR Detection System. Each reaction contained 1 µl of diluted cDNA (1:5 dilution) mixed with 6.25 µl of iTaq™ Universal SYBR® Green (BioRad) and 0.25 µl (10 mM) of each specific primer in a final volume of 12.5 µl.

The thermal conditions used were 3 min at 95 °C of pre-incubation, followed by 40 cycles at 95 °C for 10 s and 57 °C (annealing temperature) for 30 sec. Melting curve

analysis was always performed to confirm specificity of the reaction. The expression of the target genes was normalized using the elongation factor 1α (*ef1α*) gene of European seabass. Data was standardized by dividing the normalized expression values of cells from different treatments by the normalized expression values of the control group.

3.2.9 Immune-related gene expression in whole blood

For whole blood immune-related gene expression, RNA was extracted with Tri Reagent (Sigma) following the manufacturer's instructions, and resuspended in free nuclease water (Invitrogen). RNA was quantified using Take 3 Microvolume Plate (Biotek) and samples were then treated with DNase using the RQ1 RNase-free DNase kit (Promega) following manufacturer's indications. Total RNA per sample (1.5 µg) was used for cDNA synthesis, which was performed using the NZY First-Strand cDNA Synthesis Kit (NZYTech) according to the manufacturer's instructions.

A set of different primers was designed to evaluate immune-relevant gene expression profiles. The chosen genes were: *il1β*, *mmp9*, *gpx*, dicentracin (*dctr*) and *gr*. Primer sequences were designed with Primer-Blast software (NCBI) and the structural primer analysis was performed with Oligoanalyzer (IDT®). Primer sequences are listed in Table 3. Optimization of assays was carried out as described above.

Quantitative PCR reactions were carried out in an Eppendorf Mastercycle ep realplex. Each reaction contained 1 µl of diluted cDNA (1:5 dilution) mixed with 10 µl of NZYSpeedy qPCR Master Mix and 0.4 µl (10 mM) of each specific primer in a final volume of 20 µl.

The thermal conditions used were 10 min at 95 °C of pre-incubation, followed by 40 cycles at 95 °C for 15 s and annealing temperature for 1 min. Melting curve analysis was always performed to confirm specificity of the reaction. The expression of the target genes was normalized using the 40S ribosomal protein SA (*rpsa*) gene of European seabass. Standardization was carried out as described above.

Table 3. Specifications of real-time PCR assays including forward (F) and reverse (R) primers, length of amplicon, GenBank ID (NCBI), annealing temperature and PCR efficiency.

Gene name	Abbr.	GenBank	*Eff*[1]	AT[2]	Product length[3]	Primer sequence (5'-3')
elongation factor 1α	*ef1α*	AJ866727.1	96.5	57	144	F:AACTTCAACGCCCAGGTCAT R:CTTCTTGCCAGAACGACGGT
40S ribosomal protein SA	*rpsa*	HE978789.1	93.0	55	79	F: TGATTGTGACAGACCCTCGTG R: CACAGAGCAATGGTGGGGAT
interleukin 1β	*il1β*	AJ269472.1	96.7	57	105	F:AGCGACATGGTGCGATTTCT R:CTCCTCTGCTGTGCTGATGT
matrix metallo-proteinase 9	*mmp9*	FN908863.1	98.4	57	166	F:TGTGCCACCACAGACAACTT R:TTCCATCTCCACGTCCCTCA
complement factor 3	*c3*	HM563078.1	111.5	57	165	F:CAGTGGGAATCTGTGGGCTT R:GGCAAACACCTTGGCAAC
glutathione peroxidase	*gpx*	DT044993	94.2	57	176	F:GTTTGGACATCAGGAGAACTGC R:CATCGCTGGGGTATGGAAGC
hepcidin	*hamp*	DQ131605.1	94.2	57	172	F:CTGGAGGAGCCAATGAGCAA R:TGGAGAGAGCATCAGAGCAC
glucocorticoid receptor	*gr*	AY549305.1	111.2	55	110	F:AGGCATTCACCACCCCATTC R:GAAGTGACCCAGGCTGTTGA
Melanocortin 2 receptor	*mc2r*	FR870225.1	108.7	55	676	F: GGAACAGGAACCTCCACTCG R: ACCACGTGTAGCTGGAACAG
dicentracin	*dctrn*	AY303949.1	89.2	55	70	F:CTCATGGCTGAACCTGGGG R:TGGACTTGCCGACGTGAAC

[1] Efficiency of PCR reactions (represented in percentage) were calculated from serial dilutions of tissue RT reactions in the validation procedure.

[2] Annealing temperature (°C)

[3] Amplicon (nt)

3.2.10 Data and statistical analysis

Statistical analyses were performed with STATISTICA (StatSoft, Inc. 2013, version 12) for WINDOWS. Results are expressed as means ± SD of the mean. Data were analyzed for normality and homogeneity of variance and, when necessary, outliers were removed using the STATISTICA tool for outliers and extremes removal. Data were log-transformed and analyzed by one-way analysis of variance (ANOVA) (immune status-related analysis) or Multifactorial ANOVA (inflammatory response) with dietary treatment and sampling time as variables. Whenever significant differences were found among groups, a multiple-comparisons Tukey HSD test was performed to identify significantly different groups. For every test, the level of significance chosen was $p \leq 0.05$.

3.3 Results

3.3.1 **Neuroendocrine response**

3.3.1.1 Brain monoamine content

Hypothalamus. At the end of the feeding trial, NA content was higher in MET-fed fish than in those fed TRP (Appendix II, Supplementary table 1). After being i.p.-injected, the 5-HIAA/5HT ratio was higher in fish sampled at 4 h than in those sampled at 24 h post-injection, regardless dietary treatment (Appendix II, Supplementary table 1)

Telencephalon. No effects were observed in monoamine content before i.p.-injection (Appendix II, Supplementary table 2). However, DA content decreased at 24 h post-injection in HBSS-injected fish fed CTRL (Appendix II, Supplementary table 2). Moreover, DOPAC levels decreased over time and were lower in *Phdp*-injected fish than in their HBSS counterparts, regardless of dietary treatment and sampling time (Appendix II, Supplementary table 2). 5-HT and 5-HIAA levels were higher in TRP-fed fish than in MET-fed fish but no differences were observed compared to the CTRL group (Appendix II, Supplementary table 2). Fish fed TRP showed higher DOPAC/DA ratio compared to the CTRL-fed group but only in *Phdp*-injected animals (Fig. 1).

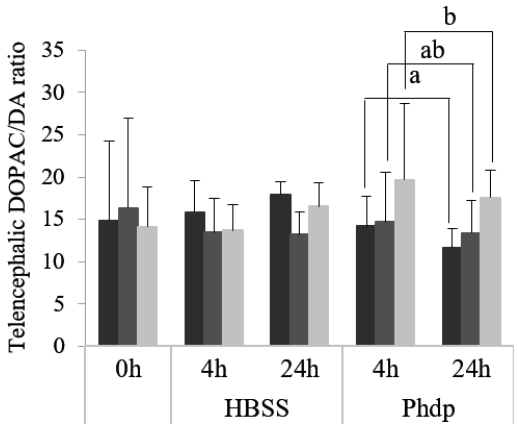

Fig 1. Telencephalic DOPAC/DA ratio in juvenile European seabass fed CTRL (■), MET (■) or TRP (■) for 14 days (0 h) and sampled at 4 h or 24 h post HBSS- or *Phdp*- i.p. injection (mean ± SD, n=6). a and b stand for significant differences between dietary treatments. One-way ANOVA (before i.p. injection) and Multifactorial ANOVA (after i.p. injection); Tukey post hoc test; p≤0.05).

Optic tectum. No effects were observed in monoamine content before i.p.-injection (Appendix II, Supplementary table 3). Following bacterial inoculation, DA was observed to be higher in *Phdp*-injected fish fed TRP than in the correspondent HBSS-injected group, regardless of sampling time (Fig. 2 A). DOPAC was higher in CTRL-fed fish injected with HBSS than in bacteria-injected fish, regardless of sampling time (Fig. 2 B). Moreover, TRP-fed fish injected with *Phdp* showed higher DOPAC levels than their

CTRL counterparts (Fig. 2 B). 5-HT was higher in TRP-fed fish compared to both CTRL- and MET-fed fish, but only in *Phdp*-injected group (Fig. 2 C) while 5-HIAA was also higher in TRP-fed fish but only compared to those fed MET and sampled at 4 h post-injection (Fig. 2 D).

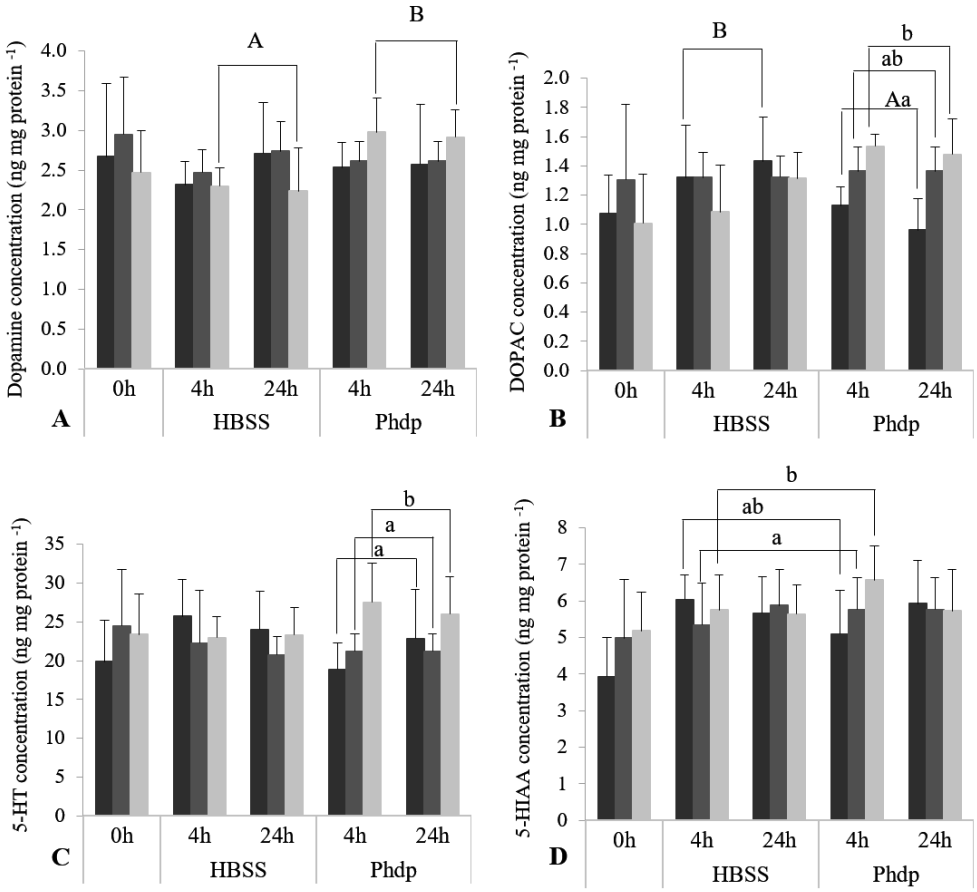

Fig. 2 Optic tectum dopamine (DA, **A**), 3,4-dihydroxyphenylacetic acid (DOPAC, **B**) serotonin (5-HT, **C**) and 5-hydroxyindoleactic acid (5-HIAA, **D**) content in juvenile European seabass fed CTRL (■), MET (■) or TRP (■) for 14 days (0 h) and sampled at 4 h or 24 h post HBSS- or *Phdp*- i.p. injection (mean ± SD, n=6). a and b stand for significant differences attributed to dietary treatments. A and B depict differences attributed to stimuli. One-way ANOVA (before i.p. injection) and Multifactorial ANOVA (after i.p. injection); Tukey post hoc test; p≤0.05).

3.3.1.2 Plasma cortisol

Fish fed the experimental diets for 14 days showed no differences on plasma cortisol levels (Fig. 3). Cortisol levels were higher in *Phdp*-injected fish fed supplemented diets and sampled at 24 h post-injection than in CTRL-fed counterparts (Fig. 3). Moreover, in CTRL-fed fish, cortisol was higher in HBSS-injected fish sampled at 24 h post-injection than in those injected with *Phdp*.

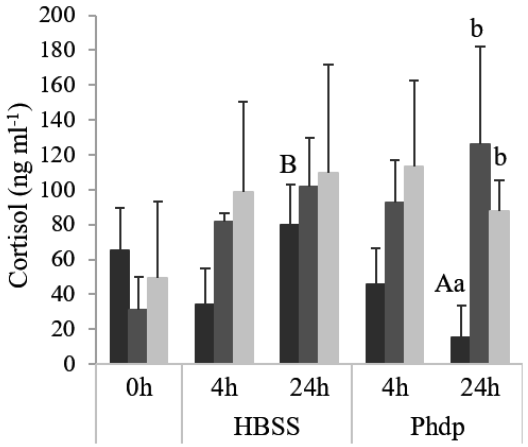

Fig. 3 Plasma cortisol levels in juvenile European seabass fed CTRL (■), MET (■) or TRP (■) for 14 days (0 h) and sampled at 4 h or 24 h post HBSS- or *Phdp*- i.p. injection (mean ± SD, n=6). a and b stand for significant differences attributed to dietary treatments. A and B depict differences attributed to stimuli. One-way ANOVA (before i.p. injection) and Multifactorial ANOVA (after i.p. injection); Tukey post hoc test; p≤0.05).

3.3.1.3 Quantitative *gr* and *mc2r* mRNA expression

Expression levels of *gr* and *mc2r* in the head-kidney were not affected by any dietary treatments (Appendix II, Supplementary table 5). However, mRNA levels of both receptors were down-regulated over time, after i.p. injection (Appendix II, Supplementary table 5). Differently, *gr* was down-regulated in blood of CTRL-fed fish sampled at 24 h compared to the same dietary group sampled at 4 h post-injection, regardless of stimulus (Fig. 4). Moreover, *gr* expression levels were lower in MET-fed fish compared to the CTRL-fed group sampled at 4 h post-injection (Fig. 4).

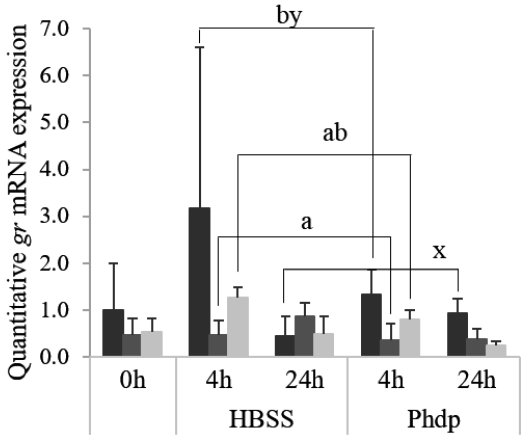

Fig. 4 Quantitative *gr* mRNA expression in blood of juvenile European seabass fed CTRL (■), MET (■) or TRP (■) for 14 days (0 h) and sampled at 4 h or 24 h post HBSS- or *Phdp*- i.p. injection (mean ± SD, n=6). x and y stand for significant differences attributed to sampling time. a and b stand for significant differences attributed to dietary treatments. One-way ANOVA (before i.p. injection) and Multifactorial ANOVA (after i.p. injection); Tukey post hoc test; p≤0.05).

3.3.2 Oxidative stress

3.3.2.1 Liver total antioxidant capacity

No significant differences were observed in TAC at the end of the feeding trial but it increased in time after i.p. injection regardless of dietary treatment and stimulus (Appendix II, Supplementary table 4).

3.3.2.2 Respiratory burst

Peripheral blood superoxide anion production was not affected by dietary treatments at the end of the feeding trial (Fig. 5). However, at 24 h post-injection, superoxide production was highest in TRP-fed fish, though values were only significantly higher than those measured in MET-fed fish (Fig. 5).

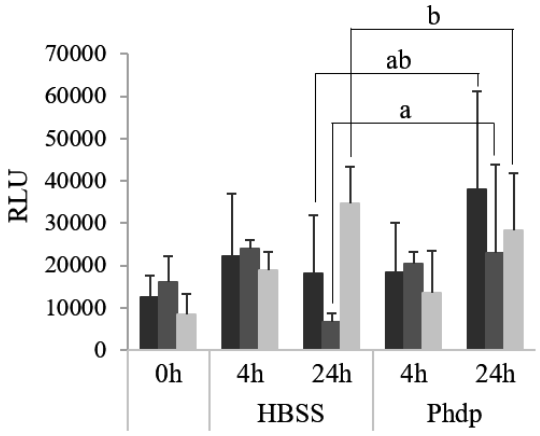

Fig. 5 Respiratory burst activity of peripheral leucocytes in juvenile European seabass fed CTRL (■), MET (■) or TRP (■) for 14 days (0 h) and sampled at 4 h or 24 h post HBSS- or *Phdp*- i.p. injection (mean ± SD, n=6). a and b stand for significant differences attributed to dietary treatments. One-way ANOVA (before i.p. injection) and Multifactorial ANOVA (after i.p. injection); Tukey post hoc test; p≤0.05).

3.3.2.3 Relative head-kidney and blood *gpx* mRNA expression

At the end of the feeding trial, *gpx* was up-regulated in the head-kidney of fish fed both supplemented diets relatively to the CTRL-fed group, while those fed TRP showed higher expression levels than MET-fed fish, too (Appendix II, Supplementary table 5). Differently, expression levels of *gpx* in blood were lower in MET-fed fish sampled at 24 h post-injection relatively to those of CTRL- and TRP-fed fish (Fig. 6).

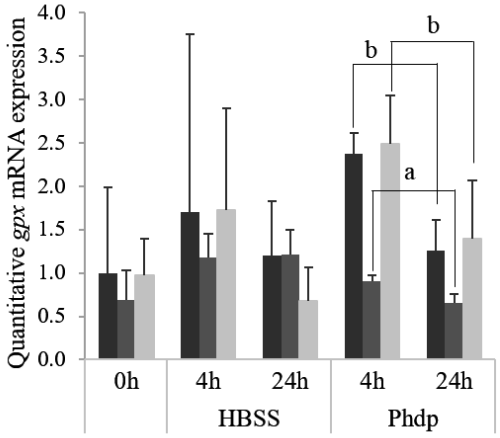

Fig. 6 Quantitative *gpx* mRNA expression in blood of juvenile European seabass fed CTRL (■), MET (■) or TRP (■) for 14 days (0 h) and sampled at 4 h or 24 h post HBSS- or *Phdp*- i.p. injection (mean ± SD, n=6). a and b stand for significant differences between dietary treatments. One-way ANOVA (before i.p. injection) and Multifactorial ANOVA (after i.p. injection); Tukey post hoc test; p≤0.05).

3.3.3 Immune response

3.3.3.1 Leucocyte migration dynamics

Peripheral blood. At the end of the feeding trial, total WBC, neutrophils and monocytes concentrations were higher in fish fed MET compared to CTRL-fed animals (Fig. 7 A, B and C, respectively). At 24 h post-injection, total WBC, neutrophils and monocytes were higher in *Phdp*-injected fish than in fish injected with HBSS (Figs….and.., respectively). Neutrophils increased in time in *Phdp*-injected fish, regardless of dietary treatment (Fig. 7 B), and monocytes concentration was lower at 24 h in HBSS-injected fish (Fig. 7 C). MET-fed fish showed higher lymphocyte numbers when injected with *Phdp*, compared to those injected with HBSS (Fig. 7 D). Furthermore, at 4 h post HBSS injection, lymphocytes were higher in TRP-fed compared MET-fed fish (Fig. 7 C). At 24 h post-injection, and regardless of stimuli, thrombocytes were lower in TRP-fed fish than in CTRL-fed fish (Fig. 7 E).

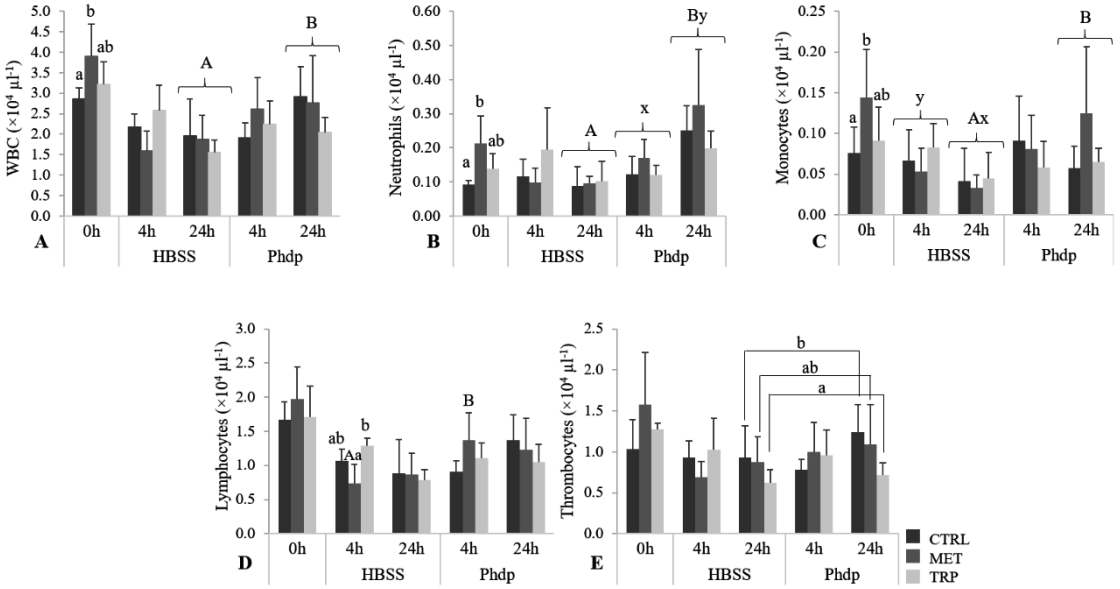

Fig. 7 Peripheral white blood cells (WBC, **A**), neutrophils (**B**), monocytes (**C**), lymphocytes (**D**) and thrombocytes (**E**) in juvenile European seabass fed CTRL (■), MET (■) or TRP (■) for 14 days (0 h) and sampled at 4 h or 24 h post HBSS- or *Phdp*- i.p. injection (mean ± SD, n=6). x and y stand for significant differences attributed to sampling time. a and b stand for significant differences between dietary treatments. A and B depict differences between stimuli. One-way ANOVA (before i.p. injection) and Multifactorial ANOVA (after i.p. injection); Tukey post hoc test; p≤0.05).

Peritoneal cavity. Total WBC, monocytes and lymphocytes concentrations were higher in *Phdp*-injected fish compared to the HBSS-injected group, regardless of sampling time and dietary treatment (Fig. 8 A, B and D, respectively). Neutrophils increased with time in both HBSS- and *Phdp*-injected groups but were lower in fish challenged with vehicle than in those challenged with *Phdp*, within each sampling time (Fig. 8 B).

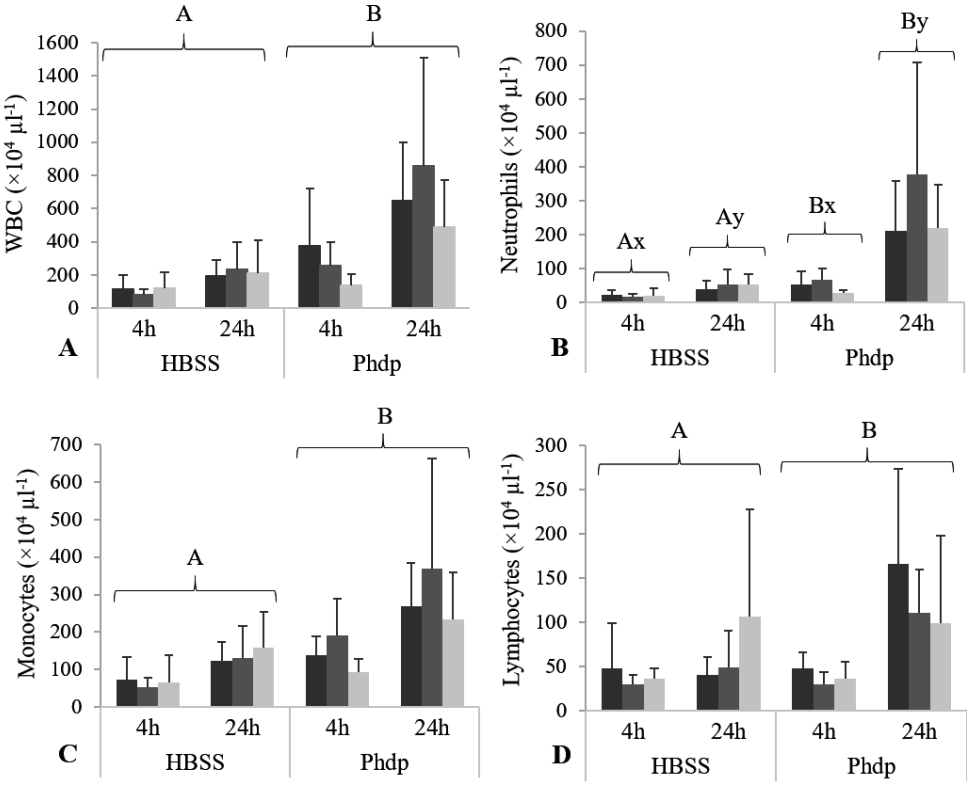

Fig. 8 Peritoneal white blood cells (WBC, **A**), neutrophils (**B**), monocytes (**C**) and lymphocytes (**D**) in juvenile European seabass fed CTRL (■), MET (■) or TRP (■) for 14 days and sampled at 4 h or 24 h post HBSS- or *Phdp*- i.p. injection (mean ± SD, n=6). x and y stand for significant differences between sampling times. A and B depict differences between stimuli. (Multifactorial ANOVA; Tukey post hoc test; p≤0.05).

3.3.3.2 Quantitative gene expression – Head-Kidney

Gene expression of *mmp9* increased with time in TRP-fed fish, irrespectively of stimulation (Fig. 9 A). However, TRP-fed group presented lower mRNA levels of *mmp9* compared to CTRL-fed fish, but only when injected with HBSS (Fig. 9 A). *c3* expression levels increased at the end of the feeding trial in fish fed TRP and MET compared to the CTRL group and, although not statistically significant, tended to be up-regulated in MET-fed fish injected with *Phdp*. (Fig. 9 B). Gene expression of both *hep* and *il1β* was not modulated by any condition tested (Appendix II, Supplementary table 5).

3.3.3.3 Quantitative gene expression – Blood

At the end of the feeding trial, *il1β* gene expression was down-regulated in fish fed TRP relatively to CTRL-fed fish (Fig. 9 C) but no further effects were observed in any other gene expression evaluated (Appendix II, Supplementary table 5). *il1β* gene expression was lower in both MET-fed and TRP-fed fish at 4 h post-injection regardless of stimulation, compared to CTRL-fed fish (Fig. 9 C). Upon HBSS-injection, *dctrn* was up-

regulated in MET-fed fish relatively to their CTRL counterparts and to the same dietary group injected with *Phdp* (Fig. 9 D).

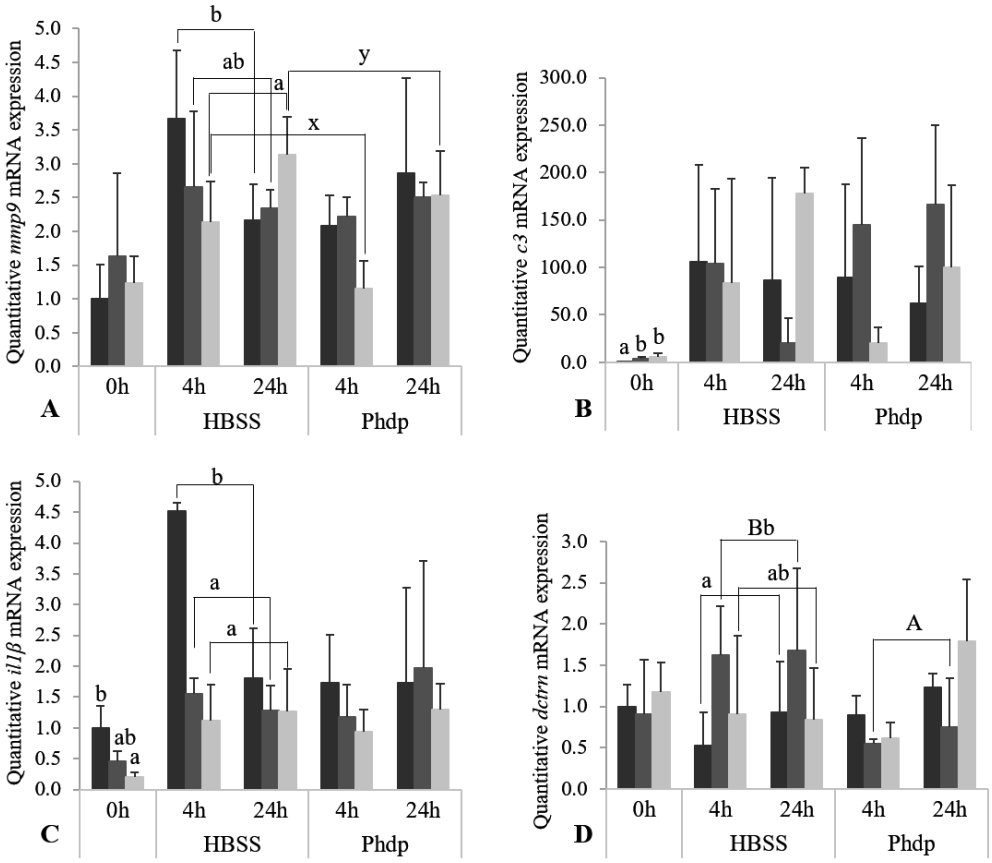

Fig. 9 Quantitative expression of immune-related genes in the head-kidney (**A** and **B**) and blood (**C** and **D**) in juvenile European seabass fed CTRL (■), MET (■) or TRP (■) for 14 days (0 h) and sampled at 4 h or 24 h post HBSS- or *Phdp*-i.p. injection (mean ± SD, n=6). Metalloproteinase 9 (*mmp9,* **A**), complement factor 3 (*c3*, **B**) interleukin 1β (*il1β*, **C**) and dicentracin (*dctrn*, **D**)**.** x and y denote significant differences attributed to sampling time. a and b stand for significant differences between dietary treatments. A and B stand for significant differences between stimuli. One-way ANOVA (before i.p. injection) and Multifactorial ANOVA (after i.p. injection); Tukey post hoc test; p≤0.05).

3.4 Discussion

The neuroendocrine–immune network is essential for restoring homeostasis upon distress and infection. Sustaining a balanced immune response that effectively clear pathogens while minimizing damage to the host is essential. It is now recognised that in teleosts the neuroendocrine and immune systems interact in a bi-directional way, similarly to that known for mammals [4]. However, this bi-directional communication has implications in physiological homeostasis and health that are yet far from being elucidated. Therefore, the present study aimed to contribute to this endeavour using a combination of a well-established inflammatory model plus a dietary intervention to assess the interaction of brain neurotransmitters, plasma cortisol levels, leucocyte migration dynamics and transcript levels of endocrine and immune-related genes. The

modulation of those intimate interactions between endocrine and immune systems by amino acids would be of major assistance to promote host health.

Outcomes from the present study indicate that i.p. injected fish (either with HBSS or UV-killed *Phdp*) were mounting a neuroendocrine response, as plasma cortisol levels increased following i.p. injection in both groups. Also, Supplementary figure 1 from the Appendix II clearly shows in all dietary treatments an increase of serotonergic activity in i.p. injected fish compared to non-injected fish. This is in agreement with previous studies, both in mammals and teleosts, which showed a cortisol-induced production after injection challenge (i.e. bacterial lipopolysaccharide (LPS) or bacteria) [33-35]. For instance, LPS induced cortisol secretion in gilthead seabream, *Sparus aurata*, at 2 and 6 h after LPS injection [36]. In the present study, cortisol increase was much higher in fish fed MET and TRP than in CTRL-fed fish, and this was especially evident 24 h post-injection. Post-injection period was also characterized by lower gr gene expression particularly in blood and HK of MET and groups, but also in TRP groups when cortisol production was highest. Similarly, the number of glucocorticoid receptors in carp decreased with increasing cortisol levels [37]. The authors suggested that this response could be related to mechanisms of negative feedback in which immune cells may become resistant to cortisol. Hypothalamus–pituitary–interrenal (HPI) axis activation induces pituitary secretion of adrenocorticotropic hormone that, in turn, induces cortisol production in HK interrenal cells through activation of *mc2r* cell receptor [38]. In our study, though amino acid supplementation did not seemed to affect *mc2r* expression levels, together with HK *gr*, it was down-regulated at 24 h post-injection, in accordance with an established negative feedback mechanism.

Blood gr down-regulation in supplemented diets is absent before the challenge, being more evident 4h after the fish were i.p. injected. In contrast, levels of cortisol in fish fed supplemented diets were much higher (2 ×, see Fig. 4) at 4 h post-injection than before the immune challenge, whereas such increase was not observed in the CTRL group. This seems to indicate that although cortisol increase is a consequence of the HPI axis activation, apparently triggered by the i.p. injection itself as denoted by HBSS-injected fish, its release to the blood is enhanced by the presence of amino acid supplementation. This increase in cortisol at 4 h in groups fed amino acid-supplemented diets, comparatively to basal levels, might be related to two triggering mechanisms: i) an enhanced inflammatory response driven by amino acid surplus or ii) increased 5-HT precursor (tryptophan) which would promote synthesis of this monoamine that, in turn, would potentiate a neuroendocrine response. According to Verburg-Van Kemenade et al. [4], an inflammatory response activates the central nervous system by means of circulating pathogen-associated molecular patterns, cytokines and prostaglandins.

These mediators then activate neuronal receptors that are directly involved in HPI axis activation [39]. Moreover, as highlighted by Tort [40], other aspects strongly denote such intimate connection: several immune and endocrine mediators share a common phylogeny and as the HK has both endocrine and lymphoid tissues and functions, this allows a close and direct communication between both systems. Considering the significantly higher amount of total circulating leucocytes in both peripheral blood and peritoneal cavity in animals fed MET compared to other groups, it seems that i.p. injection together with enhanced leucocytes proliferation increased cortisol production in MET-fed fish. Such increase might be the result of regulatory mechanisms triggered by a significant increase on circulating immune cells. This provides further evidence on the bi-directional communication between neuroendocrine and immune systems in teleosts. In TRP-fed animals, higher cortisol production might have resulted from the increased 5-HT production, as observed in TRP-fed fish. Nonetheless, results are quite intriguing, especially for tryptophan, which has been shown to reduce cortisol response to different stressors [19,41]. The fact that cortisol levels increased with TRP instead of decreasing might be associated to the nature of the stressor. In this study, the stressor used was not a physical stressor, and foreign substances in the peritoneal cavity may directly activate immune processes that affect the HPI axis.

The involvement of amino acids in the immune response has been studied mostly in mammalian models but the interest on developing functional feeds for farmed fish raised attention on the potential of amino acids as additives for fish feed [11,42-45]. Methionine is precursor of S-adenosylmethionine and cysteine which narrows the topics of studies on methionine potential as feed ingredient to polyamine turnover, methylation pathways and oxidative stress [11,46-48]. Nonetheless, modulation of polyamine turnover and glutathione biosynthesis also affect the innate immune response, as it is mostly mediated by cells and characterized by intensive production of oxygen free radicals [49]. Thus, as precursor of glutathione, a free radical scavenger, methionine could modulate oxidative status [50]. Moreover, being necessary to polyamine biosynthesis, it may improve cell proliferation and differentiation [51]. Results of the present study show an improved cellular proliferation and migration to the inflammatory focus in MET-fed fish, which is reflected in the peripheral and peritoneal leucocyte numbers. However, effects of this dietary methionine supplementation on immune response are rather ambiguous. Methionine supplementation induced HK *gpx* gene expression before the immune challenge. Being precursor of cysteine, methionine might have potentiated biosynthesis of glutathione, a co-factor in *gpx* activity. Increased enzyme production would then facilitate antioxidant activity during the inflammatory response. *gpx* gene expression was down-regulated in the blood of MET-fed fish which might result from a negative-feedback

mechanism in response to the earlier strong enzyme synthesis that seemed to take place before challenge. Contrary to what was expected, data on TAC during the inflammatory response does not depict an increase of the antioxidant power in MET-fed fish. Still, it is important to bear in mind that glutathione is only one amongst many other antioxidant molecules whose activity is also quantified by the technique used. Therefore, glutathione increase was perhaps not high enough to be detected within all other antioxidants' activities.

Despite the fact that the cell migration indicator mmp9 was not significantly affected by methionine supplementation, and the pro-inflammatory interleukin il1β was indeed down-regulated, peripheral blood cells increased in fish fed MET. As precursor of polyamines, methionine might have promoted polyamine biosynthesis and thereby increase immune cell numbers.

The role tryptophan played in brain monoamine content was far more evident than that of methionine. The majority of neurotransmitters analysed and their metabolites were more abundant in the optic tectum and telencephalon of animals fed TRP which probably contributed to boost cortisol production. The presence of serotonergic [52] and glucocorticoid receptors [53] in immune-related cells, as well as cytokine receptors in brain cells [54] clearly demonstrate the presence of signalling pathways established between the two systems. In addition, the fact that interrenal cells are located in an important lymphoid organ as the HK also evidences the closeness between the two systems. Chronic stress exposure has long been associated to immunosuppression and poorer disease resistance [55,56] as reviewed by Tort [40]. The present experiment was not designed to inflict chronic stress but fish might have perceived the injection itself as a neuroendocrine stimulus that triggered cortisol production. These conditions might have partly influenced the immune response.

Before being stimulated, TRP-fed fish had larger amounts of *gpx* and complement factor *c3* mRNA compared to fish fed CTRL. On the other hand, *il1β* gene expression was down-regulated and peripheral leucocytes respiratory burst tended to be lower than that in CTRL-fed fish. After immune stimulation tryptophan supplementation inhibited blood il1β and head-kidney mmp9 expression and decreased thrombocytes compared to fish fed a basal diet. Tryptophan immune-suppressive effects are likely to result not only of the higher cortisol levels but also of the metabolic pathway mediated by IDO, which in mammals is known to be induced by immune stimulation, converting tryptophan to molecules with anti-inflammatory properties [9,16,57]. Quinolinate, for instance, is able to inhibit T-cell proliferation and reduce apoptosis cytotoxicity, while immature lymphocytes develop into regulatory T-cells [16]. This could help to explain the down-regulation of pro-inflammatory genes and the lower numbers of circulating and peritoneal leucocytes

observed in fish fed TRP. Tryptophan failed to extend the inflammatory response of seabass on plasma humoral parameters, as it suppressed the activity of peroxidase and nitric oxide synthesis [51]. Cytotoxicity of the tryptophan metabolite 3-hydroxykynurenine is known to be mediated by hydrogen peroxide [58,59]. Hence, the tryptophan load and consequent metabolite production might have led to increased hydrogen peroxide production that, in turn, induced glutathione peroxidase synthesis, as observed in TRP-fed fish before i.p.-injection.

In conclusion, feeding seabass methionine- or tryptophan-supplemented diets for 2 weeks seems to modulate the neuroendocrine response during an inflammatory insult as it fostered plasma cortisol levels once fish were subjected to peritoneal inflammation. Changes in brain monoamine content were poorly affected by MET but were evident in TRP-fed fish, which also seem to be related to the observed stress response. Results point out at enhancing effects on innate immune response in MET-fed fish, as evidenced by an enhanced leucocyte response. Differently, tryptophan supplementation did not significantly change inflammatory markers, though immune-related gene expression was often down-regulated. Future studies on polyamine and cytokine quantification should be considered to further characterize each amino acid role during the inflammatory response in fish.

3.5 Funding

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Chapter 4

Local immune response of two mucosal surfaces of the European seabass, *Dicentrarchus labrax*, fed tryptophan- or methionine-supplemented diets

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Local immune response of two mucosal surfaces of the European seabass, *Dicentrarchus labrax*, fed tryptophan- or methionine-supplemented diets

Abstract

Immune responses relies on an adequate provision of multiple nutrients that sustain the synthesis of key effector molecules. These needs are depicted in the already reported increase of circulating free amino acids in fish under stressful conditions. Since aquaculture and the inherent fish welfare are an emergent call, the immunomodulatory effects of amino acids on gut- and skin-associated lymphoid tissues of the European seabass (Dicentrarchus labrax) were studied under unstressed conditions and after an inflammatory insult. To achieve this goal, fish were distributed in duplicate tanks (fifteen fish per tank) and were fed for 14 days with methionine or tryptophan-supplemented diets at 2× dietary requirement level (MET and TRP, respectively) or a control diet meeting the amino acids requirement levels (CTRL). Afterwards, samples of skin and posterior gut were collected from 6 fish per dietary treatment for the assessment of the immune status while the remaining animals were intraperitoneally-injected with inactivated *Photobacterium damselae* subsp. piscicida and subsequently sampled either 4 or 24 h post-injection.

The immune status of both mucosal surfaces was poorly affected, although a tryptophan effect was denoted after bacterial inoculation, with several immune-related genes up-regulated in the gut at 4 h post-injection, which seems to suggest a neuroendocrine-immune systems interaction. In contrast, skin mucosal immunity was inhibited by tryptophan dietary supplementation. Regarding methionine, results were often statistically non-significant, though increasing trends were denoted in a few parameters.

Overall, dietary methionine did not significantly affect neither gut nor skin immunity, whereas tryptophan supplementation seems to induce modulatory mechanisms that might be tissue-specific.

Key-words: functional ingredients; GALT; SALT; mucosal immunity; amino acids; European seabass (*Dicentrarchus labrax*)

4.1 Introduction

Fish health maintenance is a key aspect in aquaculture farms. Defining strategies to improve fish welfare and health is therefore of utmost importance. Being an excellent growing media, seawater around fish farms is a vehicle of bacteria outbreaks that can devastate an entire fish stock. When present in the water, pathogenic bacteria are in direct contact with the first lines of fish innate immune defences: the mucosal barriers. Hence, the inherent innate immune defences are known to be the first, quickest and strongest mechanisms of response in teleost fish, in which these animals highly rely in the course of infection [1].

Fish gut-associated lymphoid tissue (GALT), one of the mucosal immune tissues, is known to be comparatively less organized than that of mammals but it contains widespread immune cells such as lymphocytes, neutrophils and macrophages [2]. Macrophages, for instance, are located in and under the intestinal epithelium and are able to phagocytise and digest antigens to be later presented to lymphocytes in other lymphoid organs [3]. The same cells can be encountered in skin surface (named SALT) along with an array of antibodies, antimicrobial peptides, lectins and proteases [1]. A large amount and variety of microorganisms inhabit these mucosal surfaces as commensals, and they are recognized by resident immune cells in the same way pathogens are [4,5]. Thus, a delicate balance exists based on both pro-inflammatory and regulatory mechanisms that must enable a symbiotic relationship between commensal and host intestinal epithelium.

Numerous substances are being included in fish feeds as functional ingredients to improve or enhance certain traits or functions [6-10]. There is a growing interest on amino acids potential as functional ingredients and, though efforts to date have been put on mammals' research, studies on functional amino acids in fish immune response are becoming available [11-16]. Several amino acids were already shown to play important roles in the immune system. Methionine, for instance, appears to have clear pro-inflammatory effects on the juvenile Jian carp, *Cyprinus carpio*, immune response [17,18]. Decarboxylated S-adenosylmethionine is directly involved in polyamines turnover through addition of aminopropane groups and the importance of polyamines during cell proliferation is widely known [19]. This is particularly important as both innate and adaptive immunity are highly dependent on cellular responses. Methionine is also precursor of cysteine, which is one of the three glutathione constituents, and thus it is an essential element for glutathione production. However, knowledge on methionine involvement in the health of mucosal associated lymphoid tissues is scarce, and few studies have explored its nutritional importance in gut immunity, integrity and oxidative status [20]. Methionine oxidation seems

to be of key importance in intestinal goblet cells for the production of mucins involved in innate immunity, which composition is cysteine-rich [21].

Understanding the regulatory mechanisms of the immune system is necessary upon the analysis of a specific immune response. As substrate of 2, 3-indoleamine dioxygenase (IDO), tryptophan metabolic cascades lead to anti-inflammatory commands, the main players being regulatory T-cells [22-24]. Considering this, increasing tryptophan availability in the organism may be regarded as a strategy to counteract the deleterious aspects of a powerful innate immune response. Differently, when metabolized by tryptophan hydroxylase, tryptophan is converted to serotonin (5-HT). This conversion occurs in the central nervous system, where 5-HT has a role on the neuroendocrine response. However, the majority of 5-HT (95 %) is synthetized in the serotonergic nerve fibres and enterochromaffin cells of the intestinal epithelium [25]. Serotonin is a paracrine messenger in the gastrointestinal tract and, in mammals, elevated concentrations of this indoleamine have been associated with increased chemotaxis, cytotoxicity and proliferation of natural killer cells, as well as with protection from oxidative damage [26]. Less is even known about the effects of dietary amino acids levels on the skin-associated lymphoid tissue. Therefore, this study intended to unveil possible immunomodulatory effects of methionine and tryptophan on the European seabass gut- and skin-associated lymphoid tissues, both before and following an inflammatory insult. By means of peritoneal bacterial injection, we intended to induce a local inflammation, which would elicit a neuroendocrine response by activation of the hypothalamus-pituitary-interrenal axis, which might in turn modulate mucosal surfaces immune responses.

4.2 Material and Methods

4.2.1 Formulation and proximal composition of the experimental diets

Three isonitrogenous (44.9 % crude protein) and isolipidic (14.9 % crude lipid) diets were formulated with fish meal and a blend of plant feedstuffs as protein sources and fish oil as the main lipid source. The plant-protein fraction represented almost 50 % of the total feed composition. In two of these diets, $_{L}$-tryptophan or $_{L}$-methionine were added at 2 × the requirement level, at the expenses of fish meal. The non-supplemented diet was regarded as the CTRL and met the amino acids requirement levels established for European seabass [27]. The two supplemented diets were considered dietary treatments and will be referred as TRP (tryptophan-supplemented diet) and MET (methionine-supplemented diet).

More detailed information on diets composition and proximate analysis is given in Table 1. All ingredients were ground, mixed together and dry-pelleted in a laboratory pellet mill (CPM, California Pellet Mill, Crawfordsville, IN, USA). Proximate analysis of the diets was performed according to the Association of Official Analytical Chemists methods [28] and amino acids analysis was carried out according to Banuelos-Vargas [29] (Table 2).

Table 1. Ingredients and proximate composition of experimental diets

	Experimental diets		
	CTRL	**TRP**	**MET**
Ingredients (%)			
Fish meal[1]	34.1	33.5	33.2
Soybean meal[2]	15.0	15.0	15.0
Corn gluten[3]	10.0	10.0	10.0
Wheat gluten[4]	5.0	5.0	5.0
Wheat meal[5]	16.7	16.6	16.2
Fish oil	13.9	13.4	14.0
Vitamin premix[6]	1.0	1.0	1.0
Choline chloride (50 %)	0.5	0.5	0.5
Mineral premix[7]	1.0	1.0	1.0
Binder[8]	1.0	1.0	1.0
Agar	1.0	1.0	1.0
Dibasic calcium phosphate	0.84	0.91	0.96
L-Methionine[9]	—	—	1.16
L-Tryptophan[9]	—	0.52	—
Proximate analyses (% dry weight)			
Dry matter (%)	95.2	94.9	94.3
Crude protein	44.9	45.2	45.0
Crude lipid	15.5	16.5	16.9
Ash	10.5	10.4	10.5

[1]Pesquera Centinela, Steam Dried LT, Chile (CP: 71.4%; CL 9.3%). Sorgal, S.A. Ovar, Portugal
[2]Soybean meal (CP: 54.9%; CL:2.1%), Sorgal, S.A. Ovar, Portugal
[3]Corn gluten (CP: 72.2%; CL: 2.0%), Sorgal, S.A. Ovar, Portugal
[4]Wheat gluten (CP: 84.4%; CL: 2.1%), Sorgal, S.A. Ovar, Portugal
[5]Wheat meal (CP: 13.9%; CL: 1.8%), Sorgal, S.A. Ovar, Portugal
[6]Vitamins (mg kg^{-1} diet): retinol, 18000 (IU kg^{-1} diet); calciferol, 2000 (IU kg^{-1} diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.
[7]Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dicalcium phosphate, 8.02 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.4 (g kg^{-1} diet).
[8]Aquacube. Agil, UK.
[9] Feed grade amino acids; Sorgal, S.A. Ovar, Portugal

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

	Experimental Diet		
	CTRL	**TRP**	**MET**
Arginine	7.74	7.11	7.02
Histidine	3.78	3.63	4.12
Isoleucine	5.05	4.64	4.71
Leucine	9.73	9.71	9.49
Lysine	6.66	6.96	6.68
Methionine	2.57	2.42	4.95
Phenylalanine	5.39	5.16	5.16
Tyrosine	4.04	3.96	3.98
Threonine	4.68	4.38	4.51
Tryptophan	1.12	2.24	1.10
Valine	5.38	5.10	5.12
Aspartic Acid	8.20	7.54	7.60
Glutamic Acid	16.39	16.14	16.09
Serine	4.15	4.42	4.29
Glycine	3.98	4.18	4.20
Alanine	4.89	4.92	4.97
Proline	4.99	5.50	4.84

4.2.2 Bacteria inoculum preparation

Photobacterium damselae subsp. *piscicida*, strain PP3 (*Phdp*) was kindly provided by Dr. Ana do Vale (Institute for Molecular and Cell Biology, University of Porto, Portugal) and previously isolated from the yellowtail (*Seriola quinqueradiata*, Japan) by Dr. Andrew C. Barnes (Marine Laboratory, Aberdeen, UK). Bacteria cells were first cultured for 48 h at 22 °C in tryptic soy agar (Difco Laboratories) supplemented with 1 % NaCl (w/v) (TSA-1). Colonies were then inoculated into tryptic soy broth equally supplemented with NaCl (TSB-1) and incubated overnight at 22 °C. Exponentially growing bacteria were centrifuged at 3,500 × *g* for 30 min, resuspended in TSB-1 with glycerol at a final concentration of 15 % (v/v) and stored at -80 °C as stock solution. *Phdp* inocula were obtained by culturing bacteria from the stock solution as previously described, and by suspending in sterile Hank's Balanced Salt Solution (HBSS) at a final concentration of 1 × 10^6 colony forming units (CFU) ml^{-1}, according to Costas et al. [30]. Bacteria were killed by a 2 h UV-light exposure and bacterial growth was not observed when UV-killed bacteria were plated in TSA-1.

4.2.3 Fish and experimental design

This study was carried out at the Marine Zoological Station, Porto, Portugal. After two weeks of quarantine being fed a commercial diet, European seabass (274.7 ± 20.4 g) juveniles were randomly distributed into six fibreglass tanks in a seawater recirculation system (300 l; n=15; temperature: 25 ± 1 °C; salinity: 35 ppt; natural light-dark cycle). Dietary treatments were randomly assigned to duplicate tanks, each with 15 animals, and fish were fed twice a day until apparent satiety. The feeding trial lasted for 14 days and O_2, salinity, pH, temperature and water quality were monitored daily. At the end of this period, 3 fish were sampled per tank (n=6 per dietary treatment). The remaining 12 fish were intraperitoneally (i.p.) injected with either 100 µl of UV-killed *Phdp* (n=6) or HBSS (sham group, n=6) and redistributed into new 300 l tanks according to dietary treatment and stimuli (n=6 per tank, in duplicates). Thereafter, fish were sampled (n=6 per dietary treatment, sampling time and stimuli) either 4 or 24 h post-inoculation. Fish were euthanized by immersion in 2-phenoxyethanol (1,500 ppm; Sigma). Skin mucus samples were collected according to Guardiola and co-workers [31] with some modifications. Briefly, skin mucus was collected by gentle scraping the dorso-lateral surface of specimens using a cell scraper with enough care to avoid contamination with blood and/or urino-genital and intestinal excretions. Samples were then aliquoted and stored at -80 ºC until use. The posterior intestine and a skin portion were then excised, frozen in liquid nitrogen and stored at -80 °C until further assayed.

Experiments were performed by trained scientists in full compliance with national rules and following the European Directive 2010/63/EU of the European Parliament and the European Union Council on the protection of animals used for scientific purposes.

4.2.4 Mucus samples treatment

In order to get sufficient mucus for all assays, equal samples of mucus were pooled (2 fish per pool) and homogenized with 1 volume of Tris-buffered saline (TBS, 50 mM Tris–HCl, 150 mM NaCl, pH 8.0). Homogenates were vigorously shaken and centrifuged (1,500 rpm, 10 min, 4 ºC) and the supernatant lyophilized following freezing at -80 ºC. Lyophilized skin mucus powder was dissolved in Milli-Q water, and the undissolved mucus portion was isolated by centrifugation (3,500 × *g*, 10 min, 4 ºC). Protein concentration in each sample was determined according to Bradford [32] and skin mucus samples were adjusted to 500 µg protein ml^{-1}.

4.2.5 Skin mucus immune defences

i) Bacteriostatic activity

Three marine pathogenic bacteria (*Vibrio harveyi* [*Vh*], *Vibrio anguillarum* [*Va*] and *Phdp*) were used to determine the bacteriostatic activity present in skin mucus samples. Bacteria were grown in TSA plates at 25 ºC. Afterwards, fresh single colonies of 1-2 mm were diluted in 5 ml of appropriate liquid culture medium and cultured for 16 h at 25 °C at 200-250 rpm. The skin mucus bacteriostatic activity was determined using the method of Sunyer and Tort [33] with some modifications. Aliquots of 100 µl of each bacterial dilutions (1/10) were placed in flat-bottomed 96-well plates and cultured with equal volumes of skin mucus samples. The optic density (OD) of the samples was measured at 620 nm in a microplate reader (FLUOstar Omega, BMG Labtech) at 30 min intervals during 24 h, at 25 ºC. Samples without bacteria were used as blanks (negative control). Samples without mucus were used as positive controls (100 % growth or 0 % bacteriostatic activity). The percentage of bacteriostatic activity (bacterial growth inhibition) was calculated for each pool.

ii) Anti-protease activity

Total anti-protease activity was determined considering the ability of skin mucus to inhibit trypsin activity [34]. Briefly, 40 µl of skin mucus samples were incubated (10 min, 22 ºC) with 10 µl of trypsin solution (5 mg ml^{-1}). After adding 70 µl of 100 mM ammonium bicarbonate buffer and 125 µl of 0.7 % azocasein, samples were incubated (2 h, 30 ºC) and, following the addition of 250 µl of 4.6 % trichloroacetic acid (TCA), a new incubation (30 min, 30 ºC) was done. The mixture was then centrifuged (10,000 × *g*, 10 min) and the supernatants transferred to a 96-well plate in triplicate wells containing 100 µl of 0.5 N NaOH, and the OD read at 450 nm in a plate reader. In positive controls buffer replaced skin mucus (100 % protease and 0 % anti-protease activity), and in negative controls buffer replaced trypsin (0 % protease and 100 % anti-protease activity). The percentage of inhibition of trypsin activity was calculated for each pool.

iii) Protease activity

Protease activity was quantified using the azocasein hydrolysis assay according to Ross et al. [35]. Briefly, equal volume of skin mucus were incubated with 100 mM ammonium bicarbonate buffer containing 0.7 % azocasein (Sigma) for 19 h at 30 ºC. The reaction was stopped by adding 4.6 % TCA and the mixture centrifuged (10,000 × *g*, 10 min). The supernatants were transferred to a 96-well plate in triplicate wells containing 100 µl of 0.5 N NaOH, and the OD read at 450 nm in a plate reader. Skin mucus was

replaced by trypsin solution (5 mg ml^{-1}, Sigma) in the positive control (100 % of protease activity), or by buffer in the negative control (0 % activity). The percentage of protease activity was calculated for each pool.

iv) Peroxidase activity

The peroxidase activity in skin mucus samples was measured according to Quade and Roth [36]. Briefly, 30 µl of skin mucus were diluted with 120 µl of HBSS without Ca^{2+} or Mg^{2+} in flat-bottomed 96-well plates. As substrates, 50 µl of 20 mM of 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB, Sigma) and 5 mM H_2O_2 were added to each well. 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 in a plate reader. Wells containing substrates but without skin mucus were used as blanks. One unit was defined as the amount producing an absorbance change of 1 and the activity expressed as U ml^{-1}.

v) Immunoglobulin M levels

Immunoglobulin M (IgM) levels in skin mucus were analysed by an enzyme-linked immunosorbent assay (ELISA) [37]. Briefly, plate wells were coated with mucus proteins, washed 3 times with PBS-T [20 mM phosphate saline buffer (PBS) and 0.05 % Tween 20, pH 7.3], blocked for 2 h at room temperature with blocking buffer (3 % bovine serum albumin in PBS-T) and rinsed again with PBS-T. The plates were then incubated for 1 h with 100 µl per well of mouse anti-European seabass IgM monoclonal antibody (1/100 in blocking buffer; Aquatic Diagnostics Ltd.), washed and incubated with the secondary antibody anti-mouse IgG-HRP (1/1,000 in blocking buffer; Sigma). Washing, development and reading was carried out as described above. Negative controls consisted of samples without skin mucus or without primary antibody, which OD values were subtracted for each pool.

4.2.6 Gene expression

Total RNA from 6 fish per treatment was extracted from 0.5 g of posterior intestine and skin samples and was pooled in pairs (n=3). RNA was then quantified and the purity assessed by spectrophotometry; the 260:280 ratios were 1.8-2.0. The RNA was then treated with DNase I (Promega) and complementary DNA (cDNA) was synthesized from 1 µg of total RNA using SuperScript III reverse transcriptase (Invitrogen) with an oligo-dT18 primer. A set of eight different primers (Table 3) was designed to evaluate immune-relevant gene expression profiles. Efficiency of each primer pair was determined by real-time PCR according to Pfaffl [38]. Expression levels of the selected genes were analysed

by real-time PCR (ABI PRISM 7500 instrument, Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures (containing 10 µl of 2 × SYBR Green supermix, 5 µl of primers [0.6 µM each] and 5 µl of cDNA template) were incubated for 10 min at 95 ºC, followed by 40 cycles of 15 s at 95 ºC, followed by 1 min at the corresponding annealing temperature (Table 3), and then 15 s at 95 ºC, 1 min at 60 ºC and 15 s at 95 ºC. Data was analysed following the 2-ΔΔCt method [39]. For each mRNA, gene expression was corrected by the elongation factor 1-alpha (ef1α) content in each sample. Standardization was performed relatively to the mean value of the CTRL-fed group, sampled before intra-peritoneal challenge. In all cases, PCR was performed with triplicate samples. Negative controls (cDNA replaced by water) and a cDNA pool were included in all real-time PCR assays to confirm specificity and reproducibility of the reaction.

Table 3. Forward and reverse primers used for real-time PCR.

Gene	Acronym	GenBank ID	AT[1]	PL[2]	Primer sequence (5'-3')
elongation factor 1α	*ef1α*	AJ866727	57	100	F: CGTTGGCTTCAACATCAAGA R: GAAGTTGTCTGCTCCCTTGG
alkaline phosphatase	*alp*	FJ860000	57	116	F: TTACCTCTGTGGGGTCAAGG R: TAGCCCATTTGAGGATGGAG
interleukin 8	*il8*	AM490060	58	110	F: GTCTGAGAAGCCTGGGAGTG R: GCAATGGGAGTTAGCAGGAA
non-specific cytotoxic cell receptor 1	*nccrp1*	FM022070	59	110	F: TGGGGTGAGATACGTCCACT R: TGGTTTTGGTTGCTCTGACA
superoxide dismutase	*sod*	FJ860004	59	90	F: TGTTGGAGACCTGGGAGATG R: ATTGGGCCTGTGAGAGTGAG
caspase 1	*casp1*	DQ198376	58	141	F: CCAGATCGTGGGTGTTTTCT R: TCTTCAAAGCGTTGCATGAC
cyclooxygenase 2	*cox2*	AJ630649	59	149	F: AGCACTTCACCCACCAGTTC R: AAGCTTGCCATCCTTGAAGA
natural killer cell-enhancing factor A	*nkefa*	AM987213	59	145	F: CTGCCGAAGATTTCAGGAAGA R: CGCCGTGTGTCAGATACCAG
trypsin	*tryp*	AJ006882	59	90	F: GCACCATGTGCACAGTCTCT R: ACAGGATGGGGATGTTCAAG

[1] Annealing temperature (°C)

[2] Product length (nt)

4.2.7 Data and statistical analysis

Statistical analyses were performed with STATISTICA (StatSoft, Inc. 2013, version 12) for WINDOWS. Results are expressed as means ± standard deviation of the mean. Data were analysed for normality and homogeneity of variance and, when necessary, outliers were removed using the STATISTICA tool for outliers and extremes removal. Data were log-transformed and analysed by one way analysis of variance (ANOVA) (immune status-related analysis) or Multifactorial ANOVA (inflammatory response) with dietary treatment and sampling time as variables. Whenever significant differences were found among groups, a multiple-comparisons Tukey HSD test was performed to identify significantly different groups. For every test, the level of significance chosen was $p \leq 0.05$.

4.3 Results

4.3.1 *Skin mucus immune defences*

Before i.p. injection (0 h), mucus bacteriostatic activity was enhanced in TRP-fed fish, compared to the CTRL-fed group regarding *Vh* growth but no other effects were observed regarding this activity neither before nor after the immune challenge (Fig. 1).

No effects were observed on skin mucus anti-protease, protease and peroxidase activities and IgM levels before i.p. injection (Table 4). Anti-protease activity increased at 24 h post-injection in *Phdp*-injected fish regardless of dietary treatment but was higher at 4 h in HBSS-injected group than in fish i.p injected with bacteria (Table 4). IgM levels were higher at 24 h post-injection compared to levels observed at 4 h, regardless of dietary treatment and stimulus (Table 4). Protease and peroxidase activities were not affected by any treatment (Table 4).

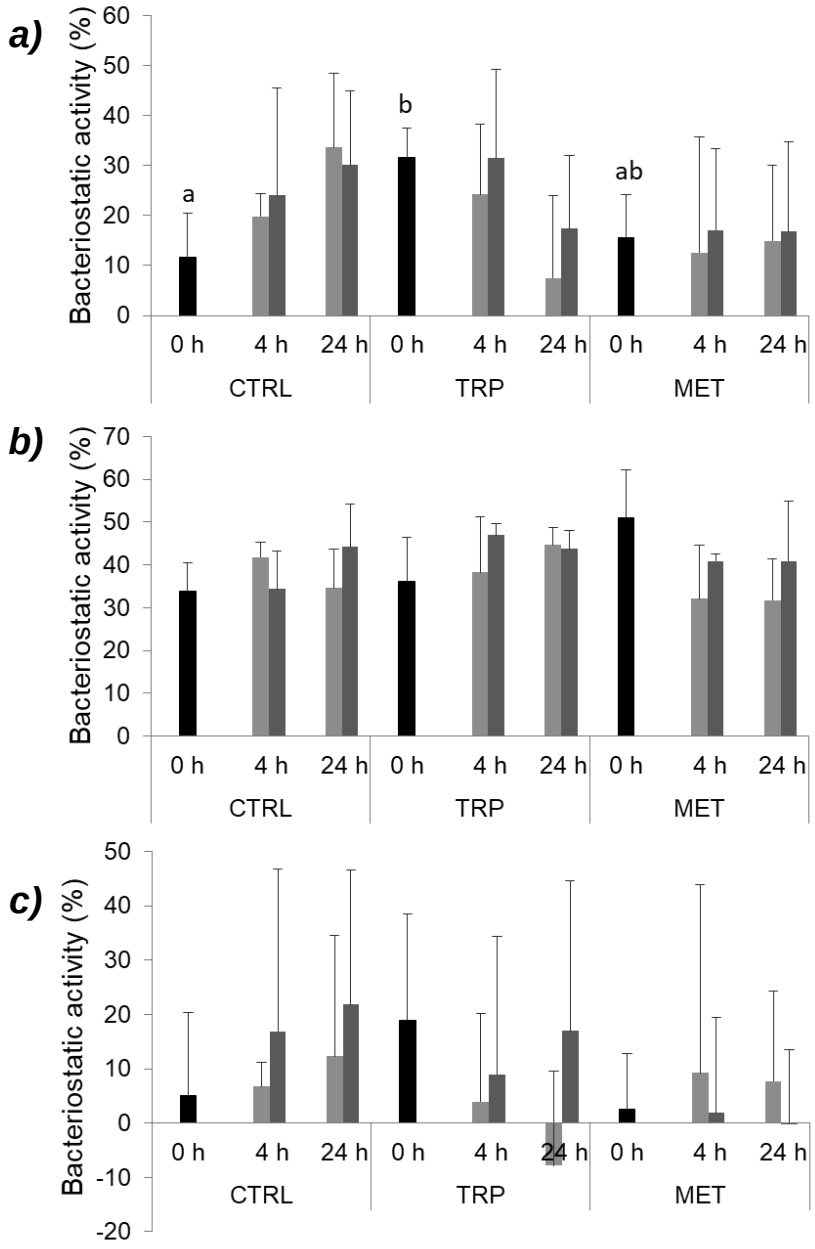

Fig. 1 Skin mucus bacteriostatic activity of European seabass, *Dicentrarchus labrax*, fed different dietary treatments and sampled at 0 h (■) and 4 or 24 h post-injection of *Phdp* (■) or HBSS (■) (means ± SD). *Vibrio harveyi* (*Vh*), *Vibrio anguillarum* (*Va*) and *Photobacterium damselae* subsp. *piscicida* (*Phdp*). Different letters stand for significant differences between dietary treatments, within each sampling time (One-way ANOVA was used to test differences before i.p. injection; Multifactorial ANOVA was used to test differences post i.p. injection; Tukey post hoc test; p≤0.05).

Table 4. Skin mucus immune parameters of the European seabass, *Dicentrarchus labrax*, at the end of the feeding trial (0 h) and at 4 or 24 h post-bacterial inoculation.

	CTRL					TRP					MET				
		Phdp		HBSS			*Phdp*		HBSS			*Phdp*		HBSS	
Two-way ANOVA	0 h	4 h	24 h	4 h	24 h	0 h	4 h	24 h	4 h	24 h	0 h	4 h	24 h	4 h	24 h
Anti-protease activity (%)	32.13 ± 0.89	28.39 ± 1.27	30.52 ± 0.65	31.53 ± 0.50	30.22 ± 3.15	32.20 ± 0.65	26.46 ± 0.20	30.88 ± 1.43	31.99 ± 0.57	32.39 ± 0.36	31.44 ± 0.71	28.08 ± 0.40	31.84 ± 2.10	30.10 ± 1.21	31.73 ± 1.42
Protease activity (%)	30.60 ± 2.39	29.69 ± 4.25	29.92 ± 6.61	34.36 ± 19.74	27.62 ± 9.32	29.69 ± 7.24	38.24 ± 4.90	29.03 ± 9.57	28.74 ± 8.45	26.65 ± 5.30	38.35 ± 23.05	29.35 ± 8.48	30.59 ± 10.27	32.04 ± 18.30	20.48 ± 8.03
Peroxidase activity (U mg^{-1} protein)	15.40 ± 4.01	11.57 ± 2.04	15.46 ± 4.75	9.94 ± 1.27	11.59 ± 0.55	14.56 ± 1.59	11.12 ± 0.64	11.22 ± 0.98	14.19 ± 2.00	12.72 ± 1.86	15.77 ± 4.44	12.27 ± 1.54	10.65 ± 0.92	14.40 ± 5.93	11.76 ± 2.36
IgM levels (OD 450 nm)	0.10 ± 0.02	0.10 ± 0.02	0.14 ± 0.03	0.14 ± 0.02	0.11 ± 0.01	0.14 ± 0.05	0.10 ± 0.01	0.11 ± 0.02	0.10 ± 0.01	0.18 ± 0.10	0.16 ± 0.05	0.11 ± 0.03	0.15 ± 0.05	0.10 ± 0.02	0.11 ± 0.03

										time × stimulus			
Multifactorial ANOVA	time	diet	stimulus	time × diet	time × stimulus	stimulus × diet	time × diet × stimulus	time		*Phdp*		HBSS	
								4 h	24 h	4 h	24 h	4 h	24 h
Anti-protease activity	<0.001	ns	<0.001	ns	0.001	ns	ns			Ax	y	B	
Protease activity	ns	ns	ns	ns	ns	ns	ns						
Peroxidase activity	ns	ns	ns	ns	ns	ns	ns						
IgM levels	0.039	ns	ns	ns	ns	ns	ns	x	y				

Values represent means ± SD (n = 3 biological replicates). x and y denote significant differences attributed to time, regardless of dietary treatment. Capital letters stand for statistically significant differences attributed to stimulus within each sampling time (One-way ANOVA was used to test differences before i.p. injection; Multifactorial ANOVA was used to test differences post i.p. injection; Tukey post hoc test; $p \leq 0.05$)

4.3.2 *Gene expression*

At the end of the feeding trial, trypsin (*tryp*) gene expression was higher in the distal gut of fish fed MET than in those fed TRP while no differences were observed in other immune-related genes analysed (Fig. 2). Alkaline phosphatase (*alp*) expression levels increased in time in TRP-fed fish injected with HBSS although it was down-regulated in the same fish compared to fish fed MET and to their *Phdp*-injected counterparts (Fig. 2a). At 4 h post-injection, natural killer cell-enhancing factor A (*nkefa*) was down-regulated in fish fed TRP compared to fish fed CTRL, regardless fish were injected with HBSS or *Phdp* (Fig. 2g). The same gene was up-regulated in time in TRP-fed fish, an effect not observed in CTRL-fed fish. *tryp* was up-regulated at 4 h post-injection in MET-fed fish regardless of stimulation, but only relatively to TRP-fed fish (Fig 2h). Regarding gene expression of caspase 1 (*casp1*, Fig. 2b), cyclooxygenase 2 (*cox2*, Fig. 2c), interleukin 8 (*il8,* Fig. 2d), superoxide dismutase (*sod,* Fig. 2e) and non-specific cytotoxic cell receptor protein 1 (*nccrp1,* Fig.2*f)*, no statistically significant effects were observed. However, levels of these genes expression tended to be higher in TRP-fed fish injected with bacteria than in the same fish injected with HBSS at 4 h post-injection and this difference tended to be larger than that observed in CTRL-fed fish.

Expression levels of *cox2* were too low to be detected by real-time PCR, therefore this gene was not included in the skin gene expression analysis. The same applies to *nkefa* and *tryp* gene expression at the end of the feeding trial. For the other analysed genes, no changes in skin gene expression were detected at the end of the feeding trial (Fig. 3).

Skin gene expression profile post-injection showed no significant alterations except for the down-regulation of *tryp* from 4 to 24 h in HBSS injected-fish, which was significant in fish fed TRP (Fig. 3g). The same group showed lower *tryp* expression levels than TRP-fed fish injected with bacteria and sampled at 24 h post-injection. Moreover, *tryp* expression levels were lower than similarly treated MET-fed fish (Fig. 3g).

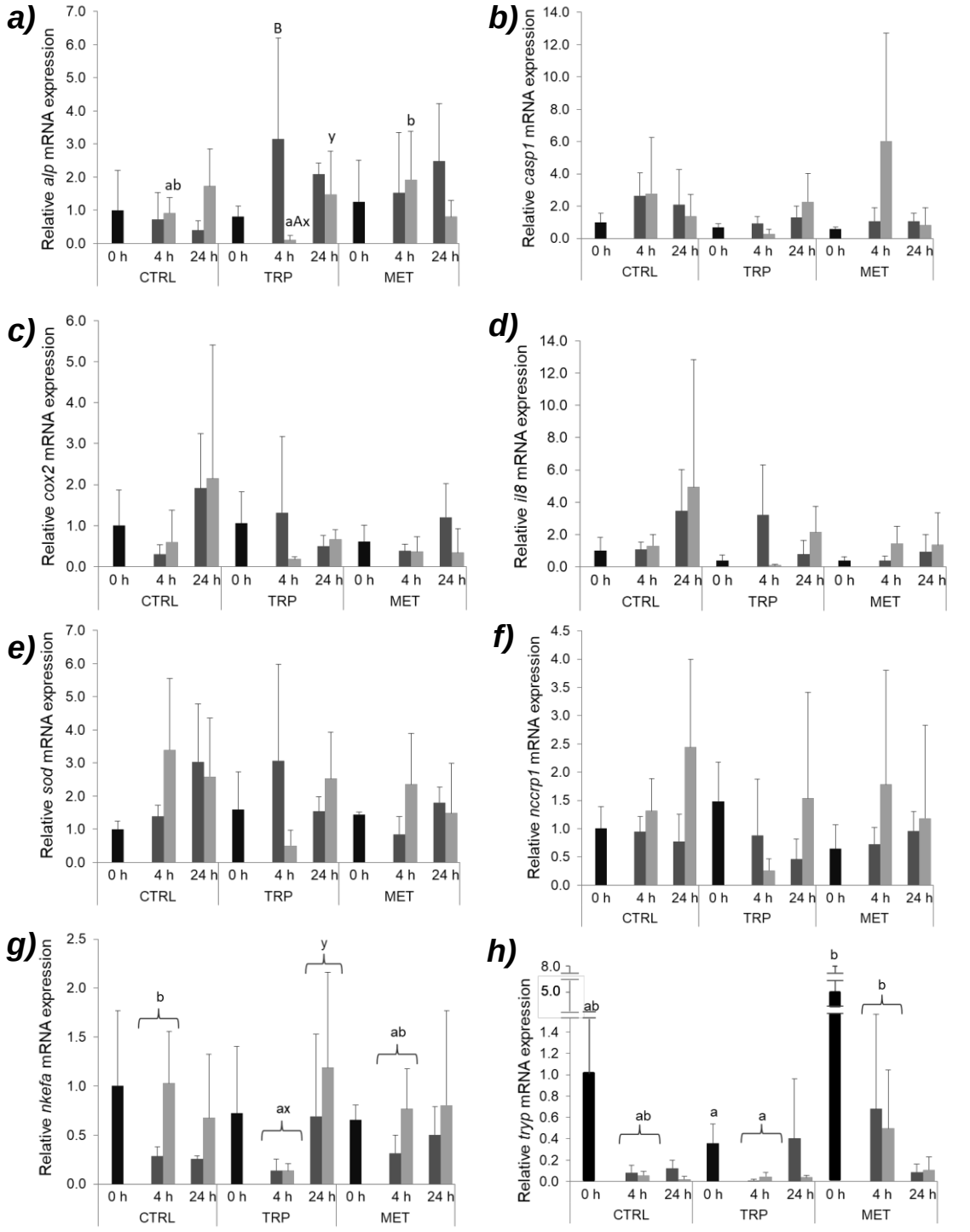

Fig. 2 Quantitative expression of immune-related genes in posterior intestine of European seabass, *Dicentrarchus labrax*, fed different dietary treatments and sampled at 0 h (■) and at 4 or 24 h post-injection of *Phdp* (■) or HBSS (■) (means ± SD). a) alkaline phosphatase (*alp*), b) caspase 1 (*casp1*), c) cyclooxygenase 2 (*cox2*), d) interleukin 8 (*il8*), e) *superoxide dismutase* (*sod*), f) non-specific cytotoxic cell receptor protein 1 (*nccrp1*), g) natural killer cell-enhancing factor A (*nkefa*) and h) trypsin (*tryp*). x and y stand for significant differences between sampling time; A and B denote significant differences attributed to stimulus; a and b stand for significant differences between dietary treatments (One-way ANOVA was used to test differences before i.p. injection; Multifactorial ANOVA was used to test differences post i.p. injection; Tukey post hoc test; p≤0.05).

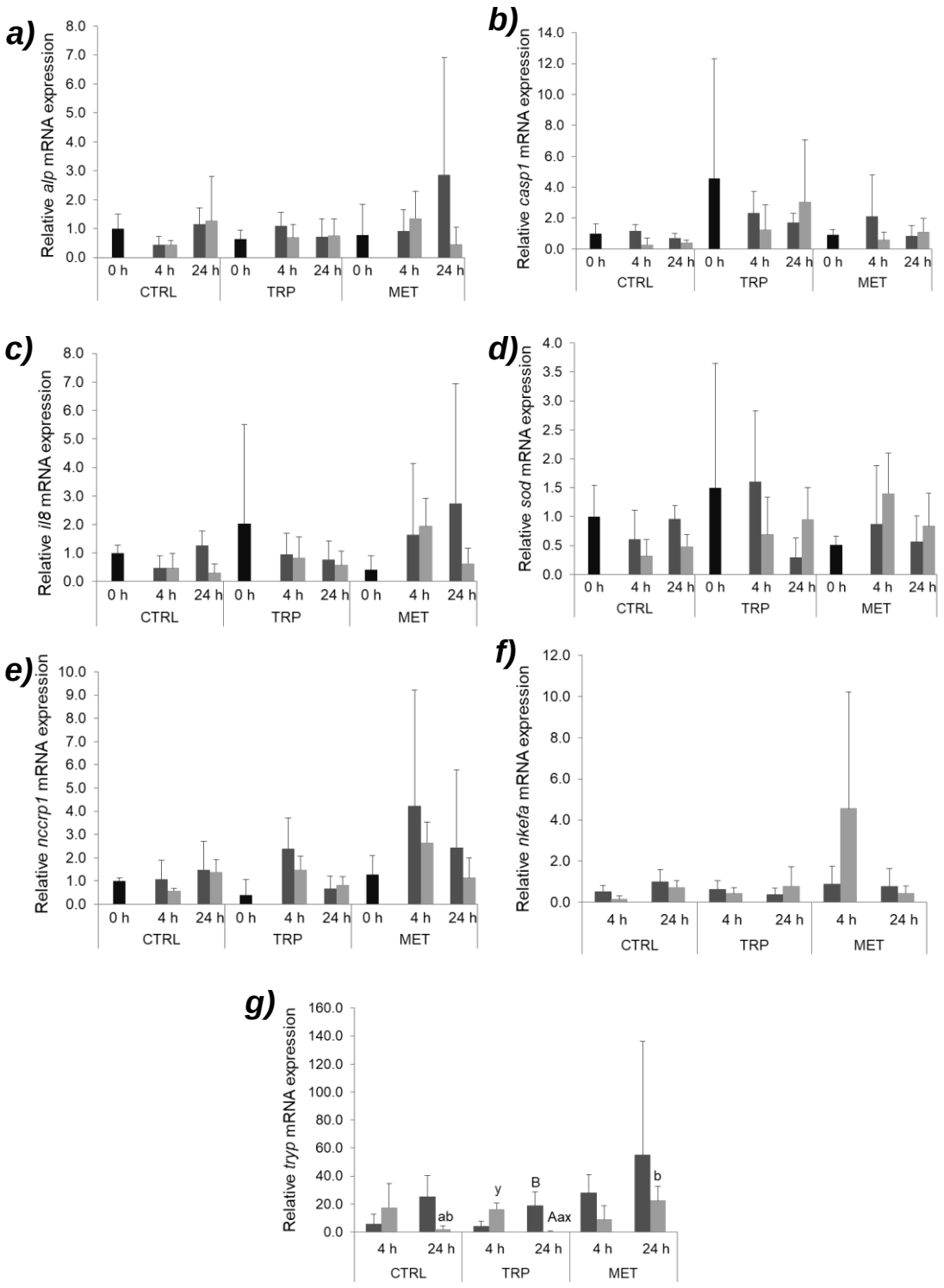

Fig. 3 Quantitative expression of immune-related genes in skin of European seabass, Dicentrarchus labrax, fed different dietary treatments and sampled at 0 h (■) and at 4 or 24 h post-injection of Phdp (■) or HBSS (■) (means ± SD). a) alkaline phosphatase (alp), b) caspase 1 (casp1), c) interleukin 8 (il8), d) superoxide dismutase (sod), e) non-specific cytotoxic cell receptor protein 1 (nccrp1), f) natural killer cell-enhancing factor A (nkefa) and g) trypsin (tryp). x and y stand for significant differences attributed to sampling time; A and B denote significant differences attributed to stimulus; a and b stand for significant differences between dietary treatments (One-way ANOVA was used to test differences before i.p. injection; Multifactorial ANOVA was used to test differences post i.p. injection; Tukey post hoc test; p≤0.05)

4.4 Discussion

Regulatory functions have long been attributed to tryptophan metabolites synthetized in the course of an immune response, but research is mostly focused in a systemic immune perspective [24,40]. Furthermore, the more expressive immunomodulatory effects of IDO-mediated tryptophan metabolism are those inflicted on cells of the specific immune response, such as T cells. However, as soon as they acquire a regulatory phenotype, these cells are responsible for the production of mediators that suppress the inflammatory response carried out by innate immune cells. Plus, one of the cell types expressing IDO is the macrophage [23]. Hence, at least indirectly, tryptophan regulatory pathways inhibit the innate immune response as well [41,42].

Though this is not one of the hottest topics in fish nutrition, tryptophan immune function has been the focus of some relevant studies. For instance, plasma lysozyme decreased in pikeperch, *Sander lucioperca,* after a long-term dietary tryptophan treatment [43]. Dietary tryptophan supplementation decreased immune cells numbers, humoral peroxidase activity and nitric oxide levels in European seabass following an immune challenge [13]. Interestingly, part of the results obtained in the present study point at an opposite direction. Although differences lack statistical significance (except in *alp* case) due to a high degree of variability, the up-regulation of several immune-related genes in the gut of bacteria-injected fish fed TRP relatively to their HBSS counterparts seems to point at an enhancing effect of tryptophan dietary supplementation on peripheral (i.e. gut) immune defences upon immune trigger. It is also important to notice that such contrast between *Phdp*-injected and sham fish was not observed in CTRL-fed or MET-fed fish. In spite of being a local insult (i.e. peritoneal cavity), the inflammatory reaction following the inactivated *Phdp* inoculation is usually characterized by intensive cell-to-cell communication, chemotaxis and inflammatory signalling molecules release [44]. Therefore, peripheral lymphoid organs such as the gut and the skin may promptly respond to this immune stimulation.

Following intestinal epithelial absorbance, circulating tryptophan can cross the blood-brain barrier and be metabolized in the central nervous system yielding 5-HT that acts both as a neurotransmitter and as a hormone [25]. However, the greatest 5-HT synthesis takes place in the enterochromaffin cells of the gastrointestinal tract [26]. Irrespective of localization, the same rate-limiting enzyme, tryptophan hydroxylase, which is not saturated at physiological tryptophan levels, mediated this reaction. Thus, 5-HT levels usually increase with tryptophan concentration. In accordance, plasma 5-HT levels in European seabass raised in response to dietary tryptophan supplementation [45]. In fact, in a study parallel to the present one, enhanced production of brain 5-HT in fish fed TRP was observed (Azeredo et al., submitted). Therefore, in spite of plasma or gut 5-HT were not analysed in this study, levels are expected to increase in TRP-fed fish and the presence of this indoleamine might have been the trigger for the increased immune-related gene expression.

Enterochromaffin and immune cells are in close contact to each other in the intestinal epithelium. Moreover, the presence of 5-HT receptors in immune system cells demonstrates the existence of inter-regulatory mechanisms between them [46-48]. In the present study, the presence of an immune stimulus (e.g. i.p. *Phdp* injection) associated to increased bioavailability of tryptophan might have led to intensified gut 5-HT production that, in turn, modulated GALT immune-related gene expression. In fact, mammals' natural killer cells proliferation and cytotoxicity was enhanced by 5-HT treatment while protecting them from oxidation [49,50]. In accordance, there seems to be an increasing trend in the immune cell marker *nccrp1* gene expression when fish were fed TRP for 14 days and were injected with UV-killed *Phdp*. Leading to local immune tolerance, *alp* is known to detoxify bacterial lipopolysaccharide, thereby assuring host-microbiota homeostasis and preventing unnecessary inflammation [51]. While the exact mechanism through which *alp* up-regulation takes place is still not known, increased expression of this enzyme is nonetheless additional information on tryptophan immunomodulation and its ability to regulate gut immune-barrier health. In addition, the

promotion of the antioxidant capacity has been denoted by Wen and co-workers [52], when grass carp, *Ctenopharyngodon idella*, fed tryptophan supplemented diets showed increased intestinal gene expression of both *sod* and glutathione peroxidase. In the present study, differently from the CTRL-fed group, *sod* gene expression was up-regulated in TRP-fed fish at 4 h post *Phdp*-injection. However, the fact that this result was devoid of statistical significance does not allow to safely conclude about tryptophan's ability to modulate the antioxidant capacity.

GALT response to the immune stimulus in TRP-fed fish was also characterized by lower transcriptional levels of *nkefa* and *tryp*, though only *nkefa* was significantly down-regulated compared to the correspondent levels in CTRL-fed fish.

Differently to what was observed in the gut mucosa, tryptophan supplementation did not alter any of the evaluated gene expression in the skin, except *tryp* which transcription was up-regulated in TRP-fed fish injected with bacteria, relatively to the sham group. Accordingly, at the end of the feeding trial, these fish showed an enhanced bacteriostatic activity in skin mucus. As in mammals, skin intraepithelial goblet cells produce mucins that compose a thin layer of mucus physically and biochemically protecting living epithelial cells [1]. It contains several antimicrobial substances such as trypsin that readily avoid pathogen adhesion, passage and proliferation or even neutralize pathogens, playing key roles as first line of defence. 5-HT dynamics in the skin, however, are poorly known, particularly in teleost fish, as no significant 5-HT synthesis occurs in this peripheral tissue [53]. Therefore, such tryptophan-enhanced immune parameters cannot be explained by 5-HT-mediated mechanisms as they might be in the gut. Differently, the enhancement of these pro-inflammatory mechanisms might have been the result of inflammatory signals originated either in the inflammatory focus (i.e. peritoneal cavity) or the gut that consequentially triggered other peripheral organs immune response. Indeed, Machado and co-workers [13] showed higher plasma anti-protease, alternative complement and bactericidal activities in European seabass fed similar tryptophan-supplemented diets for 14 days.

Differently from tryptophan, methionine immune functions have been largely explored, not only in other animal models but also in fish [13,17,54-56]. One of the more significant aspects of methionine immunomodulation is related to its ability to increase cell numbers and thereby improve systemic immune response, as observed in the present experiment and published elsewhere [13]. However, in this study methionine failed to affect seabass local innate immune responses in the gut and the skin. Indeed, few significant differences were detected at gene expression level or in mucus enzymatic activities in fish fed MET relatively to fish fed CTRL diet. Still, an increasing trend of some immune-related genes (intestinal *alp*, *casp1* and *tryp*, as well as skin *alp*, *il8* and *nccrp1* expression levels) was noticed and deserves to be further evaluated.

Data on head-kidney and blood gene expression collected in this experiment and presented elsewhere (Azeredo et al., submitted) depicted a similarly weak effect of methionine on modulating immune-related genes transcription. Pan and co-workers [57] reported a down-regulation of skin pro-inflammatory genes such as *il8*, interleukin-1β and tumour necrosis factor-α in grass carp fed graded levels of methionine hydroxy analogue-supplemented diet and intra-peritoneally infected with *Aeromonas hydrophila*, while gene expression related to regulatory cytokines was markedly stimulated. At the same time, complement factors, IgM, lysozyme and other innate immune defences were all improved in three lymphoid organs including skin. However, such response occurred 15 days after injection with live bacteria, whereas in the present study fish were sampled 4 or 24 h post-injection and the inflammatory stimulus was UV-killed *Phdp*. This might account, at least partly, to the absence of clear results.

One of the expected outcomes of supplementing methionine to fish, as well as other animals, is the improvement of the intestinal oxidative status, since cysteine is one of the constituents of glutathione [58]. As this study did not evaluate glutathione content in the gut nor any other relative molecule we restrain from making further suggestions.

Overall, while methionine dietary supplementation seems to enhance inflammation regarding a systemic response, it did not affect local immune processes, as few significant results were observed either in GALT or skin tissue. On the other hand, the

majority of the immune genes tended to be up-regulated in the posterior gut of *Phdp*-injected TRP-fed fish compared to their sham counterparts, suggesting a generally improved immune response. Still, skin mucus response being only slightly affected suggests that tryptophan immunomodulatory pathways might be tissue-specific and modes of action must be further investigated. Nonetheless, an undeniable modulatory potential exists in tryptophan that should be taken in consideration when selecting promising functional ingredients.

4.5 Acknowledgments

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Chapter 5

European seabass (*Dicentrarchus labrax*) immune status and disease resistance are impaired by arginine dietary supplementation

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Abstract

Infectious diseases and fish feeds management are probably the major expenses in the aquaculture business. Hence, it is a priority to define sustainable strategies which simultaneously avoid therapeutic procedures and reinforce fish immunity. Currently, one preferred approach is the use of immunostimulants which can be supplemented to the fish diets. Arginine is a versatile amino acid with important mechanisms closely related to the immune response. Aiming at finding out how arginine affects the innate immune status or improve disease resistance of European seabass (*Dicentrarchus labrax*) against vibriosis, fish were fed two arginine-supplemented diets (1 % and 2 % arginine supplementation). A third diet meeting arginine requirement level for seabass served as control diet. Following 15 or 29 days of feeding, fish were sampled for blood, spleen and gut to assess cell-mediated immune parameters and immune-related gene expression. At the same time, fish from each dietary group were challenged against Vibrio anguillarum and survival was monitored. Cell mediated immune parameters such as the extracellular superoxide and nitric oxide decreased in fish fed arginine-supplemented diets. Interleukins and immune-cell marker transcripts were down-regulated by the highest supplementation level. Disease resistance data were in accordance with a generally depressed immune status, with increased susceptibility to vibriosis in fish fed arginine supplemented diets. Altogether, these results suggest a general inhibitory effect of arginine on the immune defences and disease resistance of European seabass. Still, further research will certainly clarify arginine immunomodulation pathways thereby allowing the validation of its potential as a prophylactic strategy.

5.1 Introduction

The biggest challenges regarding European seabass, *Dicentrarchus labrax*, Mediterranean aquaculture are the severe bacterial outbreaks occurring throughout the year [1]. Among them, vibriosis causes the highest mortality rates and, consequently, great economic losses. *Vibrio anguillarum* is the main aetiological agent of vibriosis often leading to a septicaemia as well as haemorrhages and exophthalmia [2]. Immunization by vaccination has been applied [3], but distinct and complementary approaches are recommended in order to successfully and cost-efficiently produce European seabass. Indeed, formulation of fish feeds must address other issues concerning fish welfare and health besides promoting optimum growth. Supplementing fish diets with key ingredients is a strategy often used in aquaculture to improve a selected trait. Such diets (functional diets) can also be used to improve fish defences and thereby, avoid high mortalities during a viral or bacterial incursion episode. Vitamins, prebiotics, probiotics and pigments such as xanthophylls and astaxanthins have been utilized as supplements for these diets [4-6]. More recently, amino acids (AA) have been employed in studies of immunomodulation [7-9], but such knowledge is still scarce.

Arginine is one of the most versatile AA, hence it represents a good candidate for inclusion in functional diets. Arginine requirements among different fish species are generally high given its great contribution to proteins composition and body fluids and also the almost total absence of its *de novo* synthesis, as this is an essential AA [10]. Similarly to higher vertebrates, fish can produce nitric oxide (NO) and ornithine from arginine using the enzymes inducible nitric oxide synthase (iNOS) and arginase, respectively [11]. When produced by phagocytes, NO is used against pathogens, acting as an oxidant that compromises structures integrity and function. Such actions represent self-damage as well, and when combined with superoxide anion, the toxicity dangerously increases [12]. Furthermore, arginine can enhance cell proliferation by fuelling polyamine biosynthesis through provision of ornithine [13]. Altogether, upon infection, these metabolic pathways may stimulate the inflammatory response. However, arginine can also mediate immunosuppressive mechanisms. In mammals, T-cell activation and function is dictated by arginine metabolism in myeloid suppressor cells (MSC) [11]. These cells metabolize arginine with either arginase or iNOS according to different stimuli and the derived products are directly involved in the suppression of T-cell functions. When arginine was added both to a primary enterocyte culture media or to the diet of Jian carp, *Cyprinus carpio* var. Jian, an inhibition of the LPS-induced inflammatory response was detected [14].

While being aware of the high importance of arginine as an essential constituent for growth, it would be of major interest to fully determine the mechanisms of arginine immunomodulation. Therefore, this study aims to decipher to what extent dietary arginine supplementation may modulate the European seabass immune response and disease resistance against *Vibrio anguillarum*.

5.2 Material and Methods

5.2.1 Diet formulation

Three plant protein-based diets with a reduced inclusion level of fish meal and fish soluble protein concentrates (12.2 %) were formulated and manufactured by Sparos Lda. (Olhão, Portugal). A blend of fish oil (6.2 %): rapeseed oil (4.2 %): palm oil (4.2 %) was used as dietary lipid source. The control diet (CTRL) was formulated to include an indispensable AA concentration meeting the ideal pattern estimated for European seabass 15. The two other diets were identical to the CTRL but supplemented with L-arginine at 1 % or 2 % (Arg1 and Arg2, respectively) at the expenses of wheat gluten. Main ingredients were ground (below 250 µm) in a micropulverizer hammer mill (SH1; Hosokawa Micron, B.V., Doetinchem, The Netherlands). Powder ingredients and oils were then mixed according to the target formulation in a paddle mixer (RM90; Mainca, S.L., Granollers, Spain). All diets were manufactured by temperature-controlled extrusion (pellet sizes: 1.5 and 2 mm) by means of a low-shear extruder (P55; Italplast, S.r.l., Parma, Italy). Upon extrusion, all feed batches were dried in a convection oven (OP 750-UF; LTE Scientifics, Oldham, UK) for 4 h at 45 °C. Formulation and proximate composition of experimental diets are presented in Table 1.

Diets were analysed for total amino acid content. Diet samples were hydrolysed in 6M HCl at 116 ºC for 2 h in nitrogen-flushed glass vials. Samples were then pre-column derivatised with Waters AccQ Fluor Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) using the AccQ Tag method (Waters, USA). Analyses were done by ultra-high-performance liquid chromatography (UPLC) in a Waters reversed-phase amino acid analysis system, using norvaline as an internal standard. During acid hydrolysis 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. Tryptophan was not determined, since it is partially destroyed by acid hydrolysis. The resultant peaks were analysed with EMPOWER software (Waters, USA). The AA profile of the experimental diets is presented in Table 2.

Table 1. Ingredients and proximal composition of experimental diets.

	Experimental diets		
	CTRL	**Arg1**	**Arg2**
Ingredients (%)			
Fishmeal Super Prime[1]	10.0	10.0	10.0
Fish soluble protein concentrate 90[2]	2.0	2.0	2.0
Fish gelatin[3]	0.2	0.2	0.2
Soy protein concentrate[4]	20.0	20.0	20.0
Pea protein concentrate[5]	7.0	7.0	7.0
Wheat gluten[6]	9.6	8.6	7.6
Corn gluten[7]	13.0	13.0	13.0
Soybean meal[8]	6.0	6.0	6.0
Rapeseed meal[9]	5.0	5.0	5.0
Wheat meal	7.0	7.0	7.0
Fish oil[10]	6.2	6.2	6.2
Rapeseed oil[11]	4.2	4.2	4.2
Palm oil[12]	4.2	4.2	4.2
Vitamin and mineral premix[13]	1.0	1.0	1.0
Choline chloride	0.2	0.2	0.2
Soy lecithin	1.6	1.6	1.6
Antioxidant[14]	0.2	0.2	0.2
Mono calcium phosphate[15]	2.1	2.1	2.1
L-Arginine		1.0	2.0
L-Lysine	0.4	0.4	0.4
DL-Methionine	0.1	0.1	0.1
Proximate analyses (% dry weight)			
Dry matter	91.0	91.9	91.7
Crude protein	51.5	54.2	55.9
Crude fat	22.3	21.5	21.7
Ash	7.0	7.2	7.1
Gross Energy (kJ g^{-1} DM)	26.3	27	26.6

[1] Peruvian fishmeal: 71% crude protein (CP), 11% crude fat (CF), EXALMAR, Peru
[2] CPSP 90 fish soluble protein concentrate: 84% CP, 12% CF, Sopropêche, France.
[3] Pharma Grade bloom 240: 92% CP, LAPI Gelatine SPA, Italy
[4] Soycomil P: 65% CP, 0.8% CF, ADM, The Netherlands.
[5] Lysamine GP: 78% CP, 8% CF, ROQUETTE, France.
[6] VITAL: 85.7% CP, 1.3% CF, ROQUETTE, France.
[7] Corn gluten feed: 61% CP, 6% CF, COPAM, Portugal.
[8] Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF, SORGAL SA, Portugal.
[9] Defatted rapeseed meal: 36% CP, 2% CF, SORGAL SA, Portugal.
[10] COPPENS International, The Netherlands.
[11] Henry Lamotte Oils GmbH, Germany.
[12] Crude palm oil: Gustav Heess GmbH, Germany.
[13] Premix for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate,7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middlings.
[14] Paramega PX, Kemin Europe NV, Belgium.
[15] Monocalcium phosphate: 22% phosphorus, 16% calcium, Fosfitalia, Italy.

Table 2. Amino acid composition (mg/g diet) of experimental diets.

	Experimental diets		
	CTRL	**Arg1**	**Arg2**
IAA[1]			
Arg	41.6 ± 1.5	56.3 ± 0.7	64.8 ± 1.7
His	10.2 ± 0.4	10.5 ± 0.1	9.7 ± 0.1
Lys	31.2 ± 1.3	31.5 ± 1.5	32.8 ± 0.9
Thr	18.4 ± 0.3	18.9 ± 0.5	18.2 ± 0.1
Ile	21.3 ± 0.1	20.8 ± 0.1	20.9 ± 0.0
Leu	42.5 ± 0.0	41.2 ± 0.1	42.1 ± 0.1
Val	23.2 ± 0.1	22.9 ± 0.3	22.6 ± 0.1
Met	9.9 ± 0.3	10.0 ± 0.1	9.8 ± 0.1
Phe	23.4 ± 1.1	23.5 ± 0.1	22.1 ± 0.4
DAA[2]			
Cys	2.8 ± 0.0	2.1 ± 0.1	2.4 ± 0.0
Tyr	21.1 ± 1.0	20.1 ± 0.2	19.4 ± 0.3
Asx	44.2 ± 2.1	45.2 ± 2.2	45.8 ± 1.3
Glx	103.3 ± 3.1	103.0 ± 5.0	101.4 ± 1.9
Ala	25.9 ± 0.7	26.0 ± 0.9	26.4 ± 0.3
Gly	34.8 ± 0.7	36.2 ± 1.6	34.0 ± 0.4
Pro	34.4 ± 0.2	33.4 ± 0.3	33.2 ± 0.1
Ser	24.5 ± 0.3	25.3 ± 1.0	24.0 ± 0.4
Tau	0.7 ± 0.0	0.6 ± 0.0	0.7 ± 0.0

Trp was not analysed. Values are means ± SD.

[1]Indispensable amino acids;

[2]Dispensable AA amino acids

5.2.2 Fish

All trials were carried out at the indoor experimental facilities of the Instituto de Acuicultura Torre de la Sal (IATS-CSIC, Castellón, Spain). Non vaccinated fingerlings of 0.6 g initial body weight were purchased from a commercial hatchery (Grupo Tinamenor, Santander, Spain) and acclimatized for more than two months to IATS experimental facilities. Fish were fed over the course of this period (March-June 2014) with the CTRL diet in a flow-through system with aerated seawater under natural photoperiod (12 h light/ 12 h dark) and temperature (22.95 °C ± 0.9) at IATS-CSIC latitude (40°5N; 0°10E). Water parameters were daily monitored with oxygen levels always higher than 85 % saturation and unionized ammonia below toxic levels (< 0.05 mg l-1).

5.2.3 Feeding trial and sampling

One hundred and fifty fish of 12.17 ± 0.17 g average weight were randomly distributed into six 500 l tanks. Dietary treatments were randomly assigned to duplicate groups and fish were fed to visual satiety two times per day, 6 days per week) for 15 or 29 days. At the end of these two periods, 5 fish per tank (n = 10 per diet) were euthanized by overexposure to the anaesthetic MS-222 (Sigma, Saint Louis, USA). Specimens were

weighed and blood was collected from the caudal vein with heparinized syringes. An aliquot of fresh blood was used for the respiratory burst assay and the remaining blood was centrifuged at 3,000 × g for 20 min at 4 °C to obtain plasma. Total visceral, mesenteric fat and liver weight were recorded. Subsequently, pieces of spleen, anterior (AI) and posterior (PI) intestine were immediately taken and frozen in liquid nitrogen. Plasma and tissue samples were kept at -80 °C until further analyses. All procedures were approved by the Ethics and Animal Welfare Committee of Institute of Aquaculture Torre de la Sal and carried out in a registered installation (code 36271-42-A) in accordance with the principles published in the European animal directive (2010/63/EU) and Spanish laws (Royal Decree RD53/2013) for the protection of animals used in scientific experiments. In all lethal samplings, fish were decapitated after 3-aminobenzoic acid ethyl ester (MS-222, 100 µg ml-1) over-exposure, and all efforts were made to minimize suffering.

5.2.4 Bacterial inoculum preparation and challenge dose validation

Vibrio anguillarum serotype O1 (strain Lab 1), isolated from diseased European seabass was cultured in tryptic soy agar (TSA, Pronadisa, Madrid, Spain) supplemented with NaCl at a final concentration of 1 % (TSA-1) at 24 °C for 24 hours.

Eight different doses were tested in a pre-challenge experiment in order to validate a suitable infective dose for the bacterial challenge. Juvenile European seabass, held in 90 l tanks at an average temperature of 22.3 °C, were intracoelomically (i.c.) injected with 0.1 ml of bacterial suspensions in phosphate-buffered saline (PBS, pH 7.4) ranging from 4.7×10^3 to 1×10^6 colony forming units (CFU) ml^{-1} (8-10 fish/dose) 16. Negative control fish received 0.1 ml of PBS. Mortalities were monitored for 8 days and were considered due to V. anguillarum only if the inoculated bacterium was recovered in pure culture from internal organs. Kidney and liver samples collected from moribund fish were directly streaked onto TSA-1 plates. For identification of the pathogen, a slide agglutination test with the corresponding antiplasma was used. The dose producing mortalities between 40 and 50 % (LD40-50) was chosen for the bacterial challenges.

5.2.5 Bacterial challenge

At the end of the first feeding period (15 days), 40 fish per dietary treatment were lightly anesthetized with clove oil (1:10,000), i.c challenged with the previously determined dose of V. anguillarum and distributed in triplicate 90 l tanks (n = 10). A group of 10 fish per dietary treatment was injected with PBS, as a control of the experimental handling. Fish were fed the same diets along the post-challenge period. The second

bacterial challenge was performed 29 days after the beginning of the feeding trial, following the same procedure as in the first challenge. In both challenges, mortalities were monitored daily (every two hours) until no more mortalities were observed for a minimum of two consecutive days, so the trial was terminated 8 days post-challenge. Fish showing signs of disease (fish near the water surface, slowly swimming around the air stone or motionless at the bottom of the tank) were humanely sacrificed by overexposure to the anaesthetic as previously mentioned. Post-mortem examination was performed as described above.

5.2.6 Respiratory burst of circulating leucocytes

The respiratory burst was assessed in circulating leucocytes at the end of each feeding period following the method described by Nikoskelainen et al. 17. Briefly, 4 µl of fresh blood were added to 96 µl of HBSS (Hanks' Balanced Salt Solution, pH 7.4) in a white flat-bottomed 96-well plate and incubated with 100 µl of a freshly prepared luminol suspension (2 mM luminol in 0.2 M borate buffer pH 9.0, with 2 µg ml^{-1} PMA) for 1 h at 24–25 °C. Each sample was run in duplicate and read against a blank into which no blood was added. Luminol-amplified chemiluminescence was measured every 3 min in a plate luminescence reader (TECAN) for generation of kinetic curves. Each sample was run in duplicate and the integral luminescence in relative light units (RLU) was calculated.

5.2.7 Innate humoral parameters

Plasma bactericidal activity was measured according to Graham et al. 18 with some modifications 19. A suspension of *Photobacterium damselae* subsp. *piscicida* (20 µl, 1 × 10^6 CFU ml^{-1}) was added to 20 µl of plasma in duplicate wells of a U-shaped 96-well plate. HBSS was added instead of plasma to serve as positive control. After an incubation period of 2.5 h at 25 °C, 25 µl of 3-(4,5 dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (1 mg ml^{-1},Sigma) were added to each well and plates were incubated for more 10 minutes at 25 °C. Then, 200 µl of dimethyl sulfoxide (Sigma) were added after centrifugation at 2,000 × g for 10 min. The absorbance of the formed, resuspended formazan was read at 560 nm in a Synergy HT (Biotek) microplate reader. Total bactericidal activity is expressed as the percentage of killed bacteria, calculated from the difference between the samples and the positive control (100 % living bacteria).

Total plasma nitrite and nitrate content was measured using a Nitrate/Nitrite colorimetric kit (Roche Diagnostics GmbH, Mannheim, Germany) by adapting it to a 96-well plate and by following manufacturer's instructions. Since both these compounds are derivatives of endogenously produced NO, they are indicative of NO amount in plasma.

Briefly, 100 µl of plasma were added in duplicate to 50 µl of reduced nicotinamide adenine dinucleotide phosphate (NADPH) followed by the addition of 4 µl of nitrate reductase. A blank was produced by adding distilled water instead of plasma. Absorbance at 540 nm was read after 30 min incubation at 25 °C. Afterwards, 50 µl of sulfanilamide and an equal volume of N-(1-naphthyl)-ethylenediamine dihydrochloride were added to each well. The mixture was allowed to stand at 25 °C for 15 min and absorbance was read at 540 nm. Total nitrite levels were calculated from a previously prepared sodium nitrite standard curve.

5.2.8 Gene expression analysis

Spleen, AI and PI were taken from fish fed the experimental diets for 29 days. Total RNA was extracted using a MagMAX™-96 total RNA isolation kit (Life Technologies, Carlsbad, CA, USA). RNA yield was 50-100 µg with 260 and 280 nm UV absorbance ratios (A260/280) of 1.9-2.1. Reverse transcription (RT) of 500 ng total RNA was performed with random decamers using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. Negative control reactions were run without reverse transcriptase and real-time quantitative PCR was carried out on a CFX96 Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using a 96-well PCR array layout designed for simultaneously profiling a panel of 30 genes under uniform cycling conditions. Genes were selected for their involvement in immune response and arginine metabolism (Table 3). Among the 30 genes, 24 genes were novel for European seabass and their sequences, derived from the IATS-Nutrigroup transcriptomic database (www.nutrigroup-iats.org/seabassdb), were uploaded to GenBank (KM225766-KM225790, Appendix III, Table 1). Controls of general PCR performance were included on each array, being performed all the pipetting operations by means of the EpMotion 5070 Liquid Handling Robot (Eppendorf, Hamburg, Germany). Briefly, RT reactions were diluted to convenient concentrations and the equivalent of 660 pg of total input RNA was used in a 25 µL volume for each PCR reaction. PCR-wells contained a 2× SYBR Green Master Mix (Bio-Rad) and specific primers at a final concentration of 0.9 µM were used to obtain amplicons of 50–150 bp in length (Appendix III, Table 2).

The program used for PCR amplification included an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation for 15 s at 95 °C and annealing/extension for 60 s at 60 °C. The efficiency of PCR reactions was always higher than 90 %, and negative controls without sample templates were routinely performed for each primer set. The specificity of reactions was verified by analysis of melting curves

(ramping rates of 0.5 °C/10 s over a temperature range of 55–95 °C), and linearity of serial dilutions of RT reactions. Fluorescence data acquired during the PCR extension phase were normalized using the delta–delta Ct method (Livak and Schmittgen, 2001). B-Actin was tested for gene expression stability using GeNorm software (M score = 0.21) and it was used as housekeeping gene in the normalization procedure. Fold-change calculations were done in reference to the expression ratio between Arg1 or Arg2 and CTRL fish (values >1 indicate up-regulated genes in Arg1 or Arg2 fish; values <1 indicate down-regulated genes in Arg1 or Arg2 fish). For comparing the mRNA gene expression level of a panel of genes in a given dietary treatment, all data values were in reference to the expression level of interleukin 10 in CTRL fish, which was arbitrarily assigned a value of 1.

Table 3. Immune-related genes analysed by real-time PCR. *New sequences for European seabass are labelled with an asterisk.

Gene name	**Symbol**	**GenBank Accession number**
Argininosuccinate lyase	*ASL*	KM225766*
Argininosuccinate synthase	*ASS*	KM225767*
Arginase-2, mitochondrial	*ARG2*	KM225768*
Glycine amidinotransferase, mitochondrial	*GATM*	KM225769*
S-adenosylmethionine decarboxylase	*AMD1*	KM225770*
Ornithine decarboxylase	*ODC1*	KM225771*
Diamine acetyltransferase 1	*SAT1*	KM225772*
Spermine oxidase	*SMOX*	KM225773*
Nitric oxide-associated protein 1	*NOA1*	KM225774*
Nitric oxide-inducible gene protein	*NOXIN*	KM225775*
Nitric oxide synthase-interacting protein	*NOSIP*	KM225776*
Interleukin 1-β	*IL-1β*	AJ311925
Interleukin 8	*IL-8*	KM225777*
Interleukin 10	*IL-10*	DQ821114
Interleukin 20	*IL-20*	KM225779*
Interleukin 34	*IL-34*	KM225780*
Tumour necrosis factor-α	*TNF-α*	DQ070246
C-C chemokine receptor type 3	*CCR3*	KM225781*
C-C chemokine receptor type 9	*CCR9*	FN665390
Atypical chemokine receptor 4	*CCR11*	KM225782*
T-cell surface glycoprotein CD3 zeta chain	*CD247*	KM225783*
T-cell surface glycoprotein CD8 beta	*CD8b*	KM225784*
Myeloid differentiation primary response protein MyD88	*MyD88*	KM225785*
Myeloid cell surface antigen CD33	*CD33*	KM225786*
Macrophage colony-stimulating factor 1 receptor	*CSF1R*	KM225787*
Macrophage migration inhibitory factor	*MIF*	FN582353
Monocyte to macrophage differentiation factor	*MMD*	KM225788*
Interferon regulatory factor 8	*IRF8*	KM225789*
Nuclear factor NF-kappa-B p100 subunit	*NFKB2*	KM225790*
β-Actin	*ACTB*	AY148350

5.2.9 Statistical Analysis

Results are expressed as means ± standard error of the mean (SEM). Data were analysed for normality and homogeneity of variances and, when necessary, transformed before being treated statistically. Analysis of fish performance, respiratory burst, plasma bactericidal capacity and NO content were performed by two-way analysis of variance (ANOVA) with dietary treatment and time of sampling as main variables, whereas gene

expression data were analysed by one-way ANOVA with dietary treatment as the sole variable. Whenever significant differences were found, a multiple-comparisons Tukey HSD test was performed to identify significantly different groups. For every test, the level of significance chosen was $P < 0.05$. All data were analysed with STATISTICA (StatSoft, Inc. 2013, version 12) for WINDOWS.

5.3 Results

5.3.1 Fish performance

Final fish weight was higher in fish reared for 29 days compared to those sampled at 15 days, regardless of the dietary treatment (Table 4). By contrast, the viscerosomatic index (VSI) was significantly lower in individuals fed Arg2 compared to fish fed Arg1 at day 15, whereas an increase in VSI was observed in animals fed Arg2 from day 15 to 29. Regarding the specific growth rate (SGR) and the feed conversion rate (FCR) no differences were observed among dietary treatments. Due to technical constraints, FCR data from 15 days are not presented.

Table 4. Data on the performance of European seabass sampled 15 or 29 days after being fed three different diets.

Parameters	15 days			29 days			P-value[1]		
	CTRL	Arg1	Arg2	CTRL	Arg1	Arg2	Time	Diet	Time × Diet
Final weight (g)	15.56 ± 0.49	18.21 ± 0.89	16.18 ± 0.66	21.55 ± 1.81	22.46 ± 1.74	23.16 ± 1.27	0.00	NS	NS
VSI[2]	11.81 ± 0.56^{ab}	12.45 ± 0.43^{a}	10.76 ± 0.30^{b*}	11.88 ± 0.45	11.75 ± 0.29	12.51 ± 0.21	NS	NS	0.00
MSI[3]	5.47 ± 0.48	5.62 ± 0.34	4.68 ± 0.28	5.83 ± 0.63	4.66 ± 0.29	5.61 ± 0.37	NS	NS	NS
HSI[4]	1.95 ± 0.14	1.88 ± 0.12	1.60 ± 0.11	1.60 ± 0.10	1.90 ± 0.18	1.94 ± 0.25	NS	NS	NS
SGR[5]	1.69 ± 0.21	2.58 ± 0.34	1.80 ± 0.28	2.12 ± 0.32	2.25 ± 0.27	2.40 ± 0.20	NS	NS	NS
FCR[6]	-	-	-	0.87 ± 0.03	0.98 ± 0.04	0.92 ± 0.01	-	NS	-

Values are means ± SEM (n = 10)

[1] P values were obtained from two-way analysis of variance. Different superscript letters in each row indicate significant differences between diets, within the same sampling time; asterisks denote differences between sampling time, within the same dietary treatment (Tukey HSD post-hoc test, $P < 0.05$)

[2] Viscerosomatic index = (Viscera weight / final weight) × 100

[3] Mesenteric fat-somatic index = (Mesenteric fat weight / final weight) × 100

[4] Hepatosomatic index = (Liver weight / final weight) × 100

[5] Specific growth rate = 100*((ln (Final weight) – ln (Initial weight)) / time)

[6] Feed conversion ratio = Weight increase / (Feed intake × % dry matter)

5.3.2 Bacterial challenge

Mortality started two days after bacterial challenge, regardless of the dietary treatment or the feeding time (Figs. 1a and 1b). No mortality was observed in fish injected with PBS (data not shown). Concerning the first challenge (day 15 of feeding trial), the infective dose, which was based on the pre-challenge trial, was around 1.5 ×10^4 CFU ml-1. The highest death numbers occurred in Arg1 and Arg2 dietary groups with 86.7 % and 93.3 % of mortality, respectively (Fig 1a). High mortalities, though lower, were recorded in the CTRL group (76.7 %). As mortality was higher than expected in the CTRL group (probably due to an increase in mean water temperature - from initial temperature 22.3 °C to final temperature 23.6 ºC), a lower dose was used for the second bacterial challenge (day 29 of feeding). Fish were injected with 3 × 10^3 CFU ml^{-1} and mortality was lower than in the first challenge (Fig 1b). Fish fed Arg1 and Arg2 diets were more susceptible to the infection compared to those fed the CTRL diet, showing mortalities of 67.5 %, 77.5 % and 52.5 %, respectively.

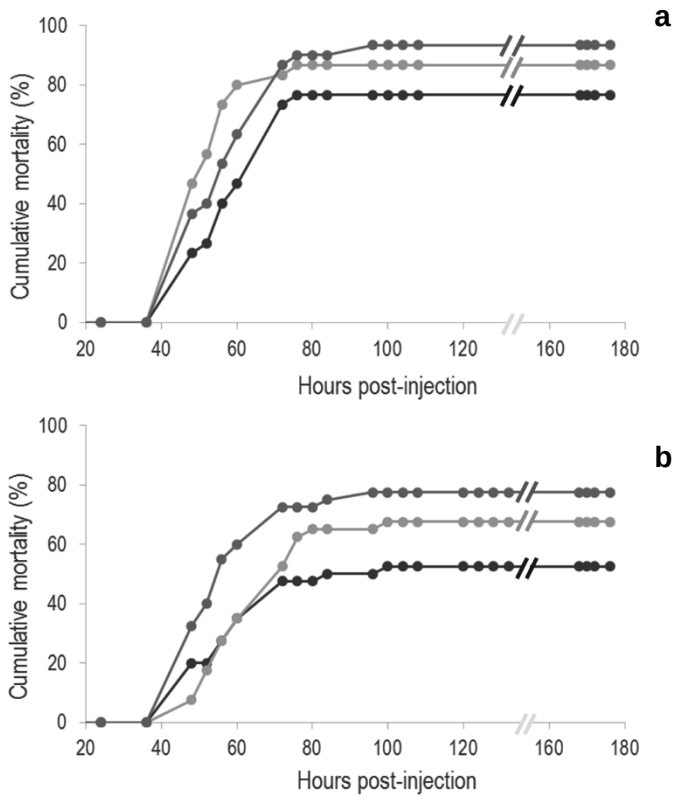

Fig. 1 Cumulative mortality of European seabass i.c. injected with *V. anguillarum* serotype O1 after 15 (1.5 × 10^4 CFU ml^{-1}, a) or 29 days (3 × 10^3 CFU ml^{-1}, b) of feeding with CTRL (black line), Arg1 (light grey line) or Arg2 (dark grey line) diets. Values are means of triplicate tanks (n = 30). SEM and sham injected fish are not presented for the clarity of the graphs.

5.3.3 Respiratory burst of circulating leucocytes

Dietary arginine significantly decreased the respiratory burst of circulating leucocytes, regardless of the supplementation level, when fish were fed for 29 days (Fig 2). Furthermore, the response of fish fed the Arg2 diet was also lower when fed only 15 days. An interaction between time and dietary treatment was also observed in fish fed Arg1 and Arg2 diets compared to individuals fed the CTRL diet at day 29.

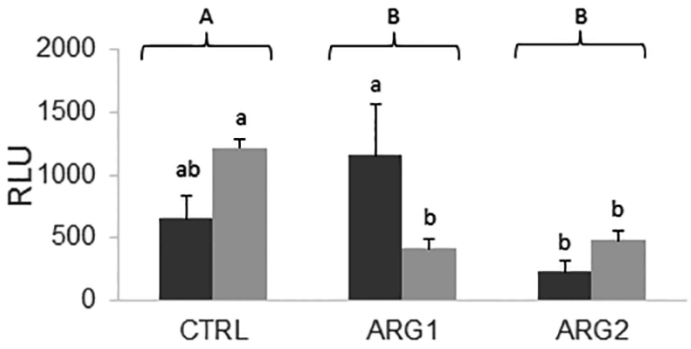

Fig. 2 Respiratory burst activity of circulating leucocytes of European seabass fed different diets for 15 (black columns) or 29 (grey columns) days. Values represent means ± SEM of relative light units (n = 6). Different capital letters stand for statistically significant differences between diets regardless of feeding time. Different low case letters stand for statistically significant differences between diets within the same feeding time (Two-way ANOVA; $P < 0.05$).

5.3.4 Innate humoral parameters

No dietary effects were observed on total plasma bactericidal activity (Fig 3a). However, a slight, but significant decrease was observed between the two feeding times in all diet groups. In contrast, decreased levels of NO were observed in Arg1 and Arg2 fish, though only significantly for the lowest supplementation level (Fig 3b). No effect of feeding time was observed on this parameter.

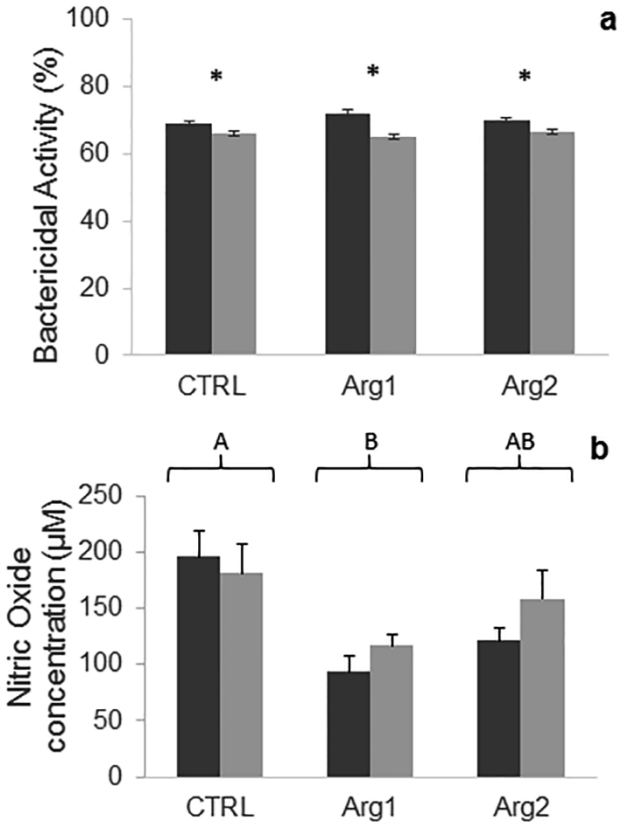

Fig. 3 Bactericidal activity (a) and nitric oxide content (b) in plasma of European seabass fed different diets for 15 (black columns) or 29 (grey columns) days. Values represent means ± SEM (n = 6). Asterisks denote differences attributed to feeding time within each diet. Different capital letters stand for statistically significant differences between diets regardless of feeding time (Two-way ANOVA; $P < 0.05$).

5.3.5 Gene expression

The relative expression of the 30 studied genes is shown in Appendix III: Tables 3, 4 and 5 (in spleen, AI and PI, respectively). To simplify data interpretation, only differently expressed genes are represented as fold change in Fig 4. Spleen retrieved the highest number of modulated genes, in comparison to both intestinal portions (Fig 4a). Arginase 2 (*ARG2*) expression was not affected in this tissue by any dietary treatment, but mRNA transcripts of the enzymes glycine amidinotransferase mitochondrial (*GATM*) and spermine oxidase (*SMOX*) were up-regulated in Arg1 fish compared to CTRL ones. Interleukin 10 (*IL-10*), membrane-bound molecules such as C-C chemokine receptor type 3 (*CCR3*) and myeloid cell surface antigen (*CD33*) transcripts were up-regulated in Arg1 fish, as well, whereas *CCR3* expression level also increased in Arg2 fish compared to CTRL fish. In contrast, S-adenosylmethionine decarboxylase (*AMD1*), *IL-10*, interleukin 20 (*IL-20*) and the atypical chemokine receptor 4 (*ACKR4*) transcripts decreased in Arg2 fish compared to CTRL diet. When comparing supplementation levels,

Arg2 fish showed lower gene expression levels of *GATM*, *SMOX*, *IL-10*, *ACKR4* and *CD33* than Arg1 fish.

Dietary treatments did not affect expression of *GATM*, *AMD1*, *SMOX*, *IL-20* nor *CCR3* in the AI, whereas a decrease in *ARG2* transcripts was observed in Arg1 fish compared to CTRL fish (Fig 4b). Furthermore, mRNA expression levels of *IL-10*, *IL-34*, *ACKR4*, T-cell surface glycoprotein *CD3* zeta chain (*CD247*) and *CD33* were down-regulated in Arg2 fish. Regarding *ACKR4* and *CD247*, expression patterns were significantly lower than both CTRL and Arg1 fish.

Regarding gene expression patterns of the PI, a significant decrease of *ARG2* transcripts was denoted on both dietary groups relatively to the CTRL group, while *CD247* gene expression decreased in Arg2 fish (Fig 4c). By contrast, nuclear factor κB (*NFKB2*) mRNA levels augmented in Arg2 fish. Moreover, the expression levels of *CD247* were lower in Arg2 fish, compared to Arg1 specimens.

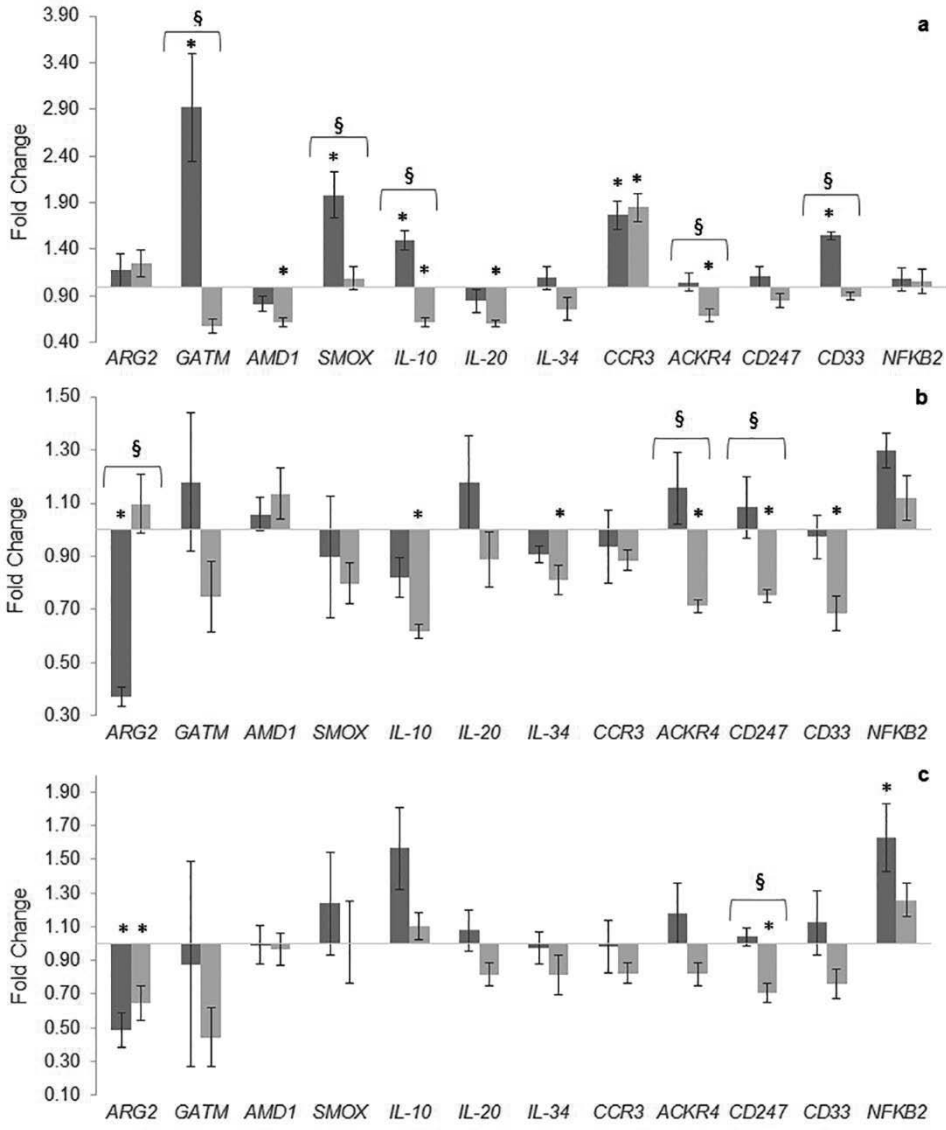

Fig. 4 Quantitative expression of immune-related genes in spleen (a), anterior (b) and posterior (c) intestine of European seabass fed different diets for 29 days. Data are presented as means ± SEM (n = 6). Bars represent fold change of Arg1 (dark grey columns) and Arg2 (light grey columns) relatively to the CTRL group, previously normalized to endogenous β-Actin expression levels. Asterisks indicate significant differences relatively to CTRL group; section sign indicates differences between arginine supplementation levels (One-way ANOVA; $P < 0.05$).

5.4 Discussion

Immunomodulation mediated by arginine has received a special attention in the last years [20-22]. Arginine is an essential AA with versatile functions including protein synthesis, production of urea, synthesis of polyamines, proline and agmatine and it is also involved in endocrine and reproduction regulatory processes [23]. Therefore, its involvement in fish immune response may be essential for managing fish health throughout the full rearing cycle. Thus, it is important to understand the delicate balance of the immune response, in which a complex network of mechanisms is orchestrated by several external and internal factors. Arginine supplementation did not affect fish growth in this study. When juvenile gold pompano, *Trachinotus ovatus*, were fed six graded levels of arginine for eight weeks, fish growth was firstly increased and then depressed with the highest supplementation levels [24]. The same effect was observed by Zhou et al. [25] in juvenile yellow catfish, *Pelteobagrus fulvidraco*, fed diets with different levels of arginine for 84 days. The fact that such increase in growth was not observed in the present study may be related to the shorter feeding time (15 and 29 days).

Several studies have considered arginine administration as a strategy that improves the immune response, and, more specifically, the inflammatory process: in fish, previous studies have shown that several factors of the innate immune response, such as the extracellular superoxide anion production, lysozyme activity and neutrophil oxidative radical production were stimulated upon dietary arginine supplementation [26]. In turbot, *Scophthalmus maximus*, high rearing density caused immunosuppressive effects that were counteracted by dietary arginine supplementation [27]. Furthermore, Chen and co-authors [28] suggested that arginine contributed to an enhanced immune response of Jian carp and higher survival against *Aeromonas hydrophila* based on the up-regulation of pro-inflammatory gene expression. However, the results of the present study showed an opposite effect of dietary arginine supplementation in European seabass. Indeed, bacterial challenges with *V. anguillarum* demonstrated a dose-dependent increased susceptibility of fish fed arginine supplemented diets. Thus, the highest mortalities occurred in the Arg2 groups and the lowest ones in CTRL fish. Mortality after bacterial challenge was also increased in golden pompano when dietary arginine supply was higher than that considered optimum [24]. This is perhaps indicative that arginine surplus might either improve or impair growth, immune response and disease resistance.

In the present study, measurements of gene expression and immune-related factors explained, to a certain extent, the observed higher disease susceptibility. One of the concurrent decreased immune factors in Arg1 and Arg2 fish was the respiratory burst of circulating leucocytes. The respiratory burst of phagocytes is part of the first line of cellular defence mechanisms and involves the production of reactive oxygen intermediates (ROI). This intracellular production of superoxide anion is a well-studied antimicrobial mechanism in fish that can be modulated by several factors [29] and therefore its decrease could be considered an immune-depression component. This is crucial in the current bacterial challenge, as V*. anguillarum* is able to inhibit leucocyte respiratory burst of European seabass [30]. Thus, those animals with a reduced ability to produce ROI would be particularly jeopardized as they would display fewer obstacles for this bacterial invasion. The current results are in accordance with those obtained for other fish species, in which extracellular superoxide anion production of head kidney phagocytes was decreased such as in channel catfish, *Ictalurus punctatus*, fed 2-4 % arginine supplemented diets, while no changes were detected in both phagocyte intracellular superoxide anion production and blood neutrophil respiratory burst [31]. By contrast, all respiratory burst values were decreased in fish fed an arginine deficient diet (0.5 %). Authors attributed the lower levels of this free radical to a non-activated cells state. However, that would explain superoxide levels similar to the control group, but not lower values. Thus, the fact that, similar to the present work, superoxide anion production was lower indicates an inhibitory effect of arginine. Arginine does not play any direct role in the respiratory burst, and it has been demonstrated that an absence of arginine has no influence on granulocyte functions, including oxidative burst, in murine and human models [32]. However, it was demonstrated *in vitro* that induction of iNOS increases ROI production in murine macrophages [33]. Thus, the lower levels of plasmatic NO also observed in our study are in accordance with lower ROI production. Another explanation for the lower respiratory burst response could be the involvement of arginine metabolism in myeloid cells [11] which impairs both cell proliferation and activation. As a consequence, the production of superoxide anion would be compromised.

Another decreased immune factor was plasmatic NO, though only significantly in Arg1 fish. At first glance, this might appear contradictory. Since NO is synthesized from L-arginine by the catalytic action of NO synthases (NOS), it would be expected to find higher levels of this molecule when its precursor is more available. However, arginine is also substrate for arginase, which converts L-arginine into L-ornithine and can also be metabolized by the enzyme *gatm* into creatine. Therefore, supplementing the diet with L-arginine would decrease the competition between the three enzymes by providing extra substrate for them. The transcriptomic results of the genes involved in these

metabolic pathways can clarify what occurred in the present study. First of all, a clear down-regulation of *ARG2* was detected in the PI of Arg1 and Arg2 fish, and also in the AI of Arg1 fish. *ARG2* is an extra-hepatic arginase involved in the production of ornithine, as precursor for polyamines, glutamate and proline, and in the regulation of arginine for NO synthesis [34]. Further, in various teleosts, liver arginase (also named ARG I) activity is induced following several weeks of fasting, as increased arginase activity may be important in AA catabolism in fasting fish [35,36]. The observed *ARG2* down-regulation may have also been affected by a negative feedback set by the increased polyamine turnover. Indeed, *SMOX*, which is involved in the recycling of spermine (a polyamine) back to spermidine, was up-regulated in the spleen and PI of Arg1 fish, although only significantly in the spleen. Accordingly, *AMD1* was equally down-regulated in the spleen of these fish, which supports this hypothesis. The transcription of *GATM* was also up-regulated in the spleen and AI of Arg1 fish, even though not significantly in the latter tissue. Consequently, an increase in creatine could be expected. Several studies have suggested an anti-inflammatory role of creatinine: *in vitro* impairment of neutrophil adhesion to human endothelial cells upon creatine supplementation [37]; inhibitory effect on the expression of pathogen receptors in the membrane of human macrophages [38]. Creatine has also been classified as antioxidant [39]. Therefore, a putative increase of creatine biosynthesis would further support our findings regarding the lower plasma NO content, but further studies are needed to corroborate this hypothesis. Concerning the third group of enzymes, NOS, no differences were observed regarding *NOSIP* transcripts, probably due to the decreased NO production, as this protein promotes de translocation of eNOS (endothelial NOS, a constitutive NOS) from plasma membrane to intracellular sites. Similarly, *NOXIN* expression was not changed, which is not unexpected, as this gene is strongly induced by different NO donors in mammals. *NOXIN* plays an anti-apoptotic role, thus when the *NOXIN* gene is inactivated or down-regulated, cells exhibit higher levels of apoptosis than their counterparts with normal levels of *NOXIN* [40]. Currently, iNOS of European seabass is unknown, therefore future studies should address if this inducible form of NOS [41] was indeed unchanged.

In mammalian models, under resting conditions, little arginine is used by myeloid cells and they do not express the major arginine metabolizing enzymes, iNOS and arginase. Thus, dietary arginine supplementation cannot enhance myeloid cell function in the absence of disease. It is only after stimulation that arginine transport into the myeloid cell is greatly increased. Some myeloid cells expressing arginase regulate T-cell function through arginine depletion. In addition, arginase is induced in myeloid cells by T-helper 2 (T_H2) cytokines, such as IL-4 and IL-13, and also by IL-6, IL-10, TGF-β, prostaglandins and catecholamines [42]. Further studies are needed to understand all

these complex interplaying regulatory factors in fish and how they are modulated after bacterial challenge to better understand the responses obtained before challenge.

The expression profile of immune-related genes differed in fish fed arginine supplemented diets and also supports the higher mortality observed in these groups. In teleosts, spleen is an important haematopoietic organ where melanomacrophages phagocytise and detain blood-borne antigens for a long period of time [43]. This tissue exhibited the highest number of differentially expressed genes, followed by AI and PI. The last portion of fish gut is characterized by a diffused presence of leucocytes in both the lamina propria and the epithelium [44], generally known as the gut-associated lymphoid tissue (GALT). The low modulation of gene expression in the PI may be related to an absence of a stimulatory event, for instance, a bacterial infection, which would certainly lead to immune cells activation. In the present study, pro- and anti-inflammatory interleukins (*IL-10*, *IL-20*, *IL-34*) were in general lower in Arg2 fish, revealing a broad inhibition of the inflammatory response, particularly in the spleen. Only *IL-10* was up-regulated in the spleen in Arg1 fish. Furthermore, along with other observed genes, its expression in the spleen of fish given the lowest supplementation was significantly higher than in Arg2 animals. The same pattern was observed in broiler chickens fed arginine-supplemented diets, in which both the expression of *IL-1β* (pro-inflammatory) and *IL-10* (anti-inflammatory) were down-regulated in a dose-dependent manner after a LPS challenge [45]. TGF-β expression, another important anti-inflammatory cytokine in fish, was up-regulated upon infection in Jian carp fed the highest level (24.5 g/kg of diet) of arginine supplementation, which translated in higher disease susceptibility after bacterial challenge [28]. Interleukins are mediators of both mounting and resolution of the inflammatory response [46]. Anti- and pro-inflammatory cytokines are essential for an efficient and correctly assembled inflammation. Anti-inflammatory cytokines are produced as a negative feedback to the secretion of other inflammatory mediators, thereby, avoiding an excessively harmful inflammatory response [47]. Furthermore, expression of arginase and iNOS is reciprocally influenced by T_H1 and T_H2 cytokines, and therefore activation of each of these cytokines may be restricted to separate subsets of myeloid-derived cells, termed alternative activation [42].

While changes in gene expression of cellular-produced compounds were more evident in the spleen, indicators of immune-related cells presence were more modulated in the AI of individuals fed the arginine diets. Indeed, mRNA levels of cell markers such as *ACKR4*, *CD247* and *CD33* dropped substantially in fish fed Arg2, suggesting that arginine was able to depress the cellular immune status of these fish, either by directly affecting cell proliferation or by impairing immune cells differentiation. The expression of *NFKB2*, a transcriptional factor directly related to the onset of the inflammatory response,

was down-regulated in chickens treated with arginine supplementation [45]. By contrast, mRNA levels of *NFKB2* were up-regulated in the PI of Arg1 fish. *NFKB2* is normally present in the cell and can be rapidly activated either as result of a cascade of events following pathogen receptors activation [48], or by inflammatory mediators such as NO or pro-inflammatory cytokines. In the present context, where no stimulus was present, the up-regulation of this gene cannot be related to the enhancement of the immune response and remains to be clarified.

The inhibitory effect of arginine supplementation was also observed in Jian carp where both *in vivo* and *in vitro* arginine supplementations counteracted LPS-induced inflammatory responses [14]. Moreover, this experiment allowed the researchers to associate the inhibitory effects of arginine to a decrease in the expression of the LPS-recognizing receptor, TLR4, and two other molecules downstream of the activated cascade. The same authors suggested this might be a “protective effect” of arginine against LPS-induced inflammation, whereas other authors referred to arginine mode of action as an “alleviation” of the immune challenge caused by *Salmonella enterica* [49]. However, this issue should be addressed with care at least in fish, since the involvement of arginine in immunity is somehow contradictory. Pro-inflammatory signals and mediators are necessary for the establishment of an efficient immune reaction against a threat; so, their inhibition should not be seen as advantageous for the host. A balanced supplementation should improve the immune response while avoiding the damages of an excessive response.

5.5 Conclusions

The present study clearly shows inhibition of some immune mechanisms in the European seabass as result of dietary arginine supplementation, which led to higher disease susceptibility. These results may be due to the direct role of arginine on cell activation and differentiation leading to a restrained humoral response, or to the action of arginine on cell communications by inhibiting the production of pro-inflammatory mediators. This study provides additional information for deciphering the two-edged effects of a dietary surplus of arginine, as arginine supplementation may produce widely different biological responses depending on the disease process where it is provided. As in human studies [42], we are therefore still in the process of identifying in fish which processes are benefited by arginine-supplemented diets. In fish, the efficiency and balance of the immune system are highly dependent and susceptible to several external and internal varying factors. It is, therefore, of utmost importance to have full knowledge of each specific situation (e.g. developmental stage, fish species, etc.) and conditions

(e.g. water temperature, densities, water quality, etc.), while supplementing aquafeeds aiming the improvement of fish health.

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Chapter 6

Dietary arginine and citrulline-mediated impairment of immune condition and inflammatory response of the European seabass, *Dicentrarchus labrax*

Dietary arginine and citrulline-mediated impairment of immune condition and inflammatory response of the European seabass, *Dicentrarchus labrax*

Abstract

The present study was designed to determine the modulatory effects of arginine and citrulline dietary supplementation on immune condition, inflammatory response and disease resistance of the European seabass, *Dicentrarchus labrax*. Four diets were manufactured: a control diet (CTRL) was formulated to meet the indispensable amino acids profile established for seabass. Based on this formulation, three other diets were supplemented with DL-Arginine at two different levels (0.5 % and 1 %, ARG1 and ARG2, respectively) and DL-Citrulline at 0.5 % (CIT). Fish were fed these diets for 2 or 4 weeks until visual satiation under controlled conditions. Blood samples were withdrawn from the caudal vein at the end of each feeding period for the evaluation of the haematological profile. Blood smears were prepared for differential cell counting and total peripheral cells were counted as well. Blood was finally centrifuged and plasma samples were stored at -80 °C until assayed. At the same time, fish from all dietary treatments were intraperitoneally-injected with live bacteria and sampled after 4, 24 our 48 h. Mortality was recorded for 3 weeks in the remaining fish. Plasma peroxidase activity, total white blood cells and lymphocytes decreased with time before bacterial challenge regardless of dietary treatment, whereas lysozyme, total white blood cells, lymphocytes, monocytes and neutrophils were all diminished in fish fed supplemented-diets after infection. Results generally point at an impairment of both the immune status and inflammatory response upon dietary supplementation of either amino acid, a scenario that might result from nitric oxide toxic effects. Accordingly, the same fish were less resistant to disease, as observed by higher mortalities of fish fed ARG1. Citrulline supplementation-induced changes were similar to those inflicted by arginine, suggesting that citrulline is probably being converted to arginine and then engaged in similar immune-impairment leading mechanisms.

Key-words: arginine; citrulline; innate immunity; leucocytes; inflammation

6.1 Introduction

In attempting to enhance health and welfare of farmed fish, efforts have been applied into the development of complementary strategies to vaccination in an environmental-friendly fashion. Immunonutrition aims at enhancing the immune system and thereby potentiate immune condition and vaccine effects. Among others, amino acids (AA) can be considered as innocuous feed additives that participate in several and relevant metabolic pathways, which presence above physiological levels might trigger or modulate immune responses [1-4]. In human research, arginine and its close involvement with trauma, infection and sepsis has received a lot of attention, mostly due to its undeniable controversy [5,6]. Indeed, the use of immune enhancing diets containing arginine surplus seems to be beneficial to the recovery of patients subjected to physical stress while it can perpetrate harmful effects under sepsis conditions [5].

Arginine requirements are often high in teleost fish, a fact that reflects the almost inexistent endogenous synthesis [4] and its great contribution to protein synthesis and body fluids. It is one of the most versatile AA, hence it represents a good candidate for inclusion in functional diets. The interest in arginine as an immune modulator has to do with important immune-related metabolic pathways in which this amino acid is a central player. Also, there is a striking reduction of arginine availability that underlies the inflammatory response and that is a consequence of lower intestinal absorption, higher immune-related protein synthesis and increased utilization by activated macrophages [7,8]. Indeed, under inflammatory conditions, fish can produce nitric oxide and ornithine from arginine, via the inducible nitric oxide synthase (iNOS) and arginase, respectively [9,10]. While nitric oxide is extensively produced by phagocytes upon infection and extends inflammatory responses, arginase activity is a classical aspect of an immune-tolerance scenario and is a crucial initial step of polyamine biosynthesis pathway and wound healing [11]. Regulation of these pathways is dependent on each specific context, immune status and triggering factor [12,13]. Therefore, different and often opposite effects are associated to the two metabolic pathways. Accordingly, research in this topic is frequently ambiguous. *In vitro* arginine supplementation to juvenile Jian carp, *Cyprinus carpio* var*. Jian*, enterocytes impaired aspects of their immune response to lipopolysaccharide stimulation [14] and a surplus of the same amino acid to European seabass, *Dicentrarchus labrax*, experimental diets generally suppressed their immune condition leading to decreased disease resistance (Chapter 5). In contrast, under stress conditions, arginine dietary supplementation enhanced nitric oxide production as well as nitric oxide synthase and lysozyme activities in juvenile Nile tilapia, *Oreochromis niloticus L*. [15].

Besides being a by-product of arginine upon conversion to nitric oxide, citrulline is also the primary endogenous source of arginine in mammals and evidences have showed the same to be true in lower vertebrates such as teleost fish, though only a small fraction of this AA pool is endogenously synthetized in fish [4,16,17].

In septic Wistar rats, citrulline dietary supplementation increased arginine circulating levels and modulated the inflammatory response by inhibiting pro-inflammatory mediators while anti-inflammatory cytokines such as interleukin 10 were not impaired [18]. NO production was not compromised in mice macrophages co-stimulated with *Mycobacterium bovis* and interferon γ, expressing the arginase gene and cultured in citrulline-supplemented media, in contrast to those maintained in arginine-supplemented media [19], suggesting these so-activated cells preferred citrulline to arginine in order to produce nitric oxide as a bactericidal mechanism.

Similar studies on citrulline contribution to the immune response are still scarce in teleost fish but Buentello and colleagues [9] showed that *in vitro* nitric oxide production was improved in channel catfish, *Ictalurus punctatus*, peritoneal macrophages upon addition of citrulline to the culture media.

Given the lack of studies on arginine effects in teleost fish immune system and their often contradictory subsets of results, it would be of major interest to further explore mechanisms of arginine immunomodulation. Hence, this study aims at evaluating short and mid-term effects of arginine and citrulline dietary supplementation on the innate immune status and inflammatory response of the European seabass. At the same time, this study is intended to determine the outcome of a 4 weeks-amino acid dietary treatment regarding fish disease resistance.

6.2 Material and Methods

6.2.1 Formulation and analytical procedures with experimental diets

Four diets were formulated and manufactured by Sparos Lda. (Olhão, Portugal). A control diet (CTRL) was formulated to include an indispensable AA profile meeting the ideal pattern estimated for European seabass [20]. Two other diets, identical to the CTRL were supplemented with $_{DL}$-Arginine and $_{DL}$-citrulline at 0.5 % dry matter (ARG1 and CIT1, respectively) at the expenses of wheat meal. A third diet was formulated by supplementing $_{DL}$-arginine at 1 % dry matter (ARG2). Main ingredients were ground (below 250 µm) in a micropulverizer hammer mill (SH1; Hosokawa Micron, B.V., Doetinchem, The Netherlands). Powder ingredients and oils were then mixed according to the target formulation in a paddle mixer (RM90; Mainca, S.L., Granollers, Spain). All diets were manufactured by temperature-controlled extrusion (pellet sizes: 1.5 mm) by

means of a low-shear extruder (P55; Italplast, S.r.l., Parma, Italy). Upon extrusion, all feed batches were dried in a convection oven (OP 750-UF; LTE Scientifics, Oldham, UK) for 4h at 45 °C. Proximate composition analysis was performed by the following methods: dry matter, by drying at 105 ºC for 24 h; ash, by combustion at 550 ºC for 12 h; crude protein (N × 6.25), by a flash combustion technique followed by gas chromatographic separation and thermal conductivity detection (LECO FP428); fat, after petroleum ether extraction, by the Soxhlet method; total phosphorus, according to the ISO/DIS 6491 method, using the vanado-molybdate reagent; gross energy, in an adiabatic bomb calorimeter (IKA).

Formulation of experimental diets and proximate analysis is presented in Table 1. Diets were analysed for total AA content. Diet samples were hydrolysed in 6M HCl at 116 ºC for 2 h in nitrogen-flushed glass vials. Samples were then pre-column derivatised with Waters AccQ Fluor Reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) using the AccQ Tag method (Waters, USA). Analyses were done by ultra-high-performance liquid chromatography (UPLC) in a Waters reversed-phase AA analysis system, using norvaline as an internal standard. During acid hydrolysis asparagine is converted to aspartate and glutamine to glutamate, so the reported values for these AA represent the sum of the respective amine and acid. Tryptophan was not determined, since it is partially destroyed by acid hydrolysis. The resultant peaks were analysed with EMPOWER software (Waters, USA). The AA profile of the experimental diets is presented in Table 2.

Table 1. Ingredients of the experimental diets.

	Experimental diets			
	CTRL	**ARG1**	**ARG2**	**CIT**
Ingredients (%)				
Fishmeal LT70 (South American)	5.0	5.0	5.0	5.0
Porcine blood meal	2.0	2.0	2.0	2.0
Poultry meal 65	5.0	5.0	5.0	5.0
Potato concentrate	10.0	10.0	10.0	10.0
Wheat glúten [1]	13.5	13.5	13.5	13.5
Corn glúten [2]	30.0	30.0	30.0	30.0
Soybean meal 48	5.0	5.0	5.0	5.0
Wheat meal	8.0	7.5	7.0	7.5
Fish oil [3]	10.0	10.0	10.0	10.0
Rapeseed oil [4]	5.5	5.5	5.5	5.5
Vitamin and mineral premix [5]	1.0	1.0	1.0	1.0
Binder (natural zeolite)	1.0	1.0	1.0	1.0
Antioxidant [6]	0.2	0.2	0.2	0.2
Sodium propionate	0.1	0.1	0.1	0.1
Monocalcium phosphate [7]	2.0	2.0	2.0	2.0
L-Arginine		0.5	1.0	
L-Citrulline				0.5
L-Histidine	0.3	0.3	0.3	0.3
L-Lysine	0.8	0.8	0.8	0.8
L-Threonine	0.2	0.2	0.2	0.2
DL-Methionine	0.4	0.4	0.4	0.4
Proximate analyses (% dry matter)				
Dry matter	96.4	95.8	96.0	96.3
Ash	5.1	5.1	5.2	5.0
Protein	51.4	51.7	53.0	52.2
Fat	18.6	18.5	18.2	18.5
Energy (kJ/g)	23.3	23.4	23.3	23.4
Phosphorus	0.8	0.8	0.8	0.8

[1] VITAL: 85.7% CP, 1.3% CF, ROQUETTE, France.

[2] Corn gluten feed: 61% CP, 6% CF, COPAM, Portugal.

[3] COPPENS International, The Netherlands.

[4] Henry Lamotte Oils GmbH, Germany.

[5] Premix for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate,7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middlings.

[6] Paramega PX, Kemin Europe NV, Belgium.

[7] Monocalcium phosphate: 22% phosphorus, 16% calcium, Fosfitalia, Italy.

Table 2. Determined amino acid composition (mg AA / g DW sample) of the experimental diets.

	Experimental diets			
	CTRL	**ARG1**	**ARG2**	**CIT**
Arginine	32.1	39.7	43.9	31.5
Histidine	17.5	17.9	17.2	16.9
Lysine	34.2	33.3	34.9	33.2
Threonine	25.0	25.2	24.6	24.0
Isoleucine	24.8	25.3	24.6	24.4
Leucine	65.2	64.5	61.2	62.6
Valine	30.4	31.1	30.6	29.6
Methionine	16.9	17.2	16.4	15.9
Phenylalanine	35.2	35.1	32.1	34.0
Cystine	5.6	4.5	5.0	5.8
Tyrosine	28.2	27.6	26.4	26.9
Aspartic acid + Asparagine	39.5	38.0	40.7	38.3
Glutamic acid + Glutamine	113.2	108.5	115.0	110.7
Alanine	33.5	33.5	32.5	31.8
Glycine	25.6	27.6	25.7	25.0
Proline	48.0	48.7	47.2	46.2
Serine	32.2	31.8	30.5	30.1
Taurine	1.5	1.4	1.6	1.4

6.2.2 Bacterial growth and inoculum preparation

Photobacterium damselae subsp. *piscicida* (*Phdp*), strain PP3, was kindly provided by Dr. Ana do Vale (Institute for Molecular and Cell Biology, University of Porto, Portugal) and isolated from yellowtail (*Seriola quinqueradiata*, Japan) by Dr Andrew C. Barnes (Marine Laboratory, Aberdeen, UK). Bacteria were routinely cultured at 22 ˚C in tryptic soy broth (TSB) or tryptic soy agar (TSA) (both from Difco Laboratories) supplemented with NaCl to a final concentration of 2 % (w/v) (TSB-2 and TSA-2, respectively) and stored at -80 ˚C in TSB-2 supplemented with 15 % (v/v) glycerol. To prepare the inoculum for injection into fish peritoneal cavities, 100 µl of stocked bacteria were cultured overnight at 22 ˚C on TSA-2. Exponentially growing bacteria were collected from the TSA-2 plates and re-suspended in sterile TSB-2. According to preliminary trials, the intended bacterial concentration to kill 50 % of the fish (LD_{50}) was obtained by absorbance reading and adjustment against its growth curve to 5 x 10^3 colony forming units (CFU) ml^{-1}. Bacteria concentration was confirmed by plating the resulting cultures on TSA-2 plates and counting of the CFU ml^{-1}.

6.2.3 Fish and experimental design

After one week of acclimatization, European seabass (8.4 g ± 0.39 g) were randomly distributed into twelve fibreglass tanks in a seawater recirculation system (200 l; n=50). Dietary treatments were randomly assigned to triplicate tanks and fish were fed three times a day by hand (9.30 am, 1.30 pm and 5.30 pm), until apparent satiety. Temperature was maintained by a water heater/cooler system, oxygen saturation was held at around 7.3 mg l^{-1} and photoperiod automatically controlled (10h dark: 14h light). Both nitrite and ammonium levels were daily recorded and its levels controlled by a water ozoniser system. Water renovations and system cleanings were performed twice a week.

6.2.4 Feeding trial

The feeding trial lasted for 2 and 4 weeks in order to assess the effect of short and mid-term AA dietary supplementation, respectively. At the end of each period, twelve fish per tank were sacrificed by anaesthetic overdose with 2-phenoxyethanol and individually weighed. Blood was collected from the caudal vein using heparinized syringes and samples from 3 fish were used to assess the haematological profile. Blood smears were prepared as well for differential cell counting. All blood samples were then centrifuged at 10,000 × *g* for 10 min at 4 ˚C and plasma pools were stored at -80 ºC.

6.2.5 Bacterial Challenge

Immediately after the 4 weeks sampling, all remaining fish were intraperitoneally (i.p.) injected with 100 µl *Phdp* (5 x 10^3 CFU ml^{-1}) and retrieved to the respective tank. Fish mortality was recorded for 3 weeks, every dead fish weighed and the respective head-kidney sampled for bacteria isolation. Feeding protocol and daily maintenance were kept while target temperature was increased to 24 °C (Temperature: 24 ± 0.5 °C; Salinity: 35 ppt; Photoperiod: 10h dark: 14h light) in a way to simulate a possible scenario during natural outbreaks.

6.2.6 Time-course trial

Simultaneously, after i.p. injection, 10 fish per tank were relocated in a comparable recirculation system (temperature: 24 ± 0.5 °C; salinity: 35 ppt; photoperiod: 10h dark: 14h light) and divided in two tanks according to dietary treatment. Fish were sampled at 4, 24 and 48 h after challenge. At each sampling time, 3 fish per tank were sacrificed by anaesthetic overdose with 2-phenoxyethanol and blood samples were collected and treated similarly to those in the feeding trial. Total peritoneal white blood cells (peritoneal

WBC) were collected according to the procedure initially described for mice by Silva et al. [21] and posteriorly adapted for fish by Afonso et al. [22]. Briefly, following fish anaesthesia and bleeding by the caudal vessel, 5 ml of cold HBSS supplemented with 30 units heparin ml^{-1} was injected into the peritoneal cavity. Then, the peritoneal area was slightly massaged in order to disperse the peritoneal cells in the injected HBSS. The i.p. injected HBSS containing suspended cells was finally collected. Total peritoneal cell counts were performed with a haemocytometer.

All protocols for haematological analysis and peripheral leucocytes assessment are described below.

6.2.7 Haematological procedures

The haematological profile comprised the analysis of the following parameters:

1) Haematocrit (Ht): The volume occupied by the red cells in a volume of whole blood, expressed as a percentage. The method consists of filling a capillary tube with blood and sealing one end of the tube with a small piece of plasticine and place the tube in the haematocrit centrifuge at 10,000 × *g* for 10 min. After that, the packed cell volume or relative percentage of red blood cells (RBC) can be obtained using a graphic reader.

2) Haemoglobin (Hb): Measured by a colorimetric method resulting from the reaction to the Drabkin's solution (SPINREACT, ref.:1001230, Spain)

3) Mean corpuscular volume: MCV (μm^3) = (Ht/RBC) × 10

4) Mean corpuscular haemoglobin: MCH (pg $cell^{-1}$) = (Hb)/ RBC × 10

5) Mean corpuscular haemoglobin concentration: MCHC (g 100 ml^{-1}) = (Hb/Ht) × 100

6) Red blood cells (RBC) and peripheral white blood cells (WBC) concentration: The solution for WBC counting resulted from a dilution 1/20 of homogenized blood in Hank's balanced salt solution (HBSS) with heparin (30 units ml^{-1}), while RBC counting resulted from a dilution 1/200 of homogenized blood in HBSS with heparin at the same concentration mentioned above. Counting was then performed in a Neubauer chamber. Values of WBC and RBC are presented in concentration, $\times 10^4$ and $\times 10^6$ cells μl^{-1}, respectively.

6.2.8 Peripheral leucocytes procedures

Blood smears were firstly fixed with formol-ethanol (10 % of 37 % formaldehyde in absolute ethanol) and afterwards stained with Wright's stain (Haemacolor; Merck). Neutrophils were identified according to their peroxidase activity, which was detected using the method described by Afonso et al. [23]. The slides were examined under oil

immersion (× 1,000) and at least 200 leucocytes were counted and classified as thrombocytes, lymphocytes, monocytes, and neutrophils. Each cell type absolute concentration and relative proportion was subsequently calculated.

6.2.9 Innate humoral parameters

Plasma bactericidal activity was measured according to Graham et al. [24] with some modifications [25]. Briefly, 20 µl of a suspension of *Photobacterium damselae* subsp. *piscicida* (1×10^6 CFU ml^{-1}) was added to 20 µl of plasma in duplicate wells of a U-shaped 96-well plate. HBSS was added instead of plasma to serve as positive control. The plate was incubated for 2.5 h at 25 °C and 25 µl of 3-(4,5 dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (1 mg ml^{-1}, Sigma) was then added to each well. After an incubation period of 10 min at 25 °C, plates were centrifuged at 2,000 × g for 10 min. Finally, 200 µl of dimethyl sulfoxide (Sigma) was added to the pellet and the absorbance of the formed, dissolved formazan was read at 560 nm. Total bactericidal activity is expressed as the percentage of killed bacteria, calculated from the difference between the samples and the positive control (100 % living bacteria).

A turbidimetric assay was used to evaluate lysozyme activity following the method as described by Costas et al. [26]. Briefly, a solution of *Micrococcus lysodeikticus* (0.5 mg ml^{-1}, 0.05 M sodium phosphate buffer, pH 6.2) was prepared. Plasma samples (15 µl in triplicates) were added to 250 µl of the above suspension in a microplate. The reaction was carried out at 25 °C and the absorbance (450 nm) was measured after 0.5 and 4.5 min. A standard curve was prepared based on serially diluted, lyophilized hen egg white lysozyme (Sigma) in sodium phosphate buffer (0.05 M, pH 6.2). The amount of lysozyme in the sample was calculated using the formula of the standard curve.

Total peroxidase activity in plasma was measured following the procedure described by Quade and Roth [27]. Briefly, 15 µl of plasma was diluted in triplicates with 135 µl of HBSS without Ca^{2+} and Mg^{2+} in flat-bottomed 96-well plates. Afterwards, 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 with the addition of 50 µl of 2 M sulphuric acid. Optical density was read at 450 nm. Wells without plasma were used as blanks. Peroxidase activity was determined by defining one unit of peroxidase as that which produces an absorbance change of 1 OD (units ml^{-1} of plasma).

All analyses were conducted in triplicates and absorbance was read in a Synergy HT (Biotek) microplate reader. Bactericidal activity, lysozyme and peroxidase protocols were previously validated for seabass according to [25].

6.2.10 Statistical analysis

All results are expressed as mean ± standard deviation (mean ± SD). Data were analyzed for normality and homogeneity of variance and, when necessary, transformed before being treated statistically. All data expressed as percentage were arcsine transformed. Data were analyzed by two-way ANOVA, with time and diet as factors, followed by Tukey post-hoc test to identify differences within experimental treatments. All statistical analyses were performed using the computer package STATISTICA 13 for WINDOWS. The level of significance used was P≤ 0.05 for all statistical tests.

6.3 Results

6.3.1 Disease resistance

Records on fish disease resistance showed that mortalities started at day 3 after bacterial inoculation, which was observed among fish from all dietary treatments except those fed CTRL (Fig. 1). At the fourth day of the bacterial challenge, mortalities in fish that had eaten ARG1, ARG2 and CIT were already more than two times those registered in CTRL-fed fish. Finally, from day 12 and until the end of the bacterial challenge, cumulative percentages were 41.7 %, 56.3 %, 39.4 % and 43.2 % in CTRL, ARG1, ARG2 and CIT, respectively.

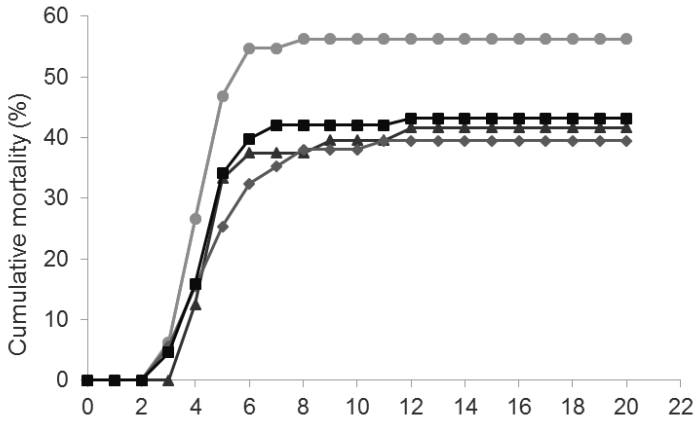

Fig.1 Cumulative mortality of European seabass fed CTRL (▲), ARG1 (●), ARG2 (♦) and CIT (■) for 4 weeks and subsequently i.p. injected with *Phdp*. Values are means of triplicate tanks (n = 22).

6.3.2 Haematology

For clarity, results regarding haematology will be divided in feeding trial and time-course trial.

i) Feeding trial. A temporal effect was observed on the Ht and the MCHC at the end of the feeding trial, in which the Ht increased while the MCHC decreased from 2 to 4 weeks, regardless of dietary treatments (Table 3).

Table 3. Haematocrit, haemoglobin, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) in European seabass fed dietary treatments for 2 weeks and 4 weeks.

	Dietary treatments							
Parameters	CTRL		ARG1		ARG2		CIT	
	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
Haematocrit (%)	24.3 ± 4.0	24.3 ± 1.0	23.9 ± 3.1	26.9 ± 2.9	24.2 ± 3.2	26.4 ± 1.3	25.8 ± 1.0	27.0 ± 3.5
Haemoglobin (g dl^{-1})	1.7 ± 0.4	1.4 ± 0.2	1.5 ± 0.2	1.6 ± 0.2	1.6 ± 0.2	1.6 ± 0.2	1.7 ± 0.3	1.7 ± 0.2
MCV (μm^3)	96.3 ± 8.2	106.3 ± 11.3	104.4 ± 32.3	98.9 ± 13.6	102.6 ± 20.6	101.8 ± 2.7	98.5 ± 9.5	108.4 ± 15.8
MCH (pg $cell^{-1}$)	6.7 ± 0.9	6.0 ± 0.9	6.7 ± 2.0	6.0 ± 1.2	6.5 ± 0.8	6.2 ± 0.9	6.5 ± 0.9	6.7 ± 1.0
MCHC (g 100 ml^{-1})	7.1 ± 1.3	5.7 ± 0.8	6.5 ± 0.6	6.1 ± 0.9	6.5 ± 1.0	6.2 ± 0.9	6.7 ± 1.1	6.2 ± 0.6

Two-way ANOVA	**P-value**			**Time**	
Parameters	Diet	Time	Diet × Time	2 weeks	4 weeks
Haematocrit (%)	ns	0.015	ns	x	y
Haemoglobin (g dl^{-1})	ns	ns	ns		
MCV (μm^3)	ns	ns	ns		
MCH (pg $cell^{-1}$)	ns	ns	ns		
MCHC (g 100 ml^{-1})	ns	0.004	ns	y	x

Values are expressed as means ± SD (n = 9). x and y denote significant differences between sampling time (Two-way ANOVA; Tukey post hoc test; p≤0.05).

Regarding peripheral cell numbers, WBC were also modulated over time irrespectively of amino acid supplementation, with fish fed experimental diets for 4 weeks showing lower numbers of circulating leucocytes (Table 4). Such decrease was particularly attributed to a general reduction in lymphocytes concentration. The remaining cell types were not further affected.

Table 4. Absolute values of peripheral blood leucocytes (total white blood cells WBC, thrombocytes, lymphocytes, monocytes and neutrophils) in European seabass fed dietary treatments for 2 weeks and 4 weeks.

Parameters	Dietary treatments							
	CTRL		ARG1		ARG2		CIT1	
	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
WBC (× 10^4 μl^{-1})	7.1 ± 1.7	6.9 ± 2.0	7.0 ± 2.1	6.1 ± 2.5	7.2 ± 1.6	5.5 ± 2.2	7.4 ± 2.1	6.2 ± 2.5
RBC (× 10^6 μl^{-1})	2.5 ± 0.5	2.3 ± 0.3	2.5 ± 0.7	2.7 ± 0.3	2.6 ± 0.3	2.5 ± 0.4	2.4 ± 0.4	2.6 ± 0.2
Thrombocytes (× 10^4 μl^{-1})	3.1 ± 0.7	3.5 ± 1.4	2.9 ± 1.1	3.5 ± 1.1	3.0 ± 1.1	3.0 ± 1.5	3.6 ± 1.0	3.6 ± 1.8
Lymphocytes (× 10^4 μl^{-1})	3.6 ± 1.3	3.3 ± 0.9	3.9 ± 1.7	2.4 ± 1.5	4.0 ± 1.3	1.8 ± 0.8	3.5 ± 1.3	2.4 ± 1.0
Monocytes (× 10^4 μl^{-1})	0.15 ± 0.08	0.15 ± 0.10	0.16 ± 0.12	0.09 ± 0.06	0.15 ± 0.05	0.16 ± 0.15	0.15 ± 0.12	0.10 ± 0.05
Neutrophils (× 10^4 μl^{-1})	0.22 ± 0.22	0.15 ± 0.13	0.12 ± 0.06	0.22 ± 0.25	0.11 ± 0.09	0.18 ± 014	0.19 ± 0.12	0.13 ± 0.08

Two-way ANOVA	**P-value**			**Time**	
Parameters	Diet	Time	Diet × Time	2 weeks	4 weeks
WBC (× 10^4 μl^{-1})	ns	0.044	ns	y	x
RBC (× 10^6 μl^{-1})	ns	ns	ns		
Thrombocytes (× 10^4 μl^{-1})	ns	ns	ns		
Lymphocytes (× 10^4 μl^{-1})	ns	<0.001	ns	y	x
Monocytes (× 10^4 μl^{-1})	ns	ns	ns		
Neutrophils (× 10^4 μl^{-1})	ns	ns	ns		

Values are expressed as means ± SD (n = 9). x and y denote significant differences between sampling time (Two-way ANOVA; Tukey post hoc test; p≤0.05).

ii) Time-course trial. Peritoneal WBC showed a decrease in time, regardless of dietary treatment, where cell counts of fish sampled at 24 and 48 h after bacterial challenge were lower than those of fish sampled at 4 h (Table 5). Ht was higher in fish sampled 48 h after i.p infection than in those sampled at 4 h (Table 5). On the other hand, Hb, MCH and MCHC were highest in CTRL-fed groups, regardless of sampling time. ARG1-fed fish showed higher MCH than those fed CTRL (Table 5). Infected fish presented lower total WBC concentration at 24 h post-infection than those sampled at 4 or 48 h, regardless dietary treatment. The highest total WBC concentration was observed in fish fed ARG1, sampled 4 h post-infection which was higher than all other groups. Differently from CTRL-fed fish, which total WBC increased with time, fish fed ARG1 had less WBC at 24 or 48 h than at 4 h post-infection (Fig. 2a). No effects were observed on RBC numbers (Fig. 2b).

Thrombocytes. The highest thrombocyte concentration was observed in ARG1-fed fish at 4 h post-infection (Fig. 2c). ARG1-fed fish sampled at 24 and 48 h later showed comparatively less circulating thrombocytes than those sampled at 4 h post-infection.

Lymphocytes. Fish fed CIT presented the lowest peripheral lymphocyte numbers but numbers were only significantly lower at 4 h post-infection than those of ARG1-fed fish (Fig. 2d). At 4 h post-infection, CIT-fed animals had less lymphocytes than fish fed ARG1 but these numbers increased at 48 h post-infection. A similar, time-dependent lymphocytosis was denoted in fish fed CTRL (Fig. 2d).

Neutrophils. Regarding neutrophils concentration, ARG1 group showed lower amounts of this cell type than CTRL-fed fish at 24 h post-infection (Fig. 2e). Moreover, a sharp decline in this cell's numbers was observed in fish fed ARG1 at both 24 and 48 h, compared to animals sampled at 4 h post-infection (Fig. 2e).

Monocytes. Finally, monocytosis was observed in fish sampled at 48 in both ARG2- and CIT-fed fish (Fig. 2f). However, though these are non-significant results, the two groups showed lower numbers of monocytes than fish fed CTRL.

Table 5. Haematocrit, haemoglobin, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) in European seabass fed dietary treatments for 4 weeks and sampled at 4, 24 or 48 h after i.p. infection with *Phdp*.

Parameters	Dietary treatments											
	CTRL			ARG1			ARG2			CIT		
	4 h	24 h	48 h	4 h	24 h	48 h	4 h	24 h	48 h	4 h	24 h	48 h
Peritoneal WBC	24.8 ± 7.3	18.0 ± 7.9	18.4 ± 6.8	29.3 ± 11.0	13.8 ± 5.3	13.5 ± 3.7	21.6 ± 5.7	21.5 ± 10.0	22.8 ± 4.0	28.3 ± 6.6	23.5 ± 3.9	17.3 ± 6.6
Haematocrit (%)	26.3 ± 1.5	26.8 ± 3.3	30.4 ± 2.5	29.0 ± 3.7	24.8 ± 1.6	27.3 ± 0.5	24.8 ± 1.6	27.3 ± 2.6	27.8 ± 1.9	26.2 ± 4.6	26.0 ± 4.0	28.2 ± 1.6
Haemoglobin (g dl^{-1})	1.6 ± 0.1	1.3 ± 0.5	1.7 ± 0.2	2.2 ± 0.6	1.6 ± 0.3	1.6 ± 0.2	1.6 ± 0.2	1.6 ± 0.3	1.6 ± 0.5	1.7 ± 0.2	1.5 ± 0.4	1.6 ± 0.4
MCV (μm^3)	107.6 ± 5.5	97.6 ± 56.4	106.1 ± 23.2	107.9 ± 14.2	85.5 ± 43.8	108.8 ± 12.7	109.1 ± 18.6	103.3 ± 17.3	109.0 ± 17.9	99.6 ± 13.5	107.7 ± 10.4	88.9 ± 44.5
MCH (pg $cell^{-1}$)	6.4 ± 0.5	5.6 ± 1.5	5.6 ± 1.1	9.00 ± 3.42	6.5 ± 0.9	6.2 ± 0.7	7.1 ± 0.9	6.1 ± 1.7	6.1 ± 1.2	6.5 ± 0.9	6.0 ± 1.3	5.8 ± 1.5
MCHC (g 100 ml^{-1})	5.9 ± 0.4	5.2 ± 1.4	5.4 ± 0.9	8.3 ± 2.7	6.2 ± 1.5	5.7 ± 0.5	6.6 ± 0.9	5.8 ± 1.0	5.7 ± 1.4	6.7 ± 1.5	5.6 ± 1.0	5.1 ± 1.1

Two-way ANOVA	**P-value**			**Time**			**Diet**			
Parameters	Time	Diet	Diet × Time	4 h	24 h	48 h	CTRL	ARG1	ARG2	CIT1
Peritoneal WBC	< 0.001	ns	ns	y	x	x				
Haematocrit (%)	0.044	ns	ns	xy	x	y				
Haemoglobin (g dl^{-1})	0.031	ns	ns	y	x	xy				
MCV (μm^3)	ns	ns	ns							
MCH (pg $cell^{-1}$)	0.005	0.047	ns	y	x	x	A	B	AB	AB
MCHC (g 100 ml^{-1})	0.001	ns	ns	y	x	x				

Values are expressed as means ± SD (n = 6). x and y denote significant differences between sampling time (Two-way ANOVA; Tukey post hoc test; p≤0.05).

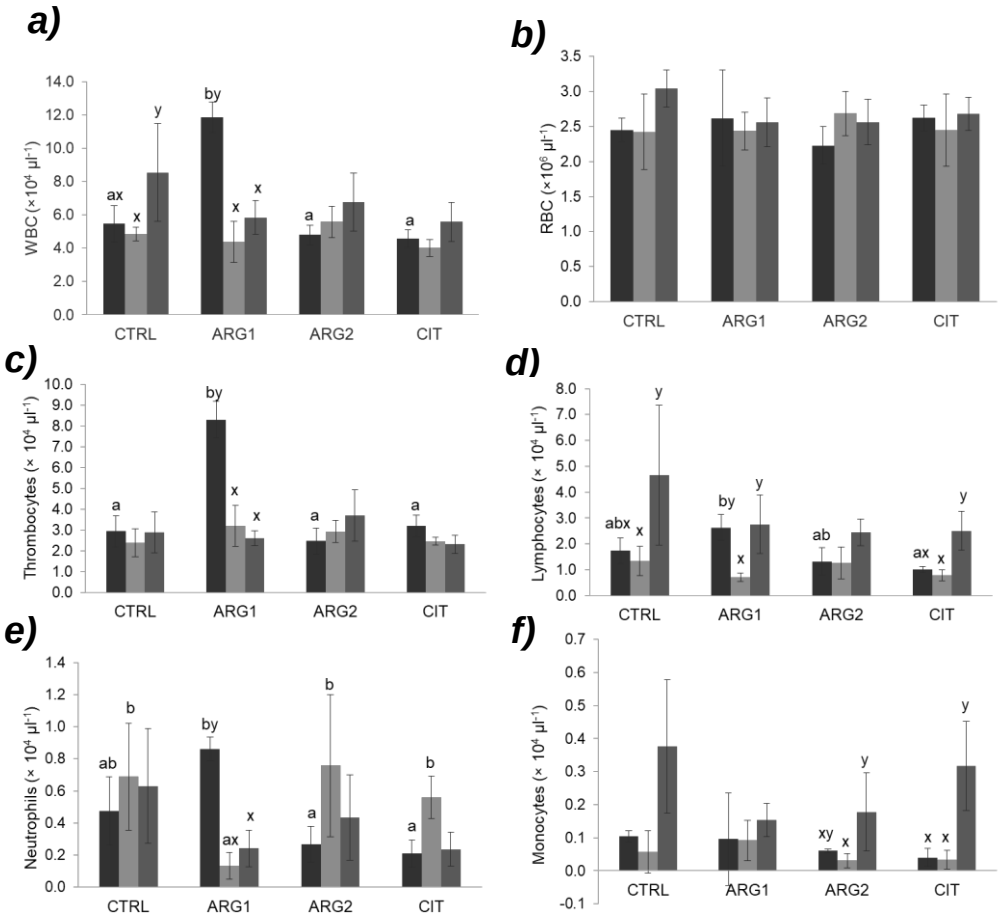

Fig. 2 Peripheral white blood cells (*a*), erythrocytes (*b*), thrombocytes (*c*), lymphocytes (*d*), neutrophils (*e*) and monocytes (*f*) in European seabass fed dietary treatments for 4 weeks and sampled at 4 (■), 24 (■) and 48 h (■) after i.p. infection with *Phdp* (means ± SD, n=6). Capital letters denote differences attributed to dietary treatments. x, y stand for significant differences attributed to time of sampling, within each dietary treatment. a and b denote differences between dietary treatments, within a given sampling time (Two-way ANOVA; Tukey post hoc test; p≤0.05).

6.3.3 Innate humoral parameters

Plasma total bactericidal activity was not altered by any tested variable, though a non-significant increasing trend seemed to take place with time post-infection, with all dietary treatments except in fish fed CIT (Fig. 4a). Regarding lysozyme, fish given CTRL presented higher plasma lysozyme content at 4 weeks than those sampled at 2 weeks, whereas such increase was not observed in the remaining groups (Fig. 3b). Infected fish fed CIT showed lower lysozyme content than those fed ARG1, but no differences were observed compared to fish fed CTRL (Fig. 4b). CIT led to a clear reduction with time of this enzyme, with fish sampled at 48 h post-infection showing lower lysozyme amount than those sampled at 4 or 24 h post-infection (Fig. 4b). Peroxidase was reduced over time, a fact particularly significant in both arginine supplementation levels, while no differences were observed with respect to the CTRL-fed group (Fig. 3c). Differently, peroxidase suffered no significant modulation after i.p. infection (Fig. 4c).

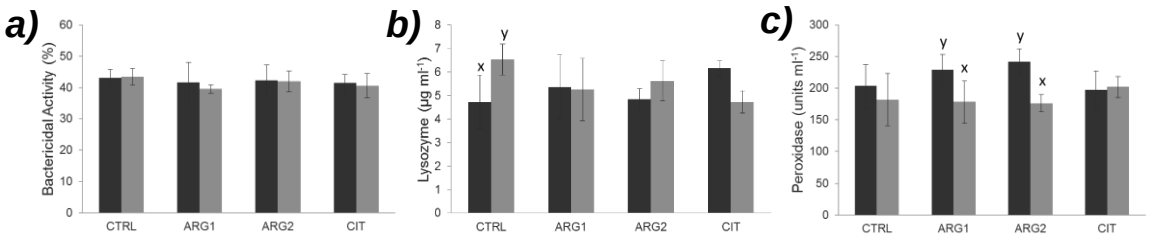

Fig. 3 Plasma total bactericidal activity (*a*), lysozyme (*b*) and peroxidase content (*c*), in European seabass fed dietary treatments for 2 (■) or 4 weeks (■) (means ± SD, n=6). x, and y stand for significant differences attributed to time of sampling within the same dietary treatment (Two-way ANOVA; Tukey post hoc test; p≤0.05).

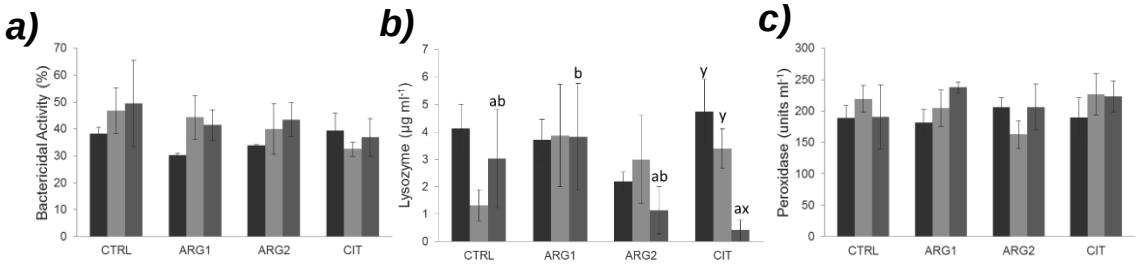

Fig.4 Plasma total bactericidal activity (*a*), lysozyme (*b*) and peroxidase content (*c*) in European seabass fed dietary treatments for 4 weeks and sampled at 4 (■), 24 (■) and 48 h (■) after i.p. infection with *Phdp* (means ± SD, n=6). x and y stand for significant differences attributed to sampling time within the same dietary treatment. a and b denote significant differences between dietary treatments within the same sampling time (Two-way ANOVA; Tukey post hoc test; p≤0.05).

6.4 Discussion

Immune-nutritional strategies to improve fish health conditions in aquaculture are a common practice nowadays that, nonetheless, requires thorough planning and adjustment to each particular situation. Physiological responses to a given dietary routine are not only species-specific, but will surely vary according to life stage, environmental and social contexts. Numerous feed additives have been tested an included in such functional feeds and naturally, different outcomes have been reported [28-33]. Arginine is perhaps one of the most intriguing nutrients given the multitude of different, frequently opposite effects perpetrated by its dietary supplementation with an immune-enhancing purpose. It is probably one of the most studied functional amino acids with deep involvement in both innate and specific responses of the immune system [6], a feature that certainly contributes to the wide array of results and that still intrigues researchers. Indeed, arginine-supplemented diets have been targeted as successful immune-enhancing strategies in humans [34-36], mice [37-39], birds [40-43] and fish [26,44,45]. However, arginine surplus may also prove to deviate immune responses in an opposite, frequently anti-inflammatory and immune-tolerance direction [14,46]. It is the metabolic pathway (i.e. NOS- or arginase-mediated) which determines arginine immunomodulatory facets [47-50].

Nonetheless, during the inflammatory process, the two enzymes individually dominate different and transient phases. Hence, arginine immunomodulation cannot be expected to be unidirectional.

Our experimental setup allowed the collection of data relative to the immune status, i.e. animals sampled after 2 or 4 weeks of dietary treatment, and to the inflammatory response at 4, 24 or 48 hours post-bacterial challenge. Present results of fish sampled before the challenge showed a light modulation of the immune response, mostly attributed to time. Plasma peroxidase, total circulating WBC and peripheral lymphocytes were all inhibited at the end of the feeding trial in comparison to the 2 weeks feeding period. However, though there is no statistical significance in it, it seems that those decreasing patterns are almost exclusive of groups fed supplemented diets (ARG1, ARG2 and CIT).

Immune pathways involving arginine metabolism are activated upon immune stimulation, i.e. enzyme expression is induced by different sets of cytokines and pathogenic stimuli and not by increased availability of substrate, hence, changes at the immune status level should not be too dramatic [51]. Furthermore, very little arginine is used by myeloid or lymphoid cells in resting conditions, as arginine transporters seem to be almost undetectable in non-activated immune cells while their gene expression is increased more than 5-fold upon cells activation, along with mRNA levels of iNOS [52]. However, studies centred in immunomodulatory aspects of arginine in resting conditions unveiled stimulatory effects on serum lysozyme activity and extracellular superoxide anion production [53], circulating monocytes, nitric oxide production and plasmatic humoral parameters [54] of the hybrid striped bass, *Morone chrysops* × *Morone saxatilis*, and turbot, *Scophthalmus maximus*, respectively. The reason behind such stimulatory effects might be the increased availability of substrate for non-myeloid arginase (constitutively expressed-arginase). Increased enzyme activity would result in a more expression of polyamine biosynthesis, and consequently, promotion of immune cells proliferation. In this study, however, increased availability of arginine (or its precursor, citrulline) led to a general leucopenia and there was no enhancement of any humoral parameter. Accordingly, Chapter 5 reported lower gene expression of several immune-related cell markers in European seabass gut and spleen subjected to dietary arginine supplementation. It is thus tempting to speculate that, instead of being metabolized by arginase, arginine was in turn converted to NO by iNOS. Conversion of arginine into NO is primarily a host-defence mechanism of phagocytes but NO cytotoxicity has been shown to mediate immune-suppressive effects regarding immune cells function and proliferation [55], particularly when arginine levels are higher than physiological concentrations [7]. NO is produced by phagocytes but it is also constitutively synthesized by endothelial and neuronal cells by other NOS isoforms (cNOS) which activity is known

to modulate immune response and inflammatory processes [56]. In the absence of an immune stimulus to induce iNOS, higher levels of circulating arginine might have increased cNOS activity leading to the observed inhibited immune defences.

Arginine is the common substrate for iNOS and arginase, regardless of its extracellular concentration. While expression levels of these enzymes in macrophages are triggered by cytokines and antigens, substrate availability regulates their activity patterns. Hrabák and co-workers have estimated that arginase is responsible for metabolizing 90 % of available arginine whereas the remaining 10 % is taken up by iNOS [57]. Hence, by competing for arginine, these enzymes regulate the activity of one another. In the presence of an immune challenge, providing seabass diets with dietary arginine surplus might i) decrease enzymes competition and potentiate both activities, in their specific timeframes; ii) saturate one of the enzymes before the other (different Km), favouring the other enzyme; iii) promote the establishment of a negative feedback mechanism led by extreme metabolite concentrations, thus inhibiting expression/activity of a given enzyme. Present results regarding the immune response to bacterial challenge were in line with those observed during the feeding trial, translating an impairment of the immune defences. Peripheral cell counts revealed a clear suppression of cellular response to the bacterial challenge that might have contributed to the observed higher fish susceptibility to disease. Indeed, an evident decrease in circulating WBC, lymphocytes, monocytes and neutrophils was particularly denoted in fish fed CIT, but also in ARG1 and ARG2 groups, though dietary supplementation did not prevent the normal increase in time of some cell types. Moreover, though not statistically significant, peritoneal WBC decrease in time was more evident in fish fed ARG1. This study seems to support the third proposed scenario, in which a cellular response impairment might have been the result of a strict control of enzymatic activity, conducted by excessive ornithine (and perhaps NO, too) that down-regulated polyamine biosynthesis, thereby preventing immune cells proliferation and differentiation. Ornithine, the resultant metabolite of arginine, is known to down-regulate arginase activity [12] when present in very high levels. In accordance, suppression of arginase 2 gene expression was observed in the posterior gut of the European seabass fed arginine-supplemented diets for 34 days [58].

The role of citrulline in the immune response is fairly unknown. Citrulline is both a precursor and a metabolite of arginine so it is inevitably involved in the same pathways. In fish, as in mammals, arginine can be produced directly from citrulline or via glutamate, though in fish such reactions occur at lower rates [17]. In the present study, the use of citrulline was based on the hypothesis that, under inflammatory conditions, citrulline could overcome increasing arginine demands. As observed in mammals, arginine availability decreases to very low levels during inflammation, but its *de novo* synthesis

does not seem to compensate such decrease, as it is severely down-regulated in the kidney [59]. Thus, feeding fish diets supplemented with this amino acid was expected to account for the increased arginine requirement. Results obtained upon citrulline dietary supplementation seem to be in line with this hypothesis, since immunomodulatory effects were similar to those observed upon arginine supplementation. Citrulline supplementation effects were actually the most evident in down-regulating immune parameters. Fish fed CIT had less total WBC, lymphocytes, monocytes and neutrophils, and lysozyme production at 48 h was strongly inhibited relatively to fish fed CTRL or ARG1. This seems to suggest that, even though CIT diet was not deficient in arginine content, i.e. contained the same arginine amount as CTRL, citrulline surplus was regarded and handled in the same way arginine surplus was. Still, further analytical support is needed. Unfortunately, similar to arginine, there is a great gap regarding knowledge on the effects of citrulline in fish immune response, but mammal models have been demonstrating its ability to mimic arginine immunomodulation, particularly the improvement of NO synthesis [60-62].

In summary, feeding seabass with arginine- or citrulline-supplemented diets for 4 weeks decreased peripheral leucocytes, mainly lymphocytes, as well as plasma peroxidase content. In accordance, inflammatory response to a bacterial challenge was weaker in terms of cellular response in fish fed supplemented diets, with the most striking effects being detected in fish fed CIT, which led to increased disease susceptibility and subsequent death. Results suggest that both in resting and in stimulation conditions, juvenile seabass immune mechanisms are impaired by arginine increasing concentration, possibly mediated by the increased production of toxic NO. Understanding which of the two enzymes activity is affected by arginine surplus in resting and stressful conditions, as well as the mechanisms that dictate this metabolic switch is a key step in establishing feeding strategies that might be species-specific.

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Chapter 7

General discussion, conclusions and future approaches

7.1 General Discussion

7.1.1 Arginine supplementation may compromise immune status and cellular-mediated immune responses

In this Thesis, the involvement of arginine and the consequent effects in fish immune responses unveiled intriguing modulatory actions of this AA that repeatedly brought down several immune parameters. The most notorious outcome of increasing arginine availability was a consistent impairment of cellular responses, regarding not only cell abundance but also immune defences resulting from their activity, as observed from the absolute quantification of cell metabolic products or gene expression analysis. Arginine dietary supplementation ultimately compromised fish disease resistance. It is therefore of utmost importance to know and understand the conditions leading to these undesirable effects when developing strategies of immunomodulation with arginine.

7.1.2 Arginine metabolism in non-inflammatory conditions

Arginine is normally incorporated in protein or broken down to ornithine for polyamine biosynthesis that sustains normal cell proliferation and division. Arginine is also a secretagogue that induces insulin and growth hormone production [1]. Moreover, NO is continuously synthetized by endothelial and neuronal nitric oxide synthase (eNOS and nNOS, respectively), thus, there is a continuous demand for arginine, too. Endothelial NO is responsible for vasodilation while neuron-produced NO role in synaptic transmission is well recognized [2]. Under normal conditions, very small amounts of arginine are used by either myeloid or lymphoid cells and expression of both immune-related enzymes, iNOS and arginase, is triggered only by inflammatory signals usually absent in normal conditions [3]. Therefore, an increase in arginine availability was not expected to significantly affect the immune status.

Unexpectedly, lower *in vitro* and *in vivo* NO production was observed in unstimulated cells and in undisturbed fish compared to leucocytes incubated in non-supplemented medium or fish fed a basal diet, as seen in Chapter 2 and Chapter 5, respectively. In the same control conditions, several immune-related genes were down-regulated by arginine supplementation to both cell culture medium and fish diets. Particularly important changes were observed regarding cellular responses. Down-regulation of polyamine synthesis-related enzymes (Chapters 2 and 5) seemed to correlate with a general reduction of circulating immune cells, as observed by lower expression levels of cell markers and peripheral leucocyte numbers (Chapters 5 and 6, respectively). Although polyamines were not quantified in the present study, arginine-mediated immunosuppressive effects might indeed been associated to an impairment of cell proliferation.

Such inhibitory effects under undisturbed state indicates that arginine is able to modulate fish immune condition, irrespectively of immune stimulation.

Arginine modulatory effects require both an increase of its intracellular levels and its metabolization by either NOS or arginase. The activity of these two enzymes activities begins to be relevant from the onset of the inflammatory response, at which time its expression is triggered, until inflammation resolution. As mentioned before, the expression of NOS and arginase in myeloid cells is dependent upon immune stimulation. However, there are other enzymes (isoforms) which constitutively metabolize arginine and which activity might have accounted for the immune-suppressive actions observed in the present study. Regardless of tissue distribution, arginase 1 and 2 were found to be constitutively expressed in several organs of carp, *Cyprinus carpio* [4], and arginase 2 and iNOS in trout [5]. Hence, it is possible that arginine added to cell cultures (Chapter 2) or given as supplement in the feed (Chapters 5 and 6) is metabolized at high rates by either eNOS, nNOS or arginases constitutively expressed. Accordingly, graded levels of dietary arginine increased plasmatic arginase and NOS activities in channel catfish, *Ictalurus punctatus*, [6] and increased NO production in mammals [7] and in fish [8]. This being the case, and in the light of the unveiled immunosuppressive effects induced by arginine, two possible scenarios should be considered: i) increased toxicity of arginine metabolites and ii) establishment of negative feedback mechanisms.

Amongst arginine metabolites, NO is surely the one with greatest potential for cytotoxicity. Though its primary function in immune defence is that of host protection, it can also deteriorate host molecules and tissues and be an inhibitor or lymphocyte replication [9-12]. Moreover, NO can inhibit ornithine decarboxylase by S-nitrosylation of a cysteine residue on its active site [13] which might consequently impair polyamine biosynthesis and, hence, cell proliferation. However, neither in Chapter 2 nor in Chapter 5 results revealed a particular increase of NO upon arginine increasing levels. NO tended to be lower in unstimulated cells treated with arginine surplus and was significantly inhibited in fish fed the lowest arginine supplementation level. However, sampling time could be a determinant and having data on earlier sampling points would perhaps elucidate NO fluctuations. It is also arguable that instead constitutive expression of arginase (which Km is higher than that of iNOS [14]) “seized” arginine away from NOS, converting it to ornithine. However, this could not be confirmed in the present Thesis, where down-regulation of polyamine-related genes was observed upon arginine addition to cell culture medium and no differences were depicted by arginase activity in arginine-rich environments (Chapter 2). In fact, arginase 2 gene expression was down-regulated in the intestine of fish fed arginine-supplemented diets (Chapter 5).

Samad and Kyeong-Jun [15] while establishing arginine requirement reported dose-dependent, immune-enhancing effects of graded levels of dietary arginine in red

seabream, *Pagrus major*. Differently, LPS-induced inflammatory response was impaired by arginine supplementation *in vivo* and *in vitro* in the intestine of juvenile Jian carp [16]. In experimental studies, tight control of every possible variable is crucial in determining cause-effect. The variety of results obtained in arginine immunomodulation studies is most probably associated to different experimental contexts. In Chapter 2, the experimental model took advantage of a complete cell culture medium, Leibovitz L-15 medium, which already contains arginine. Basal arginine concentration (2.87 mM) is actually much higher than that known as the physiological concentration of arginine in fish (≈100 μM [17,18]). Under inflammatory conditions, where extracellular levels of arginine are known to drop drastically [19], such high starting concentration might actually be beneficial. However, before immune stimulation *in vitro* cell cultures were incubated for 24 h in L-15 medium supplemented with arginine. It is therefore possible that this period of time (24 h) in the presence of such high arginine concentration (basal level in cell culture medium plus supplement) might have contributed to an initial boost on arginine-degrading enzymes activity that led to an intense production of arginine metabolites such as NO, known to impair macrophage function [20]. This hypothesis could help explain why unstimulated HKL incubated in arginine-supplemented medium showed lower NO production (measured at the end of 96 or 120 h in culture media) and down-regulation of polyamine biosynthesis-related enzymes: ornithine decarboxylase (*odc*) and adenosylmethionine decarboxylase (*amd*).

7.1.3 Arginine metabolism during an inflammatory response and effects on fish disease resistance

Arginine involvement in the onset and resolution of fish inflammatory responses was investigated in Chapters 2 and 6, while modulation of disease resistance was assessed in Chapters 5 and 6.

Several factors contribute to arginine dropping levels at the inflammatory focus: increased arginine uptake by immune cells, elevated protein synthesis, limited citrulline availability to arginine *de novo* synthesis, and lower intake and supply of arginine [21], a scenario that has been also reported in fish [22]. In low-arginine environments, competition between macrophagic iNOS and arginase becomes critical, as 90 % of arginine is taken by arginase in activated immune cells [23]. Nonetheless, immune responses do not feature a single macrophage phenotype. Instead, it gradually changes throughout the inflammatory response and sequentially predominant stimuli dictate enzymatic activity [24].

Increasing arginine availability during fish immune response (either *in vivo* or *in vitro*) was based on the hypothesis that higher substrate availability leads to higher enzymatic activity, thus to a generally enhanced inflammatory response. As arginine *de novo* synthesis happens at very low rates in teleosts, nutritional supplementation could

increase arginine physiological levels and thereby influence macrophage activity by decreasing enzyme competition. Accordingly, Buentello and Gatlin reported a dose-dependent increase in catfish plasma free arginine to dietary graded inclusion [25] and improved fish disease resistance [22]. Immunostimulatory effects were also denoted by Nile tilapia, *Oreochromis niloticus*, as arginine-supplemented diets helped fish to better cope with stress [26].

Surprisingly, in European seabass arginine surplus did not change arginase activity in stimulated HKL, neither significantly improved NO production in Chapter 2. Immune-related gene expression was also not enhanced; in fact, *cox2* was down-regulated and other inflammatory genes followed a similar trend. Gene expression related to polyamines biosynthesis (*amd* and *odc*) was similarly inhibited (Chapter 2) whereas *sat* was up-regulated from 4 to 24 h. *sat* mediates the interconversion between spermine and spermidine and therefore it is an indicator of polyamine turnover [27]. Its time-dependent up-regulation in stimulated HKL can be a marker of increased requirement for polyamines (for instance during an immune stimulus) that, if not fulfilled by newly-synthetized polyamines, is compensated by an enhanced turnover. The lack of sustained polyamine synthesis could compromise cell proliferation, required for an efficient cell response. Results obtained in Chapter 6 corroborate this hypothesis, as fish fed arginine-supplemented diets showed much lower abundance of peripheral leucocytes in response to the bacterial challenge compared to those fed the control diet.

In their review, Forlenza et al. [28] described macrophage polarization dynamics over time, using a fish model of parasitic infection. In such particular immune response, there is a clear early phase predominance of classically activated macrophages that gradually changes to a conspicuous presence of regulatory interleukin 10-producing macrophages. As mentioned before, there is a sharp decline on arginine levels during inflammatory processes, and this is partially due to macrophage uptake, whatever its phenotype is. In the three presented studies (Chapter 2, 5 and 6) arginine was made available long before the immune challenge (i.e. 24 h *in vitro* or 4 weeks *in vivo*). Therefore, arginine levels were, at least theoretically, high at the onset of immune response.

To discuss why increased arginine availability was not an advantage for NO production or cell proliferation at 4 and 24 h after the immune challenge, results have to be collectively interpreted. Data on seabass immune status and unstimulated HKL showed a significant arginine-induced immune impairment. Thus, increasing arginine as a strategy to avoid competition between iNOS and arginase and thereby enhance immune response was in vain, since at the time of immune stimulation macrophage function was inhibited and leucocyte numbers were generally lower.

Fish fed arginine supplements decreased disease resistance, which was in line with a weakened immune status and inflammatory response; thus, fish showing the poorest immune conditions were the more susceptible to disease and death.

Numerous studies on humans and other mammal's immunonutrition have reported the pros and cons of using immune-enhancing diets containing arginine. Benefits have mostly been observed in scenarios of physical stress, such as trauma or surgery [29], in which there is a profound decrease in arginine levels as well as in T cell numbers. Under these circumstances, supplementing diets with arginine is thought to normalize T cell function [30]. On the other hand, upon infection arginine enhances NOS activity, resulting in the production of NO at high rates, which has been shown to be deleterious [2,13,29].

Present studies on arginine involvement and effects on fish immune system unveiled modulatory mechanisms through which this AA impairs teleost immune defences. From down-regulating important inflammatory genes and cell markers, to reducing overall leucocyte function, arginine compromised fish immune status and thereby increased susceptibility to bacterial disease. Nonetheless, as opposite results have been obtained in other fish species and in different contexts, it is possible that changing experimental conditions could produce different outcomes.

7.1.4 Cell recruitment and further leucocyte-mediated immune response are enhanced by methionine increased availability

There is a high content of sulphur AA in substances produced in response to immune stimulation [31]. Among others, glutathione, metallothionein and several acute phase proteins are rich in sulphur AA, hence dependent on their availability. Sulphur AA requirement is therefore increased in such situations and peripheral tissues supply of AA maybe not enough to fulfil such demand [31]. Methionine is often the first-limiting AA in fish feeds formulation, whenever composition mostly relies on plant-based ingredients [32]. It is rapidly converted into S-adenosylmethionine, which participates in the synthesis of polyamines and glutathione, and in DNA methylation [31]. Thus, methionine presents itself as a functional AA with interesting features that could be used to benefit the immune response.

In Chapter 2, methionine supplementation to cell culture media unveiled stimulatory effects of this AA on innate immune defences, whereas expression of immune-related genes was not as elucidating. In accordance, in Chapters 3 and 4, addition of methionine to seabass diets in a 2 weeks-feeding trial enhanced *gpx* and *tryp* gene expression in the HK and the gut, respectively, and increased peripheral WBC abundance, particularly neutrophils. The immune status of the fish was therefore primed, mostly regarding immune cell numbers, meaning they were potentially better equipped in terms of immune defences than their CTRL-fed counterparts. Despite gene expression profile sometimes

suggesting otherwise, their immune response towards an i.p. bacterial injection was in general sustained by an enhanced inflammatory response, as shown by increased cell migration and abundance and a stronger respiratory burst (Chapter 3).

As aforementioned, the most relevant methionine mediated-effect was its ability to increase cell proliferation. Such feature is considered to be related to its involvement in polyamine turnover and methylation pathways [33]. Similar to what was observed in arginine-treated cells (Chapter 2), *odc* mRNA levels were much lower in methionine-treated cells than in those kept in L-15 medium. As this is the limiting-rate enzyme for polyamines production [34], biosynthesis of these molecules could have been compromised and, in this case, so would be cell proliferation. However, a time-dependent up-regulation of *amd* and *sat* was observed in HKL treated with the highest methionine supplementation level. Such increase was particularly high in stimulated cells, probably due to the higher requirement for cell proliferation. Therefore, although no polyamines content measurement was performed in the presented studies, an enhanced polyamine turnover could have accounted for the higher cell numbers denoted in fish fed methionine-supplemented diets in Chapter 3. This was not the first time methionine has been shown to be able to increase leucocyte counts as it has been already demonstrated in fish [35], humans [36] and mice [37].

Given that methionine is the precursor of glutathione, a superoxide anion scavenger, it was expected that superoxide anion levels would decrease in fish fed methionine-supplemented diets. However, the observed increase in respiratory burst (Chapters 2 and 3) as well as nitric oxide production (Chapter 2) could be explained by an enhanced cellular response. Respiratory burst behavior in response to methionine increased availability has been inconsistent in different studies [38-40] but none of these studies considered immune cell counts that would allow correlation between cell numbers and superoxide production.

More circulating immune cells could also explain the enhanced neuroendocrine response observed in MET-fed fish from Chapter 3. Cortisol levels were much higher in these fish than in those fed CTRL, regardless it was the sham group or the UV-killed *Phdp*-injected fish. This suggests that fish displaying an enhanced immune status, i.e. increased circulating leucocytes, might more easily react in terms of the central nervous system and develop an endocrine response, which is normally triggered to cope with physiological disturbances [41]. The hypothalamus-pituitary-interrenal (HPI) axis activation must therefore, not be regarded as a negative modulatory effect of methionine, at least in what concerns the development of the immune response. A disease resistance study would further disclose how more effectively MET-fed fish would deal with the bacterial challenge, despite higher circulating cortisol levels.

In Chapter 3, methionine was tested with regard to its effects on antioxidant capacity. Both *gpx* gene expression and TAC were analyzed and whereas TAC was not significantly affected neither before nor after immune challenge, HK *gpx* mRNA levels were much higher at the end of a 2 weeks-feeding trial in MET-fed fish than in CTRL fish. Similar results were obtained in carp fed graded levels of methionine hydroxy analogue, where gene expression of antioxidant enzymes was augmented in both HK and spleen [39]. Differently, gene expression of antioxidant enzymes was down-regulated after the immune challenge, a response that could have been associated to negative feedback mechanisms or to increased gene methylation potential.

Indeed, in contrast to what was observed in the majority of parameters evaluated, methionine treatment down-regulated several immune-related genes both *in vitro* and *in vivo* (Chapter 2 and 3, respectively Pan and co-workers [39] proposed that such general impairment of inflammatory mediators (also reported by them) could be the result of regulatory mechanisms on NF-κB. Differently, as an increase in methionine might also increase S-adenosylmethionine stores (a methyl group donor) it could increase methylation activity, as previously reported for humans [36,42] and fish [43], which in turn could affect gene transcription.

Another interesting point on methionine-induced effects was a seeming dose-dependency of some results obtained in Chapter 2, where two supplementation levels were tested (1 mM and 1.5 mM). This observation was of particular importance in inflammatory gene expression that, although devoid of statistical significance, presented a general decreasing trend from the lowest to the highest dose. In Chapters 3 and 4 methionine was tested in a single dose (2 × the requirement level of seabass) so such behavior could not be evaluated. However, the lower response seen in the methionine groups compared to the control group in some of the genes tested is in line with results of Chapter 2, corroborating the hypothesis that methionine reduces inflammatory genes, possibly within a concentration range.

7.1.5 Tryptophan surplus potentiates HPI axis activation and modulates immune response

Following dietary intake, tryptophan metabolism occurs through four different pathways: protein synthesis, kynurenine-niacin synthesis, serotonin (5-HT) synthesis and melatonin synthesis [44]. Physiological conditions and external factors then dictate tryptophan metabolic fate and the balance among these pathways. The interest on tryptophan as a functional AA is linked to two metabolic pathways that have significant immune implications. In one hand, tryptophan is the precursor of 5-HT, which is a key molecule at the critical point of HPI axis activation [45], thereby allowing to modulate neuroendocrine response. On the other hand, upon immune stimulation, tryptophan is

converted by 2, 3 indoleamine dioxygenase (IDO) to biological active molecules in activated myeloid cells that are known to modulate several immune-related mechanisms, often leading to immune-tolerance scenarios [46]. Therefore, being aware of the intimate relationship between immune and neuroendocrine systems, adding tryptophan to cell culture media or to seabass diets was expected to induce changes in the immune response, either directly (not affecting neuroendocrine mechanisms) or via HPI axis activation.

Evaluation of tryptophan-mediated neuroendocrine effects was done by quantifying brain monoamines, plasma cortisol levels and gene expression of two key receptors (*gr* and *mc2r*) in peripheral and HK immune cells (Chapter 3). Briefly, brain monoamine content was increased in terms of dopamine (DA), its metabolite 3, 4-dihydroxyphenylacetic acid (DOPAC), and 5-HT and its metabolite 5-hydroxyindoleactic acid (5HIAA). Cortisol was higher in TRP-fed fish, in particular at 24 h post *Phdp*-injection, while blood *gr* gene expression was down-regulated at the same sampling time.

Given that the first, rate-limiting enzyme of 5-HT synthesis is not saturated at tryptophan physiological concentrations, increased tryptophan availability promotes 5-HT synthesis [47]. Therefore, regarding this specific monoamine, results were according to expectations. DA and dopaminergic activity, known to have opposite effects to those perpetrated by 5-HT [48,49], similarly increased in both telencephalon and optic tectum of fish fed TRP. This rise has been suggested to be a strategy of the central nervous system to counteract stress-induced serotonergic activity and cortisol levels [48].

Brain 5-HT mode of action seems to be bidirectional, i.e. it can both stimulate and inhibit pituitary adrenocorticotropin hormone (ACTH) production according to HPI axis activation state [47]. Plasma cortisol levels in undisturbed fish was positively correlated with dietary tryptophan supplementation and 5-HT levels, whereas a negative correlation was observed in stressed fish [47,49]. In Chapter 3, seabass were in undisturbed conditions until they were i.p. injected. As cortisol, a stress indicator in fish [50], was much higher at 4 h post-injection than levels measured at the end of the feeding trial, it was assumed that there was activation of the HPI axis and the mounting of a stress response. Being so, tryptophan administration was expected to mitigate this response, or at least to slow down cortisol increase from 4 to 24 h post-inoculation. The absence of an inhibitory effect could be due to the type of stress induced. In Chapter 3, an immune challenge evoked an inflammatory response that is present throughout the sampling points which implies a great number of circulating immune cells and signalling molecules that could have counteracted 5-HT potential inhibitory effects in the brain.

Overall, despite the observed down-regulation of glucocorticoids receptor in blood cells, which could be a defence mechanism against detrimental effects of high cortisol

levels, data suggest that dietary tryptophan enhanced seabass HPI axis response to the immune trigger. Such event would help to explain the enhanced gene expression pattern observed in the posterior gut of seabass fed the same diet (Chapter 4). These results seem to indicate that with higher tryptophan availability, 5-HT-enhanced synthesis in the brain and that eventually produced by local enterochromaffin cells (not measured), might have benefited the intestinal immune response. Besides its known role in neurotransmission, 5-HT is also a paracrine messenger in the gastrointestinal tract and elevated concentrations of this indoleamine in mammals have been associated with increased chemotaxis, cytotoxicity and proliferation of natural killer cells as well as protection from oxidative damage [51].

In a similar way to what happens with arginine and its metabolizing enzyme arginase, IDO activity in macrophages was induced only upon cellular immune stimulation [46]. At this point, a rapid and strong tryptophan uptake takes place, drastically decreasing extracellular concentrations. Such phenomenon is considered, per se, an antimicrobial mechanism that prevents pathogenic activity [44] and the synthesis of downstream compounds is associated to anti-inflammatory effects such as the proliferation of regulatory T-cells and impairment of effector T-cells activity [44,52].

Although tryptophan immunomodulatory effects have been studied in mammals for a while [53-55], there is still a great gap in this topic in lower vertebrates such as teleosts. While results of this thesis often denote both inhibited and enhanced immune mechanisms, the majority of the effects reported in Chapters 2, 3 and 4 tended to be suppressive of the immune response, with important immune-related gene expression being down-regulated (*il1β* and *mmp9*), lower cell numbers and decreased TAC. While peripheral leucocytes respiratory burst was lowered by tryptophan dietary supplementation (Chapter 3), HK respiratory burst was improved in *in vitro* leucocyte cultures, irrespectively of cell immune stimulation (Chapter 2).

While interactions between neuroendocrine and immune response might have accounted for the general inhibited immune status and inflammatory response in seabass given a surplus of tryptophan, the same cause could not be attributed to results observed *in vitro* as only monolayers of HKL were used, which means there is no contact with endocrine mediators. Therefore, immunosuppression might instead be associated to IDO activity. As mentioned before, IDO-mediated tolerogenesis is mostly directed to T-cells. In the present *in vitro* study, only phagocyte responses were evaluated. Albeit the undeniable presence of lymphocytes, which are inevitably collected along with other leucocytes, their concentration was not estimated; so, their possible participation in modulating other immune cells behavior in this experimental setup cannot be disregarded. Moreover, tryptophan metabolism through the kynurenine pathway implies increased antioxidant power, as IDO takes up O_2^- as a co-factor and several downstream

metabolites exhibit antioxidant properties [44]. Hence, a decrease in superoxide levels would also be expected to be observed in Chapter 2, but it was not verified. As ATP production was much higher in tryptophan-treated cells compared to other AA treatments and L-15-treated cells, it is suggested that improvement of cells performance might have contributed to promote cell immune response, thereby attenuating immune suppressive effects of the AA per se. Nonetheless, tryptophan catabolism by IDO still poses itself as the most likely mechanism responsible for the partial impairment of immune defences observed in Chapter 2.

Along with groups fed TRP, a similar endocrine response also occurred in fish fed MET (Chapter 3), but immunomodulatory effects were quite different from those induced by tryptophan. Still, the improved cell response in fish fed MET could overcome the attenuating effects of cortisol on the developing inflammatory response. Since such cell numbers increase was not denoted in TRP-fed fish, this group might indeed have been particularly exposed to the inhibiting effects of circulating higher cortisol levels. Altogether, the activation of a neuroendocrine response seems to have stimulated fish local inflammatory response, whereas it suppressed the systemic immune response to the immune challenge.

7.1.6 Non-essential amino acids

i) Glutamine not only fuels leucocyte activity, but also induces inflammatory gene expression

Glutamine is considered a non-essential AA and is amongst the most abundant free AA in fish plasma and muscle [32]. Most importantly, glutamine is a preferred energy source of leucocytes and its utilization rate in these cells can be higher than that of glucose [56]. Glutamine provides carbon atoms to the citric acid cycle triggering ATP production [57]. This way, glutamine ensures leucocyte function and homeostasis upon immune activation. Glutamine is also precursor of glutamate which is in turn one of the three elements of the antioxidant molecule glutathione. Glutamine metabolic relevance lays also on its involvement in arginine *de novo* synthesis, where enteric glutamine can be converted to citrulline, via glutamate [21]. As a result, under certain physiological conditions such as an inflammatory response, glutamine may become conditionally-essential. In spite of this being a well-established pathway in mammals, arginine *de novo* synthesis from glutamine seems to be absent in most teleost species [58,59]. Based on these features, increased glutamine availability might promote cell metabolism and proliferation as well as antioxidant power. Scientific evidences in fish point at an enhancing effect of glutamine on glutathione stores [60-62]. Mouse T lymphocytes proliferation is dependent on glutamine availability [63] and carp intestinal structure and function are improved by glutamine dietary supplementation [64]. Glutamine addition to

different fish species diets modulated several immune mechanisms, improving serum lysozyme activity [62,65,66] and respiratory burst [65,67]. In endotoxemic rats, glutamine dietary supplementation was reported as a preventive strategy regarding inflammation, as it decreased gene expression of interleukin 1β, tumour necrosis factor α (TNFα), and interleukin 10 [68].

In Chapter 2, glutamine load was not positively correlated with polyamine synthesis-related gene expression (an indication of cell proliferation) as observed by decreased expression levels of *amd*, *odc* and *sat*, either in control or stimulated conditions. However, it is important to have in mind that the majority of our cell population are macrophages, which differently from lymphocytes, are terminally differentiated cells with low proliferative rates [69].

Chapter 2 results regarding *in vitro* glutamine treatment showed a general enhancing effect of this AA on HKL immune status/response. Cells incubated in glutamine-supplemented medium produced more NO and ATP, while respiratory burst activity was barely affected. Moreover, while no changes were observed in *il1β* gene expression, *cox2* was up-regulated in these cells, and *mif* and *tgfβ* followed a similar trend. It has been generally accepted that glutamine is involved in the regulation of numerous genes, from general metabolism to immunity, by directly interacting with AA responsive elements [57]. Dietary glutamine up-regulated *tgfβ* in the HK of Jian carp whereas *il1β* mRNA was not affected by the AA surplus [62]. Being aware of the great number of variables between that work and the present study (e.g. *in vivo* conditions, different fish species, time of administration and dose), such effects are still very similar to those presented in this Thesis. Interestingly, this up-regulation of inflammatory genes was slightly more evident in cells treated with the lowest glutamine dose. In the sea cucumber, *Apostichopus japonicas,* dietary glutamine supplementation showed positive effects on growth and coelomic fluid lysozyme activity that were dose-dependent [70].

Few *in vitro* studies were carried out to test glutamine immunomodulatory effects in fish species, even less towards gene expression modulation. In contrast to the pro-inflammatory behavior of glutamine observed in seabass leucocytes, mammalian models denote a general impairment of immune-related gene expression upon glutamine treatment [68,71,72]. These contrasts, together with the fact that macrophage function is improved in fish (Chapter 2) [62,65-67] suggest that glutamine involvement in immune and inflammatory mechanisms might be dose-dependent and species-related, and that it might also vary among fish species.

ii) Citrulline surplus mimics arginine immunomodulatory effects

Citrulline is not directly involved in host defence mechanisms, but as precursor of arginine, its availability at inflammation sites becomes conditionally relevant. Indeed, in

inflammatory conditions, fluctuations in citrulline plasma concentration are similar to those of arginine, i. e. there is a major reduction on citrulline availability [21]. In mammals, this shortage of citrulline directly affects arginine stores, as its *de novo* synthesis is compromised [73]. In addition, though a full ornithine-urea cycle as we know it for mammals is not functional in the majority of teleosts [74,75], the enzymes mediating citrulline to arginine conversion step exist and are functionally active in fish. Based on these facts, Chapter 6 of the present Thesis aimed at estimating the outcome of adding citrulline to seabass diets (not deficient in arginine) regarding their immune status and inflammatory response upon an i.p. bacterial challenge. There is a considerable lack of fish studies focused on citrulline dynamics with only a few addressing it as an arginine precursor [58,76,77]. The present findings are in line with those observed upon arginine dietary supplementation (Chapters 5 and 6), as the extra dose of citrulline significantly impaired the evaluated immune defences, particularly at the cellular recruitment level. Thus, results suggest that citrulline was assimilated by fish, converted to arginine and used to mediate the same immunomodulatory mechanisms seen with arginine supplementation. Citrulline administration to rats undergoing intestinal ischemia attenuated jejune damage by inactivating NOS and the NF-κB pathway [78] and peritoneal macrophages of obese rats developed a dose-dependent response upon citrulline supplementation showing up-regulation of interleukin 6 but down-regulation of TNFα [79].

Interestingly, changes resultant from citrulline surplus were more pronounced than those induced by arginine. Pohlenz and co-workers [77] observed a marked reduction on citrulline cell medium content after a 2.5 h bactericidal assay with channel catfish macrophages suggesting a strong usage of this AA by activated cells. In murine endotoxemia, citrulline supplementation improved intestinal microcirculation, NO production and increased arginine availability at superior rates than those induced by arginine supplementation [73]. Altogether, evidences seem to indicate that citrulline is a more efficient supplement than arginine itself.

Mammalian macrophages are able to convert citrulline to arginine, as reported by Wu and Brosnan [80] thereby prolonging NO production when intracellular arginine is low, by recycling citrulline. With respect to fish, there is one *in vitro* study in which channel catfish macrophages NO production was increased when cell culture medium was supplemented with citrulline [81], demonstrating their ability to use this AA as arginine source. Though in Chapter 6 there was no quantification of *in vivo* NO production, plasma NO levels in CIT-fed fish would probably be lower than those of CTRL-fed fish. In Chapter 5, arginine dietary treatment lowered NO production and similarly, fewer circulating effector cells in CIT-fed fish would certainly limit its production. In conclusion, citrulline supplementation to seabass diets led to similar suppressive immune mechanisms as did arginine supplementation, with a particular stronger inhibitory effect.

7.2 Conclusions

The following conclusions can be drawn from the present Thesis:

- AA *in vitro* supplementation modulates immune-related gene expression and cellular innate immune response to either *Vibrio anguillarum* or *Vibrio anguillarum* LPS in a teleost primary leucocyte culture (Chapter 2).

- AA addition to European seabass diets has immunomodulatory effects at both transcriptional and protein levels before and after an immune challenge (Chapters 3, 4, 5 and 6).

- Arginine severely compromised European seabass immune system as shown by impaired cell recruitment, immune function and down-regulation of immune-related transcripts (Chapters 5 and 6). Consequently or concomitantly, arginine inhibited the inflammatory response (Chapter 6) leading to higher disease susceptibility and death (Chapters 5 and 6).

- Chapters 3 and 4 highlighted the ability of methionine to enhance leucocyte response and migration to the inflammatory focus, whereas it did not affect peripheral lymphoid tissues immune response. *In vivo* and *in vitro* methionine treatment seemed to mildly down-regulate immune-related genes which might be associated to an enriched methylation capacity.

- Chapter 3 demonstrates the ability of inflammatory mediators to enhance the activation of the HPI axis, as cortisol levels were higher and prolonged in time in fish displaying an enhanced inflammatory response.

- Tryptophan dietary supplementation for 14 days increased brain monoamine synthesis (particularly 5-HT) and cortisol release but only after an inflammatory insult.

- Effects of tryptophan on European seabass tended to be immunosuppressive, denoting down-regulation of important inflammatory genes, and reduction of antioxidant capacity and leucocyte numbers (Chapters 2 and 3). However, it enhanced local immune-related gene expression in stimulated fish (Chapter 4), suggesting 5-HT-mediated pro-inflammatory mechanisms.

- The non-essential AA glutamine generally improved *in vitro* innate immune parameters, energy status and immune-related gene transcription of European seabass HKL (Chapter 2).

- Dietary citrulline immunomodulation was similar in nature to that observed for arginine, reducing peripheral immune cell numbers of European seabass both before and after a bacterial challenge (Chapter 6).

7.3 Loose-ends and future approaches

Results from the present Thesis bring new and relevant insights for the evaluation of arginine, methionine, tryptophan, glutamine and citrulline as potential nutraceutics for a Mediterranean farmed fish species. However, fish immunomodulation is still an emergent topic and teleosts immune mechanisms are largely unknown. Thus, conclusions are often drawn from mammalian parallelisms- Figure 1 schematically presents the major results obtained in this thesis as well as future research to be done regarding each amino acid.

Arginine-mediated effects were clearly immunosuppressive, but its inhibitory mechanisms were not unveiled. Immune cell function and proliferation impairment was suggested to be associated to toxicity of arginine metabolites but further research is needed to determine the causes. Quantification of ornithine and NO levels throughout the whole experiment (a time-course trial with several sampling points before and after the immune challenge) would clarify arginine metabolic fate. In case the observed immune depression is caused by NO or ornithine toxicity, arginine dietary administration could be tested for shorter periods of time (7 days and less) and be halted prior to the immune challenge. Testing graded levels of dietary supplementation would further enlighten arginine immunomodulatory role, as data from Chapter 5 seems to denote a dose-response effect.

Indeed, as particularly denoted in Chapter 2 *in vitro* studies, an effect of AA level seems to be present. Also, L-15 Leibovitz cell culture medium basal AA concentrations are already much higher than those observed in physiological conditions. This might have restrained leucocyte function, hence their immune response. Therefore, dose-response experiments are required for each AA, preferably added to lab-assembled cell culture media so that final AA concentrations are closer to physiological ranges.

Taking advantage of AA as immunomodulators does not imply that their properties need to exclusively enhance fish immune response by promoting the onset and development of the inflammatory response and counteract the anti-inflammatory phase. It might also be useful to restrain it and thereby avoid self-damage. In Chapters 3 and 4 methionine was shown to improve innate immune defences, whereas tryptophan could

either promote or impair immune function. Further research is needed on the simultaneous use of different AA in order to assess possible synergistic interactions upon different contexts. Such studies should therefore consider combining different non-antagonistic AA in one functional diet as well as the dietary supplementation of each AA coupled with different immune triggers: vaccination, pre- or probiotic treatments or stressful contexts such as fish transportation or high stocking densities.

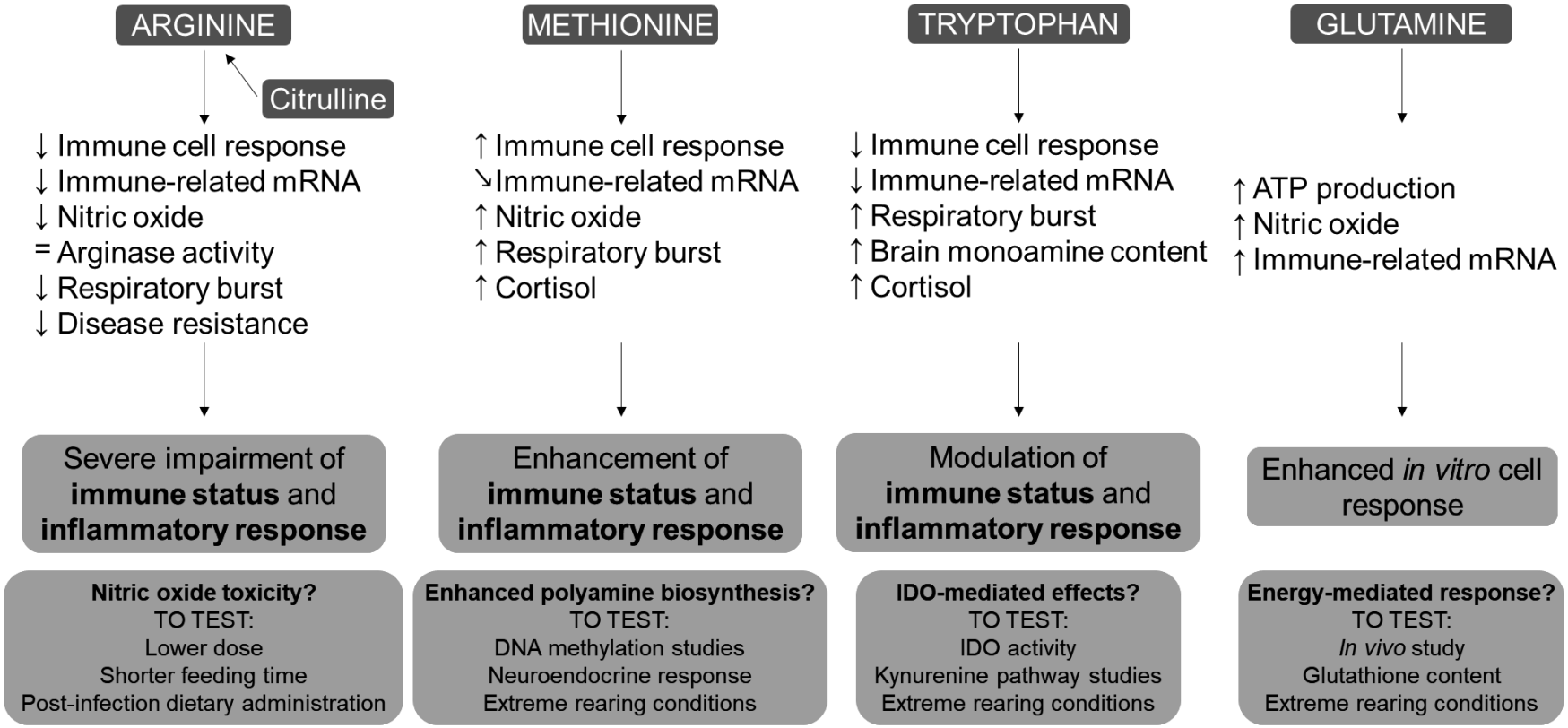

Fig. 1 Amino acid immunomodulatory actions in European seabass, general impact on immune defences and future approaches.

7.4 References

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Appendix

Appendix I

Amino acids as modulators of the European seabass, *Dicentrarchus labrax*, innate immune response: an *in vitro* approach

Table 1 A. Nitric oxide content measured as total nitrites in the supernatant of European seabass HKL subjected to experimental conditions for 72 h or 96 h.

AA	stimulus	time	NO (µM)
L-15	CTRL	72	0.54 ± 0.19
		96	0.99 ± 0.05
	vaLPS	72	0.49 ± 0.14
		96	0.77 ± 0.30
	Vang	72	1.33 ± 0.22
		96	1.75 ± 0.27
G1	CTRL	72	0.91 ± 0.23
		96	1.17 ± 0.12
	vaLPS	72	0.90 ± 0.12
		96	0.90 ± 0.12
	Vang	72	1.80 ± 0.14
		96	2.12 ± 0.19
G2	CTRL	72	0.43 ± 0.13
		96	0.80 ± 0.06
	vaLPS	72	0.67 ± 0.31
		96	1.33 ± 0.44
	Vang	72	1.21 ± 0.28
		96	2.01 ± 0.17
A1	CTRL	72	0.54 ± 0.06
		96	0.63 ± 0.12
	vaLPS	72	0.60 ± 0.06
		96	0.81 ± 0.29
	Vang	72	1.10 ± 0.32
		96	1.94 ± 0.19
A2	CTRL	72	0.51 ± 0.07
		96	0.74 ± 0.09
	vaLPS	72	0.58 ± 0.14
		96	0.92 ± 0.30
	Vang	72	1.52 ± 0.13
		96	2.18 ± 0.40
T1	CTRL	72	0.51 ± 0.17
		96	0.76 ± 0.18
	vaLPS	72	0.61 ± 0.17
		96	1.11 ± 0.48
	Vang	72	1.50 ± 0.16
		96	2.05 ± 0.49
T2	CTRL	72	0.52 ± 0.17
		96	0.76 ± 0.21
	vaLPS	72	0.68 ± 0.07
		96	1.21 ± 0.46
	Vang	72	1.54 ± 0.23
		96	2.61 ± 0.21
M1	CTRL	72	0.48 ± 0.14
		96	0.61 ± 0.07
	vaLPS	72	0.80 ± 0.03
		96	1.31 ± 0.40
	Vang	72	1.51 ± 0.33
		96	2.53 ± 0.09
M2	CTRL	72	0.45 ± 0.11
		96	0.83 ± 0.12
	vaLPS	72	1.04 ± 0.39
		96	2.68 ± 1.17
	Vang	72	1.44 ± 0.13
		96	2.67 ± 0.32

Multifactorial ANOVA	AA	time	stimulus	AA × time	AA × stimulus	time × stimulus	AA × time × stimulus
NO (µM)	< 0.001	< 0.001	< 0.001	0.003	< 0.001	ns	ns

Table S1 B	AA									time		stimulus		
	L-15	G1	G2	A1	A2	T1	T2	M1	M2	72 h	96 h	CTRL	vaLPS	*Vang*
NO (µM)	ab	c	ab	a	ab	abc	bc	abc	c	x	y	A	B	C

Table S1 C	AA × time								
	L-15	G1	G2	A1	A2	T1	T2	M1	M2
72 h	ax	b	ax	a	abx	abx	abx	abx	abx
96 h	aby	bc	bcy	a	aby	aby	abcy	abcy	cy

Table S1 D	AA × stimulus								
	L-15	G1	G2	A1	A2	T1	T2	M1	M2
CTRL	Aab	Ab	Aa	Aa	Aa	Aa	Aa	Aa	Aa
vaLPS	Aa	Aab	Aab	Aab	Aab	Aab	Aab	Bbc	Bc
Vang	B	B	B	B	B	B	B	C	B

Values represent means ± SD (n = 6 biological replicates). Different capital letters stand for statistically significant differences attributed to stimulation. a, b, c and d denote statistically significant differences between AA treatments. x and y stand for significant differences attributed to incubation time. (Multifactorial ANOVA; Tukey post-hoc test; ns: non-significant; $P \leq 0.05$).

Table 2 A. Extracellular O_2^- content in the supernatant of European seabass HKL subjected to experimental conditions for 4 h or 24 h.

AA	stimulus	time	O_2^- (nmol)
L-15	CTRL	4	3.26 ± 1.61
		24	1.98 ± 0.53
	Vang	4	2.46 ± 1.12
		24	1.17 ± 0.56
G1	CTRL	4	3.19 ± 1.44
		24	2.00 ± 0.67
	Vang	4	2.64 ± 0.86
		24	1.24 ± 0.57
G2	CTRL	4	3.07 ± 1.08
		24	2.56 ± 0.20
	Vang	4	2.80 ± 1.30
		24	1.92 ± 1.39
A1	CTRL	4	3.82 ± 0.74
		24	2.04 ± 0.44
	Vang	4	2.16 ± 1.08
		24	2.06 ± 1.11
A2	CTRL	4	4.24 ± 1.33
		24	2.87 ± 0.76
	Vang	4	3.32 ± 1.05
		24	1.78 ± 1.20
T1	CTRL	4	4.71 ± 1.44
		24	2.90 ± 1.16
	Vang	4	3.15 ± 1.34
		24	1.95 ± 1.59
T2	CTRL	4	4.23 ± 1.38
		24	3.29 ± 1.49
	Vang	4	3.61 ± 1.27
		24	2.57 ± 1.13
M1	CTRL	4	4.80 ± 1.54
		24	3.36 ± 1.30
	Vang	4	3.65 ± 1.24
		24	2.70 ± 0.66
M2	CTRL	4	4.87 ± 1.26
		24	3.61 ± 1.04
	Vang	4	3.13 ± 0.75
		24	2.61 ± 1.40

Multifactorial ANOVA	AA	time	stimulus	AA × time	AA × stimulus	time × stimulus	AA × time × stimulus
Extracellular O_2^- (nmol)	< 0.001	< 0.001	< 0.001	ns	ns	ns	ns

Table S2 B	AA									time		stimulus	
	L-15	G1	G2	A1	A2	T1	T2	M1	M2	4 h	24 h	CTRL	*Vang*
Extracellular O_2^- (nmol)	a	a	abc	ab	abcd	abcd	bcd	d	cd	y	x	B	A

Values represent means ± SD (n = 6 biological replicates). a, b, c and d denote statistically significant differences between AA treatments. x and y stand for significant differences attributed to time (Multifactorial ANOVA; Tukey post-hoc test; ns: non-significant; $P \leq 0.05$).

Table 3 A. Extracellular ATP content in the supernatant of European seabass HKL subjected to experimental conditions for 4 h or 24 h.

AA	Stimulus	Time	ATP (nM)
L-15	CTRL	4	3.83 ± 2.96 a
		24	9.49 ± 11.53 a
	vaLPS	4	10.86 ± 9.30 a
		24	42.25 ± 11.20
G1	CTRL	4	6.10 ± 2.67 abx
		24	46.29 ± 15.11 abcy
	vaLPS	4	19.86 ± 8.34 ab
		24	32.69 ± 16.16
G2	CTRL	4	6.40 ± 2.00 abx
		24	81.87 ± 46.25 Bcy
	vaLPS	4	16.93 ± 3.63 ab
		24	32.13 ± 17.64 A
A1	CTRL	4	14.74 ± 5.52 ab
		24	27.93 ± 5.08 ab
	vaLPS	4	27.23 ± 19.83 abcx
		24	65.52 ± 27.64 y
A2	CTRL	4	4.00 ± 1.16 Aab
		24	37.80 ± 18.71 ab
	vaLPS	4	56.62 ± 23.07 Bc
		24	45.71 ± 13.27
T1	CTRL	4	69.64 ± 33.68 cy
		24	30.64 ± 7.24 abx
	vaLPS	4	49.39 ± 18.86 bc
		24	38.78 ± 16.97
T2	CTRL	4	40.62 ± 24.51 b
		24	48.33 ± 16.55 bc
	vaLPS	4	34.99 ± 22.19 abc
		24	33.54 ± 10.66
M1	CTRL	4	24.51 ± 10.23 ab
		24	62.37 ± 29.20 bc
	vaLPS	4	38.83 ± 15.33 abc
		24	37.03 ± 15.29
M2	CTRL	4	13.85 ± 12.54 ab
		24	31.16 ± 12.55 ab
	vaLPS	4	20.27 ± 4.93 ab
		24	39.21 ± 20.74

Multifactorial ANOVA	AA	time	stimulus	AA × time	AA × stimulus	time × stimulus	AA × time × stimulus
ATP (nM)	< 0.001	< 0.001	ns	< 0.001	< 0.001	0.019	< 0.001

Table S3 B	AA									time	
	L-15	G1	G2	A1	A2	T1	T2	M1	M2	4 h	24 h
ATP (nM)	a	a	bcd	bc	bcd	d	cd	cd	ab	x	y

Table S3 C	AA × time								
	L-15	G1	G2	A1	A2	T1	T2	M1	M2
4 h	a	abx	abx	abc	bc	dy	cd	bc	bc
24 h	a	abcy	cy	abc	abc	abx	abc	bc	ab

Table S3 D	AA × stimulus								
	L-15	G1	G2	A1	A2	T1	T2	M1	M2
CTRL	a	abc	bcd	abc	Aabc	d	cd	bcd	ab
vaLPS	a	ab	ab	bc	Bc	bc	abc	abc	ab

Table S3 E	time × stimulus	
	4 h	24 h
CTRL	Ax	y
vaLPS	Bx	y

Values represent means ± SD (n = 6 biological replicates). Different capital letters stand for statistically significant differences attributed to stimulation. a, b, c and d denote statistically significant differences between AA treatments. x and y stand for significant differences attributed to incubation time. (Multifactorial ANOVA; Tukey post-hoc test; ns: non-significant; $P \leq 0.05$).

Table 4 A. Arginase activity in the supernatant of European seabass HKL subjected to experimental conditions for 4 h or 24 h.

AA	Stimulus	Time	Arginase activity (U L^{-1})
L-15	CTRL	4	1.02 ± 0.78
		24	1.85 ± 0.06
	Vang	4	1.17 ± 0.25
		24	2.20 ± 0.41
A1	CTRL	4	1.33 ± 0.63
		24	1.50 ± 0.71
	Vang	4	1.27 ± 0.67
		24	2.05 ± 1.39
A2	CTRL	4	1.68 ± 0.72
		24	1.51 ± 0.72
	Vang	4	1.10 ± 0.18
		24	1.63 ± 0.45

Multifactorial ANOVA	[AA]	time	stimulus	[AA] × time	[AA] × stimulus	time × stimulus	[AA] × time × stimulus
Arginase activity (U L^{-1})	ns	0.014	ns	ns	ns	ns	ns

Table S4 B	time	
	4	24
Arginase activity (U L^{-1})	x	y

Values represent means ± SD (n = 6 biological replicates). x and y stand for significant differences attributed to incubation time. (Multifactorial ANOVA; Tukey post-hoc test; ns: non-significant; $P \leq 0.05$).

Table 5 A. Expression patterns of genes involved in the immune response and polyamine biosynthesis of head-kidney leucocytes subjected to the experimental conditions for 4 and 24 h.

			Gene Expression (Fold Change)						
Treatments	Stimulus	Time	*il1β*	*cox2*	*mif*	*tgfβ*	*amd*	*odc*	*sat*
L-15	CTRL	4	0.78 ± 0.40	0.57 ± 0.22	1.00 ± 1.20	0.74 ± 0.65	0.80 ± 0.23	0.97 ± 0.23	0.77 ± 0.09
		24	0.97 ± 1.03	2.69 ± 3.54	0.30 ± 0.27	0.49 ± 0.31	1.42 ± 0.33	4.73 ± 0.70	0.56 ± 0.13 Aa
	vaLPS	4	9.30 ± 6.93	1.54 ± 1.02	0.64 ± 0.54 ab	0.88 ± 0.31	1.51 ± 0.25	1.72 ± 1.60	0.51 ± 0.20 abx
		24	0.24 ± 0.14	0.58 ± 0.52	1.74 ± 1.40	0.76 ± 0.33	1.34 ± 0.21	0.56 ± 0.17	2.01 ± 0.72 Baby
G1	CTRL	4	0.34 ± 0.19	1.46 ± 0.93	0.84 ± 0.41	1.43 ± 0.20	0.85 ± 0.09	0.36 ± 0.07	0.52 ± 0.03
		24	1.16 ± 1.41	4.19 ± 5.28	4.13 ± 1.82	1.17 ± 0.14	0.54 ± 0.16	0.06 ± 0.01	1.38 ± 0.39 ab
	vaLPS	4	10.56 ± 4.46	12.17 ± 6.14	1.52 ± 0.51 b	1.14 ± 0.24	0.61 ± 0.13	0.11 ± 0.06	0.59 ± 0.10 ab
		24	0.37 ± 0.11	1.02 ± 0.64	3.23 ± 0.48	0.93 ± 0.18	0.53 ± 0.17	0.12 ± 0.06	1.01 ± 0.30 a
G2	CTRL	4	1.31 ± 0.16	4.28 ± 1.26	0.79 ± 0.26	1.08 ± 0.50	1.23 ± 0.64	0.22 ± 0.09	0.57 ± 0.13
		24	0.59 ± 0.43	1.86 ± 1.53	1.83 ± 0.44	0.72 ± 0.21	0.48 ± 0.18	0.09 ± 0.04	1.32 ± 0.23 ab
	vaLPS	4	7.54 ± 6.09	7.31 ± 3.71	1.21 ± 0.21 b	0.79 ± 0.24	1.01 ± 0.24	0.25 ± 0.11	0.49 ± 0.13 ab
		24	0.42 ± 0.26	1.89 ± 1.02	1.04 ± 0.46	0.80 ± 0.31	0.64 ± 0.03	0.11 ± 0.03	1.33 ± 0.17 ab
A1	CTRL	4	0.80 ± 0.56	0.68 ± 0.34	1.02 ± 0.81	0.75 ± 0.42	0.50 ± 0.14	0.70 ± 0.35	0.56 ± 0.20
		24	0.08 ± 0.11	0.37 ± 0.45	0.95 ± 1.40	0.09 ± 0.09	0.49 ± 0.14	0.31 ± 0.22	0.64 ± 0.24 Aab
	vaLPS	4	7.13 ± 4.04	1.79 ± 1.00	0.41 ± 0.36 b	0.51 ± 0.46	0.61 ± 0.26	1.21 ± 0.92	0.84 ± 0.15 abx
		24	0.26 ± 0.18	0.17 ± 0.14	1.38 ± 1.30	1.12 ± 0.99	1.04 ± 0.71	0.36 ± 0.39	2.39 ± 1.56 Baby
A2	CTRL	4	0.53 ± 0.27	0.42 ± 0.42	0.07 ± 0.08	0.25 ± 0.10	0.72 ± 0.08	1.07 ± 0.92	0.68 ± 0.17
		24	0.07 ± 0.06	0.04 ± 0.04	0.50 ± 0.51	0.58 ± 0.54	0.64 ± 0.18	0.46 ± 0.27	0.82 ± 0.32 Aab
	vaLPS	4	5.09 ± 3.05	0.89 ± 0.78	0.25 ± 0.22 ab	0.43 ± 0.25	0.87 ± 0.21	0.64 ± 0.40	0.87 ± 0.23 bx
		24	0.26 ± 0.17	0.20 ± 0.10	1.05 ± 0.85	0.85 ± 0.13	1.74 ± 0.83	0.31 ± 0.19	2.87 ± 0.96 Bby
T1	CTRL	4	0.74 ± 0.15	0.84 ± 0.02	0.16 ± 0.09	0.69 ± 0.34	0.94 ± 0.45	0.43 ± 0.29	0.46 ± 0.05
		24	0.09 ± 0.05	0.31 ± 0.12	2.16 ± 1.36	0.85 ± 0.14	0.54 ± 0.01	0.13 ± 0.05	1.02 ± 0.14 ab
	vaLPS	4	7.09 ± 4.93	3.65 ± 1.10	0.52 ± 0.07 ab	0.82 ± 0.30	0.79 ± 0.37	0.28 ± 0.22	0.45 ± 0.23 abx
		24	0.29 ± 0.11	2.68 ± 0.33	2.24 ± 1.88	1.13 ± 0.11	0.82 ± 0.17	0.13 ± 0.01	2.96 ± 1.51 by
T2	CTRL	4	0.60 ± 0.44	0.52 ± 0.40	0.58 ± 0.67	0.44 ± 0.52	0.56 ± 0.21	1.16 ± 0.89	0.66 ± 0.23 x
		24	0.16 ± 0.13	0.06 ± 0.04	0.91 ± 0.89	0.93 ± 0.41	0.55 ± 0.10	0.40 ± 0.09	1.60 ± 0.41 by
	vaLPS	4	5.07 ± 4.12	1.49 ± 0.92	0.61 ± 0.66 ab	0.37 ± 0.26	0.66 ± 0.29	1.22 ± 0.77	0.38 ± 0.17 ax
		24	0.18 ± 0.16	0.24 ± 0.21	1.29 ± 1.30	0.70 ± 0.27	0.92 ± 0.20	0.27 ± 0.19	1.84 ± 0.60 aby
M1	CTRL	4	0.53 ± 0.03	1.66 ± 0.75	0.35 ± 0.12	0.54 ± 0.44	1.03 ± 0.33	0.29 ± 0.07	0.43 ± 0.03 x
		24	0.09 ± 0.07	0.52 ± 0.33	4.31 ± 3.36	0.80 ± 0.01	0.48 ± 0.15	0.07 ± 0.02	1.28 ± 0.28 aby
	vaLPS	4	8.14 ± 2.02	3.68 ± 0.48	0.25 ± 0.17 ab	0.44 ± 0.24	1.02 ± 0.38	0.53 ± 0.19	1.06 ± 0.30 b
		24	0.36 ± 0.23	2.79 ± 0.23	3.90 ± 3.16	0.97 ± 0.45	0.65 ± 0.24	0.10 ± 0.02	1.69 ± 0.35 ab
M2	CTRL	4	0.45 ± 0.13	0.25 ± 0.21	0.42 ± 0.44	0.37 ± 0.44	0.63 ± 0.11	0.49 ± 0.32	0.69 ± 0.53
		24	0.04 ± 0.04	0.21 ± 0.39	0.77 ± 0.90	0.39 ± 0.49	0.74 ± 0.07	0.68 ± 0.52	1.00 ± 0.29 ab
	vaLPS	4	9.08 ± 2.62	0.80 ± 0.88	0.24 ± 0.38 ax	0.14 ± 0.13	0.52 ± 0.19	0.40 ± 0.62	0.48 ± 0.14 abx
		24	0.30 ± 0.17	0.28 ± 0.28	2.09 ± 1.13 y	1.18 ± 0.71	2.64 ± 2.49	0.19 ± 0.11	1.84 ± 0.64 y

Multifactorial ANOVA	AA	time	stimulus	AA × time	AA × stimulus	time × stimulus	AA × time × stimulus
il1β	0.011	< 0.001	< 0.001	ns	0.026	< 0.001	ns
cox2	< 0.001	< 0.001	< 0.001	ns	ns	ns	ns
mif	0.002	< 0.001	ns	ns	ns	ns	0.037
tgfβ	< 0.001	0.030	ns	0.028	ns	0.009	ns
amd	< 0.001	ns	0.002	< 0.001	ns	0.006	ns
odc	< 0.001	< 0.001	0.025	0.004	0.031	ns	ns
sat	ns	< 0.001	< 0.001	0.004	< 0.001	< 0.001	< 0.001

Table S5 B	AA									time		stimulus		AA × time																
														4 h									24 h								
	L-15	G1	G2	A1	A2	T1	T2	M1	M2	4 h	24 h	CTRL	vaLPS	L-15	G1	G2	A1	A2	T1	T2	M1	M2	L-15	G1	G2	A1	A2	T1	T2	M1	M2
il1β	b	ab	ab	ab	ab	ab	a	ab	ab	y	x	A	B																	
cox2	bc	cd	d	ab	a	cd	ab	cd	a	y	x	A	B																	
mif	ab	b	ab	ab	a	ab	ab	ab	a	x	y																			
tgfβ	ab	b	ab	a	a	ab	a	a	a	x	y			abc	c	bc	ab	ab	abc	ab	ab	a								
amd	b	a	ab	a	ab	a	a	a	ab			A	B	ab	a	b	ab	ab	ab	ab	a	ab x	b	a	a	a	ab	ab	ab	a	by
odc	d	a	ab	bcd	bcd	abc	cd	ab	abc	y	x	B	A	ab	ab	ab	b	ab	ab	b	ab	a	b	a	a	a	a	a	ab	a	a
sat										x	y	A	B	x	x	x	x	x	x	x	x	x	y	y	y	y	y	y	y	y	y

Table S5 C	time × stimulus				AA × stimulus																	
	4		24		CTRL									vaLPS								
	CTRL	vaLPS	CTRL	vaLPS	L-15	G1	G2	A1	A2	T1	T2	M1	M2	L-15	G1	G2	A1	A2	T1	T2	M1	M2
il1β	Ax	By	By	Ax	b	ab	ab	Aab	Aa	ab	ab	Aab	Aa				B	B			B	B
cox2																						
mif																						
tgfβ		x	A	By																		
amd	y		Ax	B																		
odc					Bb	a	a	a	a	a	a	a	a	Ab	a	a	ab	ab	a	ab	a	a
sat	x	x	Ay	By				A	A					abc	ab	a	Bbc	Bc	abc	a	bc	abc

Values represent means ± SD (n = 6). Different capital letters stand for statistically significant differences attributed to stimulation. a, b, c and d denote statistically significant differences between AA treatments. x and y stand for statistically significant differences attributed to incubation time. (Multifactorial ANOVA; Tukey post-hoc test; ns: non-significant; P ≤ 0.0

Appendix II

Neuroendocrine and immune responses to inflammation engaged different fates following tryptophan or methionine treatment: tales from a teleost model

Supplementary table 1. Hypothalamic monoamine content in juvenile European seabass fed dietary treatments for 14 days (0 h) and sampled at 4 h or 24 h post HBSS- or *Phdp*- i.p. injection.

Hypothalamic monoamine content									
			NA	DA	DOPAC	5-HT	5HIAA	DOPAC/DA	5HIAA/5HT
	0 h	CTRL	$^{44.5}_{ab}$ ± 7.0	8.2 ± 0.5	0.7 ± 0.4	39.9 ± 6.9	5.2 ± 1.7	8.5 ± 4.2	12.7 ± 2.8
		MET	56.1 b ± 12.3	10.6 ± 1.9	0.9 ± 0.4	49.2 ± 9.3	6.5 ± 2.2	8.2 ± 2.3	12.0 ± 1.8
		TRP	40.1 a ± 7.4	8.8 ± 1.4	0.8 ± 0.3	37.0 ± 10.6	5.9 ± 2.6	9.2 ± 1.8	15.8 ± 3.8
CTRL	HBSS	4 h	42.4 ± 10.9	9.6 ± 1.6	0.9 ± 0.3	42.0 ± 11.6	7.9 ± 1.9	9.4 ± 1.3	19.4 ± 3.6
		24 h	40.5 ± 7.3	8.6 ± 3.2	0.9 ± 0.4	44.3 ± 15.2	8.2 ± 3.7	12.6 ± 1.9	16.9 ± 2.9
	Phdp	4 h	38.2 ± 8.9	7.2 ± 2.4	0.7 ± 0.3	36.3 ± 11.2	6.3 ± 3.1	10.0 ± 3.8	17.7 ± 3.5
		24 h	43.2 ± 6.8	8.4 ± 2.6	0.8 ± 0.2	41.6 ± 7.8	7.9 ± 1.9	9.2 ± 0.2	19.4 ± 1.8
MET	HBSS	4 h	44.9 ± 8.9	8.0 ± 3.4	0.9 ± 0.5	40.8 ± 13.0	7.5 ± 2.8	13.1 ± 2.5	18.4 ± 2.4
		24 h	45.1 ± 13.4	9.1 ± 1.7	0.8 ± 0.4	43.2 ± 10.3	6.6 ± 1.5	8.7 ± 2.5	15.0 ± 0.9
	Phdp	4 h	46.7 ± 8.1	10.2 ± 1.6	0.9 ± 0.2	43.4 ± 6.3	7.3 ± 1.6	9.7 ± 0.9	18.0 ± 2.6
		24 h	46.7 ± 6.8	9.8 ± 1.9	1.0 ± 0.2	47.4 ± 8.9	8.3 ± 3.1	10.8 ± 1.0	15.9 ± 2.2
TRP	HBSS	4 h	51.1 ± 8.3	9.8 ± 1.5	1.2 ± 0.3	48.2 ± 4.7	9.1 ± 0.9	12.4 ± 1.3	19.1 ± 2.4
		24 h	41.6 ± 4.0	9.2 ± 1.1	0.9 ± 0.2	45.6 ± 6.2	8.0 ± 1.4	9.9 ± 0.6	16.6 ± 2.6
	Phdp	4 h	48.2 ± 9.6	9.2 ± 3.1	1.1 ± 0.6	43.5 ± 14.0	9.0 ± 3.9	10.3 ± 2.8	22.2 ± 4.1
		24 h	43.7 ± 5.4	8.8 ± 3.8	1.1 ± 0.4	45.9 ± 11.1	8.0 ± 1.3	11.3 ± 3.2	16.6 ± 1.5

Multifactorial ANOVA	**P-value**							Time	
	time	diet	stimulus	time × diet	time × stimuli	diet × stimuli	diet × stimuli × time	4 h	24 h
NA	NS	NS	NS	NS	NS	NS	NS		
DA	NS	NS	NS	NS	NS	NS	NS		
DOPAC	NS	NS	NS	NS	NS	NS	NS		
5-HT	NS	NS	NS	NS	NS	NS	NS		
5HIAA	NS	NS	NS	NS	NS	NS	NS		
DOPAC/DA ratio	NS	NS	NS	NS	NS	NS	NS		
5HIAA/5HT ratio	0.001	NS	NS	NS	NS	NS	NS	y	x

One-way ANOVA	**P-value**
	diet
NA	0.025
DA	NS
DOPAC	NS
5-HT	NS
5HIAA	NS
DOPAC/DA ratio	NS
5HIAA/5HT ratio	NS

Values are expressed as means ± standard error (n=6). Noradrenaline (NA), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA). x and y identify significant differences between sampling time. a and b stand for significant differences between dietary treatments. One-way ANOVA

(before i.p. injection) and Multifactorial ANOVA (after i.p. injection); Tukey post hoc test; p≤0.05).

Supplementary table 2. Telencephalic monoamine content in juvenile European seabass fed dietary treatments for 14 days (0 h) and sampled at 4 h or 24 h post HBSS- or *Phdp*-i.p. injection.

			Telencephalic monoamine content						
			NA	DA	DOPAC	5-HT	5HIAA	DOPAC/DA	5HIAA/5HT
	0 h	CTRL	27.1 ± 1.5	3.3 ± 0.2	0.5 ± 0.3	42.5 ± 6.1	13.0 ± 1.5	14.9 ± 9.4	30.9 ± 4.5
		MET	25.2 ± 10.0	2.3 ± 1.6	0.4 ± 0.3	31.8 ± 17.7	10.6 ± 5.7	16.3 ± 10.6	33.4 ± 5.3
		TRP	26.6 ± 3.1	3.5 ± 1.2	0.5 ± 0.3	50.0 ± 14.9	17.0 ± 4.0	14.2 ± 4.7	34.9 ± 5.3
CTRL	HBSS	4 h	29.5 ± 3.7	4.1 y ± 0.9	0.6 ± 0.2	49.3 ± 9.8	18.5 ± 3.8	15.8 ± 3.7	37.8 ± 5.0
		24 h	26.9 ± 4.4	2.6 x ± 0.3	0.5 ± 0.2	44.6 ± 8.2	16.3 ± 3.2	18.0 ± 1.5	36.5 ± 1.9
	Phdp	4 h	27.0 ± 3.7	3.1 ± 0.3	0.4 ± 0.1	38.4 ± 9.3	14.3 ± 4.3	14.2 ± 3.6	37.0 ± 6.0
		24 h	27.8 ± 3.8	3.8 ± 1.1	0.4 ± 0.1	47.5 ± 7.7	16.9 ± 3.0	11.6 ± 2.3	35.5 ± 1.5
MET	HBSS	4 h	28.3 ± 4.5	4.1 ± 0.8	0.6 ± 0.2	45.2 ± 14.3	16.4 ± 4.9	13.4 ± 4.1	36.6 ± 5.2
		24 h	27.3 ± 5.7	3.2 ± 0.9	0.5 ± 0.1	41.3 ± 8.3	15.5 ± 3.6	13.2 ± 2.7	37.4 ± 3.4
	Phdp	4 h	26.5 ± 6.1	3.3 ± 0.7	0.4 ± 0.1	42.8 ± 12.0	15.0 ± 4.6	14.7 ± 5.9	34.9 ± 1.9
		24 h	28.2 ± 4.6	2.8 ± 0.8	0.3 ± 0.0	41.5 ± 5.1	14.7 ± 2.9	13.4 ± 3.8	35.3 ± 3.9
TRP	HBSS	4 h	26.1 ± 2.7	3.8 ± 0.4	0.5 ± 0.2	53.9 ± 9.8	19.0 ± 4.2	13.7 ± 3.0	35.1 ± 4.0
		24 h	26.1 ± 5.4	3.1 ± 0.3	0.5 ± 0.1	50.0 ± 10.6	17.3 ± 1.0	16.5 ± 2.8	34.3 ± 4.2
	Phdp	4 h	26.5 ± 4.1	3.3 ± 0.4	0.6 ± 0.3	48.5 ± 8.2	18.8 ± 4.6	19.7 ± 9.0	38.3 ± 4.4
		24 h	27.1 ± 4.6	2.8 ± 0.4	0.4 ± 0.0	48.1 ± 10.1	18.9 ± 0.8	17.6 ± 3.3	37.1 ± 6.1

																							diet × stimuli		
Multifactorial ANOVA	**P-value**							time		diet			stimuli		HBSS			*Phdp*							
	time	diet	stimulus	time × diet	time × stimuli	diet × stimuli	diet × stimuli × time	4 h	24 h	CTRL	MET	TRP	HBSS	*Phdp*	CTRL	MET	TRP	CTRL	MET	TRP					
NA	NS	NS	NS	NS	NS	NS	NS																		
DA	0.001	NS	NS	NS	0.005	NS	0.023																		
DOPAC	0.04	NS	0.01	NS	NS	NS	NS	y	x				B	A											
5-HT	NS	0.03	NS	NS	NS	NS	NS			ab	a	b													
5HIAA	NS	0.03	NS	NS	NS	NS	NS			ab	a	b													
DOPAC/DA ratio	NS	0.04	NS	NS	NS	0.024	NS														a	ab	b		
5HIAA/5HT ratio	NS	NS	NS	NS	NS	NS	NS																		

One-way ANOVA	**P-value**
	Diet
NA	NS
DA	NS
DOPAC	NS
5-HT	NS
5HIAA	NS
DOPAC/DA ratio	NS
5HIAA/5HT ratio	NS

Values are expressed as means ± standard error (n=6). Noradrenaline (NA), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA). x and y identify significant differences between sampling times. a and b stand for significant differences attributed to dietary treatments. A and B identify significant differences between stimuli. One-way ANOVA (before i.p. injection) and Multifactorial ANOVA (after i.p. injection); Tukey post hoc test; p≤0.05).

Supplementary table 3. Optic tectum monoamine content in juvenile European seabass fed dietary treatments for 14 days (0 h) and sampled at 4 h or 24 h post HBSS- or *Phdp*- i.p. injection.

Optic tectum monoamine content									
			NA	DA	DOPAC	5-HT	5HIAA	DOPAC/DA	5HIAA/5HT
	0 h	CTRL	22.6 ± 8.5	2.7 ± 0.9	1.1 ± 0.3	19.9 ± 5.4	3.9 ± 1.1	42.7 ± 9.3	19.2 ± 1.4
		MET	22.8 ± 3.5	2.9 ± 0.7	1.3 ± 0.5	24.5 ± 7.2	5.0 ± 1.6	43.7 ± 12.7	20.5 ± 3.3
		TRP	20.7 ± 3.6	2.5 ± 0.5	1.0 ± 0.3	23.4 ± 5.2	5.2 ± 1.1	42.0 ± 17.6	23.0 ± 6.0
CTRL	HBSS	4 h	23.3 ± 3.8	2.3 ± 0.3	1.3 ± 0.4	25.7 ± 4.8	6.0 ± 0.7	51.8 ± 12.3	23.9 ± 3.2
		24 h	21.5 ± 4.8	2.7 ± 0.6	1.4 ± 0.3	24.0 ± 4.9	5.7 ± 1.0	47.7 ± 9.6	24.0 ± 5.0
	Phdp	4 h	21.7 ± 5.3	2.2 ± 0.3	1.1 ± 0.1	18.9 ± 3.4	5.1 ± 1.2	46.0 ± 6.5	27.0 ± 4.4
		24 h	21.6 ± 6.2	2.6 ± 0.8	1.0 ± 0.2	22.8 ± 6.3	5.9 ± 1.2	42.0 ± 3.8	26.6 ± 4.1
MET	HBSS	4 h	20.3 ± 4.2	2.5 ± 0.3	1.3 ± 0.2	22.3 ± 6.8	5.3 ± 1.1	54.0 ± 14.1	24.8 ± 5.5
		24 h	20.9 ± 3.6	2.7 ± 0.4	1.3 ± 0.1	20.7 ± 2.4	5.9 ± 1.0	51.8 ± 13.2	28.8 ± 6.2
	Phdp	4 h	19.5 ± 4.9	2.2 ± 0.2	1.2 ± 0.4	18.9 ± 7.9	4.4 ± 0.7	54.3 ± 14.6	25.4 ± 8.4
		24 h	20.8 ± 4.6	2.6 ± 0.2	1.4 ± 0.2	21.2 ± 2.3	5.8 ± 0.9	52.4 ± 5.1	27.3 ± 4.4
TRP	HBSS	4 h	21.1 ± 2.8	2.3 ± 0.2	1.1 ± 0.3	22.9 ± 2.8	5.8 ± 1.0	46.4 ± 10.0	25.2 ± 4.3
		24 h	20.6 ± 1.9	2.2 ± 0.6	1.3 ± 0.2	23.3 ± 3.5	5.6 ± 0.8	51.7 ± 7.5	24.6 ± 4.8
	Phdp	4 h	23.0 ± 3.1	3.0 ± 0.4	1.5 ± 0.1	27.5 ± 5.1	6.6 ± 0.9	48.2 ± 8.1	24.2 ± 3.2
		24 h	22.2 ± 3.4	2.9 ± 0.3	1.5 ± 0.2	26.0 ± 4.9	5.7 ± 1.2	50.8 ± 6.7	22.0 ± 1.3

								time × diet						diet × stimuli					
Multifactorial ANOVA	**P-value**							4 h			24 h			HBSS			*Phdp*		
	time	diet	stimulus	time × diet	time × stimuli	diet × stimuli	diet × stimuli × time	CTRL	MET	TRP	CTRL	MET	TRP	CTRL	MET	TRP	CTRL	MET	TRP
NA	NS	NS	NS	NS	NS	NS	NS												
DA	NS	NS	NS	NS	NS	0.010	NS									A			B
DOPAC	NS	NS	NS	NS	NS	0.001	NS							B			Aa	ab	b
5-HT	NS	0.017	NS	NS	NS	0.029	NS										a	a	b
5HIAA	NS	NS	NS	0.047	NS	NS	NS	ab	a	b									
DOPAC/DA ratio	NS	NS	NS	NS	NS	NS	NS												
5HIAA/5HT ratio	NS	NS	NS	NS	NS	NS	NS												

One-way ANOVA	P-value
	Diet
NA	NS
DA	NS
DOPAC	NS
5-HT	NS
5HIAA	NS
DOPAC/DA ratio	NS
5HIAA/5HT ratio	NS

Values are expressed as means ± standard error (n=6). Noradrenaline (NA), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA). x and y identify significant differences attributed to sampling time. a and b stand for significant differences between dietary treatments. A and B identify significant differences attributed to stimuli. One-way ANOVA (before i.p. injection) and Multifactorial ANOVA (after i.p. injection); Tukey post hoc test; p≤0.05).

Supplementary table 4. Liver total antioxidant capacity in juvenile European seabass fed dietary treatments for 14 days (0 h) and sampled at 4 h or 24 h post HBSS- or *Phdp*- i.p. injection.

			Total antioxidant capacity (mM mg^{-1} protein)
	0 h	CTRL	5.9 ± 2.5
		MET	4.1 ± 2.1
		TRP	3.1 ± 0.9
CTRL	HBSS	4 h	3.5 ± 0.5
		24 h	7.4 ± 5.1
	Phdp	4 h	3.3 ± 0.9
		24 h	11.4 ± 5.9
MET	HBSS	4 h	3.5 ± 0.2
		24 h	5.2 ± 3.3
	Phdp	4 h	4.5 ± 0.6
		24 h	5.0 ± 2.5
TRP	HBSS	4 h	4.0 ± 1.1
		24 h	7.1 ± 4.0
	Phdp	4 h	3.3 ± 0.7
		24 h	5.7 ± 1.5

Multifactorial ANOVA	P-value							time	
	time	diet	stimulus	time × diet	time × stimuli	diet × stimuli	diet × stimuli × time	4 h	24 h
Total antioxidant capacity (mM mg^{-1} protein)	0.002	NS	NS	NS	NS	NS	NS	x	y

One-way ANOVA	P-value
	Diet
Total antioxidant capacity (mM mg^{-1} protein)	NS

Values are expressed as means ± standard error (n=6). x and y stand for significant differences between sampling times. One-way ANOVA (before i.p. injection) and Multifactorial ANOVA (after i.p. injection); Tukey post hoc test; p≤0.05).

Supplementary table 5. Relative neuroendocrine- and immune-related gene expression in juvenile European seabass blood and head-kidney fed dietary treatments for 14 days (0 h) and sampled at 4 h or 24 h post HBSS- or *Phdp*- i.p. injection.

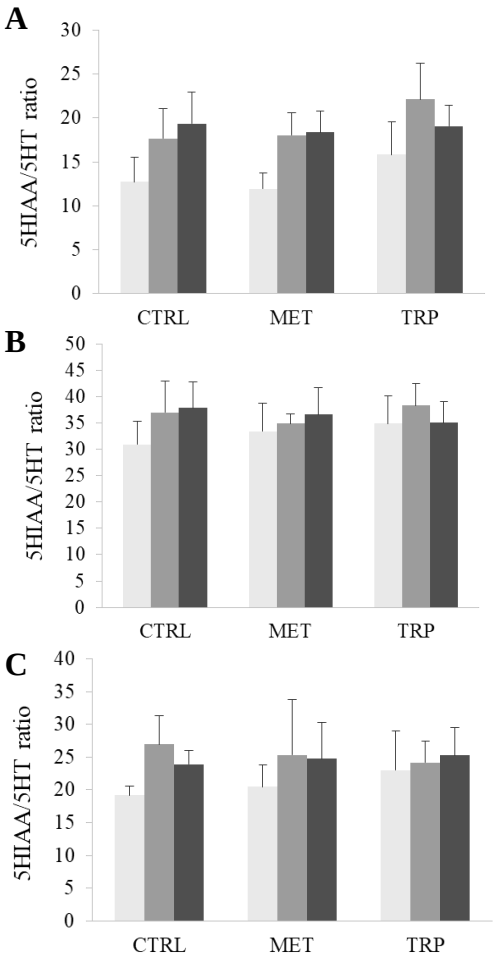
			Blood	Head-kidney				
			mmp9	*gpx*	*mc2r*	*gr*	*hep*	*il1β*
	0 h	CTRL	1.0±0.9	0.5 ± 1.0	0.8 ± 0.3	0.8 ± 0.2	1.0 ± 0.6	1.0 ± 0.7
		MET	1.1±0.2	15.2 ± 10.9	0.9 ± 0.2	0.7 ± 0.3	1.7 ± 1.1	1.9 ± 1.8
		TRP	0.9±0.7	369.1 ± 150.6	0.8 ± 0.2	0.8 ± 0.2	1.4 ± 1.0	1.1 ± 0.8
CTRL	HBSS	4 h	2.3±1.9	2968.8 ± 2153.6	0.5 ± 0.3	0.3 ± 0.3	3.8 ± 3.9	2.9 ± 2.4
		24 h	3.5±1.0	403.7 ± 346.5	0.2 ± 0.3	0.1 ± 0.2	1.9 ± 0.8	0.7 ± 0.5
	Phdp	4 h	3.4±1.7	3069.3 ± 2766.1	1.2 ± 1.0	0.9 ± 0.2	2.8 ± 3.0	1.7 ± 1.2
		24 h	3.7±1.7	2204.3 ± 2307.8	0.3 ± 0.3	0.2 ± 0.3	2.1 ± 1.1	2.5 ± 1.6
MET	HBSS	4 h	3.5±0.6	2531.3 ± 2093.9	0.6 ± 0.7	0.9 ± 1.0	2.5 ± 1.5	2.1 ± 2.0
		24 h	1.6±0.1	2544.2 ± 1735.3	0.2 ± 0.4	0.3 ± 0.4	1.2 ± 0.7	0.7 ± 0.2
	Phdp	4 h	2.7±1.1	666.9 ± 642.1	0.9 ± 0.4	1.1 ± 1.0	2.3 ± 0.2	1.4 ± 0.5
		24 h	1.7±0.7	3477.3 ± 2738.3	0.6 ± 0.6	0.5 ± 0.6	2.6 ± 2.6	1.4 ± 0.8
TRP	HBSS	4 h	1.8±0.3	1274.6 ± 1480.0	0.8 ± 0.1	0.5 ± 0.1	2.0 ± 1.2	1.3 ± 0.6
		24 h	3.8±1.3	3954.5 ± 2192.4	0.6 ± 0.9	0.5 ± 0.7	3.0 ± 3.1	2.6 ± 1.8
	Phdp	4 h	1.6±0.3	3183.4 ± 3052.3	1.1 ± 0.6	0.8 ± 0.4	3.5 ± 3.1	1.6 ± 0.9
		24 h	2.3±3.4	1130.5 ± 1086.5	0.4 ± 0.5	0.3 ± 0.3	3.0 ± 1.5	2.1 ± 1.4

	P-value							time	
Multifactorial ANOVA	time	diet	stimulus	time × diet	time × stimuli	diet × stimuli	diet × stimuli × time	4 h	24 h
mmp9	NS	NS	NS	NS	NS	NS	NS		
mc2r	0.006	NS	NS	NS	NS	NS	NS	y	x
gr	0.003	NS	NS	NS	NS	NS	NS	y	x
hep	NS	NS	NS	NS	NS	NS	NS		
il1β	NS	NS	NS	NS	NS	NS	NS		

One-way ANOVA		P-value
		Diet
Blood	*mmp9*	NS
Head-kidney	*mc2r*	NS
	gr	NS
	hep	NS
	il1β	NS

Values are expressed as means ± standard error (n=6). Metalloproteinase 9 (*mmp9*), melanocortin receptor 2 (*mc2r*), glucocorticoid receptor (*gr*), hepcidin (*hep*) and interleukin 1β (*il1β*)**.** x and y stand for significant differences between sampling times. One-way ANOVA (before i.p. injection) and Multifactorial ANOVA (after i.p. injection); Tukey post hoc test; p≤0.05).

Supplementary figure 1. Brain 5HIAA/5HT ratio in European seabass hypothalamus **(A)**, telencephalon **(B)** and optic tectum **(C)** fed different dietary treatments for 14 days (□) and sampled 4 h post i.p. injection with *Phdp* (■) or HBSS (■). Data are mean ± SD (n=6).



Appendix III

European seabass (*Dicentrarchus labrax*) immune status and disease resistance are impaired by arginine dietary supplementation

Table 1. Characteristics of new assembled sequences of European seabass according to BLAST searches.

Contig	Size (nt)	Annotation[a]	Best match[b]	E[c]	CDS[d]	GenBank
L12_87226	2192	*ASL*	XP_004550300	0.0	154-1551	KM225766
L12_83771	1616	*ASS*	XP_003445281	0.0	<1-1242	KM225767
L1_28894	366	*ARG2*	BAF46063	7e-64	1->366	KM225768
L1_75798	1283	*GATM*	XP_004066965	5e-159	<1-689	KM225769
L12_83188	1663	*AMD1*	XP_007556077	0.0	284-1294	KM225770
L1_53546	811	*ODC1*	XP_004539948	5e-90	333->811	KM225771
L1_63507	918	*SAT1*	XP_003456292	3e-36	696->918	KM225772
L12_86945	2132	*SMOX*	XP_006787188	0.0	26-1741	KM225773
L12_75839	1285	*NOA1*	XP_004571208	0.0	<1-1115	KM225774
L1_44713	512	*NOXIN*	XP_006779966	3e-63	135->512	KM225775
L12_77157	1336	*NOSIP*	XP_005464991	3e-145	119-1033	KM225776
L2_36836	616	*IL-8*	AGR27883	2e-59	98-415	KM225777
L2_54923	496	*IL-20*	XM_004545365	6e-73	<1-456	KM225779
L2_69424	989	*IL-34*	BAM36385	6e-111	<1-512	KM225780
L12_90140	4399	*CCR3*	XP_005460244	4e-147	943-1971	KM225781
L12_82260	1585	*CCR11*	CBN82022	0.0	190-1293	KM225782
L12_77739	1356	*CD247*	XP_005457870	2e-29	290-685	KM225783
L12_73038	1123	*CD8b*	CBN81109	7e-133	116-754	KM225784
L12_83581	1697	*MyD88*	ADM25313	0.0	144-1016	KM225785
L3_76602	534	*CD33*	ACQ58565	8e-79	<1->534	KM225786
L12_87296	2199	*CSF1R*	CAJ18352	2e-164	<1-819	KM225787
L12_88792	2641	*MMD*	XP_005739998	3e-157	1418-2149	KM225788
L12_87913	2359	*IRF8*	AHB59740	0.0	127-1395	KM225789
L12_87846	2214	*NFKB2*	ABP35928	1e-75	<1-504	KM225790

[a]Gene identity determined through BLAST searches:
[b]Best BLAST-X protein sequence match (lowest E value): *ASL*, Argininosuccinate lyase; *ASS*, Argininosuccinate synthase; *ARG2*, Arginase-2 mitochondrial; *GATM*, Glycine amidinotransferase, mitochondrial; *AMD1*, S-adenosylmethionine decarboxylase; *ODC1*, Ornithine decarboxylase; *SAT1*, Diamine acetyltransferase 1; *SMOX*, Spermine oxidase; *NOA1*, Nitric oxide-associated protein 1; *NOXIN*, Nitric oxide-inducible gene protein; *NOSIP*, Nitric oxide synthase-interacting protein; *IL-8*, Interleukin 8; *IL-20*, Interleukin 20; *IL-34*, Interleukin 34; *CCR3*, C-C chemokine receptor type 3; *CCR11*, Atypical chemokine receptor 4; *CD247*, T-cell surface glycoprotein *CD3* zeta chain; *CD8b*, T-cell surface glycoprotein CD8 beta; *MyD88*, Myeloid differentiation primary response protein MyD88; *CD33*, Myeloid cell surface antigen CD33; *CSF1R*, Macrophage colony-stimulating factor 1 receptor; *MMD*, Monocyte to macrophage differentiation factor; *IRF8*, Interferon regulatory factor 8; *NFKB2*, Nuclear factor NF-kappa-B p100 subunit.
[c]Expectation value.
[d]Codifying sequence.

Table 2. Forward and reverse primers for real-time PCR.

Gene name	Symbol	GenBank	*Eff*[1]	MT[2]	Product length[3]	Primer sequence
Argininosuccinate lyase	*ASL*	KM225766	94.3	79.2	54	F TGA GGC TGT GGC TGC GAG ATG C R GCT GTA GGG AAT TGT TCG TCA GGG TTG AGA
Argininosuccinate synthase	*ASS*	KM225767	97.3	79.1	52	F GCC AAT GCT GTG TAT GAG GAC CGA TA R GCC GTG CTA CCG CAG TTC C
Arginase-2, mitocondrial	*ARG2*	KM225768	88.5	81	79	F GAG CAC GGT CCT AAA GTC AT R CAA AGT CGT GGA CAG AGT AGT
Glycine amidinotransferase, mitochondrial	*GATM*	KM225769	96.5	78.5	74	F GGA ATT GAG TGG ATG CGT CGT CAT R GGG TTA GGG TCC TTG AAT GAG ATT ATG TG
S-adenosylmethionine decarboxylase	*AMD1*	KM225770	99.3	79.1	63	F CTG ACG GAA CTT ACT GGA CCA TC R CGA AGC TGA CGT AGG AGA ACT C
Ornithine decarboxylase	*ODC1*	KM225771	99.9	78.8	69	F GGG CTG TAG TTA TGA CAC TGG CAT CC R GCT GAA TCT CCA TCT TGC TTG CAC AGT
Diamine acetyltransferase 1	*SAT1*	KM225772	91.6	78.2	55	F GCA TCA TCG CTG AAA TCC AAG GAG AGA ACA R CCA ACC ACC TTC AGG CCG TCA CT
Spermine oxidase	*SMOX*	KM225773	95.6	80	57	F CAC GGC TGC CAA CCT CTG AT R CTC GTC CTC GCA CTC CAC ATA AAT G
Nitric oxide-associated protein 1	*NOA1*	KM225774	92.9	81.1	62	F CCA TCC ACA AAG CCA CCA TAT CG R GGG AAA CTT CAG CAG GTT CAG AG
Nitric oxide-inducible gene protein	*NOXIN*	KM225775	94.1	79.1	72	F AGA GGT TGG TGG AGA ACT TGG ATG GA R CGA CAG CCT TCA TCA ACA ATG TGG ATC T

Table 2. Continued.

Gene name	Symbol	GenBank	*Eff*[1]	MT[2]	Product length[3]	Primer sequence
Nitric oxide synthase-interacting protein	*NOSIP*	KM225776	97.1	78.9	66	F GTC CAA GCC CAT CAA CCC ATT CAC R CTG TCT GTC CTG CTC TTC TCA CCT C
Interleukin 1-β	*IL-1β*	AJ311925	91.4	79.2	73	F CAT GAG CGA GAT GTG GAG ATC CAA GAT R CAT TGT CAG TGG GTG GTG GGT AAT C
Interleukin 8	*IL-8*	KM225777	92.4	79	70	F CAA TCA GCA GGG ACT ACA ACA CAC A R CTG TCT GGA GGG ATG ATC CTT GAC T
Interleukin 10	*IL-10*	DQ821114	92.4	78.8	67	F CAG TGC TGT CGT TTT GTG GAG GGT TTC R TCT CTG TGA AGT CTG CTC TGA GTT GCC TTA
Interleukin 20	*IL-20*	KM225779	99.6	79	60	F GCT AGA AAT AAA GGA GGC GGC ACA GAA GG R CAG TCC AGC ACA GTG TCC AGT TCT C
Interleukin 34	*IL-34*	KM225780	97.5	79.3	58	F AGA ACC CGA CAG AGT GCC AGA GT R CAG GAG GGA TTT TGG GGA CGC ATA TC
Tumour necrosis factor-α	*TNF-α*	DQ070246	95.5	80.2	57	F TCT ACA GCC AGG CGT CGT TCA G R CCG CAC TTT CCT CTT CAC CAT CGT
C-C chemokine receptor type 3	*CCR3*	KM225781	94.1	81.1	88	F TGA CCT TCG ACC GAC ACC TA R ACA ATA CAG GAG ACT ACC GCA TAG C
C-C chemokine receptor type 9	*CCR9*	FN665390	94.7	78.4	69	F CCT GTG TGT CTG GCT TGT TTC TAC TCT C R TCG CTC TTC ACC TGG GCA AAG ATA AAC TC
Atypical chemokine receptor 4	*CCR11*	KM225782	94.8	81	67	F TAC TTC TCT TCA CCC TGC CTT TCT G R GCT GCC GAA CCC AAC TTC CA

Table 2. Continued.

Gene name	Symbol	GenBank	*Eff*[1]	MT[2]	Product length[3]	Primer sequence
T-cell surface glycoprotein CD3 zeta chain	*CD247*	KM225783	99.8	78.6	81	F CTG ATG CGT CTG AAG AGA ATG GAG GC R GTT CAA GCA CCT GGT AAG GAT CAG CAT C
T-cell surface glycoprotein CD8 beta	*CD8b*	KM225784	93.9	79.4	77	F AGT GAT CCC GCC AAC ATT ACC TCC TA R TCT TCT TAG GGC AGC GAC AGA CT
Myeloid differentiation primary response protein MyD88	*MyD88*	KM225785	89.9	79.2	71	F CCA ATT CAG GTT GAT GAG GTT GAC A R TCC TCC AGG GTG ATA CCA ATC C
Myeloid cell surface antigen CD33	*CD33*	KM225786	97.2	80.3	70	F CTG TTC ATT CAC CCA TCC TAG AG R GGT CGA ACG ATG CCA GAT T
Macrophage colony-stimulating factor 1 receptor	*CSF1R*	KM225787	92.1	80	74	F CGG GCA GGA ACA GCT AAT CTA CCA R ACT TGG GCT CAT CAC ACA CTT CAC
Macrophage migration inhibitory factor	*MIF*	FN582353	89.3	78.8	76	F GCT CCC TCC ACA GTA TTG GCA AGA T R TTG AGC AGT CCA CAC AGG AGT TTA GAG T
Monocyte to macrophage differentiation factor	*MMD*	KM225788	88.9	77.4	59	F GGT CAT CTA CTT CTT CAT CGC TGC CTC CTA R CCA ACT CTC GCA GGT TCA ACC AAG GT
Interferon regulatory factor 8	*IRF8*	KM225789	96.4	79.9	65	F TCT GAA GGC TGC CGA ATC TCC R CTG TCT GAA CTG TAT AGG GCA CCA C
Nuclear factor NF-kappa-B p100 subunit	*NFKB2*	KM225790	92.5	78.8	55	F CTG GAG GAA ACT GGC GGA GAA GC R CAG GTA CAG GTG AGT CAG CGT CAT C
ß-Actin	*ACTB*	AY148350	96.4	76.9	51	F TCC TGC GGA ATC CAC GAG A R AAC GTC GCA CTT CAT GAT GCT

[1] Efficiency of PCR reactions were calculated from serial dilutions of tissue RT reactions in the validation procedure.
[2] Melting temperature
[3] Amplicon (nt)

Table 3. Relative expression of genes involved in the immune response or arginine metabolism in the European seabass spleen after 29 days of feeding trial. Different letters denote significant differences between dietary treatments (One-way ANOVA; P < 0.05)

Genes	Diets			One-way ANOVA
	CTRL	Arg1	Arg2	
ASL	0.85 ± 0.056	1.10 ± 0.17	0.83 ± 0.077	0.21
ASS	0.15 ± 0.02	0.18 ± 0.03	0.14 ± 0.02	0.33
ARG2	0.19 ± 0.03	0.22 ± 0.03	0.24 ± 0.03	0.53
GATM	0.18 ± 0.03 ^{a}	0.70 ± 0.19 ^{b}	0.11 ± 0.01 ^{a}	0.00
AMD1	7.23 ± 0.84 ^{a}	5.92 ± 0.61 ^{ab}	4.5 ± 0.37 ^{b}	0.01
ODC1	0.5 ± 0.08	0.57 ± 0.08	0.49 ± 0.06	0.66
SAT1	2.62 ± 0.33	2.8 ± 0.37	2.65 ± 0.46	0.95
SMOX	0.3 ± 0.043 ^{a}	0.6 ± 0.07 ^{b}	0.33 ± 0.04 ^{a}	0.00
NOA1	1.04 ± 0.04	1.29 ± 0.13	1.05 ± 0.12	0.21
NOXIN	0.25 ± 0.03	0.32 ± 0.07	0.26 ± 0.03	0.63
NOSIP	5.34 ± 0.44	5.15 ± 0.46	4.54 ± 0.5	0.46
IL1β	0.04 ± 0.01	0.08 ± 0.01	0.06 ± 0.01	0.11
IL-8	0.17 ± 0.06	0.3 ± 0.05	0.14 ± 0.03	0.23
IL-10	1.02 ± 0.08 ^{a}	1.52 ± 0.11 ^{b}	0.63 ± 0.06 ^{c}	0.00
IL-20	0.63 ± 0.06 ^{a}	0.6 ± 0.09 ^{a}	0.38 ± 0.02 ^{b}	0.03
IL-34	11.76 ± 1.85	12.88 ± 1.45	8.93 ± 1.47	0.25
TNF-α	0.80 ± 0.11	0.72 ± 0.13	0.54 ± 0.04	0.23
CCR3	0.89 ± 0.10 ^{a}	1.56 ± 1.37 ^{b}	1.64 ± 0.13 ^{b}	0.00
CCR9	1.29 ± 0.19	1.50 ± 0.26	1.25 ± 0.22	0.71
ACKR4	3.49 ± 0.26 ^{a}	3.64 ± 0.34 ^{a}	2.40 ± 0.25 ^{b}	0.01
CD247	3.27 ± 0.32	3.61 ± 0.36	2.78 ± 0.24	0.18
CD8b	0.4 ± 0.05	0.38 ± 0.03	0.33 ± 0.04	0.40
MyD88	2.07 ± 0.15	2.53 ± 0.15	2 ± 0.19	0.08
CD33	45.17 ± 5.34 ^{a}	69.77 ± 2.04 ^{b}	40.92 ± 2.94 ^{a}	0.00
CSF1R	6.91 ± 1.1	6.68 ± 0.64	5.71 ± 0.67	0.54
MIF	2.5 ± 0.18	2.94 ± 0.29	2.81 ± 0.42	0.65
MMD	0.65 ± 0.04	0.67 ± 0.06	0.63 ± 0.05	0.84
IRF8	3.9 ± 0.45	4.21 ± 0.53	3.82 ± 0.43	0.84
NFKB2	10.46 ± 0.53	11.27 ± 1.25	11.01 ± 1.33	0.88

Table 4. Relative expression of genes involved in the immune response or arginine metabolism in the European seabass anterior intestine after 29 days of feeding trial. Different letters denote significant differences between dietary treatments (One-way ANOVA; P < 0.05)

Genes	Diets			One-way ANOVA
	CTRL	Arg1	Arg2	
ASL	2.26 ± 0.27	2.48 ± 0.33	1.81 ± 0.24	0.25
ASS	0.48 ± 0.05	0.54 ± 0.02	0.51 ± 0.07	0.81
ARG2	153.10 ± 16.55 ^{a}	58.02 ± 5.71 ^{b}	168.01 ± 16.81 ^{a}	0.00
GATM	446.84 ± 99.70	526.85 ± 117.12	334.53 ± 59.24	0.33
AMD1	15.84 ± 1.83	16.76 ± 0.97	17.99 ± 1.51	0.59
ODC1	2.48 ± 0.46	2.72 ± 0.77	2.18 ± 0.21	0.75
SAT1	7.43 ± 0.79	9.54 ± 1.00	7.84 ± 0.59	0.19
SMOX	9.48 ± 2.46	8.52 ± 2.16	7.56 ± 0.74	0.74
NOA1	6.66 ± 0.55	6.31 ± 0.66	6.35 ± 0.36	0.88
NOXIN	0.85 ± 0.07	0.71 ± 0.06	0.82 ± 0.05	0.25
NOSIP	27.49 ± 2.69	26.90 ± 3.44	24.65 ± 1.07	0.72
IL-1β	0.25 ± 0.10	0.24 ± 0.03	0.21 ± 0.03	0.86
IL-8	0.61 ± 0.12	0.67 ± 0.22	0.76 ± 0.11	0.82
IL-10	1.03 ± 0.13 ^{a}	0.85 ± 0.08 ^{ab}	0.64 ± 0.03 ^{b}	0.01
IL-20	0.84 ± 0.09	0.98 ± 0.15	0.74 ± 0.09	0.3
IL-34	2.97 ± 0.12 ^{a}	2.69 ± 0.10 ^{ab}	2.41 ± 0.17 ^{b}	0.04
TNF-α	2.68 ± 0.27	2.76 ± 0.26	2.24 ± 0.11	0.21
CCR3	7.93 ± 1.17	7.44 ± 1.08	7.02 ± 0.30	0.77
CCR9	7.80 ± 0.64	7.57 ± 0.89	7.07 ± 0.89	0.83
ACKR4	43.22 ± 3.18 ^{ab}	50.03 ± 5.84 ^{a}	30.84 ± 1.06 ^{b}	0.01
CD247	14.31 ± 0.46 ^{a}	16.86 ± 1.90 ^{a}	10.74 ± 0.32 ^{b}	0.00
CD8b	1.33 ± 0.08	1.02 ± 0.09	1.07 ± 0.12	0.10
MyD88	16.89 ± 3.09	15.18 ± 1.03	14.24 ± 0.66	0.59
CD33	7.34 ± 0.80 ^{a}	7.15 ± 0.60 ^{a}	5.03 ± 0.47 ^{b}	0.03
CSF1R	6.50 ± 1.11	6.09 ± 0.61	5.42 ± 0.38	0.57
MIF	57.34 ± 6.70	52.65 ± 5.30	59.67 ± 4.69	0.69
MMD	49 ± 5.51	52.47 ± 4.63	46.10 ± 3.62	0.62
IRF8	11.60 ± 1.98	11.48 ± 1.30	10.16 ± 0.60	0.7
NFKB2	24.98 ± 3.07	32.36 ± 1.63	27. 98 ± 2.11	0.12

Table 5. Relative expression of genes involved in the immune response or arginine metabolism in the European seabass posterior intestine after 29 days of feeding trial. Different letters denote significant differences between dietary treatments (One-way ANOVA; P < 0.05)

Genes	Diets			One-way ANOVA
	CTRL	Arg1	Arg2	
ASL	6.13 ± 0.79	6.40 ± 0.66	7.44 ± 1.79	0.76
ASS	1.52 ± 0.16	1.65 ± 0.21	1.46 ± 0.18	0.76
ARG2	88.79 ± 4.26 [a]	36.75 ± 9.06 [b]	46.74 ± 5.18 [b]	0.00
GATM	10.97 ± 4.80	76.46 ± 32.35	41.82 ± 21.13	0.26
AMD1	36.00 ± 2.41	35.76 ± 4.16	34.73 ± 3.47	0.96
ODC1	2.51 ± 0.49	2.48 ± 0.29	2.58 ± 0.23	0.98
SAT1	14.80 ± 2.39	17. 57 ± 2.33	14.65 ± 0.95	0.51
SMOX	22.33 ± 3.67	27.62 ± 6.82	22.48 ± 5.49	0.75
NOA1	9.06 ± 0.99	9.53 ± 1.23	9.36 ± 0.79	0.95
NOXIN	1.67 ± 0.24	1.50 ± 0.19	1.54 ± 0.10	0.78
NOSIP	41.45 ± 5.84	43.93 ± 5.46	38.90 ± 2.90	0.76
IL-1β	0.33 ± 0.09	0.33 ± 0.04	0.37 ± 0.05	0.85
IL-8	1.32 ± 0.28	1.17 ± 0.41	1.46 ± 0.42	0.87
IL-10	1.11 ± 0.22	1.73 ± 0.27	1.22 ± 0.09	0.09
IL-20	1.57 ± 0.30	1.70 ± 0.19	1.29 ± 0.11	0.43
IL-34	7.41 ± 0.97	7.21 ± 0.70	6.02 ± 0.89	0.48
TNF-α	4.09 ± 0.82	4.24 ± 0.60	4.02 ± 0.42	0.97
CCR3	15.38 ± 1.97	15.07 ± 2.38	12.67 ± 0.95	0.51
CCR9	25.58 ± 3.30	37.18 ± 6.25	22.59 ± 3.10	0.06
ACKR4	62.32 ± 7.72	73.59 ± 11.05	51.16 ± 4.28	0.13
CD247	29.12 ± 2.76 [a]	30.27 ± 1.56 [a]	20.53 ± 1.68 [b]	0.00
CD8b	2.61 ± 0.48	2.65 ± 0.69	1.89 ± 0.20	0.38
MyD88	26.13 ± 3.32	29.08 ± 2.67	24.75 ± 1.46	0.52
CD33	16.74 ± 1.83	18.84 ± 3.19	12.74 ± 1.49	0.19
CSF1R	11.43 ± 1.70	8.99 ± 0.80	7.67 ± 0.91	0.11
MIF	104.87 ± 9.33	94.40 ± 8.28	84.95 ± 4.47	0.22
MMD	62.09 ± 11.07	50.56 ± 12.24	51.71 ± 10.24	0.75
IRF8	13.24 ± 1.72	17.94 ± 2.77	15.85 ± 1.22	0.28
NFKB2	43.96 ± 7.18 [a]	71.62 ± 8.83 [a]	55.25 ± 4.31 [ab]	0.04