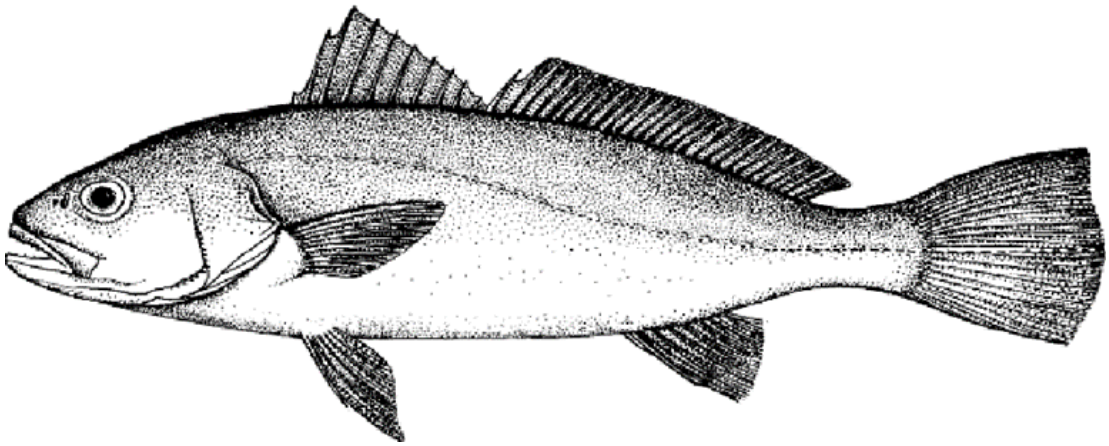
LAURA VAZ CALVINHO

Effect of phytate and phytase on the protein degradation systems and growth performance of meagre, *Argyrosomus regius* (Asso, 1801) juveniles



UNIVERSIDADE DO ALGARVE

FACULDADE DE CIÊNCIAS E TECNOLOGIA

2021

LAURA VAZ CALVINHO

Effect of phytate and phytase on the protein degradation systems and growth performance of meagre, *Argyrosomus regius* (Asso, 1801) juveniles

Mestrado em Aquacultura e Pescas

Especialidade em Aquacultura

Trabalho efetuado sob a orientação de:

Supervisor:

Ana Catarina Parreira Peres Matias, PhD

IPMA – Instituto Português do Mar e da Atmosfera /

EPPO – Estação Piloto de Piscicultura de Olhão

Co-supervisor:

Cláudia Raquel Cêa de Aragão, PhD

CCMAR – Centro de Ciências do Mar /

UAlg – Universidade do Algarve

UNIVERSIDADE DO ALGARVE

FACULDADE DE CIÊNCIAS E TECNOLOGIA

2021

Effect of phytate and phytase on the protein degradation systems and growth performance of meagre, *Argyrosomus regius* (Asso, 1801) juveniles

Declaração de autoria de trabalho:

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

(Laura Vaz Calvinho)

Vista aérea da Estação Piloto de Piscicultura de Olhão do IPMA onde foi realizado este trabalho.

Este trabalho foi financiado e realizado na Estação Piloto de Piscicultura de Olhão (EPPO) do IPMA utilizando corvinas nascidas nesta Estação e as infraestruturas e meios disponíveis para o cultivo, amostragens biológicas e análises laboratoriais. Ao IPMA reserva-se o direito de utilizar toda a informação nela contida para efeitos de publicações, relatórios da entidade financiadora e divulgação ao público em geral.

Acknowledgements

Primeiramente quero agradecer ao Dr. Pedro Pousão por me ter dado a oportunidade de realizar a Tese na EPPO, no âmbito do projeto DIVERSIAQUA II (MAR-02.01.01.FEAMP-0175).

Quero também agradecer à minha orientadora do IPMA, a Dra. Ana Catarina Matias por ter sido uma pessoa excelente e por ter estado sempre presente durante todo o meu percurso na EPPO. Muito obrigada por tudo o que me ensinou, por toda a ajuda durante as amostragens e a análise das amostras. Estou muito agradecida por tudo o que fez para que esta Tese fosse um sucesso.

À minha orientadora interna, Dra. Cláudia Aragão por me ter disponibilizado ajuda sempre que fosse necessário e por ter acompanhado o meu trabalho. Muito obrigada pela sua contribuição para o sucesso desta Tese.

Quero agradecer a todos os membros da EPPO que me receberam de braços abertos e me fizeram sentir á vontade. Especialmente à Mariza que me ensinou tudo o que tinha que saber sobre a parte prática do meu ensaio e se mostrou sempre pronta a ajudar-me. Ao Ravi, à Bárbara, à Mafalda e ao Daniel quero deixar aqui o meu agradecimento pela ajuda nas amostragens. À Lurdes, Tetyana, Beta, Papitchi, João Araújo, Isa por me terem recebido tão bem na EPPO e por todos os momentos de rizadas. Um especial agradecimento á São por todo o apoio que me deu no laboratório.

Um obrigado gigante ao meu colega Paulo por todo o apoio que me deu, por nunca me ter deixado desistir e por me ter feito acreditar que era possível. As conversas que tivemos a caminho da EPPO foram sem dúvida imprescindíveis.

Aos meus amigos quero deixar também o meu agradecimento pelo apoio incondicional.

Quero deixar um enorme agradecimento aos meus avós, à minha mãe e à minha irmã por terem tornado possível realizar os meus estudos na Universidade do Algarve e por terem estado sempre presentes em todo o meu percurso académico. À minha querida avó quero deixar o meu profundo agradecimento, estejas onde estiveres eu sei que estás a olhar por mim e muito orgulhosa de tudo o que já conquistei.

Por último, quero agradecer à Alice e ao Rui, por todo o apoio e motivação que me deram ao longo do meu percurso académico. Estarão sempre no meu coração!

Estou muito agradecida a todos os professores com quem me cruzei durante o meu percurso académico e a todas as pessoas que aqui não mencionei, mas que também fizeram parte da realização desta Tese, incutindo-me os seus conhecimentos e aprendizagens.

Abstract

The production of meagre (*Argyrosomus regius*) is of great interest in the Mediterranean region and southern Europe due to its easy adaptation to captivity, rapid growth (± 1 kg / year), high larval survival rates and tolerance to environmental stress, in terms of temperature and salinity.

The aquaculture industry is facing a huge challenge, which is to find economically and ecologically viable alternatives to fishmeal and oil. There are some plant-derived ingredients, such as soy protein or wheat gluten, which are good candidates for replacing fishmeal, however most plant-derived ingredients have nutrient imbalances, lower nutrient digestibility and anti-nutritional factors.

The present study aims to evaluate the effect of phytate, an anti-nutrient found in ingredients of vegetable origin, and phytase, an enzyme that degrades phytate, on the growth performance and protein degradation systems of meagre juveniles. A trial was carried out with 900 juvenile meagres randomly distributed through 9 tanks, in order to have triplicates for each diet. Fish were fed three experimental diets (CTRL, D1, D2) containing a high percentage of plant ingredients for 30 days. Extra amount of phytic acid (= phytate) was added to diets D1 and D2. The manufactured diets CTRL, D1 and D2 contained 0.14% dry matter (DM), 0.19% DM and 0.13% DM of phytate, respectively. Beyond phytate, diet D2 contained also 1000 FTU kg^{-1} feed of the phytase enzyme.

The addition of phytic acid to diets D1 and D2 had no significant effects on growth performance and feed utilization of juvenile meagre. The results obtained from the activity of cathepsins, the activity of the proteasome and the expression of the Psmb4 subunit of the proteasome were also not significant in the two tissues analyzed (muscle and liver). Thus, this study indicates that the amount of phytate added to the diets was not enough to affect growth performance of meagre juveniles, so the addition of phytase in the D2 diet may not had any functionality.

Keywords: Phytate, phytase, protein degradation systems, growth performance, meagre (*Argyrosomus regius*)

Resumo

A corvina (*Argyrosomus regius*, 1801) é uma espécie muito recente em aquacultura. Existe um grande interesse em produzi-la, principalmente, na região do Mediterrâneo e sul da Europa, visto ser uma espécie de fácil adaptação ao cativeiro, rápido crescimento (± 1 kg / ano), elevadas taxas de sobrevivência larvar e tolerância ao stress ambiental, em termos de temperatura e salinidade. É uma espécie de alta qualidade e sabor, muito apreciada pelos consumidores, e peixes acima de 2 kg possuem elevado valor comercial. Comparado com outras espécies, como a dourada ou o robalo, a corvina apresenta uma taxa de sobrevivência mais alta, melhor conversão alimentar e crescimento mais rápido.

Durante a fase de engorda, as corvinas requerem uma dieta com um teor de proteína superior a 44%, a fim de manter as taxas de crescimento (SGR) de 1% dia^{-1} e um teor de 17% de lípidos. As proteínas animais usadas em rações para peixes podem ser parcialmente substituídas por proteínas vegetais sem prejudicar o desempenho ou a qualidade da carne. No entanto, peixes carnívoros, como as corvinas, requerem altos níveis de proteína, e a introdução de proteína vegetal pode implicar alterações no seu metabolismo proteico e portanto no seu crescimento.

O crescimento do setor da aquacultura ao longo dos anos fez desta atividade um dos principais contribuidores, senão o principal, de peixes para consumo humano. Este grande crescimento torna a alimentação dos peixes, especialmente dos carnívoros que ainda tem por base farinha e óleo de peixe, insustentável. A indústria da aquacultura enfrenta agora o grande desafio de encontrar alternativas económica e ecologicamente viáveis para a farinha e o óleo de peixe. Certos ingredientes de origem vegetal, como a proteína de soja ou o glúten de trigo, são bons candidatos para substituir a farinha de peixe, no entanto, a maioria destes ingredientes apresentam desequilíbrios nutricionais, menor digestibilidade de nutrientes e fatores antinutricionais. Os fatores antinutricionais, por si ou por meio de produtos metabólicos, interferem na utilização dos alimentos e afetam a saúde e o crescimento dos animais.

A substituição parcial da farinha de peixe por ingredientes de origem vegetal, suplementados com aminoácidos e enzimas exógenas, que vão neutralizar o efeito dos antinutrientes, pode ser uma boa alternativa para manter taxas de crescimento compatíveis com uma produção rentável em aquacultura.

No presente estudo, foi analisado o efeito do antinutriente fitato no crescimento e nos sistemas de degradação proteica de juvenis de corvina, bem como o benefício da adição da enzima fitase como estratégia nutricional para reduzir o impacto do fitato. Para isso, foi realizado um ensaio com 900 juvenis de corvina distribuídos aleatoriamente por nove tanques, de modo a haver triplicados para cada dieta experimental. Os peixes foram alimentados até à saciedade aparente com três dietas (CTRL, D1, D2) contendo uma elevada percentagem de ingredientes de origem vegetal durante 30 dias. Foi adicionada uma quantidade extra de fitato às dietas D1 e D2. A quantidade de fitato presente nas dietas experimentais CTRL, D1 e D2 foi de 0.14% matéria seca (MS), 0.19% MS e 0.13% MS, respetivamente. Além do fitato, foi adicionada 1000 FTU kg^{-1} da enzima fitase à dieta D2. Durante o ensaio experimental, os parâmetros da água foram medidos diariamente para garantir condições ideais às corvinas e para que este não fosse o fator limitante. O oxigénio dissolvido foi mantido próximo dos níveis de saturação com auxílio de pedras de ar e renovação constante de água (5,4-7,7 mg L^{-1}). Durante o ensaio, a temperatura da água foi de 20,3 ± 2ºC.

Com base nos resultados deste estudo, o crescimento, a conversão alimentar e a utilização proteica de juvenis de corvina não foram afetados pela adição de fitato e fitase às dietas experimentais. O fato da taxa de crescimento com as dietas administradas (D1 e D2) não ter sido alterado, comparativamente ao CTRL, pode indicar que o turnover das proteínas nos dois tecidos estudados (fígado e músculo) foi idêntico, embora as taxas de metabolismo proteico apresentem diferenças entre os tecidos.

A atividade das catepsinas L, D e B, que estão envolvidas em um dos principais sistemas de degradação de proteínas (sistema autofágico-lisossomal), não apresentam alterações significativas, tanto no músculo como, no fígado das corvinas alimentadas com as duas dietas experimentais D1 e D2. A atividade do proteassoma e a expressão da subunidade Psmb4 nos peixes também não apresentaram diferenças significativas nos dois tecidos amostrados. Dobly e seus colaboradores (2004) observaram que havia uma correlação negativa entre a atividade do proteassoma no fígado e as taxas de crescimento da truta arco-íris, mas essa correlação não foi observada nos músculos. No entanto, no nosso estudo essa correlação no fígado não foi verificada, sugerindo que o efeito do fitato nesses tecidos possa ter sido fraco ou inexistente, uma vez que não houve diferenças na atividade do proteassoma nesse tecido.

Em conclusão, os resultados do presente estudo indicaram que a suplementação de 0,19% de fitato na dieta D1 não teve um efeito significativo no crescimento de juvenis de corvina, na utilização da proteína das dietas nem nos sistemas de degradação proteica estudados. A presença da enzima fitase na dieta D2 não desencadeou um efeito benéfico nos peixes. Como o fitato não teve efeito sobre os peixes, a ação da fitase poderá não ter sido relevante, pois a sua função é degradar o fitato. Além disso, o sistema autofágico-lisossomal, analisado através das catepsinas L, D e B, e o sistema ubiquitina-proteassoma, analisado através da atividade do proteassoma e da expressão da subunidade Psmb4, não mostraram ser afetados nos peixes alimentados com a dieta D1 em comparação com a dieta D2. Os resultados obtidos indicam que os juvenis de corvina são tolerantes à quantidade de fitato presente nas dietas experimentais.

Futuramente, seria interessante realizar novos testes com quantidades de fitato superiores às adicionadas neste estudo, a fim de identificar que quantidade de fitato seria necessária para provocar efeitos negativos nos peixes. Também seria interessante prolongar o tempo do ensaio para esclarecer dúvidas sobre os efeitos a longo prazo.

Palavras-chave: Fitato, fitase, sistemas de degradação proteica, desempenho de crescimento, corvina (*Argyrossomus regius*)

INDEX

LIST OF UNITS AND ABBREVIATIONS

ALS	Autophagic-Lysosomal System
CTRL	Experimental Control treatment
D1	Experimental Diet 1
D2	Experimental Diet 2
DFI	Daily Food Intake
DM	Dry Matter
EPPO	Estação Piloto de Piscicultura de Olhão
FAO	Food and Agriculture Organization
FBW	Final Body Weight
FCR	Feed Conversion Ratio
FTU	Phytase Unit
IBW	Initial Body Weight
IPMA	Instituto Português do Mar e da Atmosfera
mRNA	Messenger Ribonucleic Acid
P	Phosphorus
PER	Protein Efficiency Ratio
RFU	Relative Fluorescence Unit
SD	Standard Deviation
SEM	Standard Error of the Mean
SGR	Specific Growth Rate
UPS	Ubiquitin-Proteasome System
WG	Weight Gain

LIST OF TABLES AND FIGURES

1. INTRODUCTION

1.1. Aquaculture in the world

In 1976, the Food and Agriculture Organization of the United Nations (FAO) organized a global conference where the aquaculture sector was predicted to grow in the coming decades, acquiring strategic importance on a global scale. The reasons for this prediction were related with the positive impact through cushioning the fisheries crisis; with the need to apply quality and cheap protein production policies on a global scale; with the creation of jobs and the dynamization of subsidiary activities; with the development of isolated and more disadvantaged rural and coastal areas (FAO, 1976). At the beginning of the year 2000, FAO once again held a conference reaffirming the 1976 theses, adding that in the last three decades, aquaculture was the fastest growing food production sector in the world (FAO, 2000).

In fact, while worldwide there is a sustained increase in the production of fishery and aquaculture products, there has been, with greater intensity since the 1990s, a replacement of fishery products (catch) by aquaculture products. Global aquaculture production more than tripled in live weight volume from 34 Mt (Megaton) in 1997 to 112 Mt in 2017. The main groups of species that contributed to 75% of the aquaculture production in 2017 were seaweeds, carps, bivalves, tilapia, and catfish. In 2017, aquaculture provided more than 80 Mt of fish and crustaceans and 32 Mt of marine algae, covering around 425 cultivated species (Naylor *et al*., 2021).

1.2. Aquaculture in Portugal

The aquaculture situation in Portugal is no different from the rest of the world, as the difficulties in supplying the market through the offer of products from capture were increasing and aquaculture came to help, providing fresh and quality products to the consumer. However, the growth and modernization of the aquaculture sector, boosted by public support for investment and technical and scientific support from research institutions, was still not enough for aquaculture to reach production volumes capable of contributing, in a relevant way, to the supply of fish, significantly complementing the products from the catch. Portugal is a country with ideal conditions to further develop aquaculture production, however it is not being used for this purpose. This situation is a

matter of concern for national and local policies (REA, 2021; TECNOALIMENTAR, 2020)

Both the national strategy for the sea and the assembly of the republic consider that the aquaculture sector needs more government support and have increased the investment during the past years. Since Portugal has some maritime areas with excellent weather conditions all year round, it is a great local for the development of aquaculture. Coastal zones, lagoons and some estuaries and other more protected areas are ideal for the production of marine fauna (Figure 1.1). In some of these areas productive structures of fish, bivalves, crustaceans and algae have already been introduced (DGRM, 2020).

Among the most common species of our marine fauna (fish, molluscs and crustaceans), some have already been zootechnically worked for production in captivity and others have good characteristics to be able to become one. Such characteristics include the commercial value, adaptability to captivity and, above all, the perspectives of the domain, in the medium term, of its biological cycle (DGRM, 2020).

Figure 1.1. – Earthen ponds at IPMA's aquaculture Research Station located in Ria Formosa.

1.3. Meagre (*Argyrosomus regius*)

Meagre (Figure 1.2) is present in the Indo-Pacific, in temperate waters of the Atlantic and Pacific oceans, in lakes in the Amazon basin and in the Mediterranean (Chao, 1986;

Monfort, 2010). Southern Spain and Portugal and western France are the two regions in western Europe where meagre is most common (Monfort, 2010).

The morphological characteristics of meagre (*Argyrosomus regius*, Asso 1801) are its elongated, almost spindle-shaped and slightly compressed body, with a grayish-green or bluish back and a whitish belly. All over the body it has iridescence and a golden and silver shine. This species is robust and very agile (Piccolo *et al*., 2008, Poli *et al*., 2001).

It belongs to the Sciaenidae family that includes about 70 genera and 270 species distributed in temperate and tropical regions of the world. Members of this family are commonly called croaker, a name that comes from the sounds these fish produce when they use their swim bladder developed as a resonance chamber, thanks to the vibrations of special muscles inserted into their walls, which allow them to be identified at long distances (Lagardére and Mariani, 2006). Two very interesting characteristics of this family are that they are eurythermic and euryaline species that resist sudden changes in temperature from 10 to 30 ºC and salinity from 5 to 39 ‰, which makes them able to penetrate the mouth of rivers and lakes in estuaries, where lay their eggs. They are most often found in shallow areas and estuaries, however they can also be found at depths of 250-350 m on sandy, muddy and, in some cases, rocky bottoms (Cádernas, 2012).

Adult meagres are solitary or form in small groups, however, when the breeding season arrives, between April and June, the meagres gather in large groups to spawn. Reproduction occurs when males reach 64 cm in length (weight 4 kg) and females reach 86 cm in length (weight 7.5 kg) (García-Pacheco and Bruzón 2009). It is in coastal areas and close to estuaries where spawning takes place and larvae are transported. Juveniles explore different types of habitats, a situation that remains until adulthood and they reach sexual maturity (between 4 and 5 years of age) (Cádernas, 2012).

Meagre is considered a nocturnal fish and very voracious. In adulthood, meagre feeds on polychaetes, crustaceans, echinoderms and molluscs, in addition to other smaller fish species (clupeidos, mooges) (Jiménez *et al*., 2005). Juveniles have a low prey diversity, feeding essentially on mysids and shrimp (*Crangon crangon*). These fish are able to enter estuaries in search of their prey, even, in the case of juveniles, entering fresh water (Cabral and Ohmert 2001).

Figure 1.2. – Juvenile meagre (*Argyrosomus regius*) reared at IPMA's Aquaculture Research Station in Olhão (Portugal).

1.4. Aquaculture production of meagre

Meagre (*A. regius*) is a very recent species in aquaculture. The first commercial productions were in France in the 90s, where its captive breeding was achieved for the first time in Europe (Quémener, 2002).

There is great interest in the production of meagre in the Mediterranean region and in southern Europe (Monfort *et al*., 2010). According to FAO data (2009), the country with the largest production of meagre in the Mediterranean is Spain. The production of this species has many associated advantages, such as its easy adaptation to captivity, rapid growth (± 1 kg / year), high larval survival rates and tolerance to environmental stress, in terms of temperature and salinity (Martins *et al.,* 2019; Matias *et al*., 2020; Monfort, 2010; Ribeiro *et al.,* 2014). It is a fish species of high quality and flavor, highly appreciated by consumers, and fish above 2 kg have a high commercial value (Monfort *et al*., 2010). Compared to other species such as bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*), meagre has a higher survival rate, better feed conversion and faster growth (Ribeiro *et al*., 2013).

During the fattening phase, meagre need a diet containing a protein content greater than 44%, in order to maintain growth rates (SGR) of 1% day^{-1} (Pirozzi *et al*., 2010) and a content of 17% lipids (Panagiotidou *et al.,* 2007). Animal proteins used in their diet can be partially replaced by vegetable proteins without harming performance (Segato *et al*.

2005) or meat quality (Ben-Bani *et al*., 2018). However, carnivorous fish, such as meagre, require high levels of protein, and the introduction of vegetable protein may imply changes in protein metabolism (Tacchi *et al*., 2012).

1.5. Fish nutrition and alternative protein sources

The growth of the aquaculture sector over the years has made aquaculture one of the main contributors, if not the main one, of fish for human consumption. This large growth makes aquafeed, which is based on fishmeal and oil, unsustainable. The aquaculture industry now faces a huge challenge, which is to find economically and ecologically more viable alternatives to fishmeal and oil (FAO, 2006; Gatlin *et al*., 2007).

Fishmeal and fish oil have important properties in fish development, which are their high protein content, excellent amino acid profile, high nutrient digestibility, general lack of antinutrients, relatively low price (until recently) and their wide availability. When looking for alternatives to fishmeal and oil, it is important to consider all these properties (Gatlin *et al*., 2007).

There are some plant-derived ingredients, such as soy protein or wheat gluten, which are good candidates for replacing fishmeal, however most plant-derived ingredients have nutrient imbalances, lower nutrient digestibility and anti-nutritional factor (Dani, 2018; Estévez *et al*., 2010; Gatlin *et al*., 2007).

Anti-nutritional factors, *per se* or through their metabolic products, interfere with food utilization and the health and growth of animals (Makkar, 1993). These factors can be divided into 4 groups: A - factors that affect protein digestion, such as protease inhibitors, tannins, lectins; B - factors affecting mineral utilization, which include phytates, gossypol pigments, oxalates, glucosinolates; C - antivitamins; and D - various substances, such as mycotoxins, mimosin, cyanogens, nitrates, alkaloids, photosensitizing agents, phytoestrogens and saponins. Another way of classifying antinutrients is through their ability to resist thermal processing, being the most used treatment to prevent antinutrients from producing negative effects on animals (Rumsey *et al*., 1993; van der Peol, 1989). Some antinutrients, such as oligosaccharides, after contact with heat become more digestible (Francis *et al*., 2001).

The partial replacement of fishmeal by ingredients of vegetable origin, suplemented with amino acids, flavorings and exogenous enzymes (which will counteract the effect of

antinutrients) can be a good alternative to support growth rates that guarantee an economically viable aquaculture production.

1.6. Phytate and phytase

1.6.1. The anti-nutritional factor: Phytate

Phytate, also known as phytic acid, is the hexaphosphoric ester of cyclic hexahydric alcohol meso-inositol, present in plant seeds (Francis *et al*., 2001; Kumar *et al*., 2012). The molecular formula of phytic acid is $C_6H_{18}O_2{}^4P_6$ and its molecular mass corresponds to 660.04 g mol^{-1}. In the form of salt, phytate is the main store of phosphorus (P) in plant tissues (figure 1.3; Kumar *et al*., 2012). Since 80% of the total P in plants is in the form of phytate-P complex, it is not available to fish, due to the absence of phytases during digestion of monogastric and agastric fish (Cao *et al.*, 2007). By adding ingredients of vegetable origin in fish feeds, phytate will bind to the phosphorus molecules (Liener, 1989), turning phytate in an indigestible antinutrient to fish (Swick and Ivey, 1992). Most of the phytate-P complex formed within the body will be released into the water contributing to marine pollution with increased algae growth (Liebert and Portz, 2005). Despite having negative consequences on fish, in human food it has some positive consequences, such as antidiabetic and anticancer effects (Kumar *et al*., 2010). Phytate forms phytate-protein complexes with dietary protein, reducing the availability of the protein, which will impair fish growth, and reducing its digestibility, making it a fish excretory compound (Richardson *et al*., 1985).

Ingredients of vegetable origin commonly used in fish feed such as soyabean meal, rapeseed meal and sesame meal have 10-15, 50-75 and 24 g kg^{-1} of phytate in their constitution, respectively (Francis *et al*., 2001; Tyagi e Verma, 1998). This anti-nutritional factor negatively affects the growth of fish species cultivated in aquaculture, as proven in carp (*Cyprinus carpio*), tilapia (*Oreochromis niloticus*), trout (*Oncorhynchus mykiss*) and salmon (*Salmo salar*) (Cao *et al*., 2007). This fact is confirmed through studies previously carried out, as is the case of the study of rainbow trout fed with a diet

containing 5 g kg^{-1} of phytate and that there was, in fact, a decrease in growth (Spinelli *et al.*, 1983).

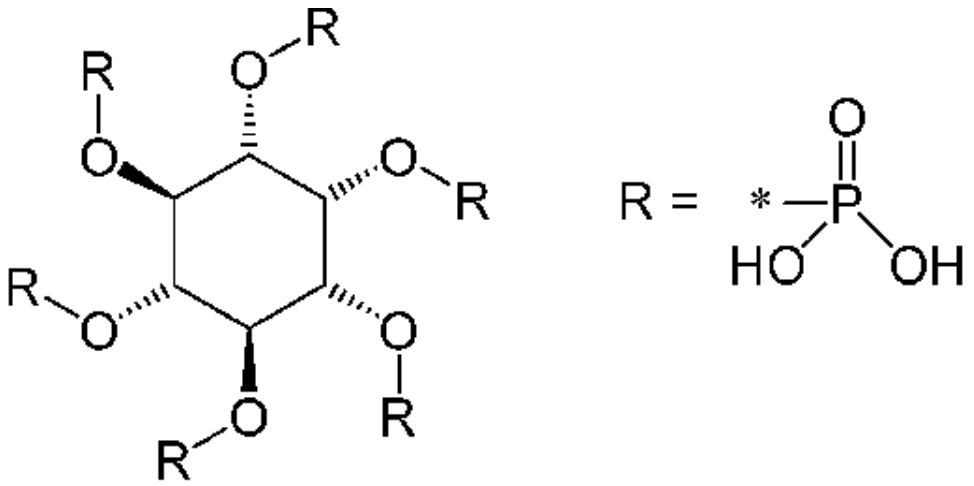


Figure 1.3. - Chemical structure of phytate (Kumar *et al*., 2010).

1.6.2. Phytase

The enzyme phytase (Figure 1.4), also known scientifically as myo-inositol (1,2,3,4,5,6) - hexaphosphate phosphohydrolase, catalyzes the hydrolysis of phytate, making P available for absorption (Baruah *et al.*, 2004; Debnath *et al*., 2005). Phytase is divided into two categories, depending on where the hydrolysis of the phytate molecule starts. If the hydrolysis occurs at the C3 position of the myo-inositol hexaphosphate ring, it is called 3-phytase (EC 3.1.3.26) or myo-inositol hexakisphosphate 3-phosphohydrolase, however if it occurs at the C6 position it is called 6-phytase (EC 3.1.3.26) or myo-inositol hexakisphosphate 6-phosphohydrolase (Cao *et al.*, 2007). Enzymes from microorganisms are considered 3-phytases, while those from higher plant seeds contain 6-phytases. (Nayini and Markakis, 1986).

Phytase activity is expressed by acronyms and they all have the same meaning, they are FYT, FTU, PU or U. A phytase unit represents the amount of enzyme that releases 1 mmol of inorganic phosphorus per minute from 0.0015 mol L^{-1} of sodium phytate at pH 5.5 and 37°C (Cao *et al*., 2007; Simons *et al*., 1990). In 1991, the first phytase, produced by the company BASF, (Natuphos) from a genetically modified strain of *A. niger* was made available on the market (Simons *et al*., 1990).

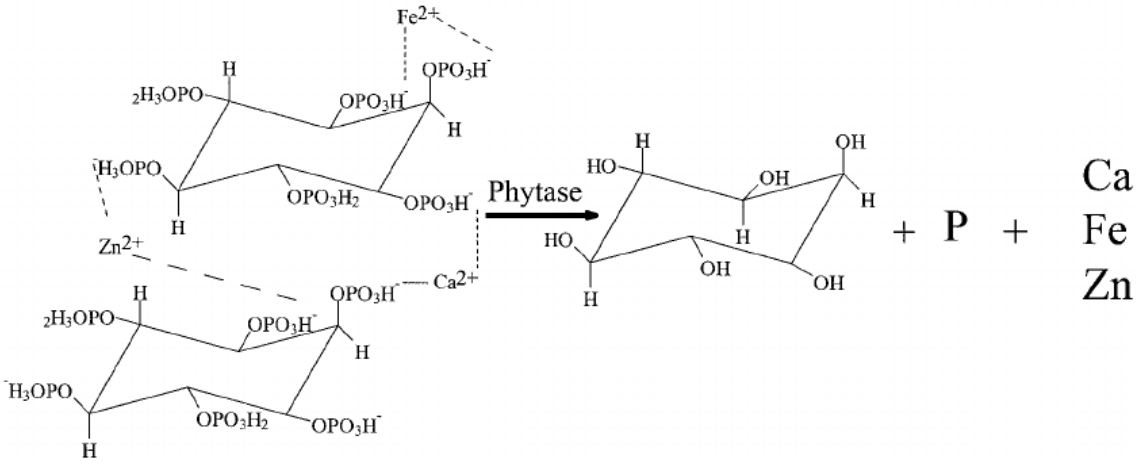


Figure 1.4. - Action of phytase (Shanmugam *et al*., 2018).

This enzyme is found in various forms of life in nature, from unicellular (microorganisms) to multicellular (plants and animals) (Cao *et al*., 2007; Kumar *et al*., 2012). Compared to the enzymatic activity of phytase in plants and microorganisms, the activity in animals is not significant (Weremko *et al*., 1997). Microorganisms, specifically filamentous fungi (*Aspergillus ficuum*, *Mucor piriformis* and *Cladosporium*), manage to have a more effective enzymatic activity than that of plants (Stefan *et al*., 2005), since their stability does not vary above pH 8 and below pH 3, and plant activity decreased dramatically at pH values below 4 and above 7.5 (Greiner and Konietzny, 2006). Another important feature of vegetable phytase is the fact that it is thermolabile which makes it sensitive to heat, when exposed to temperatures above 70ºC it becomes completely inactive (Jongbloed and Kemme, 1990). This is not the case with microbial phytase, which manages to maintain its activity after prolonged exposure to heat (Pointillart, 1988). For application in fish feed, microbial phytases will be the best option (Cao *et al.*, 2007).

As mentioned above, phytase activity is pH dependent. There are two pH values where the two major manifestations of enzyme activity are verified, pH 5-5.5 and pH 2.5. The pH of the digestive system of fish is also variable from species to species, distinguishing two groups: gastric fish and agastric fish (Cao *et al*., 2007). The pH of agastric fish is between 6.5-8.5, while gastric fish have a lower pH value than agastric fish. Previous studies have shown that the efficacy of phytase was much better in gastric, like meagre, than in agastric fish, as phytase does not have the same efficacy due to pH differences in the digestive systems of farmed fish (Ji, 1999). To solve this problem, pre-treatment of

food with phytase or the production of neutral phytase corresponding to agastric fish is applicable (Cao *et al*., 2007).

1.7. Somatic growth and protein turnover in fish

Protein turnover consists in catabolic and anabolic processes, and the balance between them results in somatic growth. The role of protein turnover is essential in the body, as it is responsible for removing defective proteins and providing amino acids for the synthesis of new proteins (Figure 1.5) (Conceição *et al*., 2008).

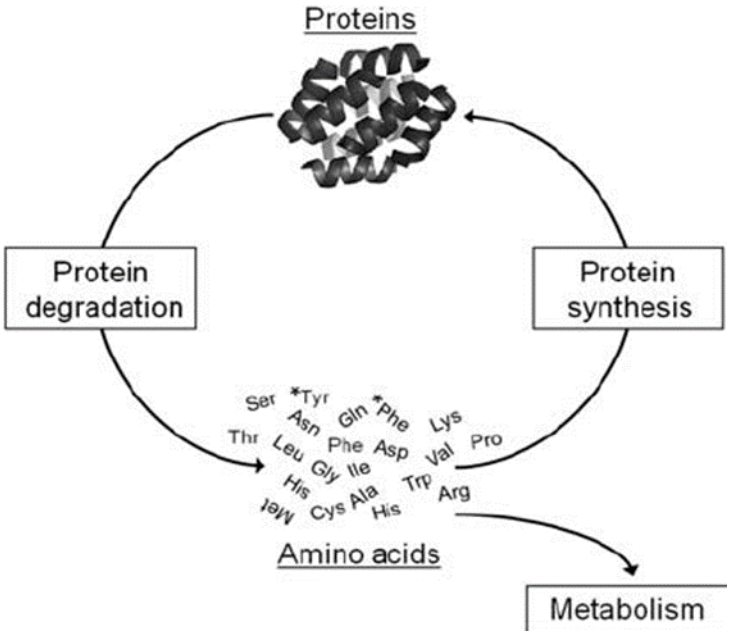


Figure 1.5. - Protein turnover (Baskin and Taegtmeyer, 2011).

Protein synthesis and degradation occurs in all organisms. When there is a small increase in synthesis or a small reduction in degradation, muscle growth increases (Houlihan *et al*., 1988, 1993; Reeds, 1989). Therefore, any stimulation of muscle growth implies that the concomitant increase in protein synthesis must be sufficient to allow both new protein deposition and an increase in protein turnover. For an increase in protein synthesis to occur, which will result in the individual's growth, it is important to consider the level of protein in the diet (Fauconneau *et al.*, 1986b), the water temperature (Fauconneau *et al*., 1986a) and the size of the food (Houlihan *et al*., 1992). All these factors are directly correlated with protein turnover, as it adapts according to the individual's diet and environmental conditions, being an indicator of metabolic plasticity (Conceição *et al*., 2001). The PI3K / AKT / TOR pathway is an important signaling pathway in cell cycle regulation (Erbay *et al*., 2003). This pathway is activated by the detection of nutrients

present in the feed eaten by fish, which, consequently, will allow the translation of mRNA and protein synthesis. This regulatory mechanism allows an organism to coordinate nutritional information to achieve balanced growth, regulating cell size and proliferation (Hietakangas and Cohen 2009). When the body does not receive enough protein from the diet, the rate of protein breakdown exceeds protein synthesis and results in muscle atrophy (Valente *et al*., 2013).

1.8. Protein degradation systems in fish

Protein degradation is regulated by several factors, such as stage of development, sexual maturation, spawning migration and changes in nutrient supply. Changes in the protein composition of fish diets can interfere with the regulation of several genes known to be involved in fish protein turnover (Tacchi *et al.,* 2012). Matias and collaborators showed that dietary taurine was able to decrease the activity of protein degradation systems in juveniles meagre, suggesting a dietary modulation of protein turnover in fish. (Matias *et al.,* 2020). Therefore, dietary components can affect protein turnover in fish, mainly in scenarios of fishmeal replacement.

Protein degradation is carried out by four proteolytic systems in eukaryotic cells: Ubiquitin-Proteosome System (UPS), Autophagy Lysosomal System (ALS), dependent Ca^{2+} and apoptosis protease systems. UPS and the ALS are the two systems that excel in fish (Kaushik and Seiliez, 2010; Seiliez *et al.,* 2014).

The UPS system involves the binding of ubiquitin molecules to the protein substrate followed by its degradation by the proteasome, that results in peptides of 7-9 amino acid residues (Ciechanover, 1994; Kaushik and Seiliez, 2010). The proteasome, also known as the 26S proteasome, has a barrel shape and is composed by two sections, the 19S subunit of the proteasome, which presents itself at the end of the complex and allows the entry of the protein to be degraded into the proteasome, and the 20S subunit of the proteasome, which is found in the middle of the complex and where the main activity of the proteasome takes place. The 20S subunit is composed of four overlapping rings, two outer rings that are comprised by 7 type α subunits, and two inner rings comprised by 7 type β subunits. The β-type subunits are responsible for proteolytic activity, namely β-1, β-2 and β-5. Psmb4, also known as 20S proteasome subunit β-7, is a β-type subunit that

is often used as a proteolytic marker of the protein degradation system in fish (Martin *et al.*, 2002; Salmerón *et al*., 2015).

The ALS system acts through a complex cellular process, where lysosomes are responsible for the degradation of the cellular material absorbed by endocytosis. Protein degradation occurring via the ALS is a result of the action of several proteases, such as cathepsins (Turk *et al.*, 2012). Cathepsins are divided into three groups: cathepsin aspartic proteases (D and E), serine cathepsins proteases (A and G) and lysosomal cysteine cathepsins (B, H, L, C, X, F, O and V) (Rossi *et al*., 2004). They all play an important role in protein turnover, however, some of them are more present in specific tissues. The cathepsins L, B, D and H are the most abundant in lysosomes and determine its proteolytic capacity (Turk *et al*., 2012). The interior of lysosomes consists of a slightly acidic pH, ideal for the activity of cathepsins, as they are unstable at neutral to alkaline pH (Turk *et al*., 2000).

Seiliez and collaborators showed that ALS contributed two to three times more to protein degradation than UPS in rainbow trout muscle cells, suggesting distinct demands in terms of protein degradation systems (Seiliez *et al.*, 2014).

2. THEME JUSTIFICATION

The partial substitution of a diet rich in fishmeal to a diet consisting essentially of vegetable ingredients, can bring some associated problems created by antinutrients that are present in diets of vegetable origin. In the present study, the effect of the antinutrient phytate on the growth performance and protein degradation systems of juvenile meagre will be analyzed. The benefit of the addition of the phytase enzyme as a nutritional strategy to reduce the impact of phytate will also be evaluated.

[This page intentionally left blank]

3. MATERIALS AND METHODS

3.1 Experimental diets

To meet the nutritional needs of juvenile meagre, three diets containing a high percentage of vegetable ingredients (CTRL, D1, D2) were formulated and manufactured by the company SPAROS, Lda. (Olhão, Portugal) (Table 3.1). After being manufactured, the biochemical composition of the diets was analyzed by SPAROS, to know the actual amounts of phytate present in the feeds administered to juvenile meagre. This analysis was important as the final amount of phytate was less than the amount of phytate initially added. In diets D1 and D2 0.4% phytate was added, however the actual amounts of phytate were: CTRL – 0.14% DM; D1 - 0.19% DM; D2 – 0.13% DM (Table 3.2). Diets D1 and D2 varied in the absence or presence of phytase. In addition to phytate, diet D2 had in its composition 1000 FTU kg^{-1} feed of phytase. Before the distribution of meagre juveniles among the experimental tanks, fish were fed with a commercial diet for three days, to allow fish acclimation to the new space. On the 4^{th} day, the commercial feed was mixed with the experimental diets (50/50) to observe the behavior of the fish to the new diets. From the 5^{th} / 6^{th} day on, fish were exclusively fed with the three experimental diets until the end of the trial.

Table 3.1. - Formulation of the experimental diets (CTRL, D1, D2).

Diets	**CTRL**	**D1**	**D2**
Ingredients (%)			
Fish meal LT70	35.0	35.0	35.0
Fish protein hydrolysate	4.5	4.5	4.5
Squid meal 83	15.0	15.0	15.0
Krill meal	15.0	15.0	15.0
Fish gelatine	3.0	3.0	3.0
Wheat meal	16.6	16.2	16.2
Vitamin and mineral premix	1.0	1.0	1.0
Vitamin E50	0.1	0.1	0.1
Antioxidant	0.2	0.2	0.2
L-Taurine	1.6	1.6	1.6
Soy lecithin	1.0	1.0	1.0
Fish oil	2.5	2.5	2.5
Rapeseed oil	4.5	4.5	4.5
Phytic acid		0.4	0.4
Phytase (FTU kg^{-1} feed)			1000.0

Table 3.2. - Biochemical composition of the tested diets (CTRL, D1, D2).

Diets	CTRL	D1	D2
Dry matter (DM), %	96.71	95.42	95.97
Crude Protein (% DM)	59.13	59.04	59.09
Crude fat (% DM)	15.81	15.75	15.70
Phosphorus (% DM)	1.33	1.39	1.39
Ash (% DM)	9.74	9.89	9.79
Gross Energy (kJ g^{-1} DM)	21.71	21.67	21.69
Phytate (% DM)	0.14	0.19	0.13

3.2 Experimental conditions

The fish trial took place at the facilities of the Aquaculture Research Station in Olhão (EPPO, Portugal), which provided the juvenile meagre used in the study.

This trial was conducted in accordance with European guidelines on the protection of animals used for scientific purposes (Directive 2010/63 / EU of the European Parliament and Council of the European Union).

Nine groups of 100 meagre juveniles (initial body weight: 4.79 ± 0.28 g) were randomly distributed into rectangular fiberglass tanks of 250 L of capacity (Figure 3.1) and supplied

Figure 3.1. - Example of the tanks used in the trial

with water from Ria Formosa (water flow rate: 360 L h^{-1}), treated prior to enter the trial tanks. Each of the three experimental diets were randomly assigned to triplicate tanks.

During the experimental trial, water parameters were measured daily to ensure that the fish had ideal conditions and that this was not the limiting factor. The dissolved oxygen was kept close to saturation levels with the help of air stones and constant water renewal (5.4-7.7 mg L^{-1}). During the trial, water temperature was 20.3 ± 2ºC. Rearing water was collected every 15 days for analysis of ammonia, as it is a product of fish excretion and whose levels depend mainly on the dietary protein and the metabolism of the fish. Ammonia levels (NH_3+NH_4^+) in the collected water were determined in the Skalar Sanplus Segmented Flow Colorimetric Autoanalyser using the Skalar method: M155-008R (EPA 350.1), by the MARINNOVA laboratory in Matosinhos. The ammonia levels in the rearing water of the experimental tanks averaged 0.71 ± 0.12 mg L^{-1}.
The trial did not have artificial light directly targeted to the experimental tanks, however the artificial light targeted to the neighboring tank was on from 7 am until 9 pm, indicating that the photoperiod of this trial was 14 h of light and 10 h of darkness. Fish were fed by hand, six times a day until apparent satiation for 30 days.

3.3 Fish sampling

At the beginning of the trial, the first 30 individuals from each tank were weighed and measured individually, and the remaining 70 weighed in groups of 10, to obtain the fish initial biomass. Throughout the fish trial, the amount of feed consumed, water temperature and dissolved oxygen and fish mortality were recorded daily. At the end of the trial, fish from all tanks were measured and weighed individually (Appendix I). In both initial and final samplings, individuals were subjected to light anesthesia (100 ppm phenoxyethanol) to facilitate fish handling, after a day of fasting (Barata *et al*., 2016).

In the initial sampling, 25 random fish were anesthetized with 700 ppm of phenoxyethanol, then immediately frozen at -20 ºC for biochemical composition analysis. In the final sampling, 10 fish per tank were also frozen at -20 ºC for biochemical composition analysis and then compared with the initial sampled group of fish. An additional group of 12 fish from each tank were randomly chosen, then placed in buckets with 700 ppm of phenoxyethanol to be subsequently sacrificed (Barata *et al.*, 2016), in

order to collect a sample of muscle (Appendix III) and liver (Appendix II) to analyze the activity of cathepsins and the proteasome and also protein expression. Both samples were frozen in liquid nitrogen, then transferred to a freezer to be kept at -80 ºC before analysis. A schematic representation of the initial and final samplings is indicated in Figures 3.2 and 3.3.

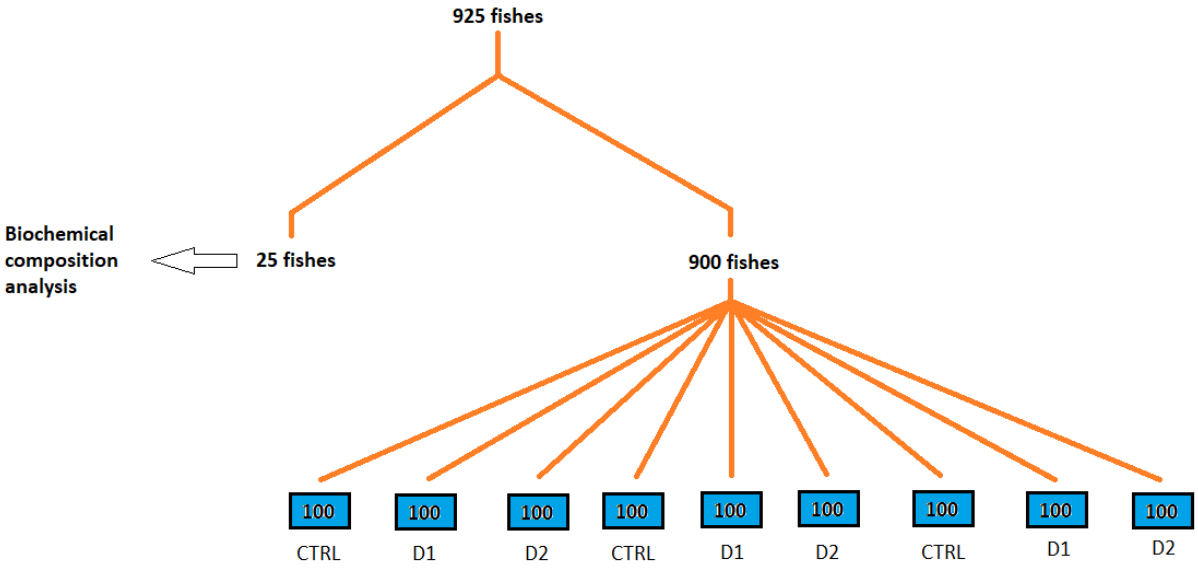


Figure 3.2. – Schematic representation of the initial sampling for each tank.

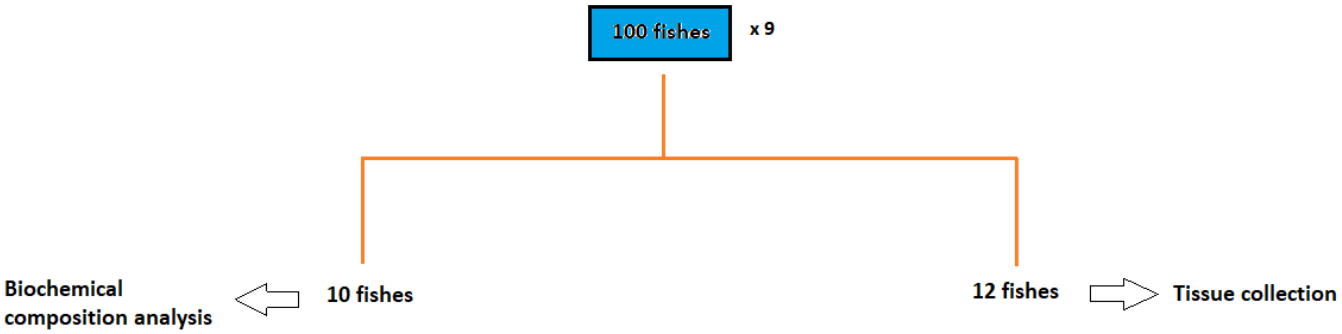


Figure 3.3. – Schematic representation of the final sampling for each tank.

3.4 Protein extraction and quantification

Liver and muscle samples were homogenized in 4 and 6 mL g^{-1} of cold lysis buffer, respectively, and proteins extracted by grinding samples with liquid nitrogen using a mortar and pestle. For protein expression analysis the lysis buffer was composed by 20 mM β-glycerophosphate, 50 mM NaF, 2 mM EDTA, 20 mM hepes, 10 mM benzamidine, 200 µM leupeptin, 10 µM trans-epoxy succinyl-L-leucy-lamido-(4-guanidino) butane, 5 mM dithiotheitol, 300 µM phenyl methyl sulfonyl fluoride (PMSF), 50 µg mL^{-1} pepstatin, 1% v/v Triton X-100, pH 7.0. For enzymatic activity analysis the lysis buffer was PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) containing 0.5 % v/v of NP40 detergent. Homogenized samples were centrifuged at 15000 *g* for 20 min at 4 ºC and supernatants transferred to clean tubes.

Total proteins were determined using the Bradford method (5000006, BioRad) (Bradford, 1976). It is a very simple and fast method, which consists of adding a solution containing the Coomassie G-250 dye to the sample to be tested, wait a few minutes of incubation, and read the absorbance at 595 nm.

This method was used for all samples in duplicate on a microplate, where 6 different concentrations of Bovine Serum Albumin (BSA) (0, 10, 20, 40, 80, 100 mg mL^{-1}) were also added to create a standard curve. The standard curve was important for determining the protein concentration of the samples.

3.5 Proteases activity

3.5.1 Cathepsins B, D and L

Cathepsins are enzymes that belong to the protease family and are present in the cell lysosome. Its function is to break peptide bonds that link specific amino acids. These enzymes play a key role in protein turnover. Cathepsins L, B and D were chosen to be analyzed in this study.

The activity of cathepsins B, L and D in liver and muscle samples was measured on 96-well black plates based on fluorometric methods. Samples were incubated with 200 µM cathepsin B substrate (Z-RR-AMC), 200 µM cathepsin L substrate (Z-FR-AMC), or 20

µM cathepsin D substrate (MCA-GKPILFFRLK (Dnp) -r -NH2) in reaction buffer (100 mM sodium acetate, 120 mM NaCl and 1 mM EDTA with pH 5 containing 4 mM DTT). The reaction buffer had an acidic pH (pH 5) to match the acidic lysosomal lumen, optimal for cathepsin activity. The inhibitor for each cathepsin was added to only one of the sample duplicates into the 96-well plate: for cathepsin D, 2 µl Pepstatin A (ALX-260-085, Enzo Life Sciences), for cathepsin L, 2 µl cathepsin L inhibitor (Z-FF-FMK) (219421, Merck) and for cathepsin B, 2 µl CA074 in DMSO (ALX-260-017, Enzo Life Sciences). After incubating for 1 h at 37 ºC, cathepsin D activity was measured in the fluorescence plate reader (Sinergy 4, Biotek) at Ex / Em 328 / 393nm. The activity of cathepsins L and B was measured using the Glomax fluorometer (Promega) at Ex / Em 365/410-460nm. The cathepsin activity recorded in the absence of the inhibitor represented the total proteolytic activity (RFU) of the sample and the activity in the presence of the inhibitor corresponded to the non-cathepsin activity (iRFU). Therefore, the RFU generated by each type of cathepsin activity was RFU-iRFU.

3.5.2 Proteasome activity

Proteasome activity was measured through the chemotryptic activity of the β-5 subunit of the proteasome. This activity was determined on 96-well black plates based on fluorometric methods. Protein samples were incubated with 100 µM proteasome substrate labelled with AMC (539142, Calbiochem) in proteasome assay buffer (20 mM Tris, 2 mM $MgCl_2$, 6% v/v glycerol, pH 7.5 containing 400 µM DTT and 800 µM ATP). The assay was conducted in the absence and presence of 10 µM proteasome inhibitor MG132 (474790, Calbiochem) to determine enzymatic activity. The reaction mixture was incubated at 37 ºC for 1 h. Released AMC was measured every 10 min, using Glomax fluorimeter (Promega) at Ex/Em 365/410-460nm. A standard curve was constructed with AMC standard ranging from 0 to 100 pmol (from BioVision proteasome activity fluorometric assay kit, k245). The proteasome activity was calculated using the following equation: B/(T2-T1) x P, B is the amount of AMC in the reaction well (pmol); T1 is the time of the first reading (min); T2 is the time of the second reading (min) P is the total protein amount added into the reaction well (mg).

3.6 Protein expression of the proteasome subunit beta type-4 by western blot

Equivalent amounts of hepatic and muscle proteins 50-75 µg were separated on 12% (w/v) acrylamide gels. Separated proteins were transferred onto a PVDF membrane (Amersham, 0.45 µm). Non-specific binding sites on the membranes were blocked with 5% (w/v) non-fat milk in TBST 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% v/v Tween 20 for 30 min at room temperature. Membranes were incubated overnight with the primary antibody Psmb4 (monoclonal mouse anti-proteasome Psmb4 subunit, Santa Cruz Biothecnology sc-390878, 1:1000) or β-actin (monoclonal mouse anti-β-actin, Santa Cruz Biothechnology sc-47778, 1:1000). After washing in TBST (3 periods, 5 min each time) the membranes were incubated with horseradish peroxidase-linked secondary antibody (Thermofisher Scientific G-21040, 1:5000) for 1h at room temperature, washed again in TBST 3 periods, 5 min each time, and protein bands detected using enhanced chemiluminescence (Roche) using the Chemidoc XRS+, BioRad. Detected bands were quantified using Imagelab 6.0 software and normalized with β-actin housekeeping protein.

3.7 Calculations

3.7.1 Fish performance calculations

The growth performance and feed utilization of meagre juveniles were calculated using the following equations:

$$Weight\ Gain\ (WG\ \%) = \left[\frac{(Final\ weight - Initial\ weight)}{Initial\ weight}\right] \times 100$$

$$Specific\ Growth\ Rate\ (SGR\ \%) = \left[\frac{(\ln Final\ weight - \ln Initial\ weight)}{T\ (days)}\right] \times 100$$

$$Feed\ Conversion\ Ratio\ (FCR) = \frac{Total\ feed\ intake\ (g)}{Total \quad weight\ gain\ (g)}$$

$$Protein\ Efficiency\ Ratio\ (PER) = \frac{weight\ gain\ (g)}{Total\ protein\ intake\ (g)}$$

3.8 Statistical analysis

The results were interpreted using the R 4.1.0 software and its RStudio tool. Tests were performed to determine if the data distribution follow the normal distribution and if there is homogeneity of variances. If the data followed the assumptions of the parametric test, one-way ANOVA factor variance test was applied. Finally, to see if there were statistically significant differences in the data, the p-value would have to be <0.05.

4. RESULTS

4.1. Growth performance and feed utilization

Throughout the experiment, no mortality was recorded in the three treatments. The introduction of the new experimental diets was successful, as they were well accepted by the fish.

The initial weight of fish had no statistically significant variations between treatments (p = 0.638), thus ensuring that the initial sampling was as homogeneous as possible. At the end of the trial, the weight was about five times higher than the initial, both in the control (25.25 ± 0.15 g), as in D1 (25.80 ± 1.28 g) and D2 (25.74 ± 1.32 g) (Table 4.1). Thus, there were no statistically significant differences between the final weight of fish in the three treatments (p = 0.86).

Regarding the specific growth rate (SGR) (Table 4.1), there were no significant differences between the control (5.62 ± 0.1% day^{-1}), D1 (5.57 ± 0.14% day^{-1}) and D2 (5.53 ± 0.18% day^{-1}) treatments (p = 0.84).

Fish fed control diet had a daily food intake (DFI) of 3.67 ± 0.03% day^{-1}, whereas the group of fish fed D1 and D2 diets were practically identical, 3.60 ± 0.1% day^{-1} and 3.59 ± 0.16% day^{-1}, respectively (Table 4.1). However, there was no statistically significant differences among the dietary treatments (p = 0.81).

Also, there were no statistically significant differences in the feed conversion ratio (FCR) between the control (0.81 ± 0.01), D1 (0.79 ± 0.04) and D2 (0.79 ± 0.05) group of fish (p = 0.898) (Table 4.1).

The protein efficiency ratio (PER) observed in the group of fish fed diet D1 was 2.25 ± 0.11, and 2.17 ± 0.04 and 2.24 ± 0.15 for the groups of fish fed control and D2 diets, respectively (Table 4.1). Nevertheless, there were not significant differences between each treatment (p = 0.739).

Table 4.1. - Growth performance and feed utilization of meagre juveniles fed experimental diets (CTRL, D1, D2)

	Diet		
	CTRL	D1	D2
IBW (g)	4.76 ± 0.09	4.77 ± 0.04	4.84 ± 0.11
FBW (g)	25.25 ± 0.15	25.80 ± 1.28	25.74 ± 1.32
WG (g)	2048.9 ± 23.54	2086 ± 121.54	2091 ± 135.98
SGR (% day $^{-1}$)	5.62 ± 0.1	5.57 ± 0.14	5.53 ± 0.18
DFI (% day $^{-1}$)	3.67 ± 0.03	3.60 ± 0.1	3.59 ± 0.16
FCR	0.81 ± 0.01	0.79 ± 0.04	0.79 ± 0.05
PER	2.17 ± 0.04	2.25 ± 0.11	2.24 ± 0.15

Values are presented as means ± SD of triplicates (n = 3). IBW – Initial body weight; FBW – Final body weight; WG – Weight gain; SGR – Specific growth rate; DFI – Daily food intake; FCR – Feed conversion ratio; PER – Protein efficiency ratio.

4.2. Cathepsins B, L and D activity

In this assay, the activity of cathepsins B, L and D was analyzed in the muscle and liver of the sampled fish (Table 4.2). Analysis of cathepsin B activity in the liver showed that the control (CTRL) exhibited a value of 165.7 ± 30.5 RFU µL^{-1} total protein, diet D1, 101.2 ± 12.4 RFU µL^{-1} total protein and diet D2 162.0 ± 33.7 RFU µL^{-1} total protein. However, the treatments did not show statistically significant differences (p = 0.191). As in the liver, the analysis of cathepsin B activity in muscle did not show statistically significant differences (p = 0.408).

After analyzing the activity of cathepsin L in the liver, it was found that the control value was 346.3 ± 44, that of diet D1 was 326.6 ± 40.9 and that of diet D2 was 383.0 ± 31.3 RFU µL^{-1} total protein. In muscle, the control result was 29.9 ± 2.4 RFU µL^{-1} total protein, the D1 diet was 32.1 ± 3.2 RFU µL^{-1} total protein and the D2 diet was 28.6 ± 1.9 RFU µL^{-1} total protein. Cathepsin L activity in liver and muscle was not affected by different dietary treatments. Regarding the activity of cathepsin D in the liver, the values were: CTRL = 45845.6 ± 5647.1 RFU µL^{-1} total protein; D1 = 47202.4 ± 5656.7 RFU µL^{-1} total protein and D2 = 50508 ± 7735.4 RFU µL^{-1} total protein and in muscle, the control presented a value of 508 ± 146.7 RFU µL^{-1} total protein, diet D1 of 460.7 ± 128.3 RFU µL^{-1} total protein and diet D2 of 855 ± 85 RFU µL^{-1} total protein. Cathepsin D activity, similarly to the activity of cathepsins B and L, showed no statistical differences among the three treatments, both in liver (p = 0.895) and in muscle (p = 0.065).

Table 4.2. - Cathepsins B, L and D activity (RFU μL^{-1} total protein) in the liver and muscle of meagre juveniles fed with the experimental diets (CTRL, D1, D2).

Tissues	**CTRL**	**D1**	**D2**
Cathepsin B			
Liver	165.7 ± 30.5	101.2 ± 12.4	162.0 ± 33.7
Muscle	31.0 ± 2.5	25.9 ± 2.6	26.6 ± 2.9
Cathepsin L			
Liver	346.3 ± 44	326.6 ± 40.9	383.0 ± 31.3
Muscle	29.9 ± 2.4	32.1 ± 3.2	28.6 ± 1.9
Cathepsin D			
Liver	45845.6 ± 5647.1	47202.4 ± 5656.7	50508 ± 7735.4
Muscle	508 ± 146.7	460.7 ± 128.3	855 ± 85

Values are presented as means ± SEM of triplicates (n = 9).

4.3. Proteasome activity

The results of the analysis of proteasome activity in the liver of fish fed the control diet was 9.5 ± 1.1 mU mg^{-1} total protein, in the group of fish fed D1 diet was 14.5 ± 3.2 mU mg^{-1} total protein and the group fed the D2 diet was 12.1 ± 1.7 mU mg^{-1} total protein. In the muscle it was as follows: CTRL = 8.4 ± 1.2; D1 = 6.1 ± 0.5 and D2 = 7.0 ± 0.8 mU mg^{-1} total protein (Table 4.3). However, in none of the analyzed tissues (liver and muscle) statistical differences were observed between experimental diets.

Table 4.3. - Proteasome activity (mU mg^{-1} total protein) in the liver and muscle of meagre juveniles fed diets CTRL, D1 and D2.

Tissue	**Diet**		
	CTRL	D1	D2
Liver	9.5 ± 1.1	14.5 ± 3.2	12.1 ± 1.7
Muscle	8.4 ± 1.2	6.1 ± 0.5	7.0 ± 0.8

Values are presented as means ± SEM of triplicates (n = 9).

4.4. Protein expression

The expression of the proteasome subunit Psmb4 was not affected by the different dietary treatments (CTRL, D1 and D2) in the liver ($p = 0.999$) (Figure 4.1) and muscle ($p = 0.990$) of fish (Figure 4.2).

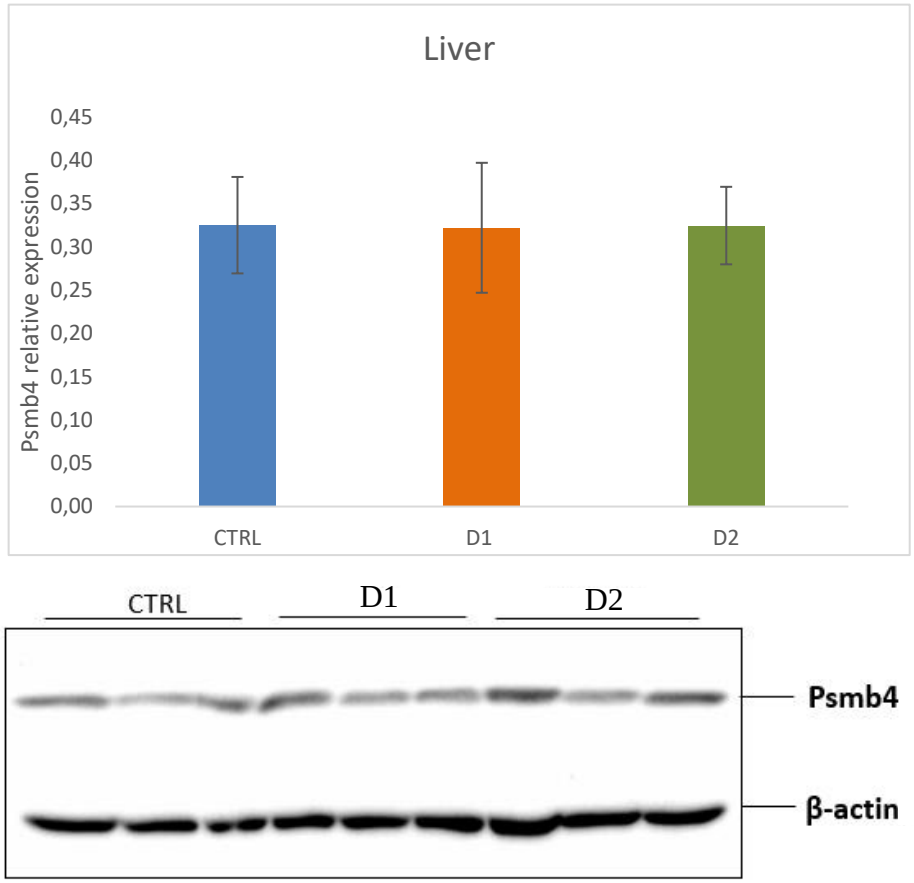


Figure 4.1 - Representative immunoblots showing Psmb4 relative protein expression in the liver of meagre juveniles fed experimental diets (CTRL, D1 and D2). β-actin was used to normalize protein signals from liver. Values are shown as mean ± SEM ($n = 3$).

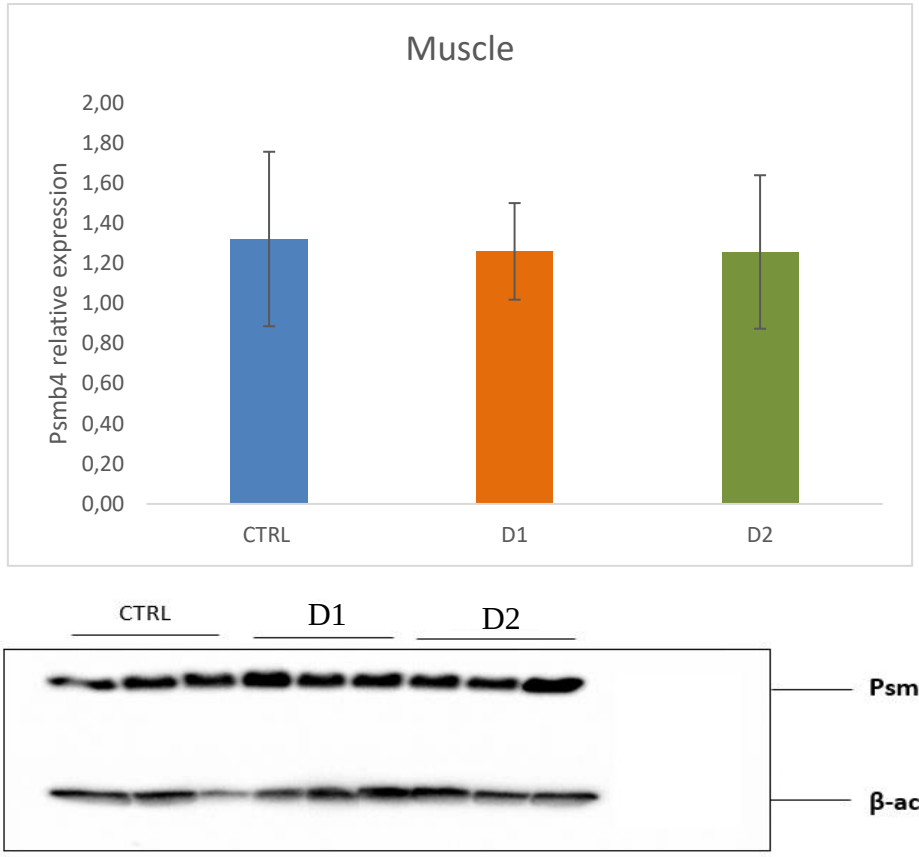


Figure 4.2. - Representative immunoblots showing Psmb4 relative protein expression in the muscle of meagre juveniles fed experimental diets (CTRL, D1 and D2). β-actin was used to normalize protein signals from muscle. Values are shown as mean ± SEM (*n* = 3).

[This page intentionally left blank]

5. DISCUSSION

The presence of phytate in plant-based diets causes difficulties in the digestion of fish, because phytate creates bonds with proteins, nutrients or minerals, and these complexes are insoluble. (Cao *et al*., 2007; Kumar *et al*., 2012; Riche and Garling Jr., 2004). This problem triggers others, such as reduced nutrient availability, which in turn will negatively affect fish growth (Kumar *et al*., 2012).

Scientific studies have shown that supplementing diets with phytase, an enzyme that degrades phytate, can inhibit the negative effects of phytate, benefiting fish growth (Castillo and Gatlin III, 2015; Kumar *et al*., 2012; Maas *et al*., 2018). However, scientific studies on the effect of phytate and phytase on meagres have not yet been carried out.

In this study, phytate and phytase presence were analyzed in the experimental diets, in order to study the impact of these ingredients in the growth performance and in the two main systems of protein degradation in vertebrates, the autophagy-lysosomal and ubiquitin-proteasome systems, in meagres juveniles. These two main pathways of protein degradation are implicated in protein turnover, which controls somatic growth in fish (Seiliez *et al*., 2014). For this reason, these pathways are important to identify the mechanisms by which phytate and phytase could modulate meagre growth.

After being manufactured, the biochemical composition of the experimental diets was analyzed, in order to know the actual amounts of phytate, crude protein, ash, crude fat, phosphorus and gross energy presents in the feeds administered to juvenile meagre. This analysis showed that the final amount of phytate was less than the amount of phytate initially added. In diets D1 and D2 0.4% phytate was added, however the actual amounts of phytate were: CTRL – 0.14% DM; D1 - 0.19% DM; D2 – 0.13% DM. The fact that the two diets do not have the same amount of phytate can be explained by the possibility that phytase enzyme in diet D2 might have reacted with phytate during the preparation of the feed. This event is supported by a study previously carried out by Sajjadi and Carter (2004). Although phytate was not added to the CTRL diet, it does contain a certain amount of phytate in its constitution that derives from the constituents of vegetable origin that are present in the diet.

Based on the results of this study, growth, feed conversion ratio and protein efficiency ratio of juvenile meagre were not affected by the addition of phytate and phytase to the

diets. Laining *et al*. (2010) observed similar results in juvenile Japanese flounders (*Paralichtys olivaceus*) that were fed diets supplemented with 0.51 and 1.04% phytate. However, there was a reduction in growth and feed intake of Japanese flounders fed diets with phytate levels above 1.35%. Denstadli *et al*. obtained results in concordance with this study. They found that there were no statistical differences in growth and feed intake in Atlantic salmon (*Salmo salar L.*), which were fed with diets containing phytate levels ranging between 0.1 and 0.47%. Sajjadi and Carter (2004) also had no effect on salmon growth after being fed diets with 0.8% phytate. Supplementation with 2000 U kg^{-1} of phytase in the salmon diet did not improve FCR and growth, as observed in the results of the present study. The studies indicated previously did not show results for the protein efficiency ratio (PER), but the study by Richardson *et al.* (1985) observed that the inclusion of 0.162 and 0.646% of sodium phytate in the diets did not affect the use of protein of juvenile chinook salmon (*Oncorhynchus tshawytscha*), only the inclusion of 2.58% of sodium phytate in the diets reduced the PER. In this sense, it is suggested that the inclusion of 0.19% of phytate was not enough to trigger negative effects on meagre juveniles, and the help of phytase was not needed to reverse its effects. The fact that the growth of fish fed the experimental diets D1 and D2 was unaffected, comparatively with the CTRL, may indicate that protein turnover in the two tissues studied (liver and muscle) did not vary (Carter and Houlihan, 2001).

The activity of cathepsins L, D and B, which are involved in one of the main protein degradation systems (ALS), was unaffected, either in the muscle or in the liver of the meagre juveniles fed with the two experimental diets D1 and D2. The autophagic-lysosomal system (ALS) degrades proteins through the action of 13 cathepsins that are found inside the lysosomes, however those that are more relevant in fish are L, B and D, especially in muscle (Chéret *et al*., 2007; Delbarre-Ladrat, 2006). In the study by Salmerón *et al*. (2015), the results obtained showed that the two protein degradation systems, autophagic-lysosomal and ubiquitin-proteasome, are regulated in a coordinated way to control the muscle growth of sea bream (*Sparus aurata*) and show evidence that feeding can alter protein breakdown in the muscle of farmed fish. Something that was not observed in our study, which may indicate that the amount of phytate present in the experimental diets was not enough to alter protein degradation. As observed for cathepsins activity, proteasome activity and the expression of Psmb4 proteasome subunit did not show significant differences in the two tissues sampled. The ubiquitin-proteasome

degradation system involves a recognition step, by the ubiquitin molecules, of the protein to be degraded and a degradation step, by the proteasome, of the labeled protein (Martin *et al*., 2002). Psmb4 is a β-subunit of the proteasome that is commonly used as a proteolytic marker of the ubiquitin-proteasome degradation system (Martin *et al*. 2002; Sálmeron *et al*., 2015). Dobly and his researchers (2004) noted that there was a negative correlation between proteasome activity in the liver and rainbow trout growth rates, but this correlation was not seen in muscle. However, in our study this correlation in the liver was not verified, suggesting that the effect of phytate in these tissues may have been weak or null since there were no differences in the proteasome activity in this tissue.

6. CONCLUSION AND FUTURE PERSPECTIVES

To our knowledge, the present work was the first that analyzed the effect of phytate and phytase supplementation on the growth and protein degradation systems of *A. regius* juveniles.

In conclusion, the results of the present study confirmed that the supplementation of 0.19% phytate in diet D1 had no negative impact in the growth performance of juvenile meagre nor in the use of dietary protein, and that the addition of phytase in diet D2 did not trigger a beneficial effect on fish. This reinforces the idea that the amount of phytate added to the diets was not enough to reduce fish growth and increase its protein degradation, as phytate had no effect on fish, the action of phytase was not necessary, since its functionality is to degrade phytate. Furthermore, the autophagic-lysosomal system, analyzed through cathepsins L, D and B, and the ubiquitin-proteasome system, analyzed through the proteasome activity and the expression of the Psmb4 subunit, did not show evidence that there was greater protein degradation in fish fed diet D1 compared to diet D2. This result is also in line with the idea that phytate had no effect on fish, as they were tolerant to the added amount.

In the future, it would be interesting to carry out new tests with higher amounts of phytate in experimental diets than those added in this study, to identify the amount of phytate needed to observe negative effects on meagre juveniles. It would also be interesting to extend the trial time to clarify doubts regarding long-term effects.

7. REFERENCES

Baruah K., Sahu N. P., Pal A.K., Debnath D. (2004). Dietary phytase: an ideal approach for a cost effective and low polluting aqua feed. NAGA World Fish centre Quarterly. 27: 15–19.

Baskin K., Taegtmeyer H. (2011). Taking pressure off the heart: the ins and outs of atrophic remodelling. Cardiovascular Research. 90: 243-250.

Ben-Bani A., Sedki S., Nhhala H., Chebbaki K., Idhalla M., Kheyyali D., Bouhaimi A. and Moukrim A. (2018). Effect of protein/lipid ratio of the diet on the growth performance and feed utilization of common meagre (*Argyrosomus regius*, Asso 1801) juveniles. International Journal of Fisheries and Aquatic Studies. 6: 259-263.

Cabral H. and Ohmert B. (2001). Diet of juvenile meagre, *Argyrosomus regius*, within the Tagus estuary. Cahiers de Biologie Marine. 42: 289-293.

Cao L., Wang W., Yang C., Yang Y., Diana J., Yakupitiyage A., Luo Z., Li D. (2007). Aplication of microbial phytase in fish feed. Enzyme and Microbial Technology. 40: 497-507.

Cárdenas S. (2012). Biología y acuicultura de corvinas en el mundo. Revista AquaTIC. 37: 1-13.

Carter C.G. and Houlihan D.F. (2001). Protein synthesis. In: Wright, P.A., Anderson, P.M. (Eds.), Fish Physiology, Nitrogen Excretion, London: Academic Press. 20: 31–75.

Chao LN. (1986). Sciaenidae. Fishes of the North-eastern Atlantic and the Mediterranean. Unesco, Paris. 865-874.

Chéret R., Delbarre-Ladrat C., Lamballerie-Anton M., Verrez-Bagnis V. (2007). Calpain and cathepsin activities on post mortem fish and meat muscles. Food Chemistry. 101: 1474-1479.

Ciechanover A. (1994). The ubiquitin-proteasome proteolytic pathway. Cell 79. 13-21.

Conceição LEC., Skjermo J., Skjak-Braek G., Verreth JAJ., 2001. Effect of an immunostimulating alginate on protein turnover of turbot (*Scophthalmus maximus L.*) larvae. Fish Physiology and Biochemistry 24: 207–212.

Conceição LEC., Morais S., Dinis MT., Rønnestad I. (2008). Tracer studies in fish larvae. In: Cyrino JEP, Bureau D, Kapoor BG (eds) Feeding and Digestive Functions in Fishes. Science Publishers, Enfield, NH, USA. 349–392.

Dani D., 2018. A review on replacing fishmeal in aqua feeds using plant protein sources. International Journal of Fisheries Aquatic Studies 6: 164–179.

Debnath D., Sahu NP., Pal AK., Jain KK., Yengkokpam S.; Mukherjee SC. (2005). Mineral status of *Pangasius pangasius* (Hamilton) fingerlings in relation to supplemental phytase: absorption, whole body and bone mineral content. Aquaculture Research. 36: 326–335.

Delbarre-Ladrat C., Chéret R., Taylor R., Verrez-Bagnis V. (2006). Trends in postmortem aging in fish: understanding of proteolysis and disorganization of the myofibrillar structure. Critical Reviews in Food Science and Nutrition. 46: 409-421.

Denstadli V., Skrede A., Krogdahl A., Sahlstrøm S., Storebakken T. (2006). Feed intake, growth, feed conversion, disgestibility, enzyme activities and intestinal structure in Atlantic salmon (*Salmo salar L.*) fed graded levels of phytic acid. Aquaculture. 256: 365-376.

DGRM (2020). Plano Estratégico para a aquicultura portuguesa. Governo de Portugal, Ministério da Agricultura e do Mar.

Dobly A., Martin S.A.M., Blaney S.C., Houlihan D.F. (2004). Protein growth rate in rainbow trout (Oncorhynchus mykiss) is negatively correlated to liver 20S proteasome activity. Comparative Biochemistry and Physiology Part A. 137: 75-85.

Erbay E., Park I-H., Nuzzi PD., Schoenherr CJ., Chen J. (2003). IGF-II transcription in skeletal myogenesis is controlled by mTOR and nutrients. Journal of Cell Biology. 163: 931–936.

Estévez A., Treviño L., Kotzamanis Y., Karacostas I., Tort, L., Gisbert, E. (2010). Effects of different levels of plant proteins on the ongrowing of meagre (*Argyrosomus regius*) juveniles at low temperatures. Aquaculture Nutrition. 17: 572-582.

FAO (1976). Report of the FAO Technical Conference on Aquaculture. Food And Agriculture Organization of The United Nations.

FAO. (2000). Aquaculture Development Beyond 2000: the Bangkok Declaration and Strategy. Conference on Aquaculture in the Third Millennium, Bangkok, Thailand. NACA, Bangkok and FAO, Rome. 27.

FAO (2006). State of World Aquaculture. FAO Fisheries Technical Paper. Rome, FAO. 500: 145.

Fauconneau B., Aguirre P., Bergot P. (1986a). Protein synthesis in early life of coregonids: influence of temperature and feeding. Ergebnisse der Limnologie. 22: 171–188.

Fauconneau B., Aguirre P., Dabrowski K., Kaushik SJ. (1986b). Rearing of sturgeon (Acipenser baeri Brandt) larvae. Protein metabolism influence of fasting and diet quality. Aquaculture. 51: 117–131.

Francis G., Makkar HPS., Becker K. (2001). Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture. 199: 197-227.

García-Pacheco MM and Bruzón MA. (2009). Gametogenic cycle and first sexual maturity size of meagre, *Argyrosomus regius*. En: 4*th* Workshop on Gonadal Histology of Fishes. Centro IFAPA El Toruño, El Puerto de Santa María, Cádiz.

Gatlin III D., Barrows F., Brown P., Dabrowski K., Gaylord TG., Hardy RW., Herman E., Hu G., Krogdahl A., Nelson R., Overturf K, Rust M., Sealey W., Skonberg D., Souza EJ., Stone D., Wilson R. & Wurtele E. (2007). Expanding the utilization of sustainable plant products in aquafeeds: a review. Aquaculture Research. 38: 551-579.

Greiner R. and Konietzny U. (2006). Phytase for food application. Food Technology Biotechnology. 44: 125–40.

Hietakangas V., Cohen SM. (2009). Regulation of tissue growth through nutrient sensing. Annual Review of Genetics. 43: 389–410.

Houlihan DF., Hall SJ., Gray C., Noble BS. (1988). Growth rates and protein turnover in Atlantic cod, *Gadus morhua*. Canadian Journal of Fisheries and Aquatic Sciences. 45: 951–964.

Houlihan DF., Wieser W., Foster A., Brechin J. (1992). In vivo protein synthesis in larval nase (*Chondrostoma nasus L.*). Canadian Journal of Zoology. 70: 2436–2440.

Houlihan DF., Pannevis M., Heba H. (1993). Protein synthesis in juvenile Tilapia *Oreochromis mossambicus*. Journal of the World Aquaculture Society. 24: 145–151.

Ji H. (1999). Anti-nutritional factors in plant based fish feed. Fish Reserve. 19: 22-24.

Jiménez MT., Pastor E., Grau A., Alconchel I., Cárdenas S. (2005). Revisión sobre el cultivo de esciénidos en el mundo, con especial atención a la corvina (*Argyrosomus regius*). Boletin - Instituto Espanhol Oceanografia 21: 169-176.

Jongbloed AW. and Kemme PA. (1990). Effect of pelleting mixed feeds on phytase activity and the apparent absorbability of phosphorus and calcium in pigs. Animal Feed Science Technology. 28: 233–42.

Kaushik SJ. and Seiliez I. (2010). Protein and amino acid nutrition and metabolism in fish: current knowledge and future needs. Aquaculture Research. 41: 322-332.

Kumar, V., Sinha, A. K., Makkar, H. P. S., Becker, K. (2010). Dietary roles of phytate and phytase in human nutrition: a review. Food Chemistry. 120: 945–959.

Kumar V., Sinha AK., Makkar HPS., Boeck G., Becker K. (2012). Phytate and phytase in fish nutrition. Journal of Animal Physiology and Animal Nutrition. 96: 335-364.

Lagardére, JP. and Mariani A. (2006). Spawning sounds in meagre *Argyrosomus regius* recorded in the Gironde estuary, France. Journal of Fish Biology. 69: 1697-1708.

Laining A., Traifalgar RF., Thu M., Komilus CF., Kader MA. (2010). Influence of dietary phytic acid on growth, feed intake, and nutrient utilization in juvenile japonese flounder, Paralichthys olivaceus. Journal of the World Aquaculture Society. 41: 5.

Liebert F. and Portz L. (2005). Nutrient utilization of Nile tilapia Oreochromis niloticus fed plant based low phosphorus diets supplemented with graded levels of different sources of microbial phytase. Aquaculture. 248: 111–9.

Liener IE. (1989). Antinutritional factors in legume seeds: state of the art. In: Huisman, J., Van der Poel, A.F.B., Liener, I.E. (Eds.), Recent Advances of Research in Antinutritional Factors in Legume Seeds. Pudoc, Wageningen. 6–14.

Makkar HPS. (1993). Antinutritional factors in foods for livestock. In: Gill, M., Owen, E., Pollot, G.E., Lawrence, T.L.J. (Eds.)., Animal Production in Developing Countries. Occasional publication. British Society of Animal Production. 16: 69–85.

Martin SAM., Blaney S., Bowman AS., Houlihan DF. (2002). Ubiquitin-proteasome-dependent proteolysis in rainbow trout (*Oncorhynchus mykiss*): effect of food deprivation. European Journal Physiology. 445: 257-266.

Martins G., Ribeiro L., Candeias-Mendes A., Diogo P., Gamboa M., Barata M., Cancela ML., Pousão-Ferreira P., Gavia PJ. (2019). Reduction of skeletal anomalies in meagre (*Argyrosomus regius*, Asso, 1801) through early introduction of inert diet. Aquaculture Research. 1-11.

Matias AC., Dias J., Barata M., Araujo RL., Bragança J., Pousão-Ferreira P. (2020). Taurine modulates protein turnover in several tissues of meagre juveniles. Aquaculture. 528.

Monfort M. (2010). Present market situation and prospects of meagre (*Argyrosomus regius*), as an emerging species in Mediterranean aquaculture. Studies and Reviews. General Fisheries Commision for the Mediterranean (GFCM). 89: 28.

Nayini N. R. and Markakis P. (1986). Phytases. In: E. Graf (ed), Phytic acid: Chemistry and Applications. Pilatus Press, Minneapolis, MN. 101–118.

Naylor L., Ronald W. Hardy, Alejandro H. Buschmann, Simon R. Bush, Ling Cao, Dane H. Klinger, David C. Little, Jane Lubchenco, Sandra E. Shumway & Max Troell. (2021). A 20-year retrospective review of global aquaculture. Nature. Vol 591.

Panagiotidou M., Chatzifotis S., Papaioannou N., Papasolomontos S., Pavlidis M., Nengas I., Mylonas CC. (2007). Effect of dietary lípid levels on growth, feed utilization, body composition and serum metabolites of meagre (*Argyrosomus regius*). Aquaculture Europe. Estambul, Turquía.

Piccolo G., Bovera F., De Riu N., Marono S., Salati F., Cappuccinelli R., Moniello G. (2008). Effect of two different proteína/fat ratios of the diet on meagre (*Argyrosomus regius*) traits. Italian Journal of Animal Science. 7: 363-371.

Pirozzi I., Booth MA., Allan GL. (2010). The interactive effects of dietary protein and energy on feed intake, growth and protein utilization of juvenile mulloway (*Argyrosomus japonicus*). Aquaculture Nutrition. 16: 61-71.

Pointillart A. (1988). Phytate phosphorus utilisation in growing pigs. In: Buraczewska L, Buraczewska S, Zebrowska T, editors. Digestive physiology in the pig. Proceedings of the fourth international seminar. Jablonna, Poland: Polish Academy of Science. 192–6.

Poli BM, Parisi G, Mecatti M, Lupi P, Iurzan F, Zampacavallo G, Gilmozzi M. (2001). The meagre (*Argyrosomus regius*), a new species for Mediterranean aquaculture.1. Morphological, merchantable and nutritional traits in a commercial wide size-range. EAS Special Publication. 29: 209-210.

Quémener L. (2002). Le maigre commun (*Argyrosomus regius*). Biologie, pêche, marché et potentiel aquacole. Éditions Ifremer, Plouzané, France. 31.

REA: Portal do Estado do Ambiente. (2021). Produção em aquicultura. Disponível em: <rea.apambiente.pt>. Acesso em: 18/12/2021.

Reeds PJ. (1989). Regulation of protein turnover. In: Campion DR, Hausman GJ, Martin RJ (eds) Animal Growth and Regulation. Plenum Press, New York. 183–210.

Ribeiro, L., Soares, F., Quental-Ferreira, H., Gonçalves, A., Pousão-Ferreira, P. (2013). Portuguese research studies: Meagre production in earthen ponds. Global Aquaculture Advocate 16. 38–40.

Ribeiro L., Moura J., Santos M., Colen R., Rodrigues V., Bandarra N., Soares F., Ramalho P., Barata M., Moura P., Pousão-Ferreira P., Dias J. (2014). Effect of vegetable based diets on growth, intestinal morphology, activity of intestinal enzymes

and haematological stress indicators in meagre (*Argyrosomus regius*). Aquaculture. 447: 116-128.

Richardson, N.L., Higgs D. A., Beames R. M., McBride J. R. (1985). Influence of dietary calcium, phosphorus, zinc and sodium phytate level on cataract incidence, growth, and histopathology in juvenile Chinook salmon (*Oncorhynchus tshawytscha*). Journal of Nutrition. 115: 553–567.

Rossi A., Deveraux Q., Turk B., Sali A. (2004). Comprehensive search for cysteine cathepsins in the human genome, Biological Chemistry. 385: 363–372.

Rumsey, G.L., Hughes, S.G., Winfree, R.A. (1993). Chemical and nutritional evaluation of soy protein preparations as primary nitrogen sources of rainbow trout (*Oncorhynchus mykiss*). Animal Feed Science Technology. 40: 135–151.

Sajjadi M. and Carter CG. (2004). Dietary phytase supplementation and the utilization of phosphorus by Atlantic salmon (*Salmo salar L.*) fed a canola-meal-based diet. Aquaculture. 240: 417-431.

Salmerón C., Navarro I., Johnston IA., Gutiérrez J., Capilla E. (2015). Characterisation and expression analysis of cathepsins and ubiquitin-proteasome genes in gilthead sea bream (*Sparus aurata*) skeletal muscle. BMC Research Notes. 8: 149.

Segato S., Corato A., Fasolato L., Andrighetto I. (2005). Effect of the parcial replacement of fishmeal and oil by vegetable products on performance and quality traits of juvenile shi drum (*Umbrina cirrosa L.*). Italian Journal of Animal Science. 4: 159-166.

Seiliez I., Dias K., Cleveland BM. (2014). Contribution of the autophagy-lysosomal and ubiquitin-proteasomal proteolytic systems to total proteolysis in rainbow trout (Oncorhynchus mykiss) myotubes. American Journal Physiology-Regulatory. Integrative and Comparative Physiology. 307: 1330-1337.

Shanmugam G. (2018). Characteristics of phytase enzyme and its role in animal nutrition. International Journal of Current Microbiology and Applied Sciences. 7: 1006-1013.

Simons, P. C.; Versteegh, H. A.; Jongbloed, A. W.; Kemme, P. A.; Slump, P.; Bos, K. D.; Wolters, M. G.; Beudeker, R. F.; Verschoor, G. J. (1990). Improvement of phosphorus availability by microbial phytase in broilers and pigs. British Journal of Nutrition. 64: 525–540.

Spinelli, J.; Houle, C. R.; Wekell, J. C. (1983). The effect of phytates on the growth of rainbow trout (*Salmo gairdneri*) fed purified diets containing varying quantities of calcium and magnesium. Aquaculture. 30: 71–83.

Stefan H., Anja K., Edzard S., Joerg B., Markus L., Oskar Z. (2005). Biotechnological production and applications of phytases. Appl Microbiol Biotechnol. 68: 588–97.

Swick, R. A. and Ivey F. J. (1992). Phytase: the value of improving phosphorus retention. Feed Manage. 43: 8–17.

Tacchi A., Secombes SJ., Bickerdike R., Adler MA., Venegas C., Takle H., Martin SA. (2012). Transcriptomic and physiological responses to fishmeal substitution with plants proteins in formulated feed in farmed Atlantic salmon (*Salmo salar*), BMC Genomics. 13: 1-21.

TECNOALIMENTAR: Revista da Indústria Alimentar. (2015). Aquicultura em Portugal: um Setor em Crescimento. Disponível em: <tecnoalimentar.pt>. Acesso em: 18/12/2021.

Turk B., Turk D., Turk V. (2000). Lysosomal cysteine proteases: more than scavengers, Biochimica et Biophysica Acta. 1477: 98–111.

Turk V., Stoka V., Vasiljeva O., Renko M., Sun T., Turk B., Turk D. (2012). Cystheine cathepsins: From structure, fuction and regulation to new frontiers. Biochimica et Biophysica Acta. 68-88.

Tyagi P. K. and Verma S. V. S. (1998). Phytate phosphorus content of some common poultry feed stuffs. Indian Journal of Poultry Science. 33: 86–88.

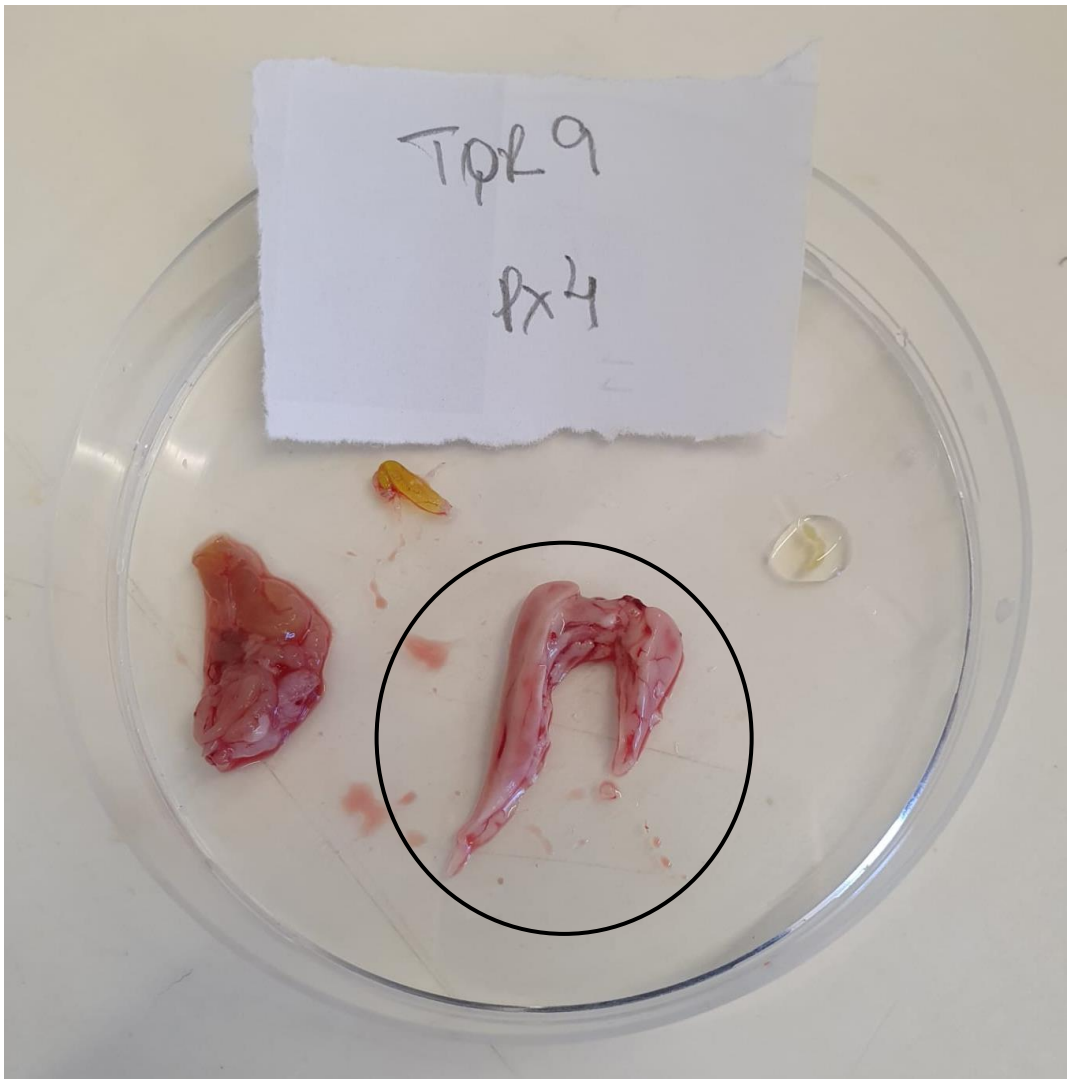
Valente L.M.P., Moutou K.A., Conceição L.E.C., Engrola S., Fernandes J.M.O. (2013). What determines growth potential and juvenile quality of farmed fish species? Reviews in Aquaculture 5: 168-193.

Van der Peol, A.F.B. (1989). Effects of processing on antinutritional factors (ANF) and nutritional value of legume seeds for non-ruminant feeding. In: Huisman, J., Van der Poel, A.F.B., Liener, I.E. (Eds.)., Recent Advances of Research in Antinutritional Factors in Legume Seeds. Pudoc, Wageningen. 213–229.

8. APPENDICES

Appendix I – Final sampling of the juvenile meagre (*Argyrossomus regius*) trial

Appendix II – Sample of a meagre liver indicated with a black circle



Appendix III – Collection of a muscle sample from a juvenile meagre