Denise Schrama

Characterization and modulation of fish allergenicity towards the production of a low allergen farmed fish

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Declaração de autoria do 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.

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“A friend is one that knows you as you are, understands where you have been, accepts what you have become, and still, gently allows you to grow”.

William Shakespeare

During this PhD thesis several scientific papers and two book chapters were published in international peer-reviewed journals:

Published scientific papers:

- **Schrama D**, Raposo de Magalhães C, Pedro M Rodrigues. *Modulação da alergenicidade em peixes de cultivo*. Studies in Social Sciences Reviews. 2022;3(4),832-845. DOI: 10.54020/seasv3n4-002

- **Schrama D,** Raposo de Magalhães C, Cerqueira M, Carrilho R, Revets D, Kuehn A, Engrola S, Rodrigues PM. *Fish processing and digestion affect parvalbumins detectability in gilthead seabream and European seabass*. Animals. 2022;12,3022. DOI:10.3990/ani12213022

- **Schrama D**[*], Czolk R[*], Raposo de Magalhães C[*], Kuehn A, Rodrigues PM. *Fish Allergenicity modulation using tailored enriched diets – where are we?* Frontiers in Physiology. 2022;13. DOI: 10.3389/fphys.2022.897168
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- Costa J, Villa C, Verhoeckx K, Cirkovic-Velickovic T, **Schrama D**, Roncada P, Rodrigues PM et al. *Are physicochemical properties shaping the allergenic potency of animal allergens?* Clinical Reviews in Allergy & Immunology. 2022;62(1):1-36. DOI: 10.1007/s12016-020-08826-1

- Pereira LN, Mendes J, **Schrama D**, Rodrigues PML. *Consumers' Willingness to Pay for Fish with a Low Allergenic Potential*. Journal of International Food & Agribusiness Marketing. 2020:1-22. DOI: 10.1080/08974438.2020.1844108

- Klueber J, **Schrama D**, Rodrigues P, Dickel H, Kuehn A. *Fish allergy management: from component-resolved diagnosis to unmet diagnostic needs*. Current Treatment Options in Allergy. 2019;6(4):322-37. DOI: 10.1007/s40521-019-00235-w

- De Magalhaes CR*, **Schrama D***, Fonseca F, Kuehn A, Morisset M, Ferreira SR, Gonçalves A, Rodrigues PM. *Effect of EDTA enriched diets on farmed fish allergenicity and muscle quality; a proteomics approach*. Food Chemistry. 2020;305. DOI: 10.1016/j.foodchem.2019.125508.
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Summary

Food allergies are a common health problem worldwide, triggering an abnormal immune response. Fish belongs to the top nine of most allergenic foods, among milk, eggs, shellfish, tree nuts, peanuts, wheat, soybeans and the most recently added sesame. The continuing increase of aquaculture production and the relatively easy access to fish worldwide, contribute to increased fish consumption which result in higher prevalence of allergies. The main allergen in fish, responsible for up to 70-95% of the allergic reactions, is a small and stable calcium-binding muscle protein named parvalbumin. This thesis was focused on parvalbumin in two economically important fish species for Southern Europe aquaculture, namely gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*). **Chapter 2** characterized this allergen, determining its structure by circular dichroism, sequencing its amino acids by mass spectrometry and analyzing its stability after fish digestion or processing. Results showed that parvalbumin represents a higher content of α-helices and some β-sheets in its secondary structure, at room temperature. Parvalbumins detection reduced throughout gastrointestinal digestion, and also several processing techniques, like salting, steaming and autoclaving showed a significant reduction ($p<0.05$) in parvalbumins detectability. In **Chapter 3,** a questionnaire conducted in Portugal was performed to evaluate consumers' willingness to pay for low allergenic fish. Results showed that not only half the consumers were willing to pay extra, but also suggested that this was explained by the presence of fish allergies in the family and by the fish unique characteristics and quality. For the modulation of fish allergenicity **Chapters 4.1, 4.2 and 4.3** analyzed this possibility by the supplementation of fish diets with additives like creatine and ethylenediamine tetraacetic acid (EDTA). Results showed a 50% reduction in fish-allergic serum Immunoglobulin-E (IgE)-reactivity when 3% EDTA was supplemented in gilthead seabreams diet. This promising result showed the possibility to modulate parvalbumin in order to decrease its allergenicity.

Keywords: Allergenicity, Creatine, EDTA, European seabass, Gilthead seabream, Parvalbumin

Resumo

Alergias alimentares são um problema a nível global para a saúde humana, induzindo uma resposta imune anormal. O peixe é um dos nove alimentos mais alergénicos, entre leite, ovos, marisco, nozes, amendoins, trigo, soja e o recentemente adicionado sésamo. O aumento continuo da produção em aquacultura e o acesso relativamente fácil a peixe globalmente, contribui para o aumento no consumo do mesmo, resultando numa maior prevalência de alergias. O principal alergénio em peixe, responsável por 70-95% das reações alérgicas, é uma proteína presente no músculo de peixe, pequena e estável que se liga ao cálcio chamada parvalbumina. Este trabalho focou-se na parvalbumina de duas espécies de peixe economicamente importantes para a aquacultura no sul da Europa, nomeadamente a dourada (*Sparus aurata*) e o robalo (*Dicentrarchus labrax*). O **Capítulo 2** caracterizou este alergénio, determinando a sua estrutura por dicroísmo circular, sequenciando os seus aminoácidos por espectrometria de massa e analisando a sua estabilidade após a digestão ou processamento do peixe. Os resultados mostraram que a parvalbumina apresenta a temperatura ambiente uma maior quantidade de hélices-α e algumas folhas-β na sua estrutura secundária. A deteção da parvalbumina diminuiu ao longo da digestão no trato gastrointestinal, e também após vários métodos de processamento, tal como a salgação, vapor e autoclave mostraram uma redução significativa ($p<0.05$) na detetabilidade da mesma. No **Capítulo 3**, um questionário conduzido em Portugal foi elaborado para avaliar a disponibilidade dos consumidores em pagar por peixes com baixo teor de alergénios. Os resultados mostraram que não só metade dos consumidores está disposto a pagar extra, mas também sugeriu que isto é explicado pela presença de alergias a peixes na família e pelas características únicas e qualidade do peixe. Relativamente à modulação da alergenicidade dos peixes, os **Capítulos 4.1, 4.2 e 4.3** analisaram esta possibilidade através da suplementação das dietas dos peixes com aditivos como creatina e ácido etilenodiaminotetraacético (EDTA). Foram realizadas análises em termos de crescimento dos peixes, da sua qualidade e eventual retenção dos aditivos no musculo dos mesmos. Foram ainda realizados estudos de expressão da parvalbumina e da reatividade das Imunoglobulinas-E (IgE) foram feitas através de géis bidimensionais (2D-DIGE) e imunoblots com soro humano (controlo e alérgicos a peixe), respectivamente. Os resultados mostraram que a adição de creatina nas duas espécies não influenciou o seu crescimento. No entanto, no caso da adição de EDTA foi necessário

adicionar um atrativo para obter o mesmo crescimento dos peixes alimentados com dietas suplementadas com este aditivo em relação ao controlo. A qualidade dos peixes não sofreu alterações com a suplementação (exceto na dourada alimentada com EDTA, aonde se verificou uma ligeira diferença na textura), nem se registou uma acumulação da creatina ou EDTA no músculo da dourada ou robalo. A análise 2D-DIGE não mostrou diferenças na expressão da parvalbumina nas duas espécies, pelo que podemos aferir que este aditivo não alterou a alergenicidade destes peixes. Observou-se, no entanto, observou-se uma redução de 50% na reatividade das IgE no soro de consumidores alérgicos a peixe, no caso das douradas alimentadas com uma dieta suplementada em 3% EDTA. Este resultado promissor mostrou a possibilidade de modular a parvalbumina de modo a diminuir a sua alergenicidade.

Palavras-chave: Alergenicidade, Creatina, Dourada, EDTA, Parvalbumina, Robalo

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Chapter 1

General Introduction

1.1 Fish consumption

Aquatic food consumption, like fish, provide important nutrients for the human population (Chen et al., 2022). High-quality proteins and protective omega-3 fatty acids (also known as n-3 polyunsaturated fatty acids) are some of the benefits of aquatic food consumption (Klueber et al., 2019). Additionally, important vitamins and minerals such as Vitamin D_3 and B_{12}, selenium, iodine, phosphorus and calcium also contribute to its high consumption rates (Khalili Tilami and Sampels, 2018). Several studies in humans proved that ingestion of fish benefits overall health and prevents chronic diseases like cardiovascular disease and diabetes mellitus type 2 (Tørris et al., 2014, Tørris et al., 2018, Tørris et al., 2017, Mohan et al., 2021). Besides this, studies with woman proved that an increased intake of fish during pregnancy reduced the risk for atopy (wheeze, eczema, food allergy) in children (Malmir et al., 2021). Neuropsychiatric disorders including depression might also be positively influenced by the ingestion of fish (Khanna et al., 2019). In 2020, worldwide aquatic food consumption reached 157.4 million tonnes of live weight (excluding 20.4 million tonnes used for non-food proposes), which represents 20.2 kg per capita (Fig. 1.1) and accounts for 17% of animal protein intake (FAO, 2022). Due to the mentioned nutritional values of seafood it is expected that its demand rises with the global population growth, which is estimated to reach 9.7 billion, by 2050 (United Nations Departement of Economic and Social Affairs PD, 2019).

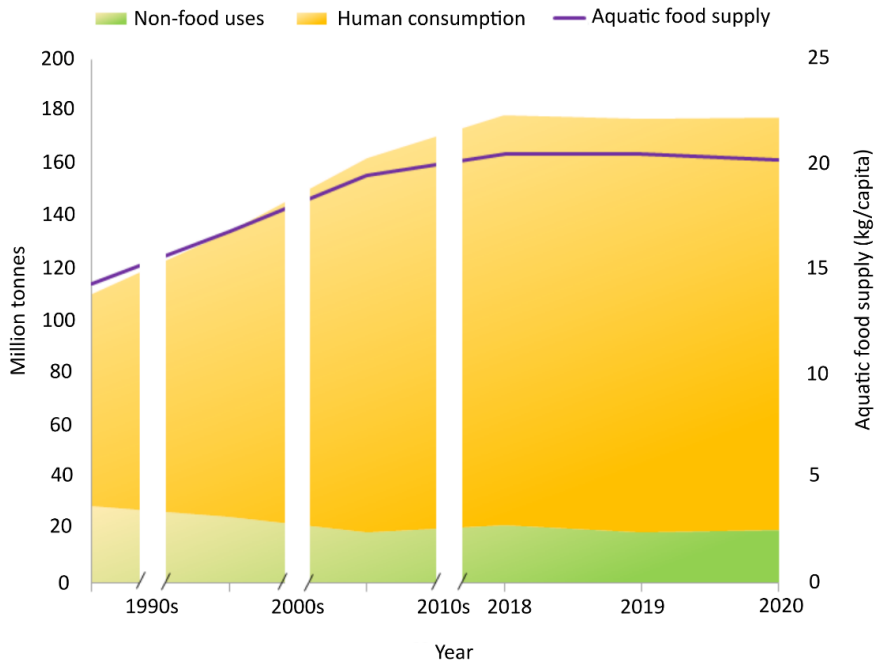

Fig. 1.1 – Global aquatic food consumption and non-food uses, coming from wild capture and aquaculture production from 1990s-2020 in million tonnes of live weight (Source: FAO, 2022).

Fish consumption varies by geographical location, such as illustrated for different continents with rates of 10.1 kg/capita/year in Africa versus 24.5 kg/capita/year in Asia in 2019 (Fig. 1.2). Even within the same continent, some countries show higher consumption of fish than others, due to traditional eating habits (e.g. in Europe: Portugal, Iceland and Norway; in Asia: Japan and Republic of Korea) or due to low accessibility of land-based protein sources (e.g. in Europe: the Faroe Islands; in North America: Greenland) (FAO, 2022). In Portugal and among others, fish contribute >10 g/day/capita to animal protein supply, which represents more than 20% (FAO, 2022). Portugal shows the highest fish consumption in the European Union (EU) with 59.9 kg/capita/year while the EU average is 24.0 kg/capita/year (European Comission, 2021a).

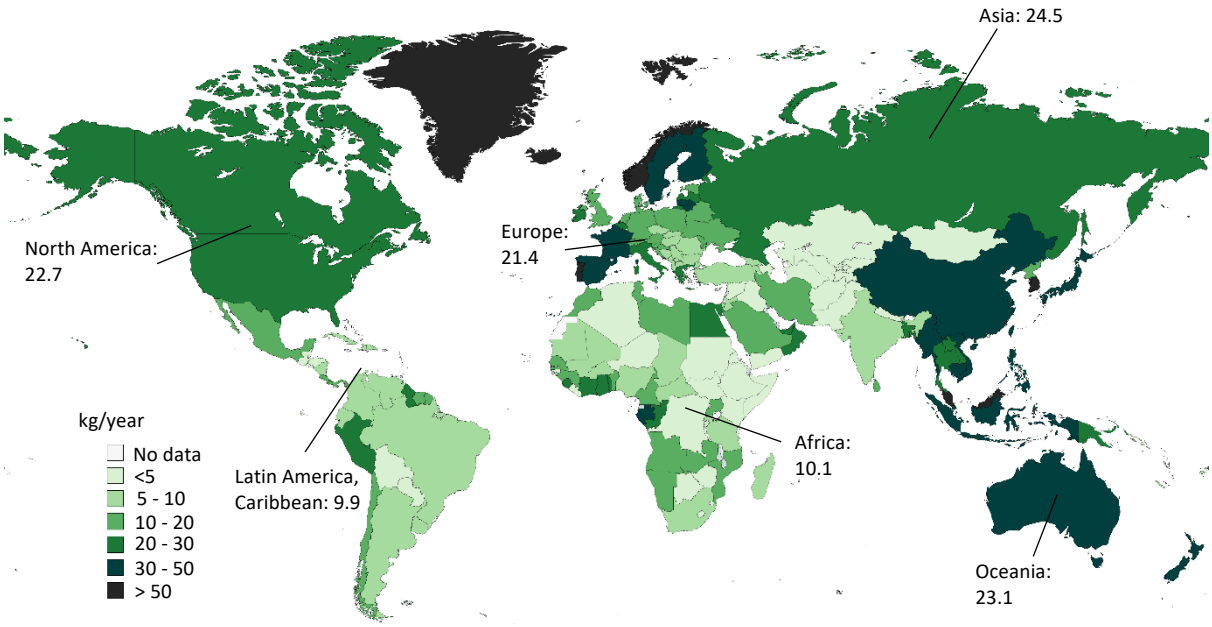

Fig. 1.2 – Total aquatic food consumption (kg/year) per capita by geographical region (average per continent) and by country (colours) from 2017-2019 (Source: FAO, 2022, created with mapchart.net).

For consumption, fish is mainly available on the market as fresh or chilled (44%), whereas 35% is frozen. After processing 11% represents prepared and preserved fish and 10% cured (FAO, 2022). Consumption of freshwater and diadromous fish represented the highest value of 8.2 kg per capita, in 2019, whereas marine finfish accounted for 6.8 kg per capita (FAO, 2022). In Europe, the top five fish species consumed are tuna (canned, mainly skipjack (*Katsuwonus pelamis)* and yellowfin (*Thunnus albacares*) (European Comission, 2021a)),

Atlantic salmon (*Salmo salar*) (European Comission, 2020), Atlantic cod (*Gadus morhua*) (European Comission, 2021b), Alaska pollock (*Gadus chalcogrammus*) and herring (*Clupea harengus*). In the USA, channel catfish (*Ictalurus punctatus*) (Billa et al., 2016), pangasius (*Pangasianodon hypophthalmus*) and Nile tilapia (*Oreochromis niloticus*) are also highly consumed besides tuna (canned, skipjack and albacore (*T. alalunga*)), Atlantic salmon (Shamshak et al., 2019), Pacific cod (*G. macrocephalus*) and Alaska pollock (NOAA Fisheries, 2020). Another important species in countries south of the equator is shark (e.g. gummy shark (*Mustelus antarcticus)* in Australia (Braccini et al., 2020) and Caribbean sharp nose shark (*Rhizoprionodon porosus*) in Brazil (Maciel et al., 2021).

1.1 Aquaculture contribution

As fish consumption will keep rising, world fisheries and aquaculture production need to increase to produce more fish. Seafood wild capture numbers reached 90.3 million tonnes in 2020 (Figure 1.3), where aquaculture production represented a record with 87.5 million tonnes (FAO, 2022). World aquaculture fish production is led by China with almost 57% (49.6 million tonnes), followed by India (8.6 million tonnes) and Indonesia (5.2 million tonnes). Production of grass carp showed the highest contribution for finfish with 10.5% (FAO, 2022). Marine and coastal aquaculture in Europe is mainly dominated by Atlantic salmon production, accounting for 2.7 million tonnes, in 2020, followed by milkfish (*Chanos chanos*) with 1.1 million tonnes. Aquaculture in Portugal is mainly focused on marine species, with a total of 3,557 tonnes of fish production annually. This PhD thesis focused on gilthead seabream (*Sparus aurata)* and European seabass (*Dicentrarchus labrax*) which accounted for an aquaculture production of 1,751 and 904 tonnes, respectively in 2020 (FAO).

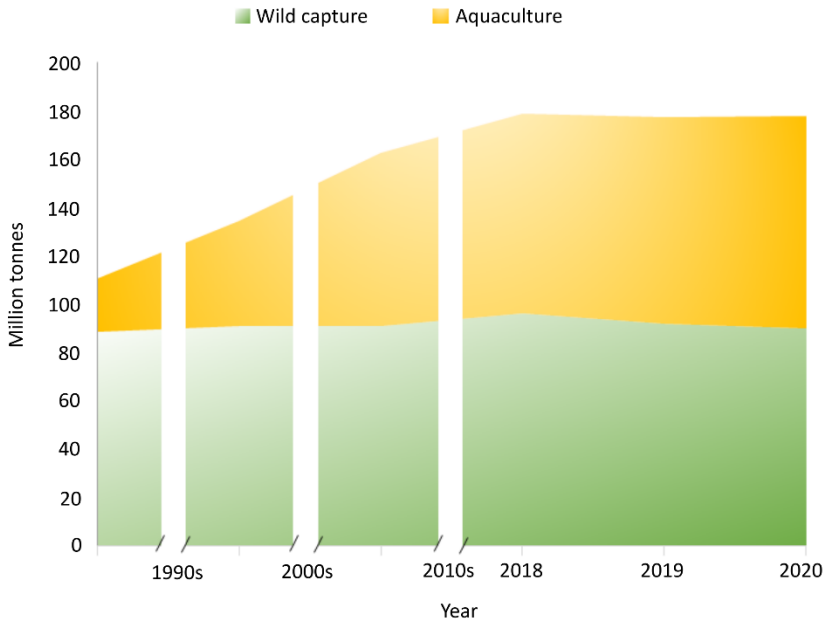

Fig. 1.3 – Global wild capture and aquaculture production from 1990-2020 in million tonnes of live weight (Source: FAO, 2022)

In 2020, aquaculture led to a production of 87.5 million tonnes, which represented 55% of seafood for human consumption. The biennial flagship report from the Food and Agriculture Organization (FAO), named the State of World Fisheries and Aquaculture (SOFIA) estimates that aquaculture production will reach 62% by 2030 (Fig.1.4) (FAO, 2022).

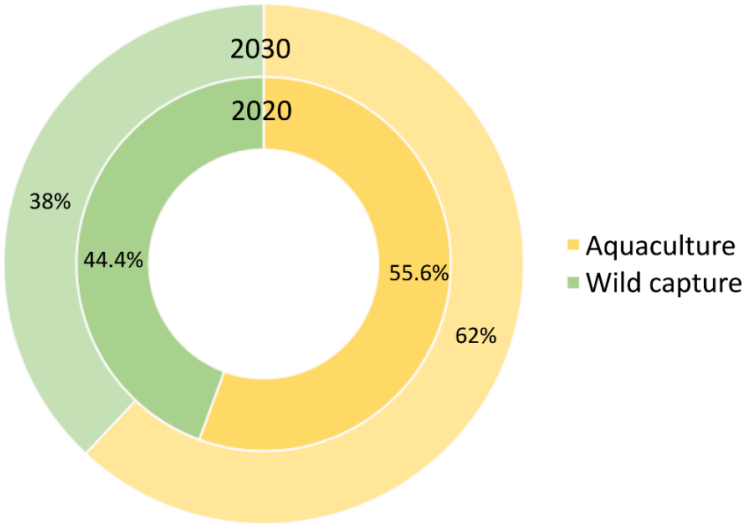

Fig. 1.4 – Estimation of global aquaculture and wild capture contribution for human consumption in 2030 (Source: FAO, 2022)

1.1.1 Gilthead seabream

The marine fish gilthead seabream (*Sparus aurata* Linnaeus, 1758), belongs to the Actinopterygii class (ray-finned fish) (Fig. 1.5). This fish occurs in the Mediterranean Sea and in the Eastern Atlantic from the strait of Gibraltar to Cape Verde, around the Canary Islands and in the Black Sea. It tolerates brackish waters explaining why it might be found in estuaries. It is mainly a carnivorous and sedentary fish. It is one of the most important fish in marine aquaculture, being one of the top farmed species in the Mediterranean Sea, especially in Greece, Turkey, Spain and Italy (Sola et al., 2007). This species can be farmed in sea cages in intensive farming, where densities (10-15 $kg.m^{-3}$) are lower than in tanks (15-45 $kg.m^{-3}$). It is highly sensitive to low water temperatures (<12°C the winter disease highlights (Schrama et al., 2017)).

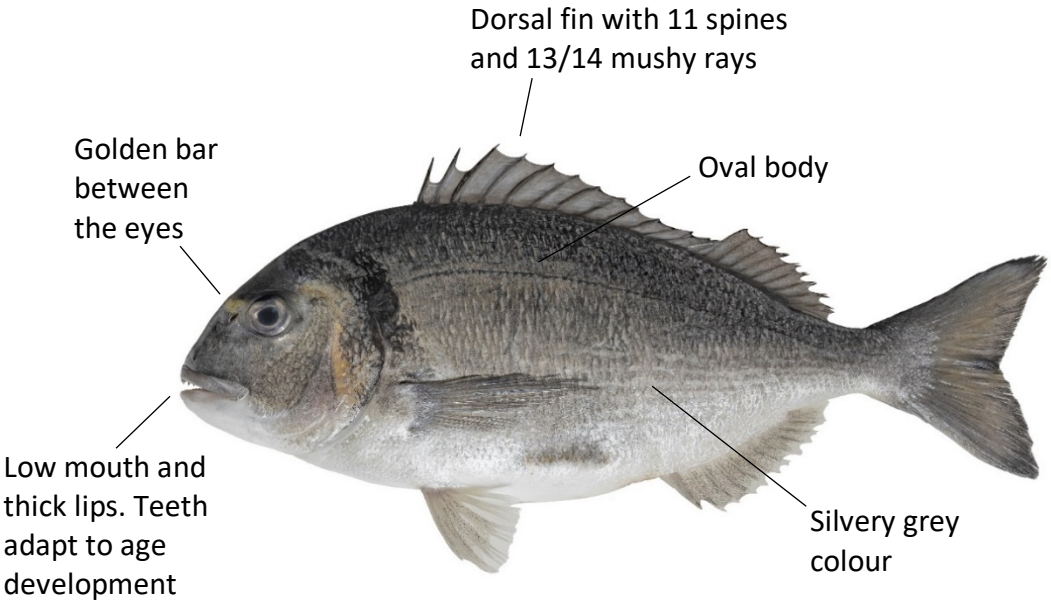

Fig. 1.5. Gilthead seabream (*Sparus aurata* Linnaeus, 1758).

1.1.2 European seabass

The marine fish European seabass (*Dicentrarchus labrax*, Linnaeus, 1758), belongs to the Actinopterygii class (Fig. 1.6). It lives in the Mediterranean Sea, and in the Eastern Atlantic from Norway to Morocco, the Canary Islands, Senegal and in the Black Sea. It tolerates brackish and even freshwater. This species is highly considered for capture and recreational fisheries, but nowadays it is clearly an aquaculture species, mainly in Mediterranean countries like Turkey, Greece, Egypt and Spain (Vandeputte et al., 2019). On growing stages of

production are mainly in sea cages, and although this species also prefers temperatures above 10°C, it does not seem to be as sensitive as gilthead seabream (Vandeputte et al., 2019).

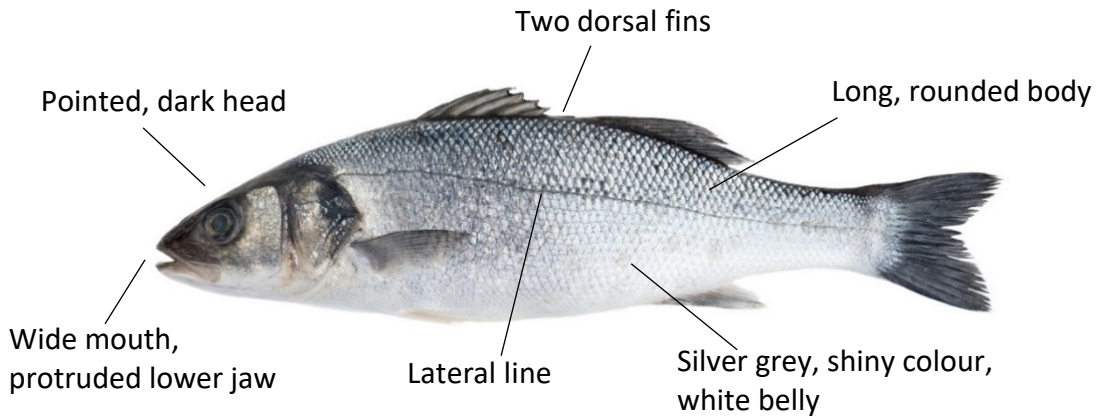

Fig. 1.6. European seabass (*Dicentrarchus labrax*, Linnaeus, 1758).

With the consumption of aquatic food, but also other types of food, an adverse reaction of the human body might be triggered, resulting in allergies against a specific food. This introduction will now be focusing on (food) allergic reactions and on the importance of understanding them better.

1.2 Allergies

In the beginning of the 20th century scientists, particularly Louis Pasteur and Paul Ehrlich, described for the first time fundamental characteristics of the immune system. A new era of vaccination medicine was heralded, though adverse reactions to vaccines could not (yet) be explained (Igea, 2013). The pediatrician Clemens von Pirquet was the first scientist to assume the immune system as responsible for these adverse reactions. He called it “allergy”, which he explained by the presence of a foreign substance triggering either a protective response leading to immunity or a harmful response leading to hypersensitivity (Igea, 2013). Noteworthy, the established definition of allergy by von Pirquet was not highly accepted by his colleagues, which later resulted in misunderstandings and misuse of the term allergy. Today, the international consensus definition of an allergic reaction is the occurrence of single or multiple symptoms which appear reproducibly due to a hypersensitive immune reaction against mostly proteins, called allergens (Igea, 2013, Ruethers et al., 2018). Allergies are a worldwide increasing health concern. They represent today the most common disease

involving chronic inflammation. In most cases, there is no causal cure available for the patients. Medical care comprises mainly symptomatic treatment with rescue medication and the set-up of avoidance measures. However, immunotherapy might be helpful in changing the immune response (refer to section 1.3.1.2). Avoidance appears feasible to a certain extent for some allergen sources, such as animal dander, foods and drugs, while others are challenging to avoid (e.g. house dust mites, tree and grass pollens) (Pawankar et al., 2013). Symptoms of an allergic reaction can be classified as either mild (e.g. sneezing or itching), moderate (e.g. conjunctivitis or swelling) or severe (e.g. anaphylactic shock).

Regarding the underlying immune mechanism, allergies might be cell-mediated or antibody-mediated. The cell-mediated response is less common. It is also referred to subtypes of Type four (IV), non-Immunoglobulin E (non-IgE) or delayed-type hypersensitivity, where the symptom onset might take 24 h and even up to several days. T cells are the key players of those reactions. Briefly, dendritic cells (allergen presenting cells) transport the allergen peptides to T cells (e.g. CD4+), where they will be recognized by specific receptors. Consequently, T cells differentiate into subtypes of T helper cells (e.g. Th1) with the help of interleukins (e.g. IL-1 or IL-12). Subsequent contact with the allergen results in the activation of macrophages by sensitized Th1 cells which remove the allergen with the help of enzymes and free radicals (Fig. 1.7A) (Punt et al., 2018). Examples of a type IV reaction are skin contact with poison plants, heavy metals, or latex. In case of the more common antibody-mediated response, also referred as type I or IgE-mediated, reactions to an allergen normally occur commonly within minutes (early response) or exceptionally, after several hours (late response). Briefly, allergens on dendritic cells are recognized by specific receptors on CD4+ T cells and consequently induce differentiation to Th2 cells. Activation and release of cytokines by Th2 cells turns a B cell into a plasma cell, which produces and releases IgE antibodies. In this process the IgE antibodies bind to Fc receptors on effector cells (mast cells, basophils and eosinophils), which are now sensitized to the specific allergen and are able to respond rapidly to a re-exposure. Activation of the mast cell/basophil induces a degranulation where histamine, platelet-activating factor (PAF) and other mediators are released, resulting in an (early) allergic reaction. Th2 cells releases IL-3 and IL-5, which activate eosinophils (leukocytes) being involved in a late allergic reaction, likewise the mast cells which also activate these white blood cells (Fig. 1.7B) (Punt et al., 2018). Examples of type I reaction are allergies due to food, drugs or pollen, among others.

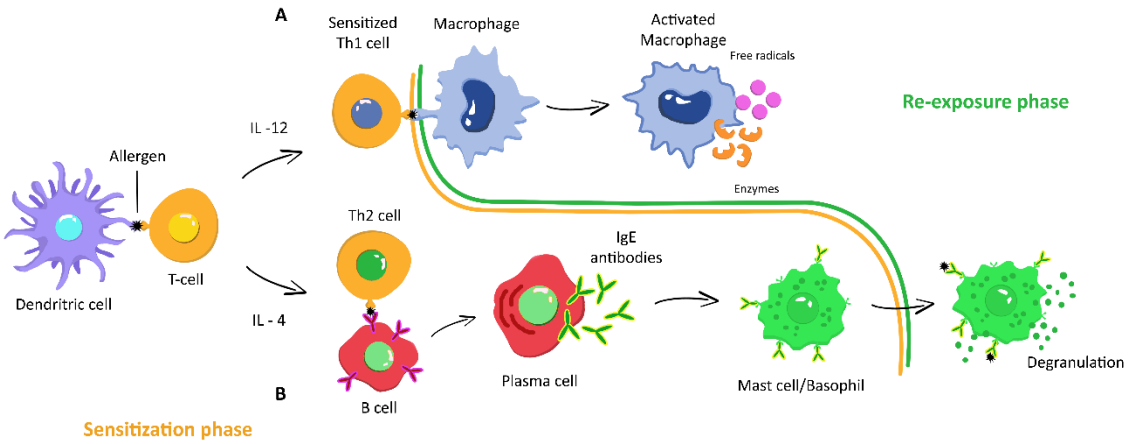

Fig. 1.7 – Summarized mechanism of hypersensitivity reactions type IV or cell-mediated (A) and type I or IgE-mediated (B). Yellow and green line differentiate between the sensitization and re-exposure phase.

1.2.1 Food allergies

The terms food intolerance and allergy are often confounded due to similarity in some foods and elicited symptoms (Muthukumar et al., 2020). Food allergy and intolerance are distinguished by being immune or non-immune mediated, respectively. Symptoms of intolerance occur mainly in the gastrointestinal tract. The most common one is milk intolerance. Rather than an active immune response to milk proteins, lactose intolerance involves gastrointestinal symptoms resulting from enzymatic malfunction in the breakdown of this disaccharide (Ortolani and Pastorello, 2006). Basically, any kind of food may trigger an allergic reaction. More than 170 foods are yet described as causes of food allergies. However, 90% of the cases are due to the top 9 food allergens, namely milk, egg, wheat, fish, shellfish, peanuts, tree nuts, soy and sesame (Vincent et al., 2021). Occupational allergy is another way of exposure to food allergens, where triggers of a hypersensitive reaction are taken up by the cutaneous or respiratory system, by workers in food processing industries (Iweala et al., 2018). The prevalence of food allergies differs between children and adults. Some common pediatric allergies such a milk and egg allergy usually disappear until school age. On the contrary, most food allergies, such as peanut, tree nut, shellfish and fish allergies, translate into adulthood (Peters et al., 2021). Currently, the prevalence of food allergies is estimated to affect ca. 8% of the children and ca. 3% of the adult population (Iweala et al., 2018). The prevalence of food allergies varies among countries and regions due to food availability or additional risk factors like early eczema (Peters et al., 2021). A classical atopic march begins

with atopic dermatitis, followed by food allergy, asthma and ultimately allergic rhinitis (Hill and Spergel, 2018).

1.2.1.1 Food allergy diagnosis

The diagnosis of food allergy is important to determine which specific food is responsible for the allergic reaction in individuals. Diagnosis is performed by a clinical history, incorporating clinical symptoms and realization of diagnostic tests (Sicherer et al., 2004). The recommended tests for food allergy diagnosis are skin prick tests (SPTs), allergen specific serum IgE (sIgE) measurements, elimination diets and oral food challenges (OFCs) (Sicherer et al., 2004). SPTs and sIgE are scientific valid tests to diagnose food allergy and are normally the first ones to be used. SPTs are inexpensive, fast, and relatively safe and performed on the arm or back of the patient. Nevertheless, this *in vivo* analysis might give some false-positive results due to non-clinically relevant IgE levels (Muraro et al., 2014). Determination of IgE levels in patient serum completes the interpretation of the results, especially nowadays, where molecular allergology, applying single allergen components instead of allergen extracts, delivers great diagnostic precision (EAACI, 2022). Nevertheless, positive predictive values from this *in vitro* assay does not always relate to clinical reactivity. Thereafter, elimination diets and/or OFCs are performed to confirm diagnostics (Muraro et al., 2014). To help allergic patients with their choice of food, accurate and extensive labelling of products is required. The Codex Alimentarius Commission was developed to create worldwide standards (FAO, 2016). However, some countries consider different allergens for labelling (Koeberl et al., 2014).

1.2.1.2 Food allergy treatment

Therapeutic treatment of food allergy received increasingly awareness in the past decade. Beyond novel biologicals (e.g. Omalizumab anti-IgE (Kopp, 2011)) the most promising approach is known as food allergen-specific immunotherapy (Kim and Burks, 2020). Food immunotherapy has been established for treatment of peanut allergy (Palforzia, Aimmune Therapeutics, Inc.), in children (Erlich, 2022). This oral immunotherapy induces desensitization, however long-term protocols and other foods are still unclear. Sublingual, subcutaneous and epicutaneous immunotherapy are subject to research but seem to lead to important side effects and will not be further discussed here (Cox and Calderon, 2010, Cox et

al., 2011, Nelson et al., 1997, Oppenheimer et al., 1992). Research studies demonstrated that immunotherapy may rise the reactivity threshold (desensitization), however no conclusions could be taken for adults (Nurmatov et al., 2017, Kim and Burks, 2020). In oral immunotherapy, regular ingestion of increasing doses, starting at milligrams (mg) to grams (g), of a specific food allergen occurs over a period of several months to years. Gradually, by increasing the dose, the maintenance phase will be reached where regular intake of the food allergen is needed (Vázquez-Cortés et al., 2020).

Other research on food allergy treatment is based on modified hypoallergenic molecules, which might result from heating milk, baking eggs or boiling peanuts, or by adjuvants (e.g. anti-IgE therapy, pre/probiotics or bacteria) (Vazquez-Ortiz and Turner, 2016). For fish allergy, research on immunization with hypoallergenic fish allergens has only been performed on murine models (Zuidmeer-Jongejan et al., 2015, Freidl et al., 2020).

Fish allergy is of relevance for the present work, which focused on two marine aquaculture species highly produced and consumed in Portugal, gilthead seabream and European seabass. More details will be provided on this topic from the next section onwards.

1.2.2 Fish allergies

Fish-allergic patients mostly suffer from IgE-mediated reactions. Noteworthy, some reactions might be misinterpreted as genuine fish allergy (e.g., fish poisoning by toxins like histidine (Feng et al., 2016) and Anisakis – a parasitic worm infecting fish muscle (Nieuwenhuizen and Lopata, 2014)). Nevertheless, non-IgE reactions such as food protein-induced enterocolitis syndrome (FPIES) especially in children (Kourani et al., 2020), and mixed reactions like eosinophilic esophagitis (Chehade and Aceves, 2010) are described. Prevalence rates differ greatly as a function of the applied study methodology. Indeed, whether data are collected based on self-report or allergy work-up (serum IgE, skin test, food challenge) account for the largest variability (Table 1.1) (Klueber et al., 2019).

Table 1.1. Fish allergies prevalence rates, in the general population but specific to age, worldwide.

Type of study	Age	Country	Prevalence rate (%)	Reference
Questionnaire	Children	Finland	7.0	(Kajosaari, 1982)
	Children	Norway	3.0	(Eggesbø et al., 1999)
	Children	Portugal	1.0	(Jorge et al., 2017)
	Children	France	0.7	(Rancé et al., 2005)
	Children	Turkey	0.3	(Orhan et al., 2009)
	Children	United States of America	0.2	(Sicherer et al., 2004)
	Adults	Portugal	0.9	(Falcao et al., 2004)
	Adults	Greece	1.5	(Hilger et al., 2017)
	Adults	United States of America	2.7	(Greenhawt et al., 2009)
Sensitization by skin and serum	Children	Finland	2.7	(Haahtela et al., 1980)
	Children	Turkey	0.2	(Orhan et al., 2009)
	Children	France	0.7	(Pénard-Morand et al., 2005)
	Adults	Germany	2.9	(Schäfer et al., 2001)
Food challenge	Children	Iceland	0.3	(Kristjansson et al., 1999)
	Adults	Denmark	0.2	(Osterballe et al., 2005)

Uptake by ingestion or contact with fish by inhalation or skin (mainly from the occupational field (Dickel et al., 2021)) might lead to clinical symptoms (itching, vomiting, bronchospasm and even anaphylaxis) within minutes (Klueber et al., 2019). It should be taken into account that symptoms from FPIES occur in the first hours after contact and symptoms might be different from IgE-mediated reactions, including pallor, lethargy and dehydration

(Kourani et al., 2020). Low amounts of fish are needed for an allergic reaction in sensitized patients, and therefore the estimated doses which triggers objective symptoms in 10% of allergic patients (cod ED_{10} from the EuroPrevall cohort) is 27.3 mg of protein (Sørensen et al., 2017). However, 5 mg of herring or cod was estimated as the lowest observed adverse effect level (LOAEL) for a clinical response, obtained by a double-blind placebo-controlled food challenge of 14 fish-allergic patients (Taylor et al., 2002).

1.3 Fish allergens

Allergens commonly occur in fish muscle and several fish allergens have been identified and registered at the World Health Organization and International Union of Immunological Societies allergen nomenclature sub-committee (WHO/IUIS) (www.allergen.org) such as, parvalbumin, β-enolase, aldolase A, collagen, in addition to others (Table 1.2). Each allergen has also been thoroughly described in the recently published Molecular Allergology User's Guide 2.0 (EAACI, 2022). Fish allergen, like vitellogenin a 118 kDa protein and precursor of yolk proteins, originated from fish roe was also described in clinical cases (Klueber et al., 2019). Notably, no cross-reactivity is documented between fish muscle and fish egg allergens.

1.3.1 Parvalbumin

The main fish allergen is parvalbumin. Fish-allergic reactions relate mostly to IgE-reactivity to this allergen (up to 70-95%), depending on the study cohort and the type of fish (Kourani et al., 2020). Parvalbumin was first identified as an allergen in Baltic cod *(Gadus callarius)* in the early 1970s, and named Gad c 1 (Aas, 1967, Aas and Jebsen, 1967). Biomolecular studies were performed on the homologue parvalbumin from Atlantic cod*,* Gad m 1 (Schrama et al., 2022b), which nowadays is used in many studies as reference (Fig. 1.8).

Parvalbumin is a small acidic white muscle protein (10-12 kDa, isoelectric point 3.5-5.0) present in all fish (Klueber et al., 2019). Parvalbumin belongs to the EF-hand family (helix-loop-helix) which bind divalent cations, and is involved in physiological functions such as calcium homeostasis related to muscle contraction/relaxation (Lewit-Bentley and Réty, 2000). Two functional EF-hand motifs (called CD and EF) bind calcium- or magnesium-ions, with different affinities, via ionic bonds with the N-terminal site being a non-functional EF-hand (AB).

Fig.1.8 - A ribbon model of the three-dimensional structure of cod parvalbumin Gad m 1 (PDB: 2MBX, www.rcsb.org)). Green spheres represent the binding sites of calcium ions (Ca^{2+}).

Affinities for Ca^{2+} are higher than for Mg^{2+} (Coughlin et al., 2007). As shown in mammals, upon muscle relaxation parvalbumin bind to Mg^{2+}. However, for muscle contraction, parvalbumin dissociates slowly from Mg^{2+} to bind Ca^{2+} and therefore decreases free myoplasmic Ca^{2+} (Berchtold et al., 2000). Parvalbumin shuttles Ca^{2+} to the sarcoplasmic reticulum (SR) Ca^{2+} ATPase pump (SERCA) which is responsible to move Ca^{2+} into the SR (Arif, 2009). Parvalbumin is a very stable protein which survives common food processing treatments, such as heating. The stability of parvalbumin in fish can be explained by its structure, which depends on calcium chelating (Tomura et al., 2008) (Table 2). Upon depletion of calcium, conformational changes of the protein might result in lower IgE reactivity (Tomura et al., 2008). Calcium chelators such as ethylene-diaminetetraacetic acid (EDTA) showed a decrease in IgE-binding ability (Jiang and Rao, 2021).

Parvalbumins are divided into the phylogenetic α- and β-lineages. While bony fish contains mostly the β-lineage, it shows higher allergenicity than the α-lineage from cartilaginous fish, due to low sequence identity (Stephen et al., 2017, Kalic et al., 2019, Kalic et al., 2022). Furthermore, the β-lineage shows a lower isoelectric point than the α one, and different isoforms/isoallergens are expressed and subsequently named β1, β2, and so on (Kuehn et al., 2014). There are substantial differences in amino acid sequences of some β isoforms, as the 64% identity between β1 and β2 from Atlantic salmon, might contribute to a higher reaction to one isoform (Sharp and Lopata, 2014). Complexes might also be formed (dimeric and polymeric), increasing the molecular weight of the parvalbumin complex (Sharp and Lopata, 2014).

Table 1.2. Parvalbumins structural properties.

Properties		Reference
Calcium-binding site modification	Chemical modification or masking of specific amino acids like arginine, perturbates parvalbumins structure and consequently its immunological reactivity	(Elsayed and Apold, 1983)
Hypoallergenicity	Uncoupling of calcium ions results in a random folding of parvalbumin, affecting the immunological reactivity	(Elsayed and Apold, 1983)
Stability by heating	Affects reversibly only part of parvalbumins structure, and therefore maintains its immunological reactivity	(Syed and Absar-ul, 2010)

Fish species like tuna and swordfish (*Xiphias gladius*) are known as large migratory species, and have a higher percentage of dark muscle compared to the white one. Dark or red muscle, are known to be used for steady swimming. On the contrary, cod, carp or herring are small sedentary with mainly white muscle, known for power swimming. Dark muscle contains more lipids and less proteins than white muscle (Dunajski, 1980), and therefore less parvalbumin and lower allergenicity, being often better tolerated by fish-allergic patients (Klueber et al., 2019, Kourani et al., 2020). Parvalbumin amounts per gram of fillet range from 1 mg to more than 2.5 mg in swordfish and Atlantic cod, respectively (Kourani et al., 2020). Also, parvalbumin content differs by muscle region, showing lower amounts at posterior (near the tail) position than anterior (near the head) and middle position (Lee et al., 2012). Furthermore, parvalbumin concentration was higher at the dorsal side instead of the ventral side, in several species (Kobayashi et al., 2016c). This main fish allergen will be the overall focus of this PhD and will be highlighted in almost all chapters.

Officially approved parvalbumins, as registered in the WHO/IUIS allergen nomenclature database, on November 2022, originate from 17 fish species (Table 1.3).

1.3.2 Enolase and aldolase

Two glycolytic enzymes, β-enolase (50 kDa) and aldolase A (40 kDa), were identified as minor fish allergens in species like, Atlantic cod, Atlantic salmon, Yellowfin tuna, Nile perch (*Lates niloticus*) and blunt snout bream (*Megalobrama amblycephala*) (Kuehn et al., 2013, Liu et al., 2011, Rosmilah et al., 2013, Tomm et al., 2013) (Table 3). Enolase binds to magnesium ions. Both enzymes are heat labile belonging to the triosephosphate isomerase (TIM)-barrel family (Kuehn et al., 2013, Klueber et al., 2019). Enolase and aldolase show (limited) cross-reactivity, yet the prevalence of IgE-binding varies with different fish species (Kuehn et al., 2013).

1.3.3 Collagen and gelatin

Collagen and its derivative, gelatin are minor allergens that can be found in connective tissues including fish skin, bone and scale (Benjakul et al., 2012). Fish collagen (300 kDa) is composed of α-chains in a triple-helical conformation. IgE-reactivity to bigeye tuna (*T. obesus*) collagen was the first to be reported (Hamada et al., 2001), followed by Pacific mackerel (*Scomber japonicus*) collagen in which 50% of fish-allergic patients showed a positive IgE-binding and cross-reactivity between 22 different fish species (Kobayashi et al., 2016b) (Table 3). Gelatin, a degradation product of collagen, might also trigger severe allergic reactions, as shown after consumption of gummi bears (Kuehn et al., 2009). In Europe, the IgE prevalence for fish gelatin was 19.3% in 62 fish-allergic patients (Kuehn et al., 2013).

1.3.4 Tropomyosin

Tropomyosin (33-39 kDa) is the main shellfish allergen. The WHO/IUIS also approved the fish homolog as an allergen (Table 3). After a first study in Mozambique tilapia (*O. mossambicus*), another study described tropomyosin as an allergen from Atlantic salmon and catfish/Pangasius (Ruethers et al., 2021).

1.3.5 Other fish allergens

Several studies identified creatine kinase (41 kDa) as being a potential allergen. Immunoblots performed with blunt snout bream (Liu et al., 2011), Atlantic salmon, catfish/Pangasius (Ruethers et al., 2021) and longtail tuna (*T. tonggol*) (Rosmilah et al., 2013) resulted in positive bands with this allergen. Another fish allergen was found in a study with

Pacific mackerel, where IgE-reactivity was shown with the 28 kDa protein identified as triosephosphate isomerase (Wang et al., 2011). Atlantic salmon and catfish did also show positive IgE-binding with this protein (Ruethers et al., 2021). Aldehyde phosphate dehydrogenase was identified as allergen after positive IgE-reactivity from cod (Das Dores et al., 2002). Fish roe may also lead to allergic reactions due to vitellogenin (118 kDa), identified in chum salmon (*O. keta*) roe (Shimizu et al., 2014), and different fish species show cross-reactivity (Table 1.3).

1.4 Cross-reactivity

Parvalbumins epitopes show cross-reactivity between different fish species, which is mainly due to high conservation of these epitopes (Dall'Antonia et al., 2011). The higher the amino acid identity between proteins the higher the cross-reactivity (Hilger et al., 2017). In case of salmon and trout parvalbumin, both from the Salmoniformes order (Table 3), high IgE cross-reactivity was detected. A comparison showed a difference in four and three amino acids in case of β1 and β2, respectively (Kuehn et al., 2011). Nonetheless, it is important to note that IgE epitopes might differ between species. Studies on cross-reactivity might be antibody based or done using fish-allergic patient serum. A large multinational cohort (n=263) with fish-allergic patients using 10 fish species, showed low allergenicity of ray parvalbumin and specific bony fish species were tolerated for up to 21% of the patients (Kalic et al., 2022). Analysis of IgE epitopes identified the highly antigenic region IV and the species-specific region I (Sharp and Lopata, 2014), explaining some cases of mono-sensitivity to salmonids (Kuehn et al., 2011). The β1-isoform is mostly responsible for allergic reactions to salmonids which seems to have a specific epitope region (Mohammadi et al., 2018, Perez-Gordo et al., 2012). Mono-sensitivities were also observed in pangasius, catfish and monkfish (*Lophius piscatorius*) (Dijkema et al., 2020).

Table 1.3. Identified fish allergens at WHO/IUIS (www.allergen.org) as of November 2022.

Taxonomic order	***Species***	***Allergen (code)***
Cichliformes	*Oreochromis mossambicus* (Mozambique tilapia)	Tropomyosin (Ore m 4)
Clupeiformes	*Clupea harengus* (Atlantic herring*)*	β-parvalbumin (Clu h 1)
	Sardinops sagax (Pacific pilchard)	β-parvalbumin (Sar sa 1)
Cypriniformes	*Ctenopharyngodon idella* (Grass carp*)*	β-parvalbumin (Cten i 1)
	*Cyprinus carpio (*Common carp*)*	β-parvalbumin (Cyp c 1) β-enolase (Cyp c 2)
Gadiformes	*Gadus callarius* (Baltic cod)	β-parvalbumin (Gad c 1)
	Gadus morhua (Atlantic cod)	β-parvalbumin (Gad m1) β-enolase (Gad m 2) Aldolase A (Gad m 3)

Table continues on the next page

Perciformes	*Lates calcarifer* (Barramundi)	β-parvalbumin (Lat c 1) Collagen α (lat c 6)
	Sebastes marinus (Ocean perch, redfish)	β-parvalbumin (Seb m 1)
	Thunnus albacares (Yellowfin tuna)	β-parvalbumin (Thu a 1) β-enolase (Thu a 2) Aldolase A (Thu a 3)
	Xiphias gladius (Swordfish)	β-parvalbumin (Xip g 1)
Pleuronectiformes	*Lepidorhombus whiffiagonis* (Megrim, whiff, turbot fish)	β-parvalbumin (Lep w 1)
	Solea solea (Sole)	Parvalbumin (Sole s 1)
Salmoniformes	*Onchorhynchus keta* (Chum salmon)	Vitellogenin (Onc k 5)
	Onchorhynchus mykiss (rainbow trout)	β-parvalbumin (Onc m 1)
	Salmo salar (Atlantic salmon)	β-parvalbumin (Sal s 1) β-enolase (Sal s 2) Aldolase A (Sal s 3) Tropomyosin (Sal s 4) Collagen α (Sal s 6) Creatine kinase (Sal s 7) Triosephosphate isomerase (Sal s 8)

Table continues on the next page

Scombriformes	*Rastrelliger kanagurta* (Indian mackerel)	Parvalbumin (Ras k 1)
	Scomber scombrus (Atlantic mackerel)	Parvalbumin (Sco s 1)
Siluriformes	*Pangasianodon hypophthalmus* (Striped catfish)	β-parvalbumin (Pan h 1) β-enolase (Pan h 2) Aldolase A (Pan h 3) Tropomyosin α (Pan h 4) Creatine kinase (Pan h 7) Triosephosphate isomerase (Pan h 8) Pyruvate kinase (Pan h 9) L-lactate dehydrogenase (Pan h 10) Glucose 6-phosphate isomerase (Pan h 11) Glyceraldehyde-3-phosphate dehydrogenase (Pan h 13)

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1.5 Fish processing and digestion

Food processing is primarily applied to increase taste and nutritional values, decrease foodborne diseases and extend shelf life (Jiang and Rao, 2021). Besides, processing facilitates transportation and turn food more convenient (Dasanayaka et al., 2020). It is important to note that fish processing might change the structure and stability of proteins like parvalbumin (Jiang and Rao, 2021). Protein denaturation (loss of tertiary and secondary structure) results often from food processing after which the protein might form aggregates (Dasanayaka et al., 2020). As a consequence, the IgE epitopes might be masked by dimers or trimmers, unmasked by exposing new allergenic regions or damaged, which results in altering the allergenicity (Costa et al., 2022). Nonetheless, studies on allergenicity are rather dependent on the extractability of the allergens after processing. Processing techniques are divided in two types, thermal and non-thermal. The conventional thermal techniques involve (moist) heating (boiling, steaming, autoclaving, smoking), canning, frying, roasting, baking or pressure cooking. The conventional non-thermal techniques involve freezing, lyophilization, fermentation, salting or ultrafiltration (Dasanayaka et al., 2020). Nowadays novel processing techniques like microwave heating, pulsed electric field, ultrasounds, magnetic fields or ultraviolet are being applied to foods (Vanga et al., 2017). Several studies demonstrated that boiling, steaming, and cooking might lead to an increased, unchanged or decreased allergenicity, depending on the fish species. A reduction in allergenicity after boiling, steaming or cooking has been observed in several fish (Bernhisel-Broadbent et al., 1992, Kubota et al., 2016, Kuehn et al., 2010), on the contrary a study with purified Atlantic cod parvalbumin showed no differences in IgE binding after boiling for 30 min at 80°C (Somkuti et al., 2012). Under similar conditions (heating at 90°C) another study with purified sardine (*Sardina pilchardus*) parvalbumin showed a slight increase in allergenicity in 10% of the patients (Mejrhit et al., 2017). Salted herring and dried cod showed a decrease in IgE binding (Sletten et al., 2010), and another example for a non-thermal technique, namely freezing, showed unchanged allergenicity in North Pacific hake (*Merluccius productus*) and other species (Dasanayaka et al., 2020). Parvalbumins stability, from gilthead seabream and European seabass, after fish processing will be highlighted in chapter 2.

Beyond the mentioned processing techniques, fish allergens are further degraded by digestion. Based on observations of resistant allergens to conditions of the gastrointestinal tract, researchers started to investigate more about the stability of allergens (Pekar et al.,

2018). Knowledge on the stability of parvalbumin after gastric and intestinal conditions, with low pH, pepsin, pancreatin and bile salts is important for several reasons, such as immunotherapy and food safety for fish-allergic patients. Gastric pH conditions in humans change according to the fasted and fed state of the stomach, varying between 1 and 5.5, respectively (Sams et al., 2016). Also, the return to basal levels depends on stomach emptying which shows inter-individual variations and depends on the type of ingested food (Sams et al., 2016). *In vitro* studies were developed to determine allergen resistance or degradation using simulated oral, gastric and intestinal fluid. Low pH values and enzyme-protein ratios were determined based on *in vivo* studies and used to simulate *in vitro* gastric conditions. Neutral pH values and 100 U pancreatin/ml and 10 mM of bile salts are recommended to use in the simulated *in vitro* intestinal conditions (Minekus et al., 2014). Studies with codfish and carp parvalbumin showed that a simulated gastric phase with pH 2.5 showed no reactivity with patient serum or antibody, respectively (Untersmayr et al., 2005, Akkerdaas et al., 2018). Parvalbumins stability, after gastrointestinal digestion, will be highlighted in chapter 2.

Knowing that parvalbumin is the main fish muscle allergen, and Portugal presents high consumption and aquaculture production of gilthead seabream and European seabass, this PhD focused on modulating allergenicity of these fishes. This will be mainly achieved by using fish nutrition which will be introduced from section 1.7 and onwards.

1.6 Fish feed supplementation

High quality fish feed is of outmost importance for a healthy farmed fish. Feeds are balanced for the best fish performance of growth and welfare. Fish diet formulations include high-quality proteins, lipids, vitamins, minerals and additives. The incorporation of additives in fish diets might be a nutritional strategy with several purposes (e.g., increasing feed intake (Dias et al., 1997), enhancing diet palatability (Bai et al., 2015) and improving lipid digestion (Morais et al., 2016), among others). The work developed in the present PhD thesis, aimed to modulate the allergenic potential of farmed fish through the inclusion of additives in aquafeeds.

1.6.1 Creatine

Dietary creatine is known to improve muscle growth and is therefore highly used as a daily supplement in humans (Burns and Gatlin, 2019). Having an important role in the energy metabolism, lately creatine has received more attention. Creatine might be synthesized intrinsically or acquired by the diet. The synthesis of creatine from the amino acids' arginine, glycine and methionine involves the enzymes glycine amidinotransferase (GATM or AGAT) and guanidinoacetate N-methyltransferase (GAMT) (Fig. 1.9). Creatine is transported by a specific transporter (CT1) to the target cells where creatine kinase (CK) is responsible for its phosphorylation into creatine phosphate producing adenosine diphosphate (ADP) and consequently consuming energy (Borchel et al., 2014) (Fig. 1.9). Upon high levels of creatine phosphate, the mitochondrial CK is able to transphosphorylate ADP to adenosine triphosphate (ATP), generating energy for several ATP-dependent processes (Sestili et al., 2016). Studies showed that creatine regulates the high ATP demanding sarcoplasmic reticulum Ca^{2+} (SERCA) pump (Negro et al., 2019). As stated before, after muscle contraction, Ca^{2+} ions are transported into the SR, which is performed by pumps, channels and buffering proteins (e.g. parvalbumin) (Butera et al., 2021). Combining the knowledge that creatine supplementation increases the production of ATP, enhancing therefore the activity of the SERCA pump, results in a more efficient decrease in the concentration of cytosolic Ca^{2+}. Therefore there is less need for Ca^{2+} buffering by parvalbumin (Xu and Van Remmen, 2021). A study performed with murine showed a decrease in parvalbumin content and an increase in the SERCA pump, when adding creatine into their diets (Gallo et al., 2008). Although the metabolism of creatine in fish is not yet fully known, it has been determined that fish muscle has a higher content of creatine than human muscle. GATM and GAMT are highly expressed in fish muscle together with creatine kinase, showing a vital metabolic function of fish muscle (Borchel et al., 2019). Studies with creatine supplementation showed improved endurance and growth performance in rainbow trout and red drum (*Sciaenops ocellatus*), respectively (McFarlane et al., 2001, Burns and Gatlin, 2019). Supplementation with creatine in gilthead seabream and European seabass diets and its effect on allergenicity will be highlighted in chapters 4.1 and 4.3.

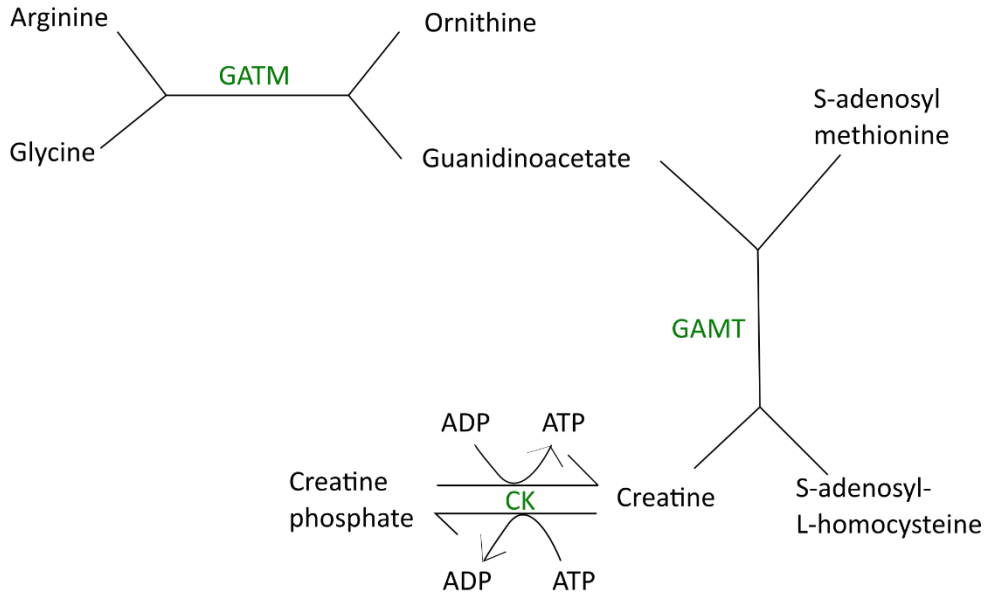

Fig. 1.9. Schematic overview of the creatine synthesis in trout. Enzymes are highlighted in green, GATM – glycine amidinotransferase, GAMT – guanidinoacetate N-methyltransferase, CK – creatine kinase. ATP – adenosine triphosphate, ADP – adenosine diphosphate (Adapted from Borchel *et al*, 2014).

1.6.2 EDTA

Ethylenediaminetetraacetic acid (EDTA) is a highly stable chelating agent of metal ions. It is used in several industries such as pharmaceutic, cosmetic and food. EDTA is one of many shelf-life enhancers in food and is known to be one of the most effective and safe additives (E385) (Wreesmann, 2011). It is authorized in the European Union (EU), according to Annex II and III of the Regulation (EC) No 1333/2008 on food additives. The maximum levels allowed in several food categories range from 75 to 250 mg/kg, and the acceptable daily intake (ADI) is set to 1.9 mg/kg of body weight (Younes et al., 2018). A study on the interaction between calcium and EDTA showed that the formation of the Ca^{2+}-EDTA complex, through proton shifts, results in a significant increase in EDTA stability (Griko, 1999). As aforementioned, parvalbumin needs two calcium ions for the EF-hand motifs to be functional, but in the presence of EDTA these ions might be chelated. The resulting rearrangement of the parvalbumin structure (demonstrated by a decrease in α-helices (Permyakov et al., 2008)) showed lower IgE-binding capacity (Bugajska-Schretter et al., 2000). Supplementation with EDTA in fish diets and its effect on allergenicity of gilthead seabream and European seabass will be highlighted in chapters 4.2 and 4.3.

1.7 Objectives

As fish allergies are a serious health problem, this PhD thesis focused on reducing the allergenic potential of gilthead seabream and European seabass by adding specific additives to the diets and by analyzing effects of processing and digestion. Therefore, the following objectives were proposed.

- Characterize gilthead seabream and European seabass parvalbumins at the biomolecular and immunological level, before and after fish processing and gastrointestinal digestion **(Chapter 2)**.
- Market study by a questionnaire in Portugal to understand consumers' willingness to pay more for a low allergenic fish **(Chapter 3)**.
- Compare the muscle protein/allergen expression profiles of fish farmed under conventional and newly established conditions by adding creatine or EDTA to the diets **(Chapters 4.1, 4.2 and 4.3)**.
- Quantify allergens and compare IgE-reactivity to parvalbumins and muscle proteins from fish farmed under conventional and the newly established conditions **(Chapters 4.1, 4.2 and 4.3)**.
- Compare sensorial and biochemical parameters of the flesh from fish raised under conventional and the newly established conditions **(Chapters 4.1, 4.2 and 4.3)**.

This PhD thesis tends to give more insight into the allergenic potential of gilthead seabream and European seabass, two economically important species in Portugal.

Chapter 2

Fish processing and digestion affect parvalbumins detectability in gilthead seabream and European seabass.

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Fish processing and digestion affect parvalbumins detectability in gilthead seabream and European seabass

Abstract

Consumption of aquatic food, including fish, accounts for 17% of animal protein intake. However, fish consumption might also result in several side-effects such as sneezing, swelling and anaphylaxis in sensitized consumers. Fish allergy is an immune reaction to allergenic proteins in the fish muscle, for instance parvalbumin (PV), considered the major fish allergen. In this study, we characterize PV in two economically important fish species for Southern Europe aquaculture, namely gilthead seabream and European seabass, to understand its stability during *in vitro* digestion and fish processing. This information is crucial for future studies on allergenicity of processed fish products. PVs were extracted from fish muscles, identified by mass spectrometry (MS), and detected by sandwich enzyme-linked immunosorbent assay (ELISA) after simulated digestion and various food processing treatments. Secondary structures were determined by circular dichroism (CD) after purification by anion exchange and gel filtration chromatography. In both species, PVs presented as α-helical and β-sheet structures, at room temperature, were shown to unfold at boiling temperatures. In European seabass, PV detectability decreased during the simulated digestion and after 240 min (intestinal phase) no detection was observed, while steaming showed a decrease ($p<0.05$) in PVs detectability in comparison to raw muscle samples, for both species. Additionally, freezing (-20°C) for up to 12 months continued to reduce the detectability of PV in tested processing techniques. We concluded that PVs from both species are susceptible to digestion and processing techniques such as steaming and freezing. Our study obtained preliminary results for further research on the allergenic potential of PV after digestion and processing.

Keywords: Parvalbumin, gilthead seabream, European seabass, gastrointestinal digestion, fish processing

2.1 Introduction

Increasing fish consumption is a viable alternative to land-animal based diets due to higher nutritional value. Easily digestible proteins of high quality, omega-3 fatty acids and vitamins are examples of the high nutritional value of fish (Pal et al., 2018). Due to this knowledge, global demand for fish and fish products is increasing every year, with aquaculture playing an important role in this prospect (Cerqueira et al., 2020b). In 2020, global fish production for human consumption reached 157 million tonnes, corresponding to 20.2 kg per capita/year (FAO, 2020). Fish consumption contributed 17% of the intake of animal proteins globally (FAO, 2020). Portugal has a long history of fish consumption and has the highest intake in the European Union (EU), with 59.91 kg/capita/year compared to an average of 23.97 kg/capita/year (European Comission, 2021a). Gilthead seabream, *Sparus aurata* and European seabass, *Dicentrarchus labrax* used in this study, are examples of two economically important species highly consumed and produced in Southern Europe.

Fish are among the most common food allergies worldwide and result from an excessive immune system response to specific proteins, called allergens (O'Brady, 2021). Avoiding fish consumption might be a constraint in countries where high exposure to fish is common (Xepapadaki et al., 2021). A better understanding of fish allergenicity of specific species could assist fish-allergic consumers in selecting the best fish for consumption (Schrama et al., 2022a, Kalic et al., 2022).

Fish parvalbumin (PV) is an allergen that is the main contributor to the clinical reactivity of fish-allergic patients. Thus, this allergen appears to be an important target to evaluate the allergenicity of fish species (WHO/IUIS, 2022). The highly stable Ca^{2+}-binding PV is an EF-hand muscle protein with a molecular weight of 10-12 kDa (Kourani et al., 2020). Indeed, muscle PV regulates intracellular Ca^{2+}-ions concentration (or Mg^{2+}-ions), involved in the process of muscle relaxation (Silva et al., 2011), via two EF-hand domains (Schwaller, 2009). Several studies on PV detection using IgG assays were performed with different fish species. PV from carp, catfish, cod and tilapia was positively identified using an anti-frog monoclonal antibody, but on the contrary, yellowfin tuna did not react (Chen et al., 2006). Using specific PV antibodies for each fish species, salmon, carp, cod, mackerel, redfish, tuna and herring PV was identified by immunoblotting (Kuehn et al., 2010). Studies on PV detection and epitopes in gilthead seabream, using mass spectrometry, were also previously reported (Carrera et al., 2012, Carrera et al., 2019). It has been suggested that PV secondary structure

suffers substantial changes upon calcium depletion, which seems to decrease its stability (Laberge et al., 1997).

Evidence showed that PVs stability decreases through its denaturation during specific digestion conditions or product processing. Allergen denaturation may lead to aggregates, which might change the way epitopes are recognized (being masked, un-masked or damaged) (Dasanayaka et al., 2020), and which may offer an accessible first step to desensitize consumers to have access to higher quality protein (Bernhisel-Broadbent et al., 1992, Pérez-Tavarez et al., 2019). In this sense, fish allergen risk assessment is important to help consumers decide if a specific fish contains a known allergen and if it might trigger an allergic reaction (Birot et al., 2017, Crevel, 2015), which is shaped by the method of detection. For instance, digestibility is one of the criteria used in fish allergy risk assessment evaluations where digested allergens are denatured or degraded. Denaturation and/or degradation of PV through the gastrointestinal tract depends on several factors, such as environmental pH and digestive enzymes (Moreno, 2007, Fernández-Tomé and Hernández-Ledesma, 2020). Additionally, the pepsin to allergen ratio is crucial for the stability of PV (Moreno, 2007). However, extractability might change and partially or totally digested allergens could be in the insoluble fraction. Another important method to analyze fish allergen risks are processing methods (e.g., heating, pressure, salting and freezing) which are used for different purposes, like improving fish quality (flavor, appearance) or extending shelf life (Dasanayaka et al., 2020). Besides this, freezing is a convenient process for consumers. Additionally, processing techniques, such as thermal and non-thermal approaches, may change PV stability through conformational changes in structure (e.g. by destroying functional epitopes) (Vanga et al., 2017). A study on cod PV stability using heat and pressure showed protein denaturation with a pressure-temperature phase diagram (Somkuti et al., 2012). In the case of the non-thermal salting process of herring, protein denaturation was also observed by SDS-PAGE (Sletten et al., 2010).

This work, therefore, aimed to evaluate PV stability upon exposure to *in vitro* simulation of the gastrointestinal tract and different fish processing techniques (e.g. heating, pressure, salting and freezing) using an in-house developed sandwich ELISA (specific for gilthead seabream and European seabass). PV conformation in native and calcium-depleted apo-form was determined by circular dichroism. With this approach we expect to lay the

molecular foundation for future research on the allergenic potential of processed fish products.

2.2 Material and methods

2.2.1 Fish and ethical legislation

Gilthead seabream (*Sparus aurata)* and European seabass *(Dicentrarchus labrax*) juveniles were sampled (after being lethally anaesthetized with MS-222 (Merck)), from rearing tanks at the Ramalhete field station (CCMAR/University of Algarve, Portugal). Research on these species reared in the same infrastructure was previously published by our group in the context of fish allergenicity (Schrama et al., 2022c, Raposo de Magalhães et al., 2020b, Schrama et al., 2018). Fillets of 6 fish and dorsal muscle samples from another three were taken and frozen at -20°C or -80°C, respectively for both species. Fish skin was removed in either species before freezing. The experiments in this project were approved by the Portuguese National Authority for the Animal health (DGAV) with permit no. 003894, following guidelines for fish welfare established in Council Directive 2010/63/EU and Portuguese legislation for the use of laboratory animals, permit number 0420/00/000-n.9909/11/2009.

2.2.2 Protein extraction

Proteins were extracted from the dorsal muscle samples of each species (three biological replicates) using a Retsch Mixer Mill MM 400 (Retsch, Haan, Germany) tissue lyser as described by Kalic et al. (2019) with some slight modifications. Briefly, one hundred mg of each muscle tissue were added to 1 steel bead of 5 mm in diameter and 1 ml of detergent lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100) as extraction buffer. Samples were homogenized for 10 min at 25 Hz, and incubated on an orbital shaker for 1 h, at 4°C, to homogenize the suspension. Following, samples were centrifuged at 20,000 xg for 15 min at 4°C. The protein content on the collected supernatant (crude extract) was quantified using the Bradford assay with bovine serum albumin (BSA) as standard protein.

2.2.3 Parvalbumin purification

As a pre-separation step, crude extracts from both species were heated at 90°C for 10 min, followed by centrifugation at 20,000 xg for 15 min (Kalic et al., 2019, Kuehn et al., 2016).

The collected supernatant was quantified using the Bradford assay with bovine serum albumin (BSA) as standard protein. To check the presence of other potential heat-resistant proteins besides PV, 10 µg of protein of each sample were loaded and separated by SDS-PAGE, using the AnykD™ Mini-Protean® TGX gels (Bio-Rad Laboratories, California, USA), for 35 min at 200 V followed by Coomassie blue staining. PV was identified by mass spectrometry and IgG-recognition using commercial anti-PV antibodies (See Supplementary Material Fig. S2.1).

To purify PV from a sample mixture (Kuehn et al., 2010), the three heated extracts per fish species were pooled. Prior to chromatography, both samples were dialyzed against 20 mM Tris, pH 8 (Amicon® Ultra-15; Merck Millipore Ltd, Cork, Ireland). Proteins were separated by anion exchange chromatography (Resource™ Q; GE Healthcare, Uppsala, Sweden), on an NGC instrument (Bio-Rad Laboratories) using an elution gradient of 0-1 M NaCl in 20 mM Tris (pH 8). The flow rate was set at 0.5 ml/min and the UV detector at a wavelength of 280 nm. PV-containing fractions were pooled, concentrated (Amicon® Ultra-15; Merck Millipore Ltd) and loaded on a high-performance gel filtration column (Superdex™ 75 10/300 GL, GE Healthcare) using 50 mM KH_2PO_4, 150 mM NaCl, pH 7, as running buffer. The flow rate was set at 0.5 ml/min and the UV detector at a wavelength of 280 nm. Purity of PV-containing fractions was verified by SDS-PAGE followed by SYPRO™ Ruby staining (Thermo Fisher Scientific, Massachusetts, USA, See Supplementary Material Fig. S2.2). Further on the manuscript, when referring to purified proteins these are the ones obtained after gel filtration chromatography.

2.2.4 Identification of parvalbumin by mass spectrometry

PVs were identified by mass spectrometry (MS) analysis. Bands were manually excised from SDS-PAGE gels from heated extracts, reduced with dithiotreitol (DTT) (10 mM, for 20 min, at 55°C) and digested with trypsin (12.5 ng/µl, 50 mM NH_4HCO_3, pH 8) for 90 min at 37°C (TrypsinGold, Promega, Madison, USA). Finally, 0.5 µl of the digested sample and 0.3 µl of the matrix solution (25 mg/ml α-cyano-4-hydroxycinnamic acid and 4 mg/ml 2,5-dihydroxybenoiquem in 50% acetonitrile containing 0.1% TFA) were spotted on a MALDI-plate (Polished steel 384 MALDI target plate, Bruker, Germany). A protein mass fingerprint (PMF) analysis was performed on the MALDI-TOF/TOF MS (Ultraflex I, Bruker Daltonics, Germany). Calibration of the MALDI-TOF-MS was done in two steps: the external one was performed by mass scale calibration using a tryptic digested BSA according to manufacturer's instructions

(Bruker Daltonics). In brief, a reference mass list of ionized peptides from tryptic digested BSA (calculated masses) was compared with the list of measured masses. A polynomial function was applied to the measured mass list to match with the reference mass list as closely as possible. This first calibration was a pre-acquisition calibration. The internal calibration was performed by the tryptic peptides coming from the auto digestion of the enzyme. They were used as second calibration for the protein mass fingerprint analysis to optimize the calibration before each request into the database. This second calibration was a post-acquisition calibration. After conversion to MS and MS/MS peak lists, the search was restricted to the Actinopterygii database (NCBI: txid7898) with 100 ppm of mass error tolerance in MS and MS/MS precursor, and 0.3 Da tolerance on MS/MS fragments.

2.2.5 Biomolecular characterization by circular dichroism

After chromatography, purified PVs were used for biomolecular characterization. Secondary protein structures of European seabass and gilthead seabream PVs were compared by circular dichroism (CD) spectroscopy (Raith et al., 2019). First, spectra were recorded at 20°C using a Chirascan CD spectrometer (Applied Photophysics, Leatherhead, UK). Afterwards, a temperature ramping from 20°C to 95°C was performed (with a ramping rate of 0.82°C per minute). After 10 min at 95°C, a subsequent scan was taken, and samples were tempered back to 20°C for a final scan to analyze the refolding capacity. All measurements were performed with a 0.1 cm optical path length quartz cell to obtain spectra in the far-UV region (180 to 260 nm) at a protein concentration of 0.2 µg/µl in 10 mM KH_2PO_4 pH 7. The CD spectra were acquired at a scan speed of 0.4 nm/s and a step resolution of 0.7 nm. Spectra were measured in five analytical replicates and averaged. PVs stability can be explained by its structure, which depends on calcium chelating. Upon depletion of calcium, conformation changes in PV might result in lower IgE reactivity. Therefore, all samples were analyzed in native conditions and after calcium depletion with 5 mM EDTA to determine the structure of PV in its apo-form (using the same conditions and settings as stated before). All spectra were blank subtracted, averaged and smoothed with Savitzky-Golay-Filter (window size 5). Results were expressed in mean residue ellipticity (Θ mdeg) at a given wavelength. Secondary structure was determined using DichroWeb using reference data set 4 as established in Sreerama and Woody (2000).

2.2.6 In vitro simulation of the gastrointestinal tract

PVs resistance was analyzed by food digestion simulating the gastrointestinal tract, as described by Minekus et al. (2014), Akkerdaas et al. (2018). The digestion parameters were determined based on physiological data. Briefly, 5 mg of crude extract (described earlier) muscle protein (three biological replicates per species) were digested in simulated gastric fluid (SGF, 6.9 mM KCl, 0.9 mM KH_2PO_4, 25 mM $NaHCO_3$, 47.2 mM NaCl, 0.1 mM $MgCl_2(H_2O)_6$, 0.5 mM $(NH_4)_2CO_3$, 15.6 mM HCl, 0.15 mM $CaCl_2(H_2O)_2$, pH 3) with 1 U pepsin per µg of protein for 2 h. Samples (100 µl) were taken after 0 min (before adding pepsin), 1, 2, 5, 10, 20, 30, 60, 90 and 120 min. Digestion was interrupted with 10 µl of NaOH 1 M and mixed thoroughly. To continue the digestion process, simulated intestinal fluid (SIF, 6.8 mM KCl, 0.8 KH_2PO_4, 85 mM $NaHCO_3$, 38.4 mM NaCl, 0.33 mM $MgCl_2(H_2O)_6$, 8.4 mM HCl, 0.6 $CaCl_2(H_2O)_2$, pH 7) was added to the remaining mixture. Pancreatin (800 U/ml) and bile salts (10 mM) were also added, and samples were taken at the same time points as stated above (digestion was interrupted by putting samples on ice). All steps were performed at 37°C with 500 rpm on a ThermoMixer® (Eppendorf, Hamburg, Germany). PVs detectability was assessed by sandwich ELISA.

2.2.7 Sandwich ELISA

For PV detection, a new sandwich ELISA was produced in our laboratory and optimized using PV antibodies from purified protein obtained from European seabass and gilthead seabream. Purified antibodies were produced in rabbits (Supplementary Material SMM2.1), immunized with the PV from both species previously purified by chromatography (Eurogentec, Seraing, Belgium). This approach achieved a high sensitivity for the detection of our native and processed fish samples (Kuehn et al., 2010). Purified antibodies were further biotinylated using the EZ-link™ Sulfo-NHS-LC-Biotinylation kit following manufacturer's instructions (Thermo Fisher Scientific). ELISA plates (F96 Maxisorp immunoplate, Thermo Fisher Scientific) were coated with anti-PV at 500 ng/well overnight at 4°C as described by Kuehn et al. (2017), Kuehn et al. (2010). Wells were blocked with 300 µl of blocking solution (3% BSA in TBS-0.05% Tween (TBS-T)) for 4 h at room temperature (RT). Samples were analyzed in duplicate. A standard curve of purified PV, from each species, was added at 1000, 200, 100, 20, 4, 2, 0.4, 0.08 ng/ml. Wells were incubated with biotinylated anti-PV (1:2000) overnight at 4°C, which was followed by streptavidin-alkaline phosphate (AP, Merck Millipore, Novagen, Darmstadt, Germany 1:1000) for 30 min at RT. For detection at 405 nm at a

multiplate reader (BioTek Instruments, Winooski, USA) alkaline phosphatase substrate solution was added to each well (p-Nitrophenyl Phosphate, pNPP, Merck) and continuous reading for 1 h with 5 min interval was performed. In between each step wells were washed with TBS-T for 5 times. Negative control was performed without adding sample to the well. LOQ and LOD were 0.06 and 0.02 ng/ml, respectively. Sample concentrations were calculated after plotting the standard values with a 4-parameter logistic curve fit using AssayFitPro for Excel.

2.2.8 Fish muscle processing and conservation

Raw fish fillets of six animals each, gilthead seabream and European seabass, were frozen at -20°C for 12 months to analyze the effects of freezing on the detectability of PV by sandwich ELISA. Three time points (0, 6 and 12 months) were chosen to extract proteins from fish muscle and to quantify PV by ELISA (described earlier). Raw muscle, at each time point, was previously processed with one of the following methods: no processing, raw (A), boiled with tap water at 95°C in a water bath for 10 min (B), autoclaved at 121°C for 30 min (C), salted with 5% NaCl (salt is used to provide flavor and when used for longer periods as preservation (Yanar et al., 2006)) followed by autoclaving at 121°C for 30 min (D) and steamed at 98-100°C for 8 min followed by autoclaving at 121°C for 30 min (E). Following, protein extracts were prepared from processed fish, including raw samples.

2.2.9 Statistical analyses

Statistical analyses were performed using R v4.1.2 (R Core Team, 2020) for MacOSX. Data from digestion and processing techniques were transformed by $\log_{10}$ prior to statistical analysis. Significant differences among digestion samples were assessed by a one-way repeated measures analysis of variance (ANOVA), after verifying residual's normality and homoscedasticity through Shapiro-Wilk and Levene's test, respectively. Pairwise comparisons were assessed by a t-test with a Bonferroni correction as post-hoc analysis. In case of a non-normal distribution a one-way repeated measures ANOVA of aligned rank transformed data was performed. This non-parametric test has been chosen over the Friedman test as it has more power and robustness when using small sample sizes (Zimmerman and Zumbo, 1993). Significant differences among processing samples were assessed by a two-way repeated measures ANOVA (a normal distribution was verified in all cases). Pairwise comparisons were

assessed by a t-test with a Bonferroni correction as post-hoc analysis. Significant differences were considered when $p<0.05$.

2.3 Results and discussion

PV, the main fish allergen, is known to be a stable protein against thermal and proteolytic degradation (Klueber et al., 2019). Nevertheless, it is also known that digestion conditions, such as low pH and proteolytic enzymes (Untersmayr and Jensen-Jarolim, 2006), and thermal and non-thermal processing might influence the structure (tertiary, secondary or primary) of PV (Jiang and Rao, 2021) and consequently change its detectability. Hence, in this study we characterized PV of two important Southern Europe fish species by determining both its structure, through circular dichroism, and its stability, after exposure to gastrointestinal tract conditions and different processing techniques, using a sandwich ELISA. It should be noted that, using this ELISA, we could only detect the reaction of PVs epitopes to our antibodies, no conclusions could be taken about allergenicity.

2.3.1 Parvalbumin purification

To visualize any protein resistant to this thermal process, protein extracts were heated at 90ºC for 10 min followed by SDS-PAGE. Subsequent PV detection with commercial antibodies was performed and shown in Supplementary Material Fig. S2.1. In case of European seabass two clear bands appear at a low molecular weight, one closer to 14 kDa and another below 10 kDa. For gilthead seabream, two bands were visible after heating close to 10 kDa. The commercial antibodies (monoclonal anti-PV Swant PV235 and anti-rabbit/mouse IgG conjugated with AP, Sigma) detected both bands in European seabass, however the upper band showed a stronger reaction than the lower. For gilthead seabream the positive bands were detected by the antibody. Therefore, we proceeded with the identification of these bands by MS and further purification steps by chromatography.

2.3.2 Parvalbumin identification

Parvalbumin sequences of gilthead seabream and European seabass are available at the Uniprot database (uniprot.org). Identification, by MALDI-TOF/TOF MS, of the two obtained bands from the gilthead seabream muscle sample (after heating, Fig. S2.1) showed 28.4% and 86.1% sequence coverage with PV from *Austrofundulus limnaeus* (Uniprot

accession number A0A2I4CT95) and *Sparus aurata* (D0VB96_SPAAU), for the upper and lower band, respectively (Fig. 2.1A). European seabass also showed two bands with low molecular weight, which were both identified as being PV from *Hypomesus transpacificus* (C3UVG3) and *Stegastes partitus* (A0A3B5AFY4) with sequence coverages of 39.4% and 35.8%, respectively (Fig. 2.1B). When using the online available BLAST software (blast.ncbi.nlm.nih.gov/Blast.cgi) against the well-known cod PV (Q90YL0) sequence coverages of 87.1%, 63.6%, 86.1% and 92.3% were identified for the upper and lower band of gilthead seabream and European seabass, respectively. Sequence identities Ca^{2+}-binding regions, known IgE-binding regions in codfish allergy, reached even up to 91.7% for gilthead seabream and 100% for European seabass, thus pointing to a possible basis for clinical cross-reactivity (Fig. 2.1). It should be noted that for the blast search only part of the sequences were used as several amino acids were not identified by MS, resulting in higher coverages except for the lower band of gilthead seabream where the whole sequence was used.

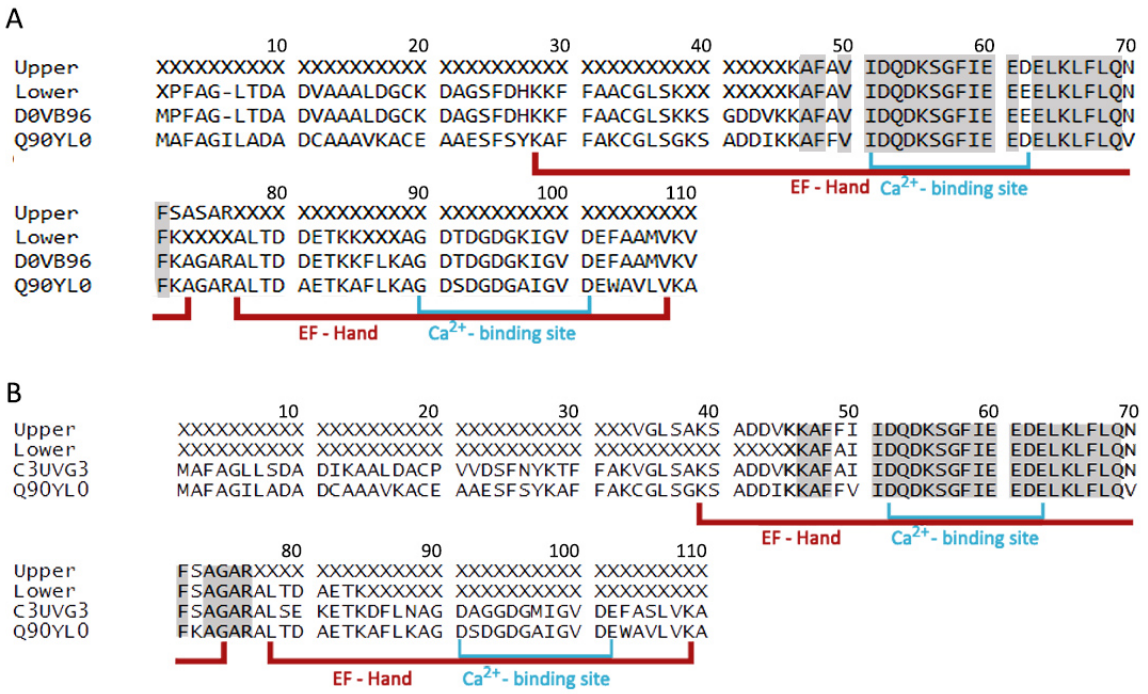

Fig. 2.1 – Parvalbumin sequences of gilthead seabream (A) and European seabass (B) obtained from positive bands presented in figure S1, in comparison to cod parvalbumin (Uniprot Q90YL0). Grey areas represent homology between sequences and X represents no MS identification.

2.3.3 Circular dichroism

After PV identification and purification, we determined the secondary structure of the main fish allergen by using the spectroscopic method of circular dichroism (CD). β-PVs structure of European seabass and gilthead seabream, determined by far-ultraviolet CD, showed a similar profile for both species in both conditions (native and apo-form) (Fig. 2.2A and 2.2B, respectively). In case of the native structure (Fig. 2.2, full lines) at room temperature (RT, 20°C, blue lines), two minima were shown in the spectra, at 208 nm and 222 nm, and a maximum below 200 nm which is typical for proteins with some β-sheets and higher content of α-helices in its secondary structure (which was confirmed by DichroWeb) (De Jongh et al., 2013). The monophasic unfolding melting point for European seabass and gilthead seabream was 69.9 ± 0.3 °C and 72.3 ± 0.1 °C, respectively, which are lower than the one determined in carp PV (Bugajska-Schretter et al., 2000). At 95°C (orange lines, Fig. 2.2), spectra showed a minimum at 204 nm and 200 nm, for European seabass and gilthead seabream respectively, which is typical of proteins with a random conformation (Kelly et al., 2005). These results are in accordance with the ones determined for carp PV (Bugajska-Schretter et al., 2000). Cooling down back to RT (green lines, Fig. 2.2) showed that PV from both species was almost able to refold back to its original structure (two minima were shown at 207 nm and 221 nm). Although we observed very similar minima and maxima wavelength values, comparing to RT structure, there was a slight shift in the zero-crossing (0 ellipticity) wavelength. We observed lower wavelengths at zero-crossing which might be due to loss of some α-helixes (De Jongh et al., 2013). This was not observed in carp PV, which was able to refold its structure after heating (Bugajska-Schretter et al., 2000).

Calcium chelator compounds like ethylenediaminetetraacetic acid (EDTA), a safe and effective food additive to extend shelf life or as an emulsifier, are suggested to decrease PV stability. In case of the calcium-depleted structure (apo-form) (Fig. 2.2, dashed lines) with 5 mM EDTA, a more notable difference was shown for the refolding capacity of gilthead seabream where a minimum of 205 nm and no stable maximum below 200 nm seems to influence slightly its structure. As such, the affected stability of PV might influence the binding to IgE and consequently modify its allergenic potential.

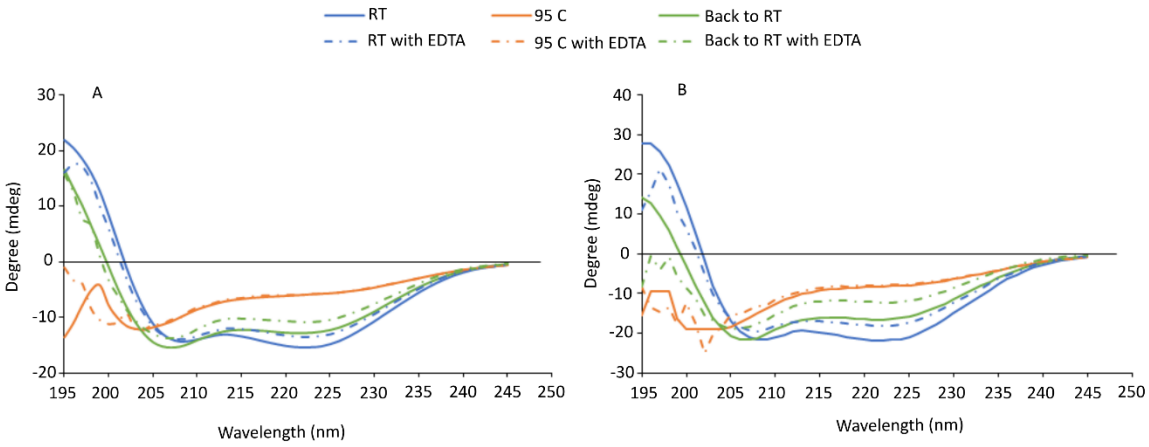

Fig. 2.2 – Far-UV circular dichroism analysis of purified β-parvalbumin of (A) European seabass and (B) gilthead seabream at RT (blue line), 95°C (orange line) and back to RT (green line). Also purified β-parvalbumin depleted with 5 mM EDTA (dashed lines) is shown for all conditions (RT, 95°C and back to RT).

2.3.4 Parvalbumin digestion

To further characterize PV, we proceeded with an *in vitro* simulation of the gastrointestinal tract to evaluate the resistance of PV under static pH conditions, with pepsin activity in the gastric phase, and with bile salts and pancreatin present in the intestinal environment (Zhang et al., 2017, Brodkorb et al., 2019). Yet, it is known that protein denaturation and degradation depends on gastric acidity and on the consequent proteolytic activity of pepsins (Untersmayr et al., 2005). Besides this, *in vivo*, a change from fasted to a fed state shifts pH from 1 to 6 and higher, respectively. As explained previously, the detectability of PV was performed using a sandwich ELISA with specific antibodies produced for the fish species being studied. Using this detection model, we were able to understand how the PV epitopes change through digestion. Our *in vitro* digestion analysis, under static conditions in the gastric phase, showed in both cases that PV detectability decreased throughout the gastrointestinal tract, however, traces were still detected after at least 210 min (Fig. 2.3 A-B). Our results confirmed that the gastric and intestinal phase degrade and denature proteins, which results in a lower affinity with the antibody (Koziolek et al., 2015). Additionally, Supplementary Material Fig. S2.3 showed that PV bands are degraded by digestion in the gastric phase. These results confirm the ones obtained by the sandwich ELISA. Significant differences in the gastric phase were determined using a One-way repeated

measures ANOVA followed by a paired *t*-test with Bonferroni correction to control a type I error when using multiple t-tests. European seabass PV, exposed to pH 3 and 1000:1 pepsin (U) to protein ratio (mg), decreased significantly after 5 min, except for 30 min which showed higher biological variation. Gilthead seabream showed a visible decrease of PV over time, but statistics only showed differences for the first minutes and after half an hour, this might be explained by a high biological variance at 0 min. After two hours of gastric phase, pancreatin and bile salts were added together with the intestinal fluid to the remaining solution. No significant differences were shown during this phase, for both species. A study performed on the stability of cod PV to gastrointestinal conditions showed a low stability after 1 min when subjected to a pH of 1.2, at 0.1 U of pepsin (De Jongh et al., 2013).

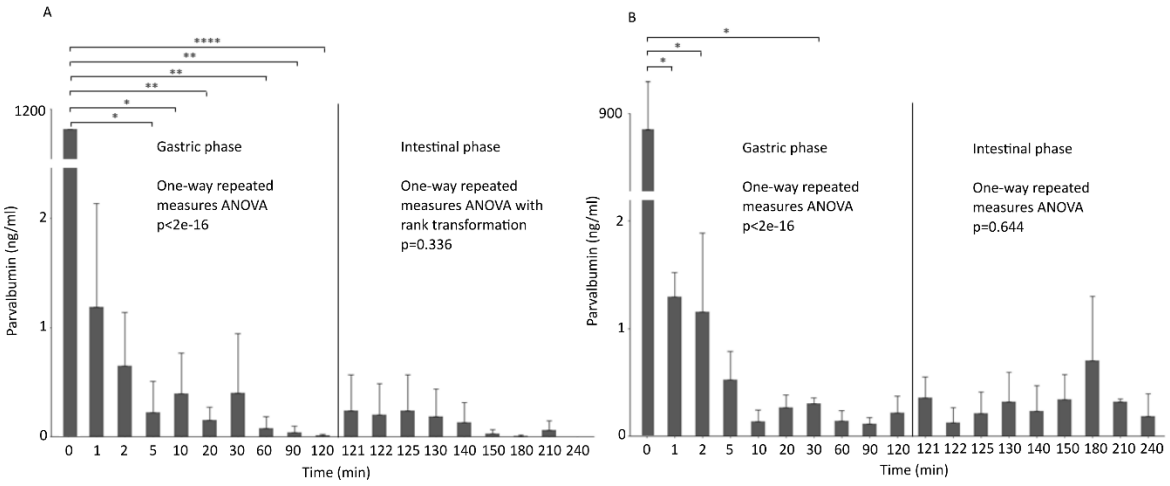

Fig. 2.3 – Bar plot of *in vitro* simulation of the gastrointestinal tract of muscle proteins (n=3) from European seabass (A) and gilthead seabream (B). Parvalbumin was quantified using a sandwich ELISA with antibodies against the analysed fish species. Asterisks indicate significant differences by paired t-test with Bonferroni correction (*p<0.05, **p<0.01, ****p<0.0001).

2.3.5 Fish processing and conservation

Besides digestion other methods might affect PV stability, such as thermal procedures which are one of the most common used techniques in fish processing. They include cooking, steaming and ultra-high heating, which might be accomplished by autoclaving (Besler et al., 2001). We used the mentioned thermal processes as follows, boiling fish muscle for 10 min at 98°C and steaming fish muscle for 8 min at 98-100 °C with or without combination of pressure by autoclaving. Besides the thermal procedures, several non-thermal processes

might be used to extend shelf live and enhance quality (Bhat et al., 2021). Well-known non-thermal techniques are freezing (Dasanayaka et al., 2020) and salting, which we used as follows, muscle samples were frozen at -20°C for 12 months and 5% NaCl was added before autoclaving, respectively. Dehydration by freezing seems to be responsible for the exposure of hydrophobic groups, which might result in structural polymers (Lee et al., 2012). After each thermal or non-thermal procedure total protein was extracted from the muscle sample, PV was detected using the produced sandwich ELISA with specific antibodies (Fig. 2.4).

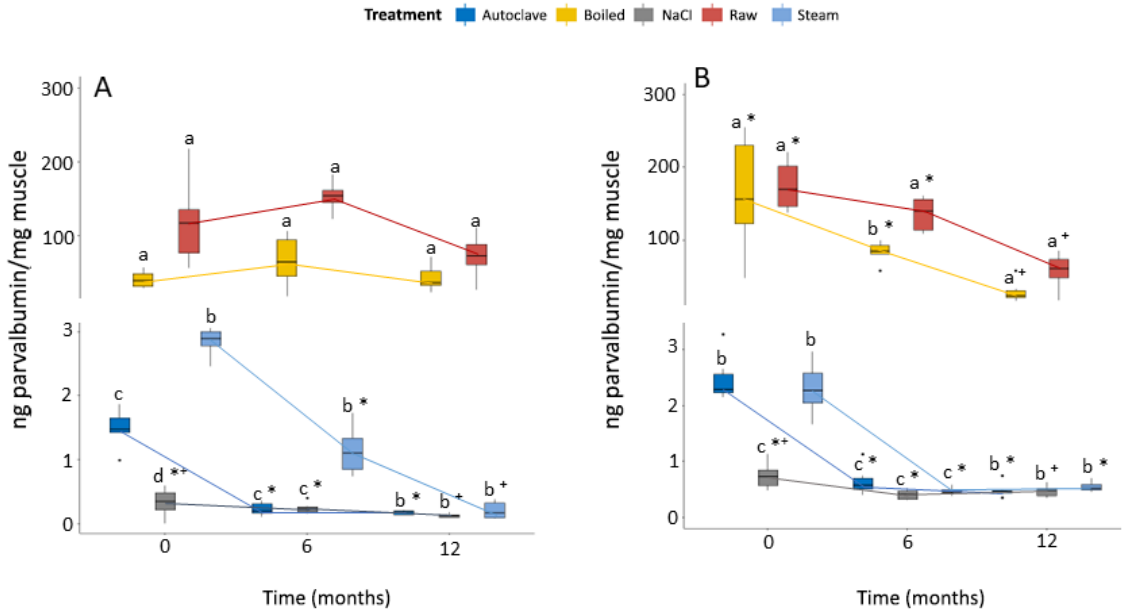

Fig. 2.4 – Box plots of European seabass (A) and gilthead seabream (B) parvalbumin conservation over time (t=0, 6 and 12 months), with different treatments (Autoclave, Boiled, NaCl, Raw and Steam). Results are shown by quartiles (from Q1 to Q3) and the horizontal line in each box represents the median value. Lines outside the boxes represent the minimum and maximum. Significant differences (two-way repeated measures ANOVA, followed by paired t-test with Bonferroni correction) are shown by letters (a, b, c, d) in between treatments at each time point and by symbols (*,⁺) in between time for each treatment.

At timepoint 0, the thermal process of heating/boiling decreased (slightly) PV detectability in comparison to raw extracts but without significant differences, for both fish species. These results were confirmed in a study with 19 fish species where a positive band for PV was shown after 15 min at 95°C (Saptarshi et al., 2014). Another study also confirmed the heat stable PV presence in 19 out of 22 fish species (except for swordfish, bigeye and yellowfin tuna) after 10 min heating at 100°C (Kobayashi et al., 2016c). It should be noted

that, although circular dichroism did show a random conformation of parvalbumin when heated to 95-100°C, it seems that the affinity for the antibodies is still intact. In case of steaming and ultra-high heating using an autoclave (with pressure just over 1 bar), PV decreased significantly in comparison to raw samples, in both species. Studies on proteins showed that pressure can affect their secondary structure (Somkuti et al., 2012). This process is used for fish canning, where elevated temperatures are used followed by packing (Dasanayaka et al., 2020). Canned tuna is an example of a processed fish species which showed a denatured PV with blocked or destroyed epitopes (Sletten et al., 2010). Non-thermal processing techniques might also denature proteins, altering their structure as described for thermal processes (Dasanayaka et al., 2020). Salting, in addition, may affect protein solubilization, resulting in aggregation or precipitation (Bhat et al., 2021). Addition of NaCl to muscle samples resulted in an even more accentuated decrease in PV content compared to raw samples, in both species used in this study. An SDS-PAGE study performed on salted herring showed a less intense band of PV (Sletten et al., 2010). In Supplementary Material Fig. S2.4, an SDS-PAGE performed with all samples showed positive bands for PV, although with less intensity for autoclave, NaCl and steamed samples, which is in accordance with our sandwich ELISA results. Additionally, it showed that the used processing techniques mainly change the structure of PV and consequently affect the epitopes. Additionally, as observed in Fig. 2.4A, for European seabass, boiled and raw samples showed no further decrease by freezing muscle at -20°C for 12 months. For gilthead seabream (Fig. 2.4B) we observed a significant reduction in PV detectability after boiling when muscle was frozen for 6 months, in comparison to raw muscle. Additionally, PV from this species reduced its detectability through time with significant lower amounts after 12 months. Moreover, freezing contributes to an even further decrease of PV after steaming, with significant differences after 6 months being at -20°C, for both species, and European seabass showed an even lower amount of PV after 12 months of freezing. Additionally, freezing up to 12 months showed significant differences in PV after autoclaving and salting, comparing to 6 months of being at -20°C. Freezing, in general, affects protein denaturation, and it seems that storage time influences the level of PV denaturation.

2.4 Conclusions

Our study about the characterization of muscle parvalbumin (PV) from two important Southern Europe fish species, European seabass and gilthead seabream, showed that this allergen has a higher content of α-helices than β-sheets. PV was not completely able to refold back to its original structure, after heating, due to the loss of some α-helices. *In vitro* digestion was able to show that PV detectability in both species decreased during digestion, suggesting that the affinity of PV antibodies decreased due to structural changes or proteolytic denaturation. Different literature showed the same results with several fish species, although PV detection was performed with other techniques (De Jongh et al., 2013, Luo et al., 2020). Different processing methods decreased the PV amount, nevertheless, variations between methods showed that salting resulted in the lowest PV detectability. Freezing over an extended time (up to 12 months) decreased PV in almost all studied processing methods, except for boiling in European seabass. The available literature on fish processing using sandwich ELISA (against mackerel PV) only used heated samples, where they did show modifications in PV with pressure cooking (Shibahara et al., 2013). This study contributed to the characterization and stability of PV, a highly allergenic protein in fish. It is important for fish consumers to have this knowledge and be able to evaluate if consumption will be safe in case of a known allergy. Nevertheless, further studies should be performed to analyze IgE-reactivity to fish muscle after being processed.

2.5 Acknowledgments

This study received Portuguese national funds from FCT – Foundation for Science and Technology through projects UIDB/04326/2020, UIDP/04326/2020 and LA/P/0101/2020 and project ALLYFISH (Ref. 16-02-01-FMP-0014, “Development of a farmed fish with reduced allergenic potential”) financed by Mar2020, in the framework of the program Portugal 2020. DS, CRM and RC acknowledges a FCT PhD scholarship with references SFRH/BD/136319/2018, SFRH/BD/138884/2018 and 2021.06786.BD, respectively. MC acknowledges a FCT contract with reference 2020.02937.CEECIND.

2.6 Supplementary Material

SMM2.1: Immunization scheme: For the production of anti-PV, purified protein was sent to Eurogentec, Belgium. Two rabbits were immunized with purified protein from gilthead seabream and another two rabbits were inoculated with purified protein from European seabass. Antibodies were produced following the classical 87-day program which consists of 4 injections and 4 bleeds. This program produces polyclonal antibodies with high affinities. At the end of the program a protein G purification was performed, by the company.

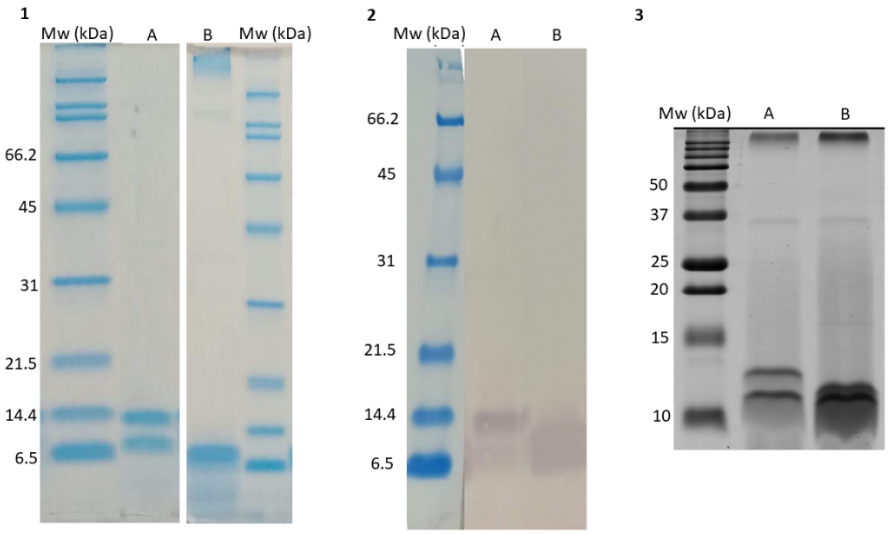

Fig. S2.1 – Parvalbumin detection after heating samples to 90°C for 10 min, 1) AnykDa™ Criterion™ TGX™ Precast SDS-PAGE (Bio-rad) with Coomassie staining, 2) Western blot using commercial antibodies (monoclonal anti-parvalbumin, Swant PV235 and anti-rabbit/mouse IgG conjugated with alkaline phosphatase, Sigma, 1:10,000 diluted in blocking buffer) and 3) 18% SDS-PAGE gels with Coomassie staining. Mw – Molecular weight (kDa). A – European seabass. B – gilthead seabream

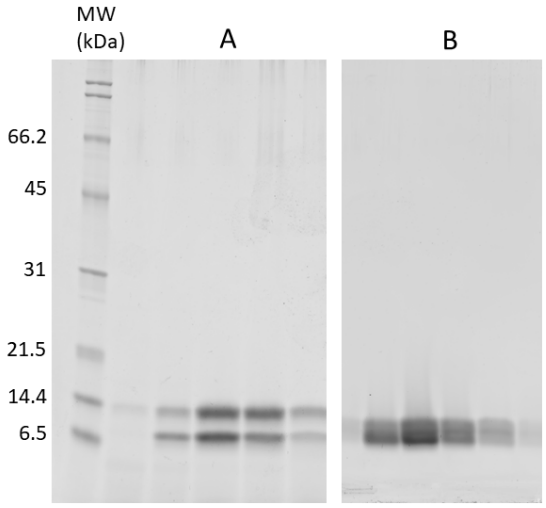

Fig. S2.2 – SYPRO ruby stained SDS-PAGE gels from several fractions after gel filtration. Mw – Molecular weight (kDa). A – European seabass and B – gilthead seabream

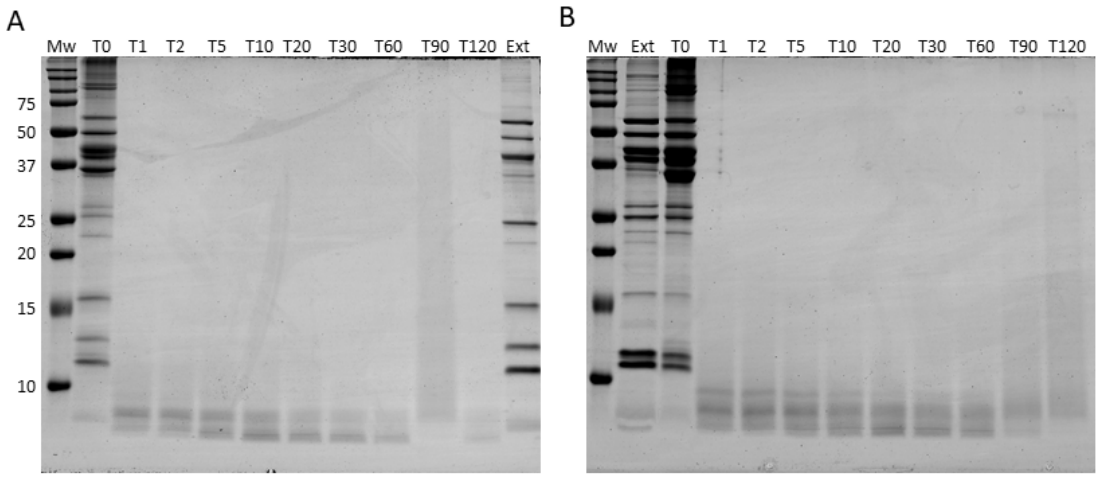

Fig. S2.3 – SDS-PAGE gels with 15% polyacrylamide from the gastric phase of the *in vitro* simulation of the gastrointestinal tract of muscle proteins (Coomassie staining). Mw – molecular weight (kDa). A – European seabass and B – gilthead seabream. Ext – crude extract

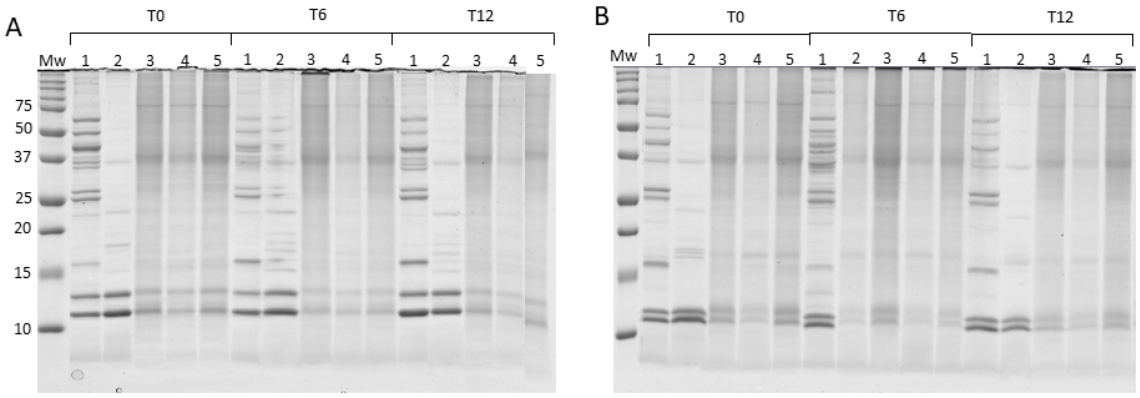

Fig. S2.4 – SDS-PAGE with 15% polyacrylamide of samples from several processing techniques without freezing (T0), and after 6 or 12 months of freezing at -20°C (T6 and T12, respectively). A – European seabass and B – gilthead seabream. 1 – Raw, 2 – Boiled, 3 – Autoclave, 4 – NaCl, 5 – Steam.

Chapter 3

Consumers' willingness to pay for fish with a low allergenic potential

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Consumers' willingness to pay for fish with a low allergenic potential

Abstract

This study explores the demand-side perspective on the factors influencing the willingness to pay (WTP) premium price for fish with a low allergenic potential (FWLAP). A survey to assess consumers' behavior and preferences for fish products was implemented in a representative sample of the Portuguese general population (*n*=640). The contingent valuation method was applied to elicit consumers' WTP. Results indicated that only half of the consumers were willing to pay higher prices for FWLAP. Econometric results suggested that WTP for FWLAP is explained by the presence of fish-allergy pathologies in the household, and the fish unique characteristics and quality.

Keywords: consumer acceptance; contingent valuation method; fish allergies; premium price; willingness to pay.

3.1 Introduction

General allergic diseases are increasing worldwide (Pawankar et al., 2012), affecting about 30% of the population (Sánchez-Borges et al., 2018). Allergic diseases may include symptoms like asthma, life-threatening anaphylaxis or rhinitis, and may be caused by drugs, insects and food, among others (Pawankar et al., 2012, Sánchez-Borges et al., 2018). An allergy occurs when an immunologic mechanism initiates a hypersensitive reaction (Johansson et al., 2004). Food allergies are steadily on the rise and show a prevalence between 5% and 10%, which varies between countries, age of the studied population and type of food (Savage and Johns, 2015). Nowadays, it is estimated that up to 3% of adults and up to 8% of children are affected worldwide by some food allergy related pathology (Tordesillas et al., 2017). Prevalence on food allergies in the Portuguese adult population was determined by Lozoya-Ibáñez et al. (2016) through a survey questionnaire by phone, showing

that 6% of the sample self-reported adverse reactions upon ingestion of at least one foodstuff. The most common type of related food allergic reaction is known as an IgE-mediated reaction, in which symptoms usually develop in minutes or seconds after ingestion of the allergic food. These symptoms can vary from an itchy red rash, swelling, wheezing, nausea, abdominal pain or diarrhea to anaphylaxis which, without proper medical treatment, can be life threatening. Fish is one of the eight (among milk, eggs, peanuts, tree nuts, shellfish, soy and wheat) most common triggers for food allergies and shows a life-time prevalence of 2.2% in Europe (Nwaru et al., 2014b). Prevalence seems to differ on the amount of fish consumption with countries like Australia, Norway, Spain and Portugal among those with high ingestion of fish (Pascual et al., 2008). In the case of a fish ingestion related allergy, clinical reactions usually manifest rapidly with single or several organs being affected and clinical symptoms ranging from mild to severe anaphylaxis (Sharp and Lopata, 2014). These allergic responses are due to ingestion, contact, inhalation or even through food cross-contamination of fish products. According to the Food and Agriculture Organization (FAO) of the United Nations, consumption of fish keeps increasing and even exceeded the annual consumption of meat from terrestrial animals, growing from 9.0 kg in 1961 to 20.2 kg in 2015 (per capita/year). This high demand for fish requires a fast growth of aquaculture industry. In 2016, aquaculture produced up to 80 million tons of fish (FAO, 2018). Aquaculture, with an average annual growth during the period 2000-2016 of 5.8%, continues to be the fastest major food production industry. Fish consumption is considered a healthy way of protein intake. Essential amino acids, lipids (polyunsaturated fatty acids - PUFAs), high-quality proteins and micronutrients (e.g., vitamin A, iron, zinc) are present in fish in considerable amounts (Kawarazuka and Béné, 2011, Kuehn et al., 2014, Schrama et al., 2018). Fish as a food source are subjected to high quality standards such as food safety, fish welfare and quality (Schrama et al., 2018, Silva et al., 2012a). In order to reach even more people worldwide it would be of great interest to produce fish with a low allergenic potential (FWLAP); as for now, the only way to avoid severe reactions is evading fish from the diet. Ninety-five percent (95%) of fish allergic reactions are due to a protein present in high amounts in white fish muscle named β-parvalbumin. Other proteins such as collagen, fish gelatin and aldehyde phosphate dehydrogenase have also been identified as minor allergens (Griesmeier et al., 2010). Studies targeting β-parvalbumin to modulate fish allergenicity have been reported by adding specific ingredients to fish diets like creatine, to reduce the concentration of the allergenic protein in

the muscle (Schrama et al., 2018) or ethylenediaminetetraacetic acid (EDTA) to promote a less allergenic form of this protein (Raposo de Magalhães et al., 2020b). The main goal of both these studies is the production of a FWLAP (Raposo de Magalhães et al., 2020b, Schrama et al., 2018).

In order to determine acceptance and willingness to pay (WTP) for FWLAP, a proper marketing research study should be performed. As a result, this study produces insightful market information to support marketing decision-making for both producers and marketers of aquaculture fish products.

Consumer's WTP for food products has long attracted the interest of researchers ((Boccaletti and Nardella, 2000, Emunu et al., 2012, Gil et al., 2000, Hjelmar, 2011, Kalogeras et al., 2009, Khan et al., 2018, Kyriakopoulos and Ophuis, 1997, Shirai, 2010, Zander and Feucht, 2018) among others), with a rising number of studies published in this area. However, despite this increase, few studies have focused explicitly on fish products for food consumption (Alfnes et al., 2006, Davidson et al., 2012, Pereira et al., 2017, Quagrainie, 2006), and to the best of our knowledge, none has been devoted to FWLAP. Many studies suggest that sociodemographic variables usually determine the consumers' WTP with different types of food products. For example, Khan et al. (2018) concluded that sociodemographic variables, such as age, education, income, residence location and household's size, significantly explain the WTP for pesticide-free fruits. Hjelmar (2011) found that education and income significantly influence the WTP for organic food products. Emunu et al. (2012), who studied Canadian consumers' WTP for omega-3 enhanced beef, pork and chicken, found that households' size, income, and history of purchasing an omega-3 product are willing to pay a premium for omega-3 meat. In the Japanese market, Shirai (2010) studied which food elements related to premium price. Shirai (2010) found that fish is included in the categories that could most achieve a premium price. In addition, Shirai (2010) discovered that the three food characteristics most related to a premium price were safety, freshness and good taste. Although research about the WTP for fish and seafood products is scarce, there are studies reporting the effect of sociodemographic variables on consumer's WTP. Quagrainie (2006) found that age, gender, race and household size significantly explain the WTP for a retail pack of individual quick-frozen 6-fillets of catfish through the USA grocery market channels. Zander and Feucht (2018) concluded that age, education and income significantly determine the WTP for sustainable seafood made in Europe. Differing from previous studies, Davidson et al.

(2012) reported that Hawaii consumers have the WTP a premium for wild-caught over farm-raised fish, as well as for fresh over frozen fish; while Alfnes et al. (2006) concluded that consumers have the WTP significantly more for salmon fillets with normal or above-normal redness, as compared with paler salmon fillets. In a different line of research, Mejrhit et al. (2018) studied the association between fish consumption and self-reported fish and shellfish allergy. They found a strong inverse relationship between these variables, which means that consumers who self-reported this allergy tend to consume a lower quantity of fish than others. Finally, research review revealed that there is a single study conducted in the surveyed population that deals with the WTP for fish products (Pereira et al., 2017). This study aimed to propose a marketing strategy for a new functional fish, for which factors determining the acceptance of a new functional seabream, as well as the WTP for this fish, were analyzed. Their results revealed that the most appreciated functional ingredients of seabream were their compounds, namely "has more Omega-3," "has more iodine" and "is less allergenic". They did not explore determinants of the WTP but estimated that consumers were willing to pay on average 7.8% more for a functional seabream than for a conventional farm-raised seabream. Thus, as far as we know, there is extensive study about consumer's WTP for a wide range of food products, but no information about consumer's WTP for FWLAP.

Specifically, the objectives of this study are to: (1) assess Portuguese consumers' intent to consume FWLAP; (2) estimate their WTP for FWLAP; (3) identify consumer segments according to their WTP; (4) identify which consumer groups (gender, age, …) would be most responsive to pay a premium price for FWLAP; and (5) examine determinants that influence consumers' WTP toward FWLAP. Since this food product is under development, it is not yet traded in the market. For this reason, it is extremely important to measure individuals' acceptance of a fish with that specific improvement and elicit the WTP for it by consumers.

The rest of the article is organized as follows. First, the materials and methods section describes important aspects of the sampling design, the method used to elicit the consumer's WTP, the survey instrument and the methods of data analysis, including the specification of regression model. The subsequent section encompasses the results for both the descriptive statistics and the regression analysis, together with the corresponding discussion of the most relevant results per variable and their categories. Finally, the main conclusions of this research are stated, together with its benefits, chances for improvement and future research lines.

3.2 Materials and methods

3.2.1 Sampling design

The target population of this study was 8.7 million Portuguese adults, aged 18 and older (Portuguese Statistical Office, 2012). The stratified random sampling method was used to select a representative sample of the population with regard to gender, age group and region. Furthermore, we have decided to proportionally allocate the global sample size to each stratum. This decision facilitated the statistical analysis, since all sampling units had equal probabilities of selection (Coelho et al., 2016). Sample size calculations used the population size in each stratum from the Portuguese Censos 2011 (Portuguese Statistical Office, 2012), the probability of consuming the new fish as the main variable of interest and the mean of this variable as the target parameter. Using the well-known formulae of the stratified sampling with proportional allocation (Coelho et al., 2016), a global sample size of 640 individuals was obtained, using a confidence degree of 95% and a relative precision of 6.25%.

3.2.2 Elicitation of consumers' WTP

The contingent valuation method (CVM) was used in this study to elicit consumers' WTP for FWLAP. This well-known survey-based method has been used for eliciting consumers' perceived value on private goods and services, namely in regards to food products (Khan et al., 2018, Kalogeras et al., 2009, Emunu et al., 2012, Zander and Feucht, 2018) and recreational fishing (Olaussen and Liu, 2011). We have decided to use the CVM because it has been used to elicit individuals' hypothetical WTP in the absence of a real purchasing situation (Boccaletti and Nardella, 2000). Since a FWLAP is not yet traded in the Portuguese food market, the CVM is operationalized with survey questions to measure individuals' WTP for the improvement of reducing the allergenic content in sea bream. In addition, we have chosen this method because it directly asks individuals about their WTP a premium price, which provides insight into the valuation on the product value (Zander and Feucht, 2018) and simplifies the analysis based on the net value of WTP (Khan et al., 2018). Finally, the CVM was chosen over other methods widely used to measure WTP (e.g. discrete choice experiment) because it is an easy method to implement in telephone interviews.

Although the CVM was considered the most appropriate method for this study, it is important to note that it is not free from limitations and criticisms (Hausman, 1993, Pomerehne and Hart, 1997). Two main limitations are highlighted in the framework of this study. The first is related to developing a scenario (a detailed description of the new food product not currently offered in the market) sufficiently comprehensible, clear, and meaningful to respondents, who must clearly understand the changes in characteristics of the food product that is being valued. In order to mitigate this drawback of the CVM, the following description about the proposed changes in a conventional sea bream was included in the questionnaire: "*Now we are going to introduce you to a new concept - fish with a low allergenic content. A fish with a low allergenic content is a fish that does not cause an allergic reaction to those suffering from this pathology*." The second limitation of the CVM is the fact that the method provides hypothetical answers to hypothetical questions, which means that respondents may supply answers different from their real behavior. This situation may induce respondents to overlook their budget constraints and consequently overestimating their stated WTP (Pomerehne and Hart, 1997). We assume that these limitations did not have significant consequences in the respondents' reported behavior since Portuguese consumers frequently experience real purchasing situations of specific food products with a reduced content (e.g. reduced-sugar, reduced-fat or reduced-gluten food). However, a slight overestimation of the WTP must be considered.

The CVM was implemented in this study through the payment card valuation technique, following some examples of application of this technique in similar market research studies (Khan et al., 2018, Zander and Feucht, 2018). This technique was chosen since it can be implemented using a close-ended question in simple framework. In practice, respondents only have to select a choice among easily comparable premium price alternatives of the FWLAP. This was a mandatory condition in order to run a telephone survey, which enables us to observe a random and representative sample of Portuguese adults living all over the country. The main research question used to elicit the Portuguese consumers' WTP for FWLAP is the following close-ended question:

> *Based on your reference price for 1 kg of farm-raised sea bream, how much would you be willing to pay for 1 kg of farm-raised seabream with a low allergic content?*

Please give me your answer in one of the following categories: 1=Not pay more; 2=1-5% more; 3=6-10% more; 4=11-15% more; 5=16-20% more; 6=21-25% more; 7=26-30% more; 8=31-35% more; 9=36-40% more; 10=41-45% more; 11=Pay more than 45%.

Respondents were then asked to state the nearest WTP value among premium price categories previously described and predefined. This CVM question did not include any reference price as to: 1) avoid any influence that a reference price might have on the consumers' WTP by influencing their reference prices; 2) reduce the measurement error of the premium price that each consumer is willing to pay; and 3) produce useful information for fish producers and retailers, as it allows them to establish the premium price for the FWLAP compared to their conventional fish prices. The first two reasons are supported by the evidence that reference prices are typically generated by the prices of analogous products or products that solve similar problems (Park et al., 2011), which differ significantly in Portugal. For example, the price of the farm-raised seabream fresh fish differs according to the region, the retailer and the size of the fish.

3.2.3 Survey instrument

A questionnaire was designed to gauge Portuguese consumers' fish consumption habits as well as acceptance and WTP for FWLAP. The instrument consisted of five sections. Fish consumption questions, including frequency of consumption and type of fish consumed (fresh, dried and frozen), were included in the first section. The following section included questions related with fresh fish consumption habits. Thereafter, the third section of the questionnaire was focused exclusively on FWLAP. This section started with a clear description of FWLAP, followed by questions regarding the acceptance (probability of consumption and recommendation) of this new food and reasons for or against its consumption. The CVM was included in this section as well. This section ended with two questions about the minimum and maximum cut-off prices that consumers were willing to pay for FWLAP in order to contextualize their response to the CVM. In the fourth section, respondents were asked to rate the extent of the importance they attach to a set of 12 items in their motivation to buy FWLAP. The items were adapted from prior studies (Pereira et al., 2017). A five-point Liker scale anchored from "not at all important" (1) to "very important" (5) was used to measure

the items of the fourth section. The last section of the questionnaire included questions concerning sociodemographic characteristics about respondents, including gender, age (measure in years), marital status, education attainment, and household dimension (measure in number of members). Questions about both self-reported fish allergy and health problems due to allergies in the household were also included in this section. These two questions were coded as dummy variables where positive answers were represented by 1. Due to both the aim of this paper and economy of space, this paper reports results based only on part of data collected through the questionnaire.

A group of four aquaculture and fisheries experts were then invited to assess the content validity of the instrument and requested to edit and improve those questions or items to increase their clarity and readability. After this task, but before the implementation of the national survey, the researchers conducted a pre-test of the questionnaire on a convenience sample of consumers to avoid ambiguous, vague and unfamiliar concepts. Based on the results of the pretest, the questionnaire was concluded with minor changes.

3.2.4 Data collection

The data collection was performed in November and December 2019 by a market research company. Two trained interviewers collected the data by telephone interview using a Computer Assisted Telephone Interviewing system. Telephone numbers were generated by a computer algorithm to ensure a random sample using a Random Digit Dialing method. Calls were made between 6 p.m. and 10 p.m. when residents were most likely to be at home. The response rate for eligible households contacted by telephone was 25.8%, using the standard definition of the American Association for Public Opinion Research (AAPOR, 2016). Up to two callbacks were made when there was no answer or the line was busy on the previous call. Only one interview per household was conducted and each respondent answered the survey designed for this study after an eligibility check. The eligibility check, based on the target population and the sampling design, aimed to confirm if the phone number belonged to a household and if at least an adult member of the household satisfied the open strata (i.e. with sample size not completely observed) in terms of gender, age group and region. The selection of the person to interview was based on the next household person's birthday, whenever more than one adult member satisfied the eligibility criteria. The telephone interview script

was developed by that company and, on average, each telephone call lasted 12 minutes. A pilot test of the whole survey process was performed in October 2019 by the company. Only after being approved by the research team, through this pilot test, could the company start the fieldwork.

The survey consisted of three parts:

i) General welcome of the participant. After the eligibility check and the selection of the interviewee, the purpose of the study was explained and each respondent was asked if s/he agreed to participate in the study. Moreover, all respondents were informed that there were no wrong answers, and we assured them about the anonymity and the confidentiality of the data.

ii) Implementation of the survey questions. The subject for all sections of the questionnaire was introduced to respondents before questioning to provide context for the respondent. The scales of measurement were also clearly presented in each question.

iii) General thank you for the cooperation and goodbye. At this stage the interviewees were also informed that they could be contacted again in the future for quality control purposes.

The quality control and monitoring of fieldwork was conducted both through direct supervision of the telephone interviews and listening in on 20% of the realized phone calls by a third-party. The later procedure checked if the interviews were conducted and if the answers were correctly registered into the database. Both procedures contributed to detect possible fraud and to reduce measurement errors. Data validation was performed with the use of a computer application developed for this purpose to prevent incorrect data entry or data inconsistencies. The principles of anonymity, confidentiality and individual privacy were assured along the study. No incentive (monetary or non-monetary) was given to the respondents.

3.2.5 Data analysis

Three approaches were applied for data analysis. First, both a descriptive and inferential analysis were applied. Beyond a descriptive profile of the sample, estimation of parameters (proportions, means and quantiles) of key variables was done and statistical tests were applied to analyze if statistically significant differences exist between groups of individuals. An estimative of the average premium price for consumers' WTP for FWLAP was done using a weighted average based on midpoints of each class. Since the target variables do not follow a Normal distribution in each group, non-parametric tests were used. Comparisons of the consumer's acceptance of a fish with a reduced allergenic content by gender (male and female), age group (18-24, 25-34, …, 65+), holding fish allergenicity (with and without allergenicity) and holding health problems due to allergies (with and without health problems) were performed using both the Mann-Whitney and the Kruskal-Wallis tests. All these analyses were performed using the IBM SPSS Statistics version 25.

The second analysis generated homogenous clusters of consumers in terms of WTP for FWLAP. A two-stage clustering process was applied to generate and select the number of clusters (Everitt et al., 2011). First, a hierarchical clusters analysis using the squared Euclidean distance as a similarity measure and the Ward's method as agglomerative algorithm was conducted. The number of clusters were chosen based on the dendrogram, R-squared statistic and analysis of distances between subgroups in each step of the algorithm. The results suggested that a three-cluster solution was the most appropriate, because their centroids were significantly different. In addition, this solution was revealed appropriate in terms of clusters' interpretation and meaningfulness. Second, a non-hierarchical *K*-means clustering technique for a definitive classification of cluster membership for each respondent was used. Readers interested in learning more about these techniques and its applications are referred to Everitt et al. (2011). The three clusters were labeled as '1-no WTP' (49.1%), '2-medium WTP' (35.7%) and '3-high WTP' (15.2%), based on their mean WTP for FWLAP. To profile the identified clusters in terms of a set interest variables (e.g. probability of consumption, probability of recommend, price), group differences were assessed using both the Chi-square and the Kruskal-Wallis tests.

The third analysis assessed the determinants of the WTP for FWLAP. Since the previous analysis generated a segmentation variable, which classifies consumers into three

ordered WTP levels, we decided to use this outcome as the dependent variable in the regression model, in order to give consistency to our results. Considering the discrete and ordinal character of the WTP segmentation variable, an ordered logit or probit regression model is recommended (Cameron and Trivedi, 2010, Long and Freese, 2006). In general, ordinal regression models estimate two set of parameters: a set of coefficients used to predict the latent variable based on the explanatory variables, and a set of cut-points which are the thresholds between the categories of the dependent variable. In this research we have used an ordered logit model, following previous research about consumers' WTP (Khan et al., 2018). The ordered logit model can be specified as:

$$y_i^* = \mathbf{x}_i'\boldsymbol{\beta} + u_i, \quad (i = 1, \dots, n) \tag{1}$$

where y_i^* is a latent variable, representing the WTP for FWLAP of the i-th consumer, $\mathbf{x}_i'$ is the vector of k independent variables, $\boldsymbol{\beta}$ is the vector of coefficients, and u_i is a disturbance term with a logistic distribution and null expected value. This model puts the observed variable, y_i, with M categories equal to j if $\tau_{j-1} < y_i^* \leq \tau_j$, where $\tau_0 = -\infty$ and $\tau_M = +\infty$. Then, $P(y_i = j|\mathbf{x}) = P\left(\tau_{j-1} < y_i^* \leq \tau_j|\mathbf{x}\right) = F\left(\tau_j - \mathbf{x}_i'\boldsymbol{\beta}\right) - F\left(\tau_{j-1} - \mathbf{x}_i'\boldsymbol{\beta}\right)$, where $F(.)$ is a logistic distribution function. Both the coefficients and the $M - 1$ thresholds are estimated using the maximum likelihood method (Long and Freese, 2006). The ordered logit model assumes that coefficients are equal for all categories of the dependent variable. This assumption, called proportional odds assumption (or parallel lines assumption), means that each probability curve differs only in being shifted to the left or right (Long and Freese, 2006). Although the proportional odds model is the most common used ordered logit model when the dependent variable has a natural order in its values, that assumption is very restrictive and often violated because it is common at least one coefficient to differ across the categories of the dependent variable (Williams, 2006). In order to overcome the proportional odds assumption and allow the effects of the explanatory variables to vary with the point at which the categories of the dependent variable are dichotomized, the partial proportional odds (PPO) model was used. Other reasons for choosing this model were its superior parsimony and ease of interpretation when compared to other non-ordinal models, such as the multinomial logit model. The PPO model is a special case of the generalized order logit model where coefficients may differ only for those variables for which proportionality does not hold (Williams, 2006). Interpretation of the estimated PPO model coefficients are similar to those of a series of binary logistic

regressions. The first set of coefficients contrasts category 1 (no WTP) with categories 2 (medium WTP) and 3 (high WTP); while the second set of coefficients contrasts categories 1 and 2 with category 3. Hence, coefficients greater than zero indicate that higher values on an independent variable make it more likely that the consumer will be in a higher category of the dependent variable than the current one, whereas coefficients less than zero indicate that higher values on an independent variable increase the likelihood of being in the current or a lower category of the dependent variable. The Stata version 11 was used to estimate the PPO ordered logit model (*gologit2* command). The *autofit* option was also used since its greatly simplifies the process of identifying PPO models that fit the data (Williams, 2006).

The selection of variables used in the econometric model was guided by the literature review on the determinants of WTP (dependent variable) of other food products than FWLAP, as referred in the Introduction section, with the specific characteristics of the food product under study. Table 3.1 defines and describes the set of independent variables included in the econometric model, along with their categories and reference categories, if applicable. The set of explanatory variables can be divided in two groups: a group of control variables composed of individual and household characteristics, and a group of variables indicating reasons for consumer's intention of FWLAP. Thus, the econometric model tests if these reasons are determinants of the WTP, such as Portuguese consumers who have household members self-reporting fish allergy have higher WTP for FWLAP.

Table 3.1. Independent variables and their categories

Name	**Description**	**Categories and reference category**
Individual and household characteristics		
Gender	Gender of the respondent	male (ref.), female
Age	Age of the respondent	not applicable
Marital status	Marital status of the respondent	single (ref.), married or living in a common law, divorced or separated, widowed
Education	Education of the respondent	low (ref.), medium, high
Household size	Number of members in the household	not applicable
Fish weekly consumption	Frequency of fish consumption per week	not applicable

Reasons for consumption fish with low allergenic potential (dummy variables)		
Fish-allergy problems	Household with members self-reporting fish allergy	no (ref.), yes
Health-allergy problems	Household with members self-reporting health problems due to allergies	no (ref.), yes
Fish low allergenic	Fish that does not cause allergic reactions to fish allergy sufferers	no (ref.), yes
Fish species maintenance	Fish created in order to no jeopardize the maintenance of the species	no (ref.), yes
Fish unique characteristics	Fish with unique internal characteristics (nutritional aspects)	no (ref.), yes
Fish excellent quality	Fish created in controlled conditions which ensure excellent quality levels	no (ref.), yes
Fish other reasons	Other reasons (e.g. good taste, good price/quality ratio, on selling at fresh fish markets, similar price to conventional fish)	no (ref.), yes

Note: ref. – reference category.

3.3 Results and discussion

The results of this study were divided into three main parts. First, the sample profile was presented. Next, the WTP was studied. The consumer segments according to their WTP were identified and profiled in this part. Lastly, the determinant attributes that drive the consumers' stated WTP for different premium prices of FWLAP were identified using an ordered logit regression model.

3.3.1 Sample

Table 3.2 presents the main characteristics of the study sample along with Portuguese general population. The sample matched the Portuguese general population aged 18 or more in what concerns region, age and gender (stratification variables) and presented similarities in other variables (marital status and household dimension). However, our sample has a larger proportion of individuals with medium (secondary education or professional training) and higher education (university degree such as bachelor, master or doctorate) level. In summary, more than half of the sample are female (53.3%) and married or living in a common law

(59.8%). The largest proportion of respondents have a medium level of education (42.7%), live in a household with 3 to 4 elements (49.3%) and are aged 65 or over (23.1%). Our representative sample allows to estimate that 4.5% of the Portuguese adult population self-reported fish allergies and 32.2% self-reported health problems due to allergies.

Table 3.2. Study sample characteristics and comparison with Portuguese general population aged 18 or more.

Characteristic	**Sample (*n*=640)**	**PT general population aged 18 or more* (*N*=8,657,240)**
Gender (%)		
Female	53.3	53.0
Male	46.7	47.0
Age group (%)		
18 – 24	9.5	9.4
25 – 34	16.6	16.5
35 – 44	18.4	18.5
45 – 54	17.2	17.2
55 – 64	15.2	15.2
65 +	23.1	23.2
Mean age (standard deviation)	47.8 (16.6)	49.4 (18.5)
Region (%)		
North	32.7	34.7
Centre	23.3	22.4
Lisbon	25.3	26.6
Alentejo	8.0	7.3
Algarve	6.3	4.3
Azores	2.2	2.2
Madeira	2.3	2.5
Marital status (%)		
Single	25.5	27.4
Married or living in a common law	59.8	56.9
Divorced or separated	8.1	6.8
Widowed	6.6	8.9
Educational attainment (%)		
Low (less than secondary)	26.4	62.1
Medium (secondary or professional training)	42.7	19.1
High (Bachelor, Master or Doctorate)	30.9	18.8
Household size (%)		
1 to 2 elements	43.7	53.0
3 to 4 elements	49.3	40.5
5 or more elements	7.0	6.5
Mean household size (standard deviation)	2.8 (1.2)	2.6 (n.a.)

Household (%)		
With self-reported fish allergy	4.5	n.a.
Without self-reported fish allergy	95.5	n.a.
Household (%)		
With self-reported health problems due to allergies	32.2	n.a.
Without self-reported health problems due to allergies	67.8	n.a.

* Source: Census 2011. n.a. – not available.

3.3.2 Distribution of WTP

The estimated distribution of Portuguese consumers' response about WTP premium price for FWLAP over conventional fish is presented in Figure 3.1. Results show that almost half of the consumers (49.1%) are not willing to pay a premium price for FWLAP. However, 18.9% of the consumers have the WTP 1-5% higher prices for FWLAP, 16.7% are willing to pay 6-10% higher prices and 15.2% have the WTP at least 11% more. We estimate that, on average, Portuguese consumers have the WTP 5% more for FWLAP. Thus, appropriate premium prices for FWLAP are considered to be around 5%. This result suggests that there is a small margin between the price of conventionally and low allergenic fish.

A deeper analysis of the WTP distribution presented in Figure 3.1 suggests that there may be consumer segments with the same profile. Thus, a hierarchical cluster analysis was performed to classify the respondents into mutually exclusive groups based on their WTP premium price for FWLAP. Three consumer types were identified: a segment with "no WTP" comprising 49.1% of the respondents, a segment with "medium WTP" (i.e. 1-10% WTP more) accounting for 35.7% of the respondents, and a segment of 15.2% with "high WTP" (i.e. additional WTP more than 11%). These results are in line with previous studies, since other authors also identified the same type of WTP segments (Zander and Feucht, 2018). As shown in Table 3.3, statistically significant differences were found among these segments of respondents.

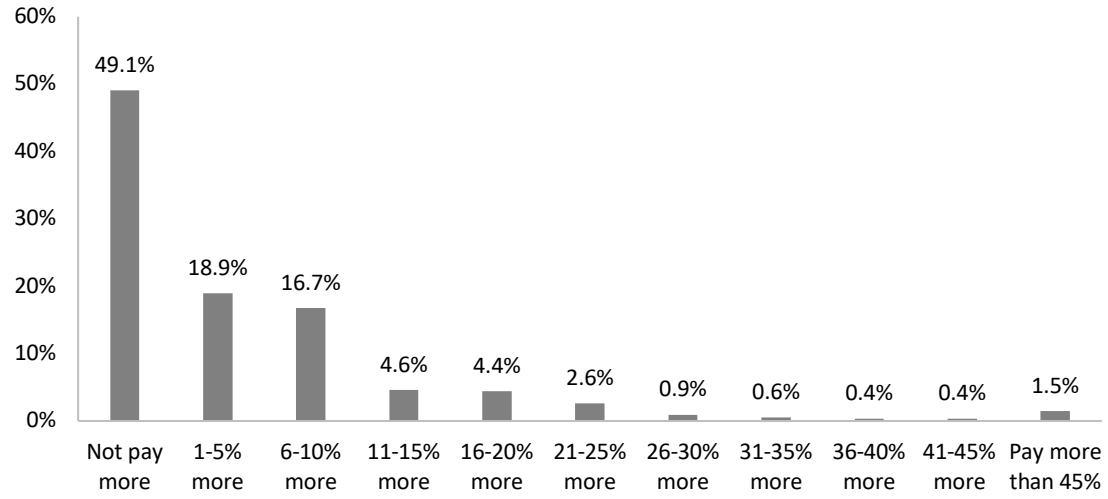

Fig. 3.1. Distribution of WTP

As expected, "no WTP" consumers reported the lowest average probability of consumption (34.3%) and recommending (36.4%) FWLAP as well as the lowest average minimum (€3.9) and maximum (€14.2) cut-off price they were willing to pay for a kilo of farm-raised sea bream. However, it is important to highlight that the reported probability of consumption and recommendation of these consumers are not very close to zero, which means that there is a non-negligible proportion of consumers – about one-third – that are receptive to consume and available to recommend the FWLAP, if it would be traded in the Portuguese food market at the same price of the conventional fish. In addition, results presented in Table 3.3 reveal that the average minimum and maximum cut-off prices are directly associated with the consumers' WTP, since these average prices increase with the consumers' WTP. This outcome means that the segments with higher WTP are also those that are made up of consumers who are willing to pay a higher average price for a farm-raised sea bream.

Table 3.3 also shows that 35.8% of the Portuguese consumers stated intention to consume FWLAP. This result is quite surprising, since only 4.5% of the Portuguese population self-reported problems with fish allergies. It would be expected that only the population with this kind of pathology could be interested in consuming the new FWLAP. Instead, this fraction of the population is even larger than the one that declared health problems due to allergies in general (32.2%). For this reason, that result is indeed promising, especially because 39.8% of the overall consumers intent to recommend FWLAP to family and friends.

Table 3.3. Results of consumer type segmentation.

Variables	No WTO (49.1%)	Medium WTP (35.7%)	High WTP (15.2%)	Overall (100.0%)	*p*-value KW test
Probability of consume (%)	34.4	49.0	51.6	35.8	<0.001
Probability of recommend (%)	36.4	53.5	55.9	39.8	<0.001
Maximum price (€)	14.2	16.5	19.4	15.9	<0.001
Minimum price (€)	3.9	4.9	6.3	4.7	<0.001

Note: Mean values are reported in this table. KW – Kruskal-Wallis test.

Table 3.4 presents the sociodemographic profile of those three segments of consumers. Results reveal that there are not statistically significant differences among the segments with respect to all sociodemographic variables, except to age (p<0.05). On one side, the "no WTP" segment comprises consumers significantly older than in other segments. In addition, this segment includes the highest percentage of married (64.8%) and low educated (28.8%) consumers. On the other side, most of the "high WTP" consumers are women (53.0%), married (56.6%) and with medium education level (51.8%). This segment also presents the lowest average frequency of weekly consumption of fish (3.1 times a week), although it is not statistically different than in other segments.

Table 3.4 Demographic characteristics of the consumer segments.

Variables	No WTP (49.1%)	Medium WTP (35.7%)	High WTP (15.2%)	*p*-value
Gender (%)				0.193 §
Female	47.6	41.8	53.0	
Male	52.4	58.2	47.0	
Marital status (%)				0.112 §
Single	20.2	32.1	30.1	
Married or living in a common law	64.8	56.5	56.6	
Divorced or separated	7.5	6.2	4.8	
Widowed	7.5	5.2	8.4	

Educational attainment (%)				0.109 §
Low	28.8	25.3	19.3	
Medium	43.8	38.7	51.8	
High	27.3	36.1	28.9	
Age (mean)	49.2	45.7	45.6	0.046 ‡
Household size (mean)	2.9	2.8	2.9	0.996 ‡
Weekly consumption of fish (mean)	3.2	3.3	3.1	0.657 ‡

Note: § χ^2 test. ‡ Kruskal-Wallis test.

3.3.3 Determinants of WTP

Table 3.5 illustrates the determinants of consumers' WTP for fish with reduced allergic content. First of all, results of a global test of the proportional odds assumption (LR $\chi^2_{(16)} = 22.96$, $p = 0.115$) did not clearly show that all variables meet this assumption. In addition, the Brant test suggested that five variables might violate the parallel lines assumption ($p < 0.10$). Thus, results presented in Table 3.5 are based on the PPO model estimated by maximum likelihood using the iterative *autofit* procedure (Williams, 2006). Table 3.5 presents the estimated coefficients of the explanatory variables, as well as its robust standard errors. Estimated coefficients of the variables 'fish excellent quality', 'fish other reasons', 'female', 'widowed' and 'medium education' are different across the categories of the dependent variable, which means that these variables violate the proportional odds assumption.

Table 3.5 reveals that there are two variables that have a significantly positive effect on the WTP: 'fish-allergy problems' and 'fish unique characteristics'. It means that consumers belonging to a household with members self-reporting fish allergy tend to have higher WTP for FWLAP. This is in line with our expectations of higher *a priori* WTP by fish-allergic-related consumers. Results also indicate that consumers' perception that the fish has unique internal characteristics (nutritional aspects) significantly determines a higher WTP. The coefficient of 'fish excellent quality' is positive but declines across cut-points, while the coefficient of 'fish other reasons' is negative but gets larger across cut-points. This means that consumers' perception that the fish is created in controlled conditions ensuring excellent quality levels, make it more likely that the consumers will be in a higher category of WTP than the current

one (no WTP). On the other hand, other reasons for consumption of FWLAP (e.g. good taste, appealing external characteristics of the fish, good price/quality ratio, on selling at fresh fish markets, similar price to conventional fish) increase the likelihood of being in the current (medium WTP) or in a lower category (no WTP).

Finally, it is interesting to notice that the individual and household characteristics do not significantly explain the WTP for FWLAP. This result can be explained by the niche market that FWLAP will fulfill. It means that this new product should be targeted to consumers with specific needs and preferences, mostly related with free-allergic and/or unique-featured products. Hight quality, good taste and appealing external characteristics of the fish (color, smell, eyes, etc.) are the most important unique-features of fish that consumers with fish allergies or a WTP are seeking. Indeed, studies suggest that sociodemographic variables usually determine the consumers' WTP, but with different types of food products (Hjelmar, 2011, Khan et al., 2018, Quagrainie, 2006, Zander and Feucht, 2018). Nevertheless, to our knowledge, there are no studies regarding the determinants of WTP for food products with a low allergenic potential.

Table 3.5. Determinants of WTP (ordered logit estimation of the PPO model)

	No WTP		**Medium WTP**	
Variables	**Coefficient**	**SE**	**Coefficient**	**SE**
Fish-allergy problems	1.095***	0.389	1.095***	0.389
Health-allergy problems	0.019	0.190	0.019	0.190
Fish low allergenic	–0.012	0.175	–0.012	0.175
Fish species maintenance	–0.082	0.190	–0.082	0.190
Fish unique characteristics	0.470**	0.211	0.470**	0.211
Fish excellent quality	0.798***	0.190	0.262	0.259
Fish other reasons	–1.562	1.111	–12.374***	0.446
Female	0.128	0.183	–0.357	0.251
Age	–0.009	0.008	–0.009	0.008
Married	–0.345	0.241	–0.345	0.241
Divorced	–0.532	0.441	–0.532	0.441

Widowed	–0.280	0.463	0.550	0.543
Medium education	0.001	0.238	0.499	0.309
High education	0.091	0.269	0.091	0.269
Household size	–0.105	0.079	–0.105	0.079
Fish weekly consumption	0.036	0.052	0.036	0.052
Constant	0.316	0.514	–1.304**	0.533

Note: PPO model: partial proportional odds model, given high WTP. The standard errors (SE) of the coefficients are robust. Nagelkerke's $R^2 = 0.130$, MacFadden's $R^2 = 0.059$. Log-likelihood= -506.6. Wald test $\chi^2_{(21)} = 1{,}362.1$, $p < 0.001$. $AIC = 1{,}059.2$. *$p < 0.1$, **$p < 0.05$, ***$p < 0.01$.

3.4 Conclusion

This study gives some important insight into Portuguese consumers' consumption intentions for FWLAP and their WTP a premium price for that new fish. This study also examines the factors that explain the WTP for FWALP. The CVM was used to estimate the Portuguese consumer's WTP for FWLAP.

Results of the survey revealed that 36% declared intention to consume and 40% to recommend FWLAP. The results of the study also suggested that Portuguese consumers are generally split about their WTP for FWLAP, with 51% of respondents willing to pay a premium price for the fish product. The Portuguese consumers are willing to pay a premium price for FWLAP of approximately 5%, but 15% of the consumers have the WTP a price at least 11% higher for FWLAP than for a conventional fish. Thus, results clearly showed that there is a large consumer segment with "no WTP" and a small segment with a "high WTP" for FWLAP.

The WTP data that was pooled into categorical levels (segments) was then used to estimate an ordered logit model. The econometric results strongly indicated that the consumers' WTP for FWLAP is explained by the presence of fish-allergy problems in the household. This means that households with members self-reporting a fish allergy are seeking the benefits of the fish, despite their type of allergy. The results also revealed that other factors such as the fish unique characteristics and quality have effects on their WTP. This shows that consumers value fish of high quality, whether they belong to a household with members who are allergic or not. All these results lead us to conclude that the market segments with WTP associate the FWLAP with added health benefits, even though there may not be any for some consumers. Surprisingly, the sociodemographic characteristics of the

Portuguese consumers, such as gender, age, marital status, education and household size, were not gauged with any statistical significance.

According to these findings, marketing strategies for FWLAP in the Portuguese market should emphasize the quality of the new fish and the unique characteristics of the product associated with the support of a label of food with a low allergenic potential. Those findings also recommend that decisions on FWLAP pricing strategies should be carefully designed and might have to account for the sensitivity of consumers to the higher prices of the new food product compared with its conventional analogue. The distribution chains should be confined to the retailing spots most preferred by consumers: hypermarkets and municipal markets. Additionally, relevant information on the health benefits made by the food product (low allergenic potential) should be clearly communicated by setting up point-of-purchase displays and an informative website, and running digital marketing campaigns.

To the authors' knowledge, this study is the first attempt to estimate the consumers' WTP for FWLAP. The information generated in this study can play an important role in the decision-making process of investment in this new fish for human consumption but can also give useful insights on how to effectively design and implement successful marketing strategies for FWLAP.

As mentioned, our findings are based on a couple of assumptions. Some of the limitations regarding the methods, the data and analysis could be met in further research. First, this study assumes a hypothetical character of the CVM, since the new FWLAP is not yet commercialized in the market. Further research on WTP for FWLAP may include a choice experiment conducted in-store if the product is already being commercialized. This method allows consumers to reveal their preference for the product based on the product's attributes while the researcher has the ability through the design of the choices to contrast certain product attributes such as price. Second, this study did not include a question to measure the average expected price that consumers were willing to pay for conventional fish, although that reference price was referred in the question used to elicit the consumers' WTP for FWLAP. Future research should include that question, because we realized during this study that the questions about the minimum and maximum cut-off prices that consumers were willing to pay for FWLAP are not enough to contextualize their response to the CVM. Third, this study also suffers from the measurement error, since it is well-known that there is always a gap between stated and real WTP in this type of research. Finally, the results presented in

this article are based on a survey of Portuguese consumers. Future studies should include consumers from other countries.

3.5 Acknowledgments

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Chapter 4

Dietary supplementation for the production of a low allergenic farmed fish

Chapter 4.1

Dietary creatine supplementation in gilthead seabream (*Sparus aurata*): Comparative proteomics analysis on fish allergens, muscle quality and liver

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Dietary creatine supplementation in gilthead seabream (*Sparus aurata*): Comparative proteomics analysis on fish allergens, muscle quality and liver

Abstract

The quality of fish flesh depends on the skeletal muscle's energetic state and delaying energy depletion through diets supplementation could contribute to the preservation of muscle's quality traits and modulation of fish allergens. Food allergies represent a serious public health problem worldwide with fish being one of the top eight more allergenic foods. Parvalbumins, have been identified as the main fish allergen. In this study, we attempted to produce a low allergenic farmed fish with improved muscle quality in controlled artificial conditions by supplementing a commercial fish diet with different creatine percentages. The supplementation of fish diets with specific nutrients, aimed at reducing the expression of parvalbumin, can be considered of higher interest and beneficial in terms of food safety and human health. The effects of these supplemented diets on fish growth, physiological stress, fish muscle status, and parvalbumin modulation were investigated. Data from zootechnical parameters were used to evaluate fish growth, food conversion ratios and hepatosomatic index. Physiological stress responses were assessed by measuring cortisol releases and muscle quality analyzed by *rigor mortis* and pH. Parvalbumin, creatine and glycogen concentrations in muscle were also determined. Comparative proteomics was used to look into changes in muscle and liver tissues at protein level. Our results suggest that the supplementation of commercial fish diets with creatine does not affect farmed fish productivity parameters, or either muscle quality. Additionally, the effect of higher concentrations of creatine supplementation revealed a minor influence in fish physiological welfare. Differences at the proteome level were detected among fish fed with different diets. Differential muscle proteins expression was identified as tropomyosins, beta enolase, and creatine kinase among others, whether in liver several proteins involved in the immune system, cellular processes, stress and inflammation response were modulated. Regarding

parvalbumin modulation, the tested creatine percentages added to the commercial diet had also no effect in the expression of this protein. The use of proteomics tools showed to be sensitive to infer about changes of the underlying molecular mechanisms regarding fish responses to external stimulus, providing a holistic and unbiased view on fish allergens and muscle quality.

Keywords: Creatine, aquaculture, gilthead seabream, parvalbumin, proteomics, fish nutrition, muscle quality.

4.1.1 Introduction

Proteins from fish are becoming an important and healthier alternative to protein from other animals. Essential amino acids, polyunsaturated fatty acids, micronutrients and high quality proteins are present in fish in considerable amounts (Beveridge et al., 2013, Kuehn et al., 2014). Due to the high amount of consumption, fish supplies are needed to increase rapidly (Thurstan and Roberts, 2014) and subsequently aquaculture is a fast growing industry. Farmed fish are subjected to important standards such as food safety, fish welfare and muscle quality (Silva et al., 2012a) with the evaluation of farmed fish quality and safety being of great concern regarding human health and acceptance by consumers (with added value for the sustainability of aquaculture industry). There are many external and internal influencing factors that affect the freshness quality of fish but texture of muscle, *rigor mortis* and pH play a critical role in the evaluation of fish quality (Wang et al., 2015a). Nutrients or special ingredients in diets are known to influence fish quality, as well as pre-slaughter stress and post-mortem processes (Silva et al., 2012a, Wang et al., 2015a). Information regarding the use of dietary supplements as finishing strategies to modulate post-mortem degradation of overall flesh quality criteria in seabream muscle is extremely scarce. It is known that proper slaughter techniques, can spare the muscle's energy reserves and delay post-mortem degradation processes, with positive impact on flesh quality. Recently, cutting-edge technologies such as proteomics have been emerging as a valuable tool in both aquaculture products analysis and food allergens studies (Hoffmann-Sommergruber, 2016, Rodrigues et al., 2012, Silva et al., 2014a). Proteomics provides a deeper knowledge of an organism's physiological state by comparing changes in the proteome of a tissue, fluid or cell at a given moment (Piras et al., 2016, Rodrigues et al., 2012).

Fish allergies show a point prevalence of 0.6% for all aged population in Europe and a lifetime prevalence of 2.17% (Nwaru et al., 2014a), in case of children this can reach 8% worldwide (Sicherer and Sampson, 2014). In 95% of the cases, fish allergies are due to the parvalbumin protein, the major allergen in fish (Kuehn et al., 2010). Enolase, aldolase and gelatin were also identified as minor fish allergens (Kuehn et al., 2014). Parvalbumins are proteins with a low molecular weight (10-12 kDa), acidic (pH 3.5-4.5), very stable, calcium-binding (Ca^{2+}) and are present in higher amount in white muscle (Van Do et al., 2005) compared to dark one. Parvalbumins are divided into two lineages, α and β, but it has been shown that the majority of fish species parvalbumins belong to the β lineage (Lindstrom et al., 1996), being considered as the more allergenic one (Griesmeier et al., 2010). Various isoforms of parvalbumin have been identified depending on fish species (Beale et al., 2009) and developmental stage (Huriaux et al., 2002).

Parvalbumin is a protein present in fish muscle, where creatine is used as a molecule to enhance bioenergetics. In vertebrates, creatine takes part in the ATP (adenosine triphosphate)/PCr (phosphocreatine) phosphate energy system, being an important substrate to increase ATP by the breakdown of PCr (Kraemer et al., 2013). The endogenous synthesis of creatine is regulated by the AGAT (L-arginine:glycine amidinotransferase) enzyme, transferring the amidino group of arginine to glycine, producing L-ornithine and guanidinoacetic acid, which is methylated, resulting in the production of creatine (Kraemer et al., 2013). In fish, creatine has been found in white muscle both in its free and phosphorylated forms and is present in higher amounts than in mammals (Danulat and Hochachka, 1989, Hunter, 1929). It contributes to the relaxation and contraction of the muscle. Creatine use has not been studied extensively in fish nutrition, but phosphocreatine has been addressed in various experiments of fish exercise (McFarlane et al., 2001). In juvenile rainbow trout, creatine supplementation showed to contribute most to a higher endurance during a fixed velocity test without differences observed in total creatine content in muscle (McFarlane et al., 2001). Also, supplementation with creatine in zebrafish showed a significant difference in lean body mass compared to control (Domas et al., 2016). The muscle mechanism of relaxation and contraction might suffer changes if the expression of specific proteins involved in the kinetics of calcium, like parvalbumin, are modified (Gallo et al., 2008). These authors studied the effect of added creatine in the diets of rats showing a significant decrease in skeletal muscle parvalbumin content (Gallo et al., 2008). In that study the authors

hypothesized that elevating the capacity for high energy phosphate shuttling through creatine loading, alleviates the need for intracellular Ca^{2+} buffering by parvalbumin and increases the efficiency of Ca^{2+} uptake by Ca^{2+}-ATPases.

In this study, commercial fish diets for gilthead seabream (*Sparus aurata*) were supplemented with different percentages of creatine (2%, 5% and 8%) and its effects on muscle quality, proteome and parvalbumin modulation were analyzed. The liver proteome was also studied due to its central role in the majority of key metabolic processes.

4.1.2 Material and methods

4.1.2.1 Fish and rearing conditions

For this trial, 24 gilthead seabream per tank were reared in 500 L conical tanks, with natural flow-through seawater at the Ramalhete experimental station of the University of Algarve, Faro, Portugal (from July till September). Tank triplicates were used for each tested diet. Fish with an initial body weight of 170 ± 1.4 g, were fed twice a day by hand, *ad libitum* and kept with natural temperature (23.3 ± 0.93 °C, with minimum and maximum of 21.2 and 25.0 °C, respectively), artificial aeration (dissolved oxygen above 5 mg L^{-1}), and salinity (33.2 ± 2 ‰). Tanks were exposed to natural environmental and photoperiod conditions. The experiment was performed according with the fish welfare regulations established in the EU, Council Directive 2010/63/EU and Portuguese legislation for the use of laboratory animals, permit number 0420/00/000-n.9909/11/2009.

4.1.2.2 Experimental diets

Fish were fed a control diet (CTRL), similar to a commercial feed (Supplementary Material - Table S4.1.1), formulated based on estimated requirements of *Sparus aurata* (Sparos, Lda., Olhão, Portugal). Three different concentrations of creatine were added to the control diet in order to get experimental diets with 2, 5 and 8% of creatine (Sparos, Lda., Olhão, Portugal). The procedures for production follows the methods described in Schrama et al. (2017) with slight modifications. The creatine was mixed with fish oil fraction in concentrations (2, 5 and 8%) according to each target formulation and added under vacuum coating conditions in a Pegasus vacuum mixer (PG-10VCLAB, DINNISEN, The Netherlands).

4.1.2.3 Sampling

After 69 days of trial, all fish (72 per diet) were sampled. Twelve fish per tank were randomly picked and lethally anesthetized with MS-222 (Sigma Aldrich). Blood was immediately withdrawn from the caudal vein using heparinized syringes and liver collected and weighted for hepatosomatic index. Muscle samples were taken from the right dorsal. All samples were frozen in liquid nitrogen and stored at -80 ºC till further analysis. The remaining twelve fish of each tank were sampled in ice and water for fish quality measurements. Fillets of five fish were preserved on ice for instrumental texture analysis (within 24 hours after slaughtering). Four fish were maintained on ice for *rigor mortis* assessment and proximate composition determination. Three fish were used for muscle pH determinations. All fish were weight and measured.

4.1.2.4 Cortisol measurements

Blood samples were centrifuged at 3000 xg for 20 min and plasma collected and frozen in liquid nitrogen and stored at -80°C till further analysis. Plasma cortisol were then determined using a commercially available ELISA kit (RE52061, IBL International), previously validated for *Sparus aurata* (Lopez-Olmeda et al., 2009) with a sensitivity of 2.5 ng ml^{-1}, and intra and inter-assay coefficients of variation (CV) of 2.9 and 3.5 %, respectively.

4.1.2.5 Parvalbumin and creatine concentration in muscle

Parvalbumin and creatine concentration were both determined using commercially available kits following manufacturer's instructions (Fish-Check ELISA kit, Bio-Check, UK and Creatine assay kit, Sigma Aldrich, respectively).

4.1.2.6 Glycogen determination in muscle

Glycogen, a carbohydrate, is the most important energy source in post-mortem muscle and was determined only in control and creatine 8% dietary treatments using the method described by Viles and Silverman (1949). Results were expressed as µg per mg of muscle (dry weight).

4.1.2.7 Metabolic fingerprinting by solid phase transmissive Fourier Transform infrared (FT-IR) spectroscopy

Liver tissue is frequently used as an index of nutritional status in fish. Therefore, liver tissues of 5 fish per tank (i.e. 15 fish per dietary treatment) from control and creatine 8% (as stated before only the highest supplementation with creatine was analyzed) dietary treatments were lyophilized. Using an agate pestle and mortar as described by (Silva et al., 2014a), each liver sample was mixed with KBr (following a ratio of 500 mg KBr per 5 mg of sample) until homogenous. The main absorption bands were attributed to the corresponding biomolecules according to (Silva et al., 2014a).

4.1.2.8 Texture analysis

From each raw fillet (5 fish per tank) a muscle section (with skin) of approximately 3 x 2 x 1.2 (height) cm was taken for texture profile analysis (TPA) on a TA.XT*plus* analyzer (Stable Micro Systems, Surrey, UK) equipped with a load cell of 30kg. The muscle pieces were compressed twice with a 50 mm diameter cylindrical metal probe (P50) at a constant speed of 2 mm/s up to 40% of the fillet height. Measurements were done at room temperature (approximately 20°C). The primary characteristics hardness, springiness, adhesiveness and cohesiveness were determined. Chewiness (secondary characteristic) was calculated as the product of hardness, cohesiveness and springiness (Hyldig and Nielsen, 2001, Careche and Barroso, 2009).

4.1.2.9 Rigor mortis and pH

Rigor mortis is one of the indexes of fish quality and it was determined using both sides of the fish at 0, 1, 2, 4, 6, 8, 24, 48 and 72h after slaughter, as described by Matos et al. (2010). Fish were handled carefully in order to prevent secondary effects on the development of the *rigor* state. Determination of the pH values of the fish muscle was done at 0, 1, 2, 4, 6, 8, 24 and 48h after slaughter, using a pH meter (Eutech waterproof pH spear). At each time a new incision in the flesh of the same fish was made.

4.1.2.10 Protein extraction and CyDye labeling

For a total protein extraction, muscle samples were individually homogenized with an Ultra-Turrax IKA T8 (IKA-WERG) in a DIGE buffer (7M urea, 2M thiourea, 4% CHAPS, 30 mM Tris, pH 8.5) containing 1 mM EDTA and 1% (v/v) protease inhibitor. Homogenates were centrifuged at 13000 x g for 10 min at 4ºC to pellet insoluble material. The resulting supernatants were quantified using Quick Start™ Bradford Protein Assay with bovine albumin as standard (Bio-Rad).

In order to simplify the protein mixture and the analysis, a fractionation approach of the muscle tissue was performed. Fractionation allows to increase the number of visualized proteins and to raise the concentration of low-abundance proteins by depletion of the highly abundant myofibrillar proteins (Silva et al., 2010, Silva et al., 2012a). Proteins from the sarcoplasmic fraction of the muscle were extracted using a lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10 mM DTT) containing 0.5% (v/v) protease inhibitor. An Ultra-turrax IKA T8 (IKA-WERG) was used to homogenize the samples and after a 30 min settle on ice centrifugation occurred for 20 min at 11200 x g at 4°C. The resulting supernatants were then depleted of non-protein contaminants using a ReadyPrep™ 2D Clean-up kit (Bio-Rad) and resuspended in DIGE buffer. Proteins were quantified as described before.

For DIGE minimal labeling, after pH adjustment of protein extracts to pH 8.5 by addition of 0.3 M NaOH, 50 µg of proteins were labeled with 400 pmol of fluorescent amine reactive cyanine dyes freshly dissolved in anhydrous dimethyl formamide following manufacturer's instructions (5 nmol minimal labelling kit, GE Healthcare). Three samples per dietary treatment were labelled with Cy3 and three with Cy5 to prevent confounding of an eventual "dye effect" with the biological effect we want to measure. An internal control consisting of equal quantities of protein from all samples was labelled with Cy2.

Liver samples were individually homogenized using DIGE buffer as described above for total protein extraction. The resulting supernatants were then depleted of non-protein contaminants using a ReadyPrep™ 2-D Cleanup kit (Bio-Rad) and proteins were quantified and labeled as previously described.

4.1.2.11 Two-dimension gel electrophoresis

Labeled proteins were first separated according to their isoelectric point on 24 cm Immobiline™ Drystrip with a pH 3-7 linear gradient (GE healthcare) as parvalbumin – the target protein – has an known acidic PH of ≈ 4 (Van Do et al., 2005). In case of liver samples a pH of 4-7 linear gradient has been chosen following the findings of Richard et al. (2016) in which this pH ensure the best compromise between high coverage and good protein separation. For each strip, 50 µg of protein of one sample from each dietary treatment plus 50 µg of internal standard, diluted in rehydration buffer (ReadyPrep 2-D starter kit, Bio-Rad) to a final volume of 450 µl, were loaded overnight in an IPG box (GE Healthcare) by passive rehydration. Isoelectric focusing was performed using an Ettan™ IPGphor™ 3 isoelectric focusing unit (GE Healthcare), at 20°C.

In muscle samples voltage gradually raised from 0 V to 500 V over the course of 1 h, kept constant at 500 V for 1 h, then gradually raised to 1,000 V over the course of 1 hour and finally gradually raised to 8,000 V over the course of 3 hours, finishing with a step of 5 h 40 min at a constant voltage for a total of 60,000 V.h, with a maximum current of 75 µA per strip. In liver samples voltage gradually raised from 0 V to 250 V over the course of 4 h, then gradually raised to 1000 V over the course of 6 h and finally gradually raised to 8,000 V over the course of 3 h 40 min, finishing with a step of 3 h 20 min at a constant voltage of 8,000 V for a total of 48,000 V.h, with a maximum current of 75 µA per strip.

After separation of proteins in the first dimension, focused proteins were reduced for 15 min in 6 ml of equilibration buffer (6 M urea, 50 mM Tris-HCl, pH 8.8, 2% (w/v) SDS, 30% (v/v) glycerol) with 2% (w/v) DTT, and then alkylated for 15 min in 6 ml of equilibration buffer with 2.5% (w/v) iodoacetamide. The equilibrated strips were then placed onto 12.5% acrylamide gel cast between low fluorescence glass cassettes (EttanDALT six gel caster system, GE Healthcare), and sealed with 0.5% (w/v) agarose in 1x electrophoresis buffer (25 mM Tris, 192 mM Glycine, 0.1% (w/v) SDS) and a trace of bromophenol blue. Proteins were separated according to their molecular weight in a second dimension by SDS-PAGE, using an EttanDALT system under constant amperage of 10 mA per gel for 1 h followed by constant amperage of 40 mA per gel until the bromophenol blue dye front reached the bottom of the gels. The electrophoresis buffer was used at 1x concentration in the lower chamber and 2x concentration in the upper chamber.

4.1.2.12 Gel image acquisition and analysis

Obtained gels were scanned with a Typhoon Trio™ Variable Mode Imager (GE Healthcare) using three laser emission filters (520BP40 for Cy2, 580BP30 for Cy3, 670BP30 for Cy5) at a resolution of 100 µm. Image analysis was performed using the SameSpots™ Software (TotalLab). A filter of average normalized volume <=100,000 and a spot area <=500 was used to eliminate small impurities before analysis. Preliminary assessment of data quality was performed using Principal Component Analysis with autoscaling. 2DE gel analysis was performed following guidelines of Silva et al. (2014b).

4.1.2.13 Protein identification by mass spectrometry

Spots with a significant difference by one-way variance of significance (ANOVA (p<0.05)) were manually excised from the preparative gels. Also a false discovery rate (FDR) of q<0.05 was applied to minimize the number of false positives. Protein spots from the muscle samples were identified at the GIGA proteomics facility (Liège University, Liège, Belgium) while protein spots from liver samples were sent and analyzed at the Center of Biosustainability (Technical University of Denmark, Hørsholm, Denmark). At the GIGA proteomics facility trypsin in gel digestion was performed in 96 well plate format on the working station Janus (Perkin Elmer). Spots were washed twice for 5 min in 50 µl of 50 mM ammonium bicarbonate on a shaker. Wash was discarded and 50 µl of 50% acetonitrile/50 mM ammonium bicarbonate was added for 5 min on a shaker, this step was repeated once. Fifty microliter of 10 mM DTT was added per well and left on a shaker for 45 min at 56°C. The DTT was removed and 40 µl of 55mM iodoacetamide was added and mixed for 1 min. The plate was incubated at 20°C for 1 hour. Liquid was discarded and 50 µl of 50% acetonitrile/50 mM ammonium bicarbonate was added for 5 min on a shaker (twice). Wash was discarded and 50 µl of 50% acetonitrile/50 mM ammonium bicarbonate was added for 5 min on a shaker (twice). Sixty microliters of 100% acetonitrile were added for 5 min on a shaker. Liquid was discarded and the step repeated. Spots were dried for 1 hour at 40°C followed by 1 hour at 20°C. Trypsin was prepared in 25 mM ammonium bicarbonate (10 ng/µl) and 3 µl was added, mixed for 1 min and incubated for 1 hour at 4ºC followed by 4 hours at 37ºC. After digestion, 18 µl of 1% formic acid was added to the gel pieces and incubated for 30 min at 40°C on a shaker. PMF and MSMS analysis was performed on a MALDI-TOF-TOF-MS UltrafleXtreme (Bruker). Automatic spectra acquisition was piloted with the software Flex control™ vs 3.4

and real time analysis by Flex analysis™ vs 3.4 (Bruker). Search on databases were managed in real time with BioTools™ vs 3.2 (Bruker) on the Mascot server vs2.2.06. Search was performed on SwissProt database restricted to Actinopterygii taxonomies with 100 ppm of mass error tolerance in MS and MSMS precursor and 0.3 Da tolerance on MSMS fragments. A second search was made with the same parameters on NCBI database restricted to Actinopterygii taxonomies.

At the Center of Biosustainability protein spots were identified by LC-MS/MS after proteins were cleaved by trypsinization. The procedures for the identification of proteins followed the protocol as described in Moreira et al. (2017). Raw data files Protein identification was obtained using the Protein Lynx Global Server (PLGS) software v2.5.3 (Water corporation) using the in-build MS^E search function against the databases generated from UniProt from the taxonomy Actinopterygii. In the database a list of known contaminants was added. The search parameters were trypsin as enzyme, carboxamidomethyl on cysteine as fixed modification and oxidation of methionine as partial modification while allowing one missed cleavage.

4.1.2.14 Statistical analysis

Statistical significance was assessed using a one-way analysis of variance (ANOVA (p<0.05)) followed by a post-Hoc Tukey (p<0.05). In case of glycogen determination and FTIR analysis a Student's T-test was performed (p<0.05). Cortisol and r*igor mortis* data were previously transformed by log and arcsine square root, respectively. Normality and homoscedasticity assumptions were previously checked using Shapiro-Wilk and Levene's tests, respectively (p<0.05). In case of cortisol analysis, a Grubbs' test was performed prior to statistical significance analysis. Results are presented in mean ± standard error of the mean (S.E.M.). All statistical analyses were performed using the R project for statistical computing (version 3.5.0) and GraphPad® v6.0 for windows was used for chart building and figures layout.

4.1.3 Results and discussion

4.1.3.1 Zootechnical analysis

In this study, at a performance level, fish fed with the different tested diets, show similar growth after 69 days of trial. The box plots show the initial distribution of the fish, with a mean body weight of 170 ± 1.4g and a final body weight of 281 ± 4.15g (Figure 4.1.1). In table 4.1.1, zootechnical results show the initial and final body weight (IBW and FBW, respectively), weight gain per day, specific growth rate (SGR) per day, thermal growth coefficient (TGC), feed conversion rates (FCR) and feed efficiency (FE) for all treatments without any significant differences among treatments and no mortalities were registered.

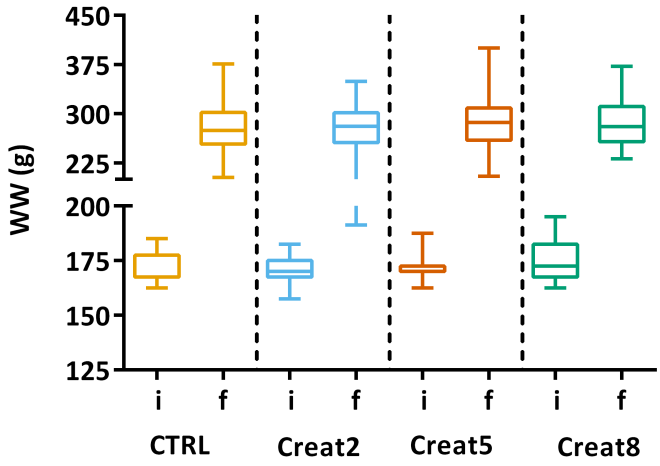

Fig. 4.1.1. Box plots of the fish weight distributions. Plot showing the distributions of wet weight (WW) of fish for each treatment (n=72) at the start of the trial (i-initial) and after 69 days (f-final). Results are shown by quartiles and the horizontal line in each box shows the median. No significant differences were observed among the treatments (one-way ANOVA, p>0.05).

Moreover, hepatosomatic index (HSI) results (Figure 4.1.2) show no significant differences among treatments suggesting that supplementation with creatine have not altered the energy reserves in these fish.

Creatine supplementation does not seem to affect fish growth and the efficiency of feed is very similar between the different concentrations tested. These results are contradictory to those reported in a previous study performed in Red drum (*Sciaenops ocellatus*) for 7 weeks, where this species has benefit from dietary creatine supplementation in practical diets with improvements observed in weight gain and feed efficiency.

Nevertheless, this trial was conducted under stressful conditions of low salinity which might have contributed to the differences to our outputs. Also, this fish species is produced in higher temperatures, which are related with higher intrinsic energy demands. The regulation and usage of creatine is known to be dependent on the body temperature, which in fish is dependent on the surrounding temperature (Burns and Gatlin, 2016). In rainbow trout, for instance, Borchel et al. (2014) showed differences in their gene-expression regarding acclimation temperatures lined up with creatine expression, supporting the previous statement. Regarding our study, this raises the question if creatine supplementation, has led to a higher muscle power output (to some extent) as seen in mice (Gallo et al., 2008) rather than to an effective effect on growth parameters.

Table 4.1.1: Fish performance parameters. Table with initial body weight (IBW), final body weight (FBW), weight gain per day, specific growth rate (SGR) per day, thermal growth coefficient (TGC), feed conversion rate (FCR) and feed efficiency (FE) calculated per treatment (n=72) at the end of the trial (69 days). Values are expressed as mean ± standard deviation. Statistics by ANOVA show no significant differences (p>0.05).

Diet	IBW (g fish^{-1})	FBW (g fish^{-1})	%IBW/ day Weight Gain[a]	%/day SGR[b]	TGC[c] (10^{-3} $g^{1/3}$ ºC^{-1} day^{-1})	FCR[d]	FE[e]
Ctrl	172 ± 3	278 ± 3	0.89 ± 0.01	0.69 ± 0.01	0.20 ± 0.001	1.66 ± 0.03	0.60 ± 0.01
Creatine2	171 ± 2	278 ± 4	0.91 ± 0.05	0.71 ± 0.03	0.20 ± 0.009	1.63 ± 0.10	0.61 ± 0.04
Creatine5	172 ± 2	285 ± 10	0.95 ± 0.06	0.73 ± 0.04	0.21 ± 0.012	1.58 ± 0.07	0.64 ± 0.03
Creatine8	175 ± 1	286 ± 12	0.91 ± 0.09	0.71 ± 0.06	0.21 ± 0.018	1.66 ± 0.08	0.60 ± 0.03

[a] Weight gain per day, calculated as $((BM_f - BM_i) * 100)/(BM_i * t_f)$, where BM_f and BM_i are the final and initial biomass, respectively and t_f are the days of the trial.

[b] Specific growth rate, calculated as SGR (% per day) = 100 * ((Ln (FBW) – Ln (IBW)) / t_f), where FBW and IBW are the final and initial fish body weight, respectively and t_f are the days of the trial.

[c] Thermal growth coefficient, calculated as TGC (10^{-3} $g^{1/3}$ ºC^{-1} day^{-1}) = [($\sqrt[3]{FBW}$-$\sqrt[3]{IBW}$)/(T x t)] x 1000, where FBW and IBW are the final and initial fish body weight, respectively, T is the mean temperature and t are total days of the trial

[d] Feed conversion ratio, calculated as FCR = FC / (BM_f – BM_i), where FC is the feed consumption and BM_f and BM_i are the final and initial biomass, respectively.

[e] Feed efficiency, calculated as FE = (BM_f – BM_i) / FC, where BM_f and BM_i are the final and initial biomass and FC is the feed consumption

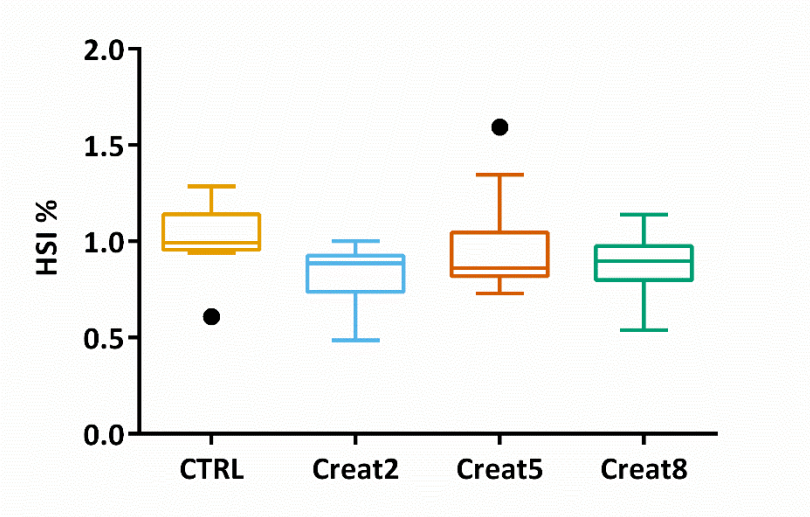

Fig.4.1.2. Box plot of the fish hepatosomatic index distribution. Plot showing the distributions of fish hepatosomatic index estimated from individual measurements (n=15 per treatment) at the end of the trial. Results are shown by quartiles and the horizontal line in each box shows the median. No significant differences were observed among treatments (one-way ANOVA $p>0.05$).

4.1.3.2 Metabolites

To analyze the first stress barrier in fish, cortisol levels were determined using an ELISA test. Our findings show that fish fed with 8% supplementation creatine diets show significantly lower values of cortisol than fish fed with control diets and fish fed with 5% supplementation of creatine (p=0.006 and p=0.014, respectively; one-way ANOVA followed by post-Hoc Tukey) (Figure 4.1.3). As mentioned above, if higher levels of creatine in the diet are related to a higher endurance capacity, as shown by McFarlane et al. (2001) with rainbow trout, one would expect fish fed creatine supplemented diets to show lower increases in plasma cortisol concentrations than fish fed a non-supplemented diet. As so, feeding periods, normally related with increases of cortisol levels, would originate less individual arousal and more ability to adjust their performance and behavior. Hence, lower cortisol levels would be detected with loading creatine diets. Lower arousal lined with reduced cortisol levels were seen by Sanchez et al. (2009) in seabream, when subjected to a random vs schedule feeding times.

Nevertheless, there are various factors that alter cortisol levels in addition to stress which has been often reviewed [several factors have been referred, that might influence the divergent concentrations reported over similar conditions, within and between fish species (see review from Ellis et al. (2012)]. Excess cortisol levels have been associated with poor

growth in goldfish, despite normal food intake (Bernier et al., 2004). In our trial, although fish show high values of cortisol, physiological stress does not seem to be affected. In fact, increasing creatine concentration, seems to decrease fish stress levels. Supporting such statement, in our study, fish almost doubled body weight during the trial with an expectable FCR (Santos et al., 2010).

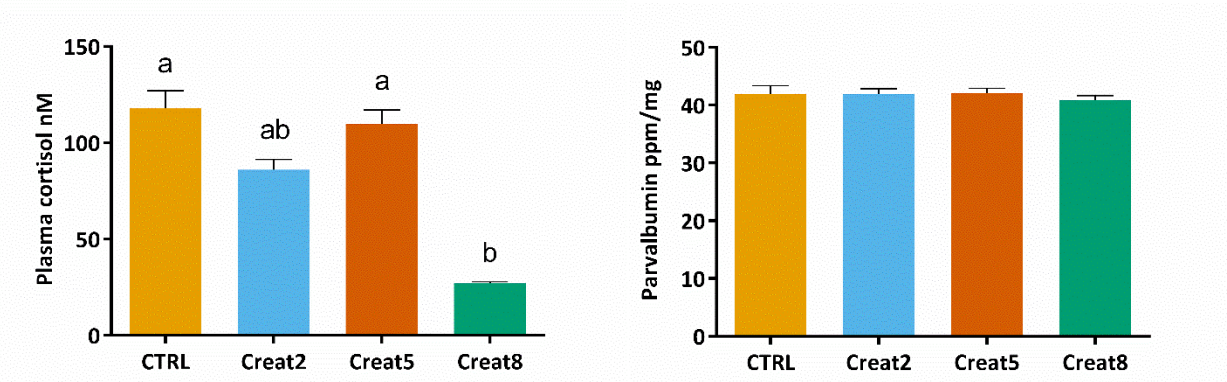

Fig. 4.1.3. Cortisol (nM) concentration in plasma and Parvalbumin (ppm/ng) concentration in muscle of gilthead seabream after 69 days of trial. Values are means (n=15) and error bars represent standard deviation. Different letters represent significant differences (one-way ANOVA followed by post-Hoc Tukey, $p<0.05$).

Parvalbumin concentrations in fish muscle were determined using a commercially available ELISA kit which is designed for cod fish. After a blast search (blast.ncbi.nlm.nih.gov), 71% of identity with *Sparus aurata* was obtained for β-parvalbumin. Although we cannot conclude this assay to be quantitative for this species, our results show no significant differences in the parvalbumin concentration in muscle of fish fed with the different tested diets (Figure 4.1.3). We hypothesize that creatine loading would decrease the expression of parvalbumin, therefore decreasing the allergenic status of fish. According to our research outputs, creatine supplementation seems not to modulate parvalbumin expression in the muscle of gilthead seabream. These results were further validated by our comparative proteomics results and explained further down. Parvalbumin is involved in the muscular system of contraction / relaxation and plays a role in the whole fish performance. It was found that fastest sprinters release energy faster because of the release of ATP-enzyme creatine kinase (Knight, 2012). Our dissonant findings may be explained by the lack of an environmental or artificial challenge (e.g. nutritional or a stressful condition) that could increase fish activity and energy release, hence the parvalbumin concentration. Previous research regarding the expression of parvalbumin in fish has been driven mainly to determine

the allergenic cross-reactivity between fish species (Van Do et al., 2005, Kuehn et al., 2010) or linked to a performance test after a stressor situation (Knight, 2012, Seebacher and Walter, 2012) which can sustain the previous statement. Nevertheless, we show the first evidences about the effect of creatine supplementation on parvalbumin modulation in non-stressed fish.

Creatine is naturally present in fish muscle (200-700 mg/100 g (Oehlenschläger, 2014), nevertheless it is important to address whether fish diet supplementation with creatine would lead to accumulation of this compound in the muscle. Using a commercially available colorimetric assay, we did not obtain significant differences between conditions (Figure 4.1.4). With these results we show that diet creatine supplementation in fish, up to 8%, does not result in an accumulation of this supplement in the muscle of these vertebrates, after 69 days of feeding. Not having similar research regarding this issue in fish, we can only speculate that creatine is entirely processed on daily basis fish activity. It should be noticed that the muscle of fish fed the control diet showed insignificant higher expression of creatine when compared to supplemented diets. Such fact suggests that a longer trial would accentuate the differences reflecting the effect of supplementation, nevertheless this implicates more driven research to confirm such indication.

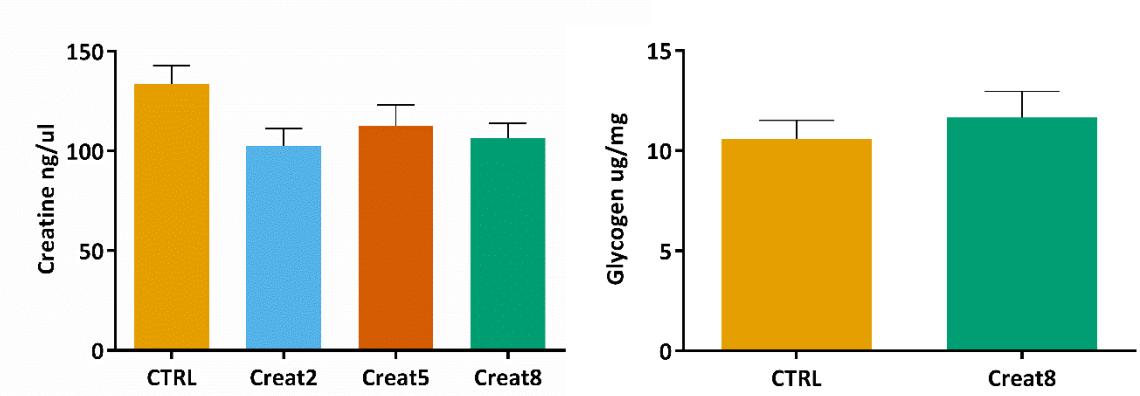

Fig. 4.1.4. Creatine (ng/µl) concentration in muscle and glycogen (µg/mg) concentration of lyophilized muscle of gilthead seabream after 69 days of trial. Values are means (n=9) and error bars represent standard error of the mean (SEM). No significant differences were detected (one-way ANOVA, p>0.05 and Student's T-test, p>0.05).

The energy reserves in fish muscle were analyzed by determining the glycogen content (Figure 4.1.4). This assay was only performed using the muscle from fish fed with 0% (control) and 8% supplemented creatine diets. No significant differences were obtained between these two conditions (Student's T-test, p>0.05), showing that creatine supplementation in the diet

does not affect the energy state of the fish muscle. In case differences between the highest concentration and control samples were observed, analysis would be performed on the other supplementation percentages. To the best of our knowledge, the effect of creatine on glycogen content was correlated for the first time in fish but in humans, was shown that creatine increases glycogen storage in muscle (van Loon et al., 2004). In fish, the expression of creatine was reported to be positively correlated with energy demand (Borchel et al., 2014) which is known to be associated with higher glycogen levels (Silva et al., 2012b). The level of locomotory activity in fish is recognized to be higher than in other vertebrates. Accordingly, several research, mainly in humans, have reported that the supplementation by creatine per se is not sufficient to alter muscle glycogen content after intense exercise. The same research suggests that only a supplementation with creatine plus a carbohydrate is capable of sparing glycogen by decreasing the reliance on glycolysis (Robinson et al., 1999, Roschel et al., 2010). Nevertheless, in fish this compounds mix have not been tested yet and research should be conducted to draw any firm conclusions.

4.1.3.3 Metabolic fingerprinting by FT-IR spectroscopy

Analysis of the FT-IR (Fourier-transform infrared spectroscopy) dataset shows that the biggest differences are found at 1000 cm^{-1} and 1450 cm^{-1}, which correspond to IR absorptions attributable to carbohydrates and lipids, respectively (Figure 4.1.5). In the case of carbohydrates (*e.g*. glycogen), the feed with 8% supplementation of creatine does not alter the hepatic glycogen reserves, since fish fed with supplemented diet showed higher carbohydrate stores than the control ones. Between 1700 and 1800 cm^{-1} (peaks associated to lipids (triglycerides, cholesterol esters and fatty acids)) it seems like that control fish presents higher lipid reserve than fish supplemented with creatine. Several researches have been conducted in mammals showing that creatine supplementation does improve glucose tolerance and glycogen content, but it seems not enough to enhance the lipid profile in healthy individuals (van Loon et al., 2004, Op't Eijnde et al., 2006, Gualano et al., 2008). Also, in birds, creatine supplementation showed to be more beneficial in the energy metabolism by reducing the muscle glycolysis, rather than on its antioxidant activity (Wang et al., 2015b). The principal component analysis (PCA) of all spectra shows the biological variability, with some control fish representing a comparable statistical profile to 8% creatine supplemented fish. This spatial representation reveals how misunderstanding are the exact mechanisms

underlying creatine-dependent carbohydrates and lipids profiles. These synergic adaptations have yet to be clarified, however our results point out in the same direction as the prementioned studies. Notwithstanding the lack of evidences regarding the metabolic fingerprinting we would suggest further studies to investigate any indirect effect of creatine supplementation on the lipid and carbohydrate profile as a result of individuals being able to catabolize better the food nutrients. Such research could have important repercussions for the augment of farmed fish performance and aquaculture productivity.

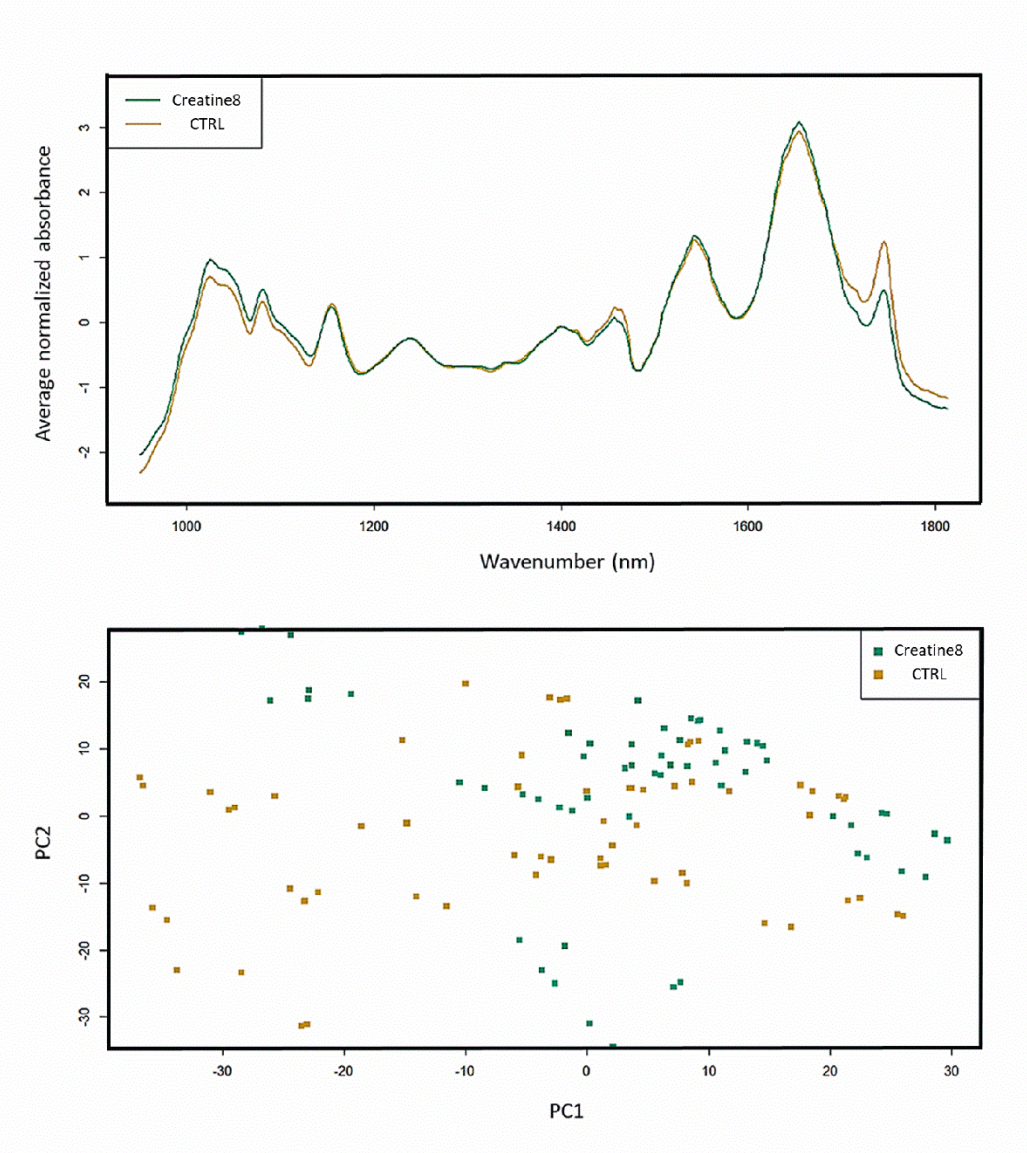

Fig. 4.1.5. FT-IR spectra of liver of gilthead seabream. Data represents the mean (n=15) for the absorption curves in the 900-1800 cm^{-1} range. Spectra and respective PCA are shown, where control is yellow and creatine 8% is green. Differences (although not significant) are observed in the carbohydrate (1000 cm^{-1}) and lipid zone (1450 cm^{-1} and 1700 to 1800 cm^{-1}).

4.1.3.4 Muscle quality analysis

The quality of fish flesh is often seen by the structure and muscle quality parameters, among others like properties of skin, eyes, gills and mucus. Textural analysis, provide important parameters of muscle quality, which are indirectly correlated with pH and *rigor mortis* (downstream indicators of the energetic status of muscle) (Silva et al., 2012b). More, both *rigor mortis* and muscle pH have been used as stress indicators in several fish species (Ribas et al., 2007, Lefèvre et al., 2008, Acerete et al., 2009). pH is known to have a great effect on conformation, thermal denaturation, and rheological properties of fish muscle proteins, particularly myosin (Tadpitchayangkoon et al., 2010). Overall, the present study seems to indicate that creatine supplementation, maintains the energy reserves of the muscle with the enhancement of the creatine levels, with potential improvement of the flesh quality. The pH measured varied between 5.0 and 6.6 over a time course of 72 hours as shown in Figure 4.1.6. For all the time points measured, except after 48 hours, distinct pH between experimental diets were found. Control diet revealed a basal pH of 6.2 (t=0) which was significantly different from the other diets. Then, immediately after slaughtering, supplemented diets seemed to improve energy reserves (even though the lack of differences between glycogen levels as pre-explained). In humans, creatine ingestion contributed for a higher rate of phosphocreatine re-synthesis and higher pH 30 sec after an intensive exercise (Yquel et al., 2002) and in rats, after an intermittent exercise, creatine loading seems to spares glycogen content (Roschel et al., 2010). Throughout storage time, pH values should decrease but our data show a constant variation of pH which is not reported in other studies (Silva et al., 2012b, Matos et al., 2013). However, the authors state that despite this variability, fish are less stressed - indicated by the lower cortisol levels and expressed higher pH after 8h of slaughtering. Also, it seems that higher the supplementation, higher the muscle energy reserves, higher the pH and faster the rigor mortis (at least to some extent). Such condition is known, as pre-mentioned, linked with better texture and fish muscle quality. In accordance, a previous study, reported a correlation between higher energy reserves in muscle with a higher pH at time of death (Silva et al., 2012b).

Regarding *rigor mortis* outputs, after 6 hours, the majority of the fish showed such condition value higher than 80% (Figure 4.1.7). Gilthead seabream with creatine supplementation shows a typical evolution of *rigor mortis*, although some slight differences can be observed after 4, 8 and 24 hours for the 5% supplementation showing less *rigor*

compared to the other creatine supplementation concentrations (one-way ANOVA followed by post-Hoc Tukey, p<0.05). The reasons for such high "resistance" to *rigor* of gilthead seabream, are unknown by the authors and need to be further investigated. After 8 hours, a significant difference was observed for the 8% supplementation showing more *rigor* than the control diet (one-way ANOVA followed by post-Hoc Tukey, *p*<0.05). Similar studies with higher energy reserves in muscle due to glycogen supplementation showed that the *rigor* status of fish have a similar pattern regarding the *rigor* continuum with our trial (Silva et al., 2012b).

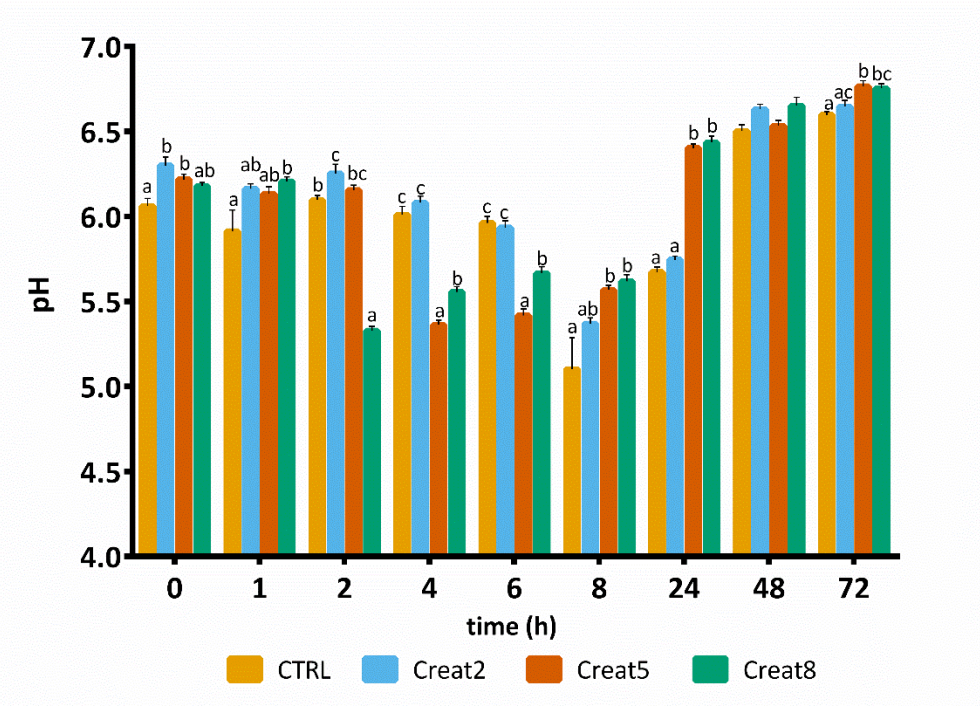

Fig. 4.1.6. – pH value of muscle of gilthead seabream fed supplemented diets with creatine. Values are means (n=9) and error bars represent standard error of the mean (SEM). Different letters represent significant differences (one-way ANOVA followed by post-Hoc Tukey, *p*<0.05). Time is set in hours.

Additionally, a Texture Profile Analysis (TPA) using several mechanical parameters to assess the quality of the fish flesh (see review from (Cheng et al., 2014)) was performed (Table 4.1.2). Briefly, hardness can be defined as the strength needed to compress the muscle between molars, adhesiveness is known as the stickiness of the muscle to a surface (palate and/or teeth), springiness can be defined as the ability of the muscle to return to the original shape, cohesiveness is known as the force needed to rupture the muscle filaments and chewiness defined as the energy needed to chew the muscle for swallowing (Hyldig and Nielsen, 2001, Careche and Barroso, 2009). In this study an effect of creatine supplementation

on these parameters was not observed. No specific literature regarding texture and fish fed supplemented diets with creatine was found. Nevertheless, the available literature, mostly regarding postmortem storage and pre-slaughter stress (Suárez et al., 2005, Álvarez et al., 2008, Ayala et al., 2010), about flesh quality are *per se* contradictory, since sample preparation and sample size can easily have an effect on the repeatability, reliability and accuracy of texture parameters. Despite the care with sample preparation, the perception and measures of the texture are known to be differently affected by chemical constituents and nonhomogeneous distribution of fat, moisture and collagen of fish (Cheng et al., 2014). Overall, our results suggest that a higher sample size or possibly a longer sampling period would possibly accentuate texture differences. Higher supplementation reveals a tendency to better textural properties, demonstrated by higher values of the hardness, springiness, cohesiveness and chewiness, then suggesting evidences of enhanced muscle uptake of creatine. However, more research has to be done in understanding the role of texture and structure on fish flesh quality.

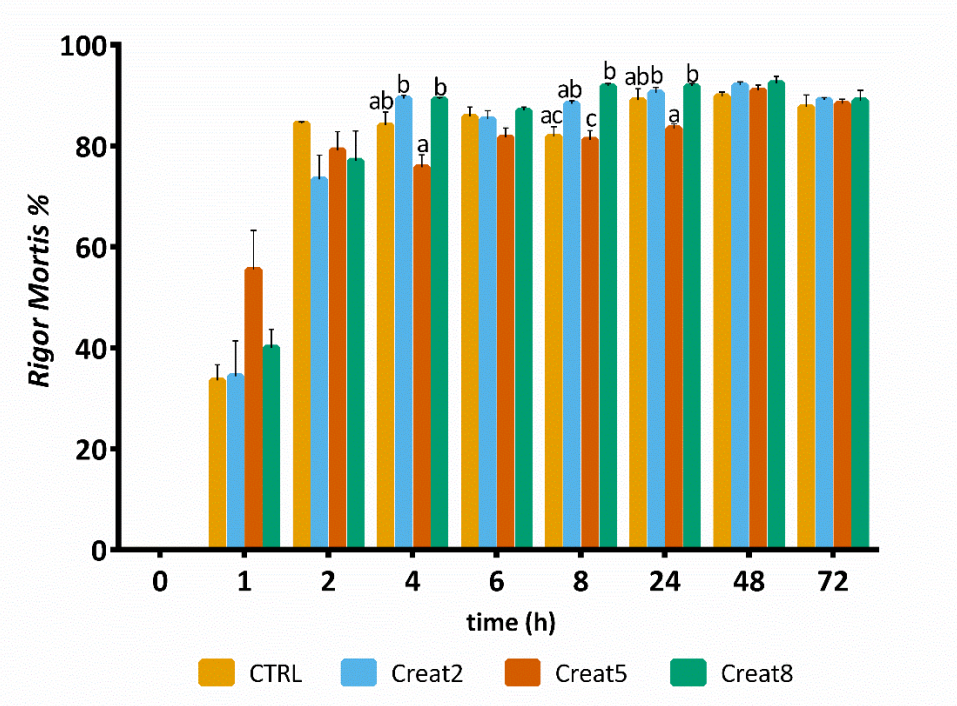

Fig. 4.1.7. *Rigor mortis* of gilthead seabream fed supplemented diets with creatine. Values are means (n=12) and error bars represent standard error of the mean (SEM). Different letters represent significant differences (one-way ANOVA followed by post-Hoc Tukey, $p<0.05$). Time is set in hours.

Table 4.1.2. Texture analysis of gilthead seabream muscle (flesh) after 69 days of trial. Values are means (n=15) ± standard deviation. No significant differences were found between the treatments for each parameter (ANOVA p>0.05).

Diet	Hardness (N)	Adhesiveness (g.sec)	Springiness	Cohesiveness	Chewiness
Ctrl	25.65 ± 4.60	-0.15 ± 0.06	0.63 ± 0.07	0.43 ± 0.04	6.84 ± 1.41
Creatine2	27.55 ± 5.14	-0.15 ± 0.06	0.63 ± 0.05	0.44 ± 0.04	7.59 ± 1.31
Creatine5	26.84 ± 7.25	-0.21 ± 0.15	0.62 ± 0.04	0.43 ± 0.05	7.04 ± 1.40
Creatine8	27.42 ± 4.33	-0.18 ± 0.10	0.65 ± 0.06	0.45 ± 0.03	7.94 ± 1.26

4.1.3.5 Proteomics

The gel images of both gilthead seabream muscle proteomes of total protein extraction and sarcoplasmic fraction are presented in Figure 4.1.8. Within the range of pH 3-7 we were able to successfully identify 127 protein spots (Supplementary material table S4.1.2). Despite the wide distribution of muscle proteins on pH above 7 recognized for seabream (Martin-Perez et al., 2012), the region of interest for the majority of allergenic proteins is acid supporting the pH range selected in adequacy to the objective of this study. As so, this narrow pH was chosen to be able to get a good protein separation in the parvalbumin region, minimizing the number of overlapping spots and eliminating the usual vertical streaking of the alkaline zone. Proteins with significant differences in expression level between tested diets are shown in table 4.1.3 with information on the protein names, accession number, molecular weight (Mw), isoelectric point (pI), score, number of peptides, coverage, fold change, false discovery rate (*q* value) and expression for each of the identified protein spots. Regarding β-parvalbumin modulation, no effect of creatine supplementation was observed in the expression of this protein, confirming the ELISA assay results. Parvalbumins, represent the major allergens for 95% of fish-allergic patients suffering hypersensitivity to fish. Nevertheless, the cross-reactivity of parvalbumin allergenicity was shown to vary between fish species (Van Do et al., 2005, Kobayashi et al., 2016c) and that allergenicity increases with parvalbumin content. The modulation of parvalbumin or its isoforms by augment of creatine concentration by oral supplementation was reported in mammals (Gallo et al., 2008, Bonilla and Moreno, 2015). The principle beyond such research intended to demonstrate that elevating the capacity for high-energy phosphate shuttling,

through creatine supplementation, buffers parvalbumin expression, decreasing the allergenicity of fish. Our findings do not support such research since 1) there was no difference in creatine muscle accumulation among diets, suggesting that supplemented creatine is used on daily basis on fish activities; 2) there was no increment of energy reserves expressed by glycogen content; 3) there was no differences on parvalbumin concentration measured by ELISA. As mentioned, increasing the trial duration could refine the specific effect of creatine supplementation on fish energy metabolism. Fish could also have adapted to the daily amount of creatine given in the diet, causing protein conformational changes masked by the increase of pH as result of supplement concentration (Tadpitchayangkoon et al., 2010).

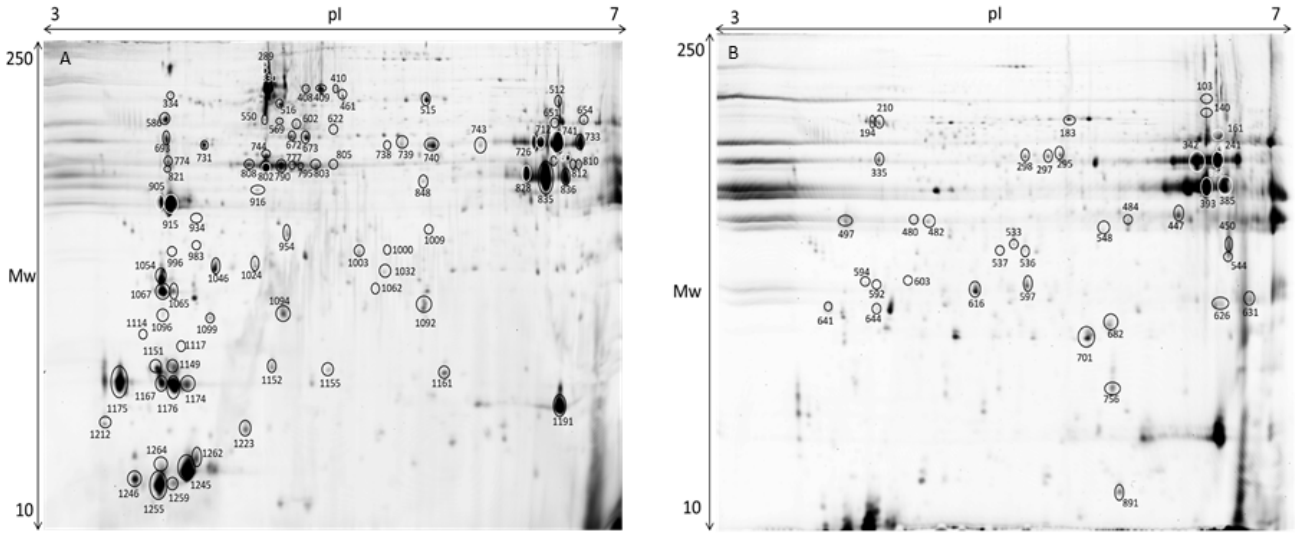

Fig. 4.1.8. Representative 2D-DIGE gel of protein extraction of muscle of gilthead seabream in a pH range of 3-7. A – Total protein extraction, B – Sarcoplasmic fraction gel. Protein identifications of significantly different spots (one-way ANOVA and post-Hoc Tukey $p<0.05$) are shown in table 4.1.3.

Although we did not observe any significant differences in the previous discussed analysis, we found some minor effects of creatine supplementation in muscle proteome. As expected in muscle tissue proteome characterization, the majority of the identified spots are myosin, actin and tropomyosin. In case of spot 461 a blast search (http://blast.ncbi.nlm.nih.gov/) identified myosin binding protein H. This protein is up-regulated with 2% of creatine and is important for the myosin bundles in the thick filaments (Silva et al., 2012a). Moreover, beta enolase (Spot 651), a protein related with energy metabolism (Richard et al., 2016) shows to be up-regulated with 2% enrichment.

Table 4.1.3. Protein identification of muscle proteins in gilthead seabream. Mw – molecular weight, pI – isoelectric point, T/C – theoretical/calculated.

Biological process	Spot	Uniprot / NCBI	Protein name	Score	Mw T/C	pI T/C	Peptides	Coverage (%)	ANOVA	Tukey´s Test (*q* value)	Fold change	Expression
Energy metabolism	461	I3K2Y8	Uncharacterized protein OS *Oreochromis niloticus* (after blast 29-08-2017 myosin binding protein H [*Fundulus heteroclitus*]	1045	56203/ 63857	5.5/ 5.5	7	13	0.042	0.038	2.50	CR2>CTRL> CR8>CR5
	497	P84335	Tropomyosin alpha 1 chain OS *Liza aurata*	15521	32709/ 34421	4.49/ 4.7	69	65	0.031	0.045	1.60	CTRL>CR2> CR5>CR8
	641	P82159	Myosin light chain 1 skeletal muscle isoform OS *Liza ramada*	3451	20054/ 23673	4.34/ 5.0	7	36	0.010	0.042	1.95	CR8>CR2> CTRL>CR5
	651	B5DGQ7	Beta enolase OS *Salmo salar*	13013	47257/ 49981	6.65/ 6.4	19	23	0.026	0.048	1.78	CR2>CTRL> CR5>CR8
	1032	P24722	Creatine kinase testis isozyme OS *Oncorhynchus mykiss*	492	42976/ 27461	6.2/ 5.7	1	3	0.0005	0.002	3.27	CR5>CR2> CTRL>CR8
	1094	gi|47221502	Unnamed protein product [*Tetraodon nigroviridis*] - after blast 29-08-2017 PREDICTED: phosphatidylethanolamine-binding protein 1 [*Xiphophorus maculatus*]	182	20800/ 21800	7.7/ 5.4	4	20	0.006	0.011	1.31	CR2>CTRL> CR5>CR8
Cell process /stress response	1062	L0R689	Heat shock protein 27 Fragment OS *Gymnocephalus cernuus*	422	13341/ 25307	5.5/ 5.7	4	30	0.036	0.040	1.70	CTRL>CR5> CR2>CR8

Myosin light chain (spot 641) showed up-regulation in fish fed 8% creatine supplementation, supporting the idea that phosphorylation shuttling driven by creatine loading modulates contractile activity regardless the suboptimal Ca^{2+} concentrations (aka parvalbumin concentration). With 5% creatine supplementation, a creatine kinase was identified as spot 1032. This protein belongs to the phosphotransfer network, which is important in the ATP/ADP gradients in the muscle (Silva et al., 2012a). Such specific outputs, to some extent support the findings in rainbow trout. The authors suggest that muscle seems to be independent of the import of creatine, instead it seems to produce creatine by itself (Borchel et al., 2014). Such possibility is supported by general higher locomotor activity of fish, making it energetically more beneficial to synthesize creatine at the place of usage instead of shuttling. That means, enriched creatine diets might be used for different biological processes rather than only for energetic pathways, as supported by the lack of difference in glycogen reserves. In the control fish tropomyosin alpha (spot 497), which plays an important role in muscle contraction, was up-regulated. Additionally, spot 1062 was identified as heat shock protein 27 (hsp27). The hsp27 regulates the changing of actin filaments (Kayhan and Duman, 2010) and acts like a chaperon in case of cell damage (Schrama et al., 2017). Overall, creatine supplementation in the diets seems to influence the muscle homeostasis.

Liver proteins were extracted to verify if the supplementation with creatine would affect the expression of proteins in this tissue, by increasing or lowering energy reserve and/or if lipid metabolism and stress proteins would be triggered. Thirty-six proteins, from a linear gradient of pH 4-7, with significant expression differences were identified, as shown in Figure 4.1.9 and Table 4.1.4, respectively. The pH selected assign the best ratio high protein coverage / good protein separation in seabream (Richard et al., 2016). Proteome analysis of control group supports the ability of these fish to activate proteins from the lipid metabolism [Apolipoprotein A (Apo-A) and 14 kDa Apo-A (Moreira et al., 2017)]. Additionally, differently expressed proteins in this experimental diet show to be involved in the immune system [Transferrin (Stafford and Belosevic, 2003); Fibrinogen beta chain (Xie et al., 2009)] and biological thermal adjustment [wap65 (Sha et al., 2008)]. Such proteins were up-regulated in control conditions, suggesting that creatine supplementation might influence the general health and biological system and lipid metabolism of these fish. Although the existence of these proteins being indicative (to some extent) of an effect of creatine supplementation, our findings could be merely arbitrary and should be founded with further applied studies.

Proteins involved in cell processes and/or stress responses was shown to be up-regulated in all conditions. Nevertheless, 9 of 11 differentially expressed proteins between experimental conditions were up-regulated in liver of fish fed with creatine supplementation (Figure 4.9; Table 4.4). Specific examples are heat shock 70 kDa and heat shock cognate proteins (Schrama et al., 2017), Peroxiredoxin (Richard et al., 2016), chloride intracellular channel (Averaimo et al., 2010) and cytidine deaminase (Richard et al., 2016) which can indicate that creatine supplementation activates biological processes to protect against any cellular damage. Several proteins related to the cytoskeleton were up-regulated indifferently between the different diets, therefore indicating that the biological processes related with such function are triggered in all fish and not specifically due to creatine supplementation [glial fibrillary protein (spots 517 and 992), tropomyosin (spots 914 and 915), intermediate filament protein (spot 541), keratin (spots 590 and 1072), cytochrome c oxidase (spot 1298) and beta enolase (spot 526)]. Three proteins related with divergent metabolic processes were only up-regulated in fish subjected to enriched diets (described in Table 4.4). To some extent, these results show that liver metabolic pathways seem to be influenced by creatine enrichment nonetheless to further understand these changes more investigation needs to be conducted.

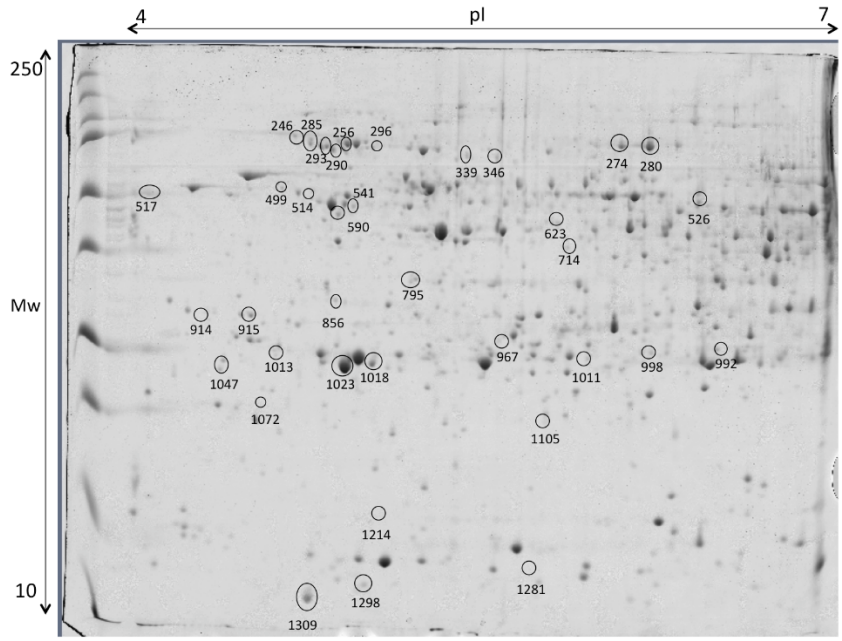

Fig.4.1.9. Representative 2D-DIGE gel of liver of gilthead seabream in a pH range of 4-7 on a 12.5% polyacrylamide gel. Protein identifications of significantly different spots (one-way ANOVA and post-Hoc Tukey $p<0.05$) are shown in table 4.4.

Table 4.1.4. Protein identification of liver proteins in gilthead seabream. Mw–molecular weight, pI–isoelectric point, T/C–theoretical/calculated.

Metabolism	Spot	Uniprot / NCBI	Protein name	Score	Mw T/C	pI T/C	Peptides	Coverage (%)	ANOVA	Tukey's Test (*q* value)	Fold change	Expression
Immune system	246	F8U094	Warm temperature acclimation like protein Fragment OS *Epinephelus bruneus*	990	42159/ 64966	5.46/ 4.8	4	7	0.001	0.0001	2.08	CTRL>CR5> CR2>CR8
	274	F2YLA1	Transferrin OS *Sparus aurata*	31467	74234/ 63269	5.88/ 5.7	36	44	0.0008	0.0004	1.62	CTRL>CR2> CR8>CR5
	280	F2YLA1	Transferrin OS *Sparus aurata*	47042	74234/ 63269	5.88/ 5.8	59	69	0.02	0.0207	1.57	CTRL>CR2> CR8>CR5
	285	C0L788	Warm temperature acclimation related 65 kDa protein OS *Sparus aurata*	8120	49126/ 62438	5.34/ 4.9	7	16	0.0006	0.0001	2.55	CTRL>CR2> CR8>CR5
	290	C0L788	Warm temperature acclimation related 65 kDa protein OS *Sparus aurata*	11826	49126/ 61617	5.34/ 4.9	12	24	0.0003	0.0000	1.94	CTRL>CR2> CR5>CR8
	293	F8U094	Warm temperature acclimation like protein Fragment OS *Epinephelus bruneus*	2250	42159/ 62438	5.46/ 4.9	8	11	0.001	0.0005	2.06	CTRL>CR2> CR8>CR5
	339	A0FJG5	Fibrinogen beta chain OS *Larimichthys crocea*	1013	55585/ 60807	5.89/ 5.2	4	8	0.002	0.0012	1.88	CTRL>CR2> CR8>CR5
	346	A0FJG5	Fibrinogen beta chain OS *Larimichthys crocea*	978	55585/ 60008	5.89/ 5.3	6	9	0.002	0.0016	1.57	CTRL>CR8> CR2>CR5
	795	Q7ZU45	Tetratricopeptide repeat protein 25 OS *Danio rerio*	134	55545/ 36781	8.70/ 5.1	1	2	0.002	0.0043	2.08	CR5>CR8> CR2>CTRL
Cell process /stress response	256	Q9I8F9	Heat shock 70 kDa protein 1 OS *Oryzias latipes*	1382	70307/ 63269	5.31/ 4.9	2	4	0.0009	0.0034	1.72	CR5>CR2> CTRL>CR8
	296	Q90473	Heat shock cognate 71 kDa protein OS *Danio rerio* GN hspa8	266	70930/ 61617	4.99/ 5.0	3	6	0.03	0.0356	1.28	CR5>CR2> CR8>CTRL
	499	Q6P3H7	Histone binding protein RBBP4 OS *Danio rerio*	946	47621/ 53272	4.56/ 4.7	6	30	0.002	0.0015	1.42	CTRL>CR5> CR8>CR2

	514	Q0GYP4	Trypsinogen II OS *Sparus aurata*	18962	26240/ 51881	4.98/ 4.8	16	49	0.016	0.0088	1.48	CTRL>CR2> CR8>CR5
	714	G3PT17	Uncharacterized protein OS *Gasterosteus aculeatus* PE 4 SV 1 (after blast on 28-04-2017 26S proteasome non-ATPase regulatory subunit 13 [*Anoplopoma fimbria*])	2099	43327/ 41432	5.95/ 5.6	14	26	0.045	0.0369	1.17	CR8>CR2> CR5>CTRL
	856	C3KGT8	Coatomer subunit epsilon OS *Anoplopoma fimbria*	2111	34041/ 33528	4.75/ 4.9	10	23	0.026	0.0427	1.32	CR8>CR2> CR5>CTRL
	967	M4AWP5	Chloride intracellular channel protein [*Xiphophorus maculatus*]	327	28409/ 28988	5.84/ 5.3	6	30	0.044	0.0399	1.46	CR8>CR5> CR2>CTRL
	998	Q4QY74	Chymotrypsin B like protein Fragment OS *Sparus aurata*	646	23818/ 27132	7.03/ 5.7	5	28	0.003	0.0061	3.25	CR8>CR2> CR5>CTRL
	1011	Q98TJ6	Glutathione S transferase Fragment OS *Platichthys flesus*	6467	14570/ 26077	5.65/ 5.6	9	28	0.049	0.0247	2.63	CR2>CR8> CR5>CTRL
	1105	G3Q5U8	Uncharacterized protein Fragment OS *Gasterosteus aculeatus* (after blast on 28-04-2017 Peroxiredoxin-1 [*Anoplopoma fimbria*])	523	22120/ 20014	6.6/5.2	5	29	0.047	0.0278	1.27	CR5>CR2> CTRL>CR8
	1281	F1QSJ0	Cytidine deaminase OS *Danio rerio*	3713	14325/ 10891	7.55/ 5.2	2	17	0.018	0.0238	1.58	CR8>CR2> CTRL>CR5
Cytoskeleton	517	P48677	Glial fibrillary acidic protein Fragment OS *Carassius auratus*	426	42578/ 51200	4.73/ 4.8	1	3	0.002	0.0020	1.93	CTRL>CR2> CR8>CR5
	526	B5DGQ7	Beta enolase OS *Salmo salar*	972	47257/ 49207	6.65/ 5.9	3	8	0.015	0.0103	1.44	CTRL>CR2> CR8>CR5
	541	P18520	Intermediate filament protein ON3 OS *Carassius auratus*	1416	57753/ 49207	4.95/ 4.9	16	23	0.023	0.0369	1.43	CR5>CR2> CTRL>CR8
	590	Q6NWF6	Keratin type II cytoskeletal 8 OS *Danio rerio*	3453	57723/ 47292	4.94/ 4.9	19	28	0.015	0.0403	1.67	CR5>CR8> CR2>CTRL

	914	Q7T3F0	Tropomyosin 4 OS *Danio rerio*	770	28484/ 30161	4.43/ 4.5	10	26	0.015	0.0178	1.53	CTRL>CR2> CR5>CR8
	915	P13104	Tropomyosin poa 1 chain OS *Danio rerio*	1828	32702/ 30970	4.5/ 4.6	7	11	0.048	0.0426	1.21	CR8>CR2> CR5>CTRL
	992	P48677	Glial fibrillary acidic protein Fragment OS *Carassius auratus*	176	42578/ 27132	4.73/ 6.0	1	3	0.03	0.0494	1.14	CR8>CR2> CTRL>CR5
	1072	W5N831	Uncharacterized protein OS *Lepisosteus oculatus* (after blast on 28-04-2017 Keratin, type I cytoskeletal 19 [*Alligator mississippiensis*])	327	88937/ 21668	4.67/ 4.6	5	4	0.003	0.0023	1.78	CTRL>CR2> CR8>CR5
	1298	P80972	Cytochrome c oxidase subunit 5A 1 mitochondrial Fragment OS *Thunnus obesus*	5414	2402/ 10329	4.28/ 4.9	1	50	0.007	0.0097	1.53	CTRL>CR5> CR8>CR2
Lipid metabolism	1018	O42175	Apolipoprotein A I OS *Sparus aurata*	22152	29615/ 26077	5.03/ 5.0	31	68	0.004	0.0058	1.79	CTRL>CR5> CR2>CR8
	1023	O42175	Apolipoprotein A I OS *Sparus aurata*	28332	29615/ 25396	5.03/ 4.9	59	70	0.003	0.0081	2.12	CTRL>CR8> CR2>CR5
	1047	Q5KSU1	Apolipoprotein A IV4 OS *Takifugu rubripes*	2047	28474/ 25734	4.59/ 4.6	4	12	0.039	0.0278	1.8	CTRL>CR2> CR8>CR5
	1309	Q4QY86	Putative uncharacterized protein OS *Sparus aurata* (after blast on 28-04-2017 14 kDa apolipoprotein [*Epinephelus bruneus*])	12510	15857/ 9797	5.03/ 4.8	9	48	0.006	0.0137	2.19	CTRL>CR8> CR2>CR5
Metabolic pathway	623	Q66I24	Argininosuccinate synthase OS *Danio rerio*	504	47099/ 46671	6.46/ 5.5	5	7	0.01	0.0112	1.32	CR5>CR2> CR8>CTRL
	1013	Q1MTI4	Triosephosphate isomerase A OS *Danio rerio*	2170	26836/ 26776	4.72/ 4.7	6	25	0.002	0.0023	2.03	CR2>CR5> CR8>CTRL
	1214	G3PDP5	Uncharacterized protein OS *Gasterosteus aculeatus* (after blast on 28-04-2017 bifunctional protein GlmU-like [*Salmo salar*])	1661	15732/ 13637	5.34/ 5.0	1	9	0.016	0.0462	1.73	CR8>CR2> CR5>CTRL

4.1.4 Conclusion

The findings of the present study show disparities with previous studies in mammals in which creatine loading improves muscle performance (e.g. fatigue resistance, contraction efficiency; strength gain and muscle growth). In fish, driven research is scarce but our data suggests that creatine enrichment up to 8% does not seem to have an effect in major biochemical and quality aspects of fish. We observed however that cortisol levels are lower in the highest percentage of creatine supplementation, making possible to infer about the effect of creatine enrichment on fish physiological primary response. Notwithstanding with the lack of evidences regarding the effect of creatine supplementation in fish, our comparative proteomic studies has shown proteins involved in the energy homeostasis and muscle contraction mechanisms of this tissue. In liver the majority of proteins involved in immune system, lipid metabolism, metabolic pathways and cell processes suggests to be modulated by creatine supplementation. Such fact endorses to our theory that supplemented creatine is not load directly in muscle but is rather shuttled to other biological tissues and processes. Moreover, our research does not show direct evidences pointing to a modulation of allergenicity in fish fed with creatine enriched diets. Deeper research is needed to understand the co-regulation between supplemented creatine and parvalbumin, as well as to tune creatine supplementation regarding aquaculture sustainability. Hence, it is important to refer that a proper economical study should be performed to evaluate the balance between the costs of enrichment diets and how it will improve aquaculture productivity (e.g. growth, reproductive success, welfare, muscle quality, among other). Overall, this study proves the sensibility of proteomics to detect changes in fish tissues (muscle and liver) submitted to enriched diets where no apparent changes were detected by standard biochemical and quality analysis. Proteomics is however an expensive technique and therefore not always accessible in all laboratories but being such a sensitive technology, it is an invaluable tool for an untargeted and unbiased assessment of the impact of exogeneous stimuli on fish metabolism. Such possibility enables approaches towards knowledge discovery which are less hypothesis-driven and more data-driven. Nonetheless it should be taken in mind that the lack of information of most fish genomes databases can be the major drawback and the major reason for a low protein identification rate in fish.

4.1.5 Acknowledgments

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4.1.6 Supplementary Material

Table S4.1.1. Ingredients and proximate composition of the control diet

Ingredients	CTRL (%)
Fishmeal LT[a]	10.00
Fishmeal 60[b]	10.00
Porcine blood meal	5.00
Soy protein concentrate[c]	10.00
Wheat gluten[d]	10.00
Corn gluten[e]	7.25
Rise protein concentrate	3.50
Soybean meal[f]	10.00
Rapeseed meal	4.00
Wheat meal	12.00
Fish oil[g]	14.50
Vit&Min Premix[h]	0.15
Soy lecithin[i]	2.00
Antioxidant	0.40
Dicalcium phosphate[j]	0.50
L-Lysine[k]	0.50
DL-Methionine	0.20
Creatine (g/kg)[l]	0.00

Proximate composition	
Dry Matter (DM) (%)	95.39 ± 0.04
Crude protein (%DM)	49.28 ± 0.14
Lipid (%DM)	20.37 ± 0.31
Ash (%DM)	8.39 ± 0.06
Gross energy (kJ/g DM)	23.43 ± 0.07

a Peruvian fishmeal LT: 71% crude protein, 11% crude fat, EXALMAR, Peru.
b Fish by-products meal: 540 g Kg-1 CP, 80 g kg-1 CF, COFACO, Portugal.
c Soycomil P: 65% CP, 0.7% CF, ADM, The Netherlands.
d VITEN: 85.7% CP, 1.3% CF, ROQUETTE, France.
e GLUTALYS: 61% CP, 8% CF, ROQUETTE, France.
f Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF, SORGAL, Portugal.
g Henry Lamotte Oils GmbH, Germany
h PVO40.01 SPAROS standard premix for marine fish, PREMIX Lda, Portugal.
i Yelkinol AC (65% phospholipids): 750 g Kg-1 CF,ADM, The Netherlands
j Dicalcium phosphate: 18% phosphorus, 23% calcium, Fosfitalia, Italy.
k L-Lysine HCl 99%: Ajinomoto Eurolysine SAS, France
l Creatine monohydrate: Sigma-Aldrich, USA~

Main ingredients were grinded (below 250µm) in a micropulverizer hammer mill Hosakawa, model #1 (Hosokawa Micron Ltd., United Kingdom). These triturated ingredients were then mixed accordingly to the target formulation in a Double-helix Mixture TGC, model 500L (TGC Extrusion, France), to attain a basal mixture (no oils were added at this stage). All diets were manufactured by extrusion (pellet size 5.0 mm) by means of a pilot-scale twin-screw extruder CLEXTRAL BC45 (Clextral, France) with a screw diameter of 55.5 mm and temperature ranging 105 – 110ºC. Upon extrusion, all batches of extruded feeds were dried in a convection oven (OP 750-EF, LTE Scientifics, United Kingdom) for 2 hours at 60ºC. After this process, pellets were left to cool at room temperature, and subsequently the creatine was mixed with fish oil fraction in concentrations (2, 5 and 8%) according to each target formulation and added under vacuum coating conditions in a Pegasus vacuum mixer (PG-10VCLAB, DINNISEN, The Netherlands) respective mixture.

Table S4.1.2 - Protein identification of muscle proteins in gilthead seabream. Mw – molecular weight, pI – isoelectric point, T/C – theoretical/calculated, FDR – false discovery rate

Spot	Uniprot / NCBI	Protein name	Score	Mw T/C	pI T/C	Peptides	Best sequence	Coverage (%)
103	gi|359390897	Muscle-type creatine kinase [*Sebastes inermis*]	345	42900/ 141400	6.23/ 6.35	4	R.GTGGVDTASVGGVFDISNADR.L	15
140	gi|410910532	Predicted: creatine kinase M-type-like isoform 1 [*Takifugu rubripes*]	51	42400/ 132500	6.44/ 6.35	2	K.SFLVWVNEEDHLR.V	10
161	gi|41056111	Phosphoglucomutase-1 [*Danio rerio*]	351	61100/ 109200	5.69/ 6.4	4	K.LSLCGEESFGTGSDHIR.E	10
183	gi|327243042	Transferrin [*Sparus aurata*]	42	74200/ 126900	5.9/ 5.8	1	K.ASSIEQYYGYAGAFR.C	2
194	gi|224551742	Warm temperature acclimation-related 65 kDa protein [*Sparus aurata*]	345	49100/ 121600	5.36/ 4.85	5	R.VHLDAITSDNAGNMYAFR.G	11
210	gi|224551742	Warm temperature acclimation-related 65 kDa protein [*Sparus aurata*]	404	49100/ 119000	5.36/ 4.9	7	K.ELHSEVDAVFTYQDHLYMIK.D	17
241	gi|31322099	Creatine kinase muscle isoform 2 [*Chaenocephalus aceratus*]	328	42700/ 114000	6.47/ 6.4	3	K.TFLVWVNEEDHLR.V	14
289	gi|1351868	Actin, alpha skeletal muscle	571	41900/ 107800	5.11/ 5.3	7	K.DLYANNVLSGGTTMYPGIADR.M	26
295	gi|323650066	Mitochondrial aldehyde dehydrogenase [*Perca flavescens*]	43	48200/ 98000	5.24/ 5.75	1	K.TIPIDGDYFCYTR.H	3
297	gi|47217288	Unnamed protein product [*Tetraodon nigroviridis*] (after blast on 27-04-2017 PREDICTED: methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial-like [*Takifugu rubripes*])	76	54500/ 98000	6.83/ 5.7	1	K.AISFVGSNSAGEYIYER.G	3

298	gi|188036012	Chain A, Crystal structure of seabream antiquitin and elucidation of its substrate	200	55100/ 96000	5.83/ 5.6	5	R.VTQATLAEYEETVQK.T	15
330	gi|6653228	Skeletal alpha-actin [*Sparus aurata*]	610	41800/ 99300	5.17/ 5.3	8	K.SYELPDGQVITIGNER.F	27
334	gi|410060781	Tropomyosin [*Oreochromis mossambicus*]	239	32600/ 80100	4.52/ 4.7	5	K.TIDDLEDELYAQK.L	13
335	gi|410906157	Predicted: ATP synthase subunit beta, mitochondrial-like [*Takifugu rubripes*]	936	55100/ 88100	4.96/ 4.9	9	R.DQEGQDVLLFIDNIFR.F	27
336	gi|31322099	Creatine kinase muscle isoform 2 [*Chaenocephalus aceratus*]	174	42700/ 88100	6.47/ 6.4	3	K.TFLVWVNEEDHLR.V	9
342	gi|410910532	Predicted: creatine kinase M-type-like isoform 1 [*Takifugu rubripes*]	367	42400/ 90000	6.44/ 6.3	3	R.GTGGVDTASVGGVFDISNADR.L	12
385	gi|47210809	Unnamed protein product [*Tetraodon nigroviridis*] (after blast on 27-04-2017 PREDICTED: beta-enolase [*Larimichthys crocea*])	263	47100/ 69500	5.97/ 6.4	4	K.LAMQEFMILPVGAANFHEAMR.I	16
393	gi|432941989	Predicted: aldo-keto reductase family 1 member B10-like [*Oryzias latipes*]	336	35600/ 66600	6.02/ 6.35	5	K.AIGISNFNKEQIEAILNKPGLK.Y	22
408	gi|317418695	Myosin binding protein H [*Dicentrarchus labrax*]	182	57800/ 82800	5.72/ 5.45	3	R.RPGNFDGGVYSCK.A	7
409	gi|317418695	Myosin binding protein H [*Dicentrarchus labrax*]	255	57800/ 82800	5.72/ 5.5	4	R.QICVQGICSLEIR.R	11
410	gi|348532430	Predicted: myosin-binding protein H-like [*Oreochromis niloticus*]	210	53900/ 82800	6.63/ 5.55	3	R.QICVQGICSLEIR.K	10
447	gi|47210809	Unnamed protein product [*Tetraodon nigroviridis*] (after blast on 27-04-2017 PREDICTED: beta-enolase [*Larimichthys crocea*])	577	47100/ 62400	5.97/ 6.25	7	K.AGYPDKIIIGMDVAASEFYR.S	17
450	Q1MTI4	Triosephosphate isomerase A OS *Danio rerio* GN tpi1a PE 2 SV 1	13910	26836/ 33019	5.58/ 6.45	21	N.A.	32
480	B5DGM7	Fructose bisphosphate aldolase A OS *Salmo salar* PE 1 SV 1	504	39531/ 38996	8.3/ 5.0	2	N.A.	6

482	gi|432922703	Predicted: protein ADP-ribosylarginine hydrolase-like [*Oryzias latipes*]	50	38900/ 62400	4.95/ 5.2	1	R.VPYNPEGTGCGAAMR.S	4
484	Q6AZW2	Alcohol dehydrogenase NADP A OS *Danio rerio* GN akr1a1a PE 2 SV 2	551	36738/ 34902	7.32/ 6	1	N.A.	2
512	gi|47210809	Unnamed protein product [*tetraodon nigroviridis*] - after blast 21-04-2017 PREDICTED: beta-enolase [*Larimichthys crocea*]	431	47100/ 75000	5.97/ 6.4	6	K.AGYPDKIIIGMDVAASEFYR.S	20
515	gi|327243042	Transferrin (*Sparus aurata*)	294	74200/ 77500	5.9/ 5.9	6	R.CLVEGAGDVAFIK.H	10
516	gi|410905149	Predicted: heat shock cognate 70 kDa protein-like [*Takifugu rubripes*]	388	71200/ 76300	5.17/ 5.4	5	K.GPAVGIDLGTTYSCVGIFQHGK.V	11
533	gi|47207795	Unnamed protein product [*Tetraodon nigroviridis*] (after blast on 27-04-2017 S-formylglutathione hydrolase [*Larimichthys crocea*])	73	29700/ 49300	5.35/ 5.55	1	K.AGSQIPAAEHGIIIIAPDTSPR.G	8
536	I3KUW7	Uncharacterized protein Fragment OS *Oreochromis niloticus* GN LOC100705384 PE 4 SV 1 (after blast on 28-04-2017 Phosphoglycolate phosphatase [*Nothobranchius rachovii*])	3228	33907/ 30807	5.55/ 5.5	3	N.A.	20
537	H2TC41	Uncharacterized protein Fragment OS *Takifugu rubripes* GN LOC101079900 PE 4 SV 1 (after blast on 28-04-2017 Electron transfer flavoprotein subunit alpha, mitochondrial [*Fundulus heteroclitus*])	1668	34642/ 30807	5.57/ 5.4	7	N.A.	22
544	Q1MTI4	Triosephosphate isomerase A OS *Danio rerio* GN tpi1a PE 2 SV 1	17307	26836/ 31674	4.72/ 6.45	38	N.A.	32
548	gi|348514003	Predicted: fructose-1,6-bisphosphatase isozyme 2-like [*Oreochromis niloticus*]	207	36700/ 56000	6.74/ 6.0	2	R.VPFVVGSPDDVNEYLSFVK.K	9
550	gi|148228513	Actin, alpha skeletal muscle 2 [*Xenopus laevis*]	820	42000/ 69100	5.1/ 5.3	8	R.KDLYANNVLSGGTTMYPGIADR.M	27
569	gi|30268605	Skeletal alpha-actin type 2b [*Coryphaenoides armatus*]	646	41900/ 68000	5.11/ 5.4	7	K.DLYANNVLSGGTTMYPGIADR.M	27

586	gi|3063940	Slow myotomal muscle tropomyosin [*Salmo trutta*]	278	32600/ 76300	4.56/ 4.7	5	K.TIDDLEDELYAQK.L	13
592	A5PLK2	Phospholysine phosphohistidine inorganic pyrophosphate phosphatase OS *Danio rerio* GN lhpp PE 2 SV 1	231	29605/ 27572	5.0/ 4.8	2	N.A.	7
594	Q6UFZ3	14 3 3 protein gamma 1 OS *Oncorhynchus mykiss* PE 2 SV 1	9942	28378/ 27958	4.7/ 4.75	7	N.A.	28
597	gi|410921770	Predicted: guanidinoacetate N-methyltransferase-like [*Takifugu rubripes*]	441	26800/ 40600	5.74/ 5.6	7	K.MFEETQVPHLLQAGFK.K	20
602	gi|1339977	Skeletal myosin heavy chain, partial [*Thunnus thynnus*]	118	90696/ 52778	5.48/ 5.3	17	K.KQADSVAELGEQIDNLQR.V	35
603	P84335	Tropomyosin alpha 1 chain OS *Liza aurata* PE 1 SV 1	144	32709/ 26819	4.49/ 4.6	4	N.A.	12
616	gi|312840387	Carbonic anhydrase [*Trematomus bernacchii*]	84	28200/ 38900	5.64/ 5.45	2	K.YPAELHLVHWNTK.Y	10
622	Q90339	Myosin heavy chain fast skeletal muscle OS *Cyprinus carpio* PE 2 SV 2	856	221461 /54234	5.4/ 5.4	15	N.A.	6
626	O57656	Glycerol 3 phosphate dehydrogenase NAD cytoplasmic OS *Takifugu rubripes* GN gpd1 PE 3 SV 1	1372	38053/ 27193	6.48/ 6.4	5	N.A.	15
631	Q90XG0	Triosephosphate isomerase B OS *Danio rerio* GN tpi1b PE 2 SV 1	13408	26810/ 26819	6.5/ 6.5	21	N.A.	33
644	O42175	Apolipoprotein A I OS *Sparus aurata* GN apoa1 PE 2 SV 1	19805	29615/ 25022	5.0/ 4.9	24	N.A.	65
654	gi|410921908	Predicted: phosphoglucomutase-1-like [*Takifugu rubripes*]	256	61000/ 69100	6.31/ 6.55	3	K.LSLCGEESFGTGSDHIR.E	10
672	gi|348515631	Predicted: desmin-like [*Oreochromis niloticus*]	206	54100/ 63600	5.2/ 5.4	4	R.LQEEIHQKEEAENNLSAFR.A	14
673	gi|348515631	Predicted: desmin-like [*Oreochromis niloticus*]	254	54100/ 61600	5.2/ 5.45	3	K.VSDLNQAVNKNNDALR.Q	10

682	gi|222088001	Adenylate kinase 1-2 [*Epinephelus coiodes*]	319	21200/ 31400	8.77/ 5.95	4	K.ATEPVIAFYEGR.G	24
693	gi|3063940	Slow myotomal muscle tropomyosin [*Salmo trutta*]	214	32600/ 71400	4.56/ 4.7	3	K.TIDDLEDELYAQK.L	13
701	gi|348534170	Predicted: protein DJ-1-like isoform 1 [*Oreochromis niloticus*]	353	19800/ 26400	6.11/ 5.8	4	K.QGPYDVVLLPGGMPGAQNLAESPAVK.E	32
712	gi|47210809	Unnamed protein product [*tetraodon nigroviridis*] - after blast 21-04-2017 PREDICTED: beta-enolase [*Larimichthys crocea*]	610	47100/ 60600	5.97/ 6.35	7	K.AGYPDKIIIGMDVAASEFYR.S	22
726	gi|47210809	Unnamed protein product [*tetraodon nigroviridis*] - after blast 21-04-2017 PREDICTED: beta-enolase [*Larimichthys crocea*]	699	47100/ 59600	5.97/ 6.3	7	R.AAVPSGASTGVHEALELR.D	17
731	gi|66267496	Zgc: 111961 [*Danio rerio*]	592	55000/ 58600	5.13/ 4.9	9	R.LVLEVAQHLGENTVR.T	28
733	gi|47210809	Unnamed protein product [*tetraodon nigroviridis*] - after blast 21-04-2017 PREDICTED: beta-enolase [*Larimichthys crocea*]	573	47100/ 60600	5.97/ 6.5	6	R.AAVPSGASTGVHEALELR.D	17
738	gi|939317735	PREDICTED: myosin heavy chain, fast skeletal muscle-like, partial [*Maylandia zebra*]	112	47917/ 47333	5.51/ 5.6	10	R.NSQRVIDSMQSTLDAEVR.S	48
739	gi|768961769	PREDICTED: myosin heavy chain, fast skeletal muscle-like, partial [*Takifugu rubripes*]	89	118546 /47981	5.16/ 5.6	14	K.KDIDDLELTLAK.V	27
740	gi|348514660	Predicted: alpha-enolase-like isoform 1 [*Oreochromis niloticus*]	588	47200/ 58600	5.97/ 5.9	7	R.AAVPSGASTGIYEALELR.D	21
741	gi|47210809	Unnamed protein product [*tetraodon nigroviridis*] - after blast 21-04-2017 PREDICTED: beta-enolase [*Larimichthys crocea*]	657	47100/ 59600	5.97/ 6.4	6	R.AAVPSGASTGVHEALELR.D	17
743	Q4S1B0	Pyruvate kinase Fragment OS *Tetraodon nigroviridis* GN GSTENG00025632001 PE 3 SV 1	3153	58040/ 46692	7.5/6	18	N.A.	23
744	gi|70778800	Actin, alpha skeletal muscle [*Danio rerio*]	821	41900/ 55800	5.18/ 5.3	8	K.DLYANNVLSGGTTMYPGIADR.M	27

756	gi|45387807	Cofilin-2 [*Danio rerio*]	217	18600/ 16800	8.85/ 5.95	3	R.YGLYDATYETK.E	18
760	gi|359390897	Muscle-type creatine kinase [*Sebastes inemis*]	507	42900/ 48100	6.23/ 6.4	3	K.RGTGGVDTASVGGVFDISNADR.L	15
774	gi|335955228	Tropomyosin [*Epinephelus bruneus*]	133	21800/ 54000	4.61/ 4.7	2	R.KLVIIEGDLER.T	13
777	gi|94469901	Fast/white muscle troponin T larval isoform [*Sparus aurata*]	339	27300/ 52200	10.04 /5.4	5	K.SALSSMGSNYSSHLQR.A	24
790	gi|225716056	Actin, alpha cardiac [*Esox lucius*]	847	41900/ 52200	5.11/ 5.4	8	R.KDLYANNVLSGGTTMYPGIADR. M	27
795	gi|225716056	Actin, alpha cardiac [*Esox lucius*]	618	41900/ 52200	5.11/ 5.45	7	K.SYELPDGQVITIGNER.F	27
802	gi|70778800	Actin, alpha skeletal muscle [*Danio rerio*]	858	41900/ 52200	5.18/ 5.3	8	K.DLYANNVLSGGTTMYPGIADR.M	27
803	gi|94469899	Fast/white muscle troponin T adult isoform [*Sparus aurata*]	436	27700/ 51400	9.91/ 5.45	5	K.SALSSMGSNYSSHLQR.A	19
805	gi|1020396140	PREDICTED: homeobox protein cut-like 1 isoform X1 [*Sinocyclocheilus grahami*]	66	163214 /42449	5.79/ 5.5	70	R.QDETEQSRK.K	7
808	gi|6653228	Skeletal alpha-actin [*Sparus aurata*]	537	41800/ 53100	5.17/ 5.15	7	R.VAPEEHPTLLTEAPLNPK.A	27
810	gi|371901819	Creatine kinase [*Platichthys flesus*]	557	43000/ 48900	6.23/ 6.5	5	R.GTGGVDTASVGGVFDISNADR.L	20
812	gi|348510129	Predicted: nucleoside diphosphate kinase A-like [*Oreochromis niloticus*]	414	17000/ 49700	7.77/ 6.5	5	R.MMLGETNPADSKPGSIR.G	38
821	gi|348525612	Predicted: tropomodulin 4-like [*Oreochromis niloticus*]	232	38100/ 54000	4.54/ 4.65	3	K.GNSHVEFLSIAATR.S	13
823	gi|410903169	Predicted: nucleoside diphosphate kinase B-like [*Takifugu rubripes*]	401	16900/ 13500	7.72/ 6.4	4	K.YMSSGPVLAMVWEGQNIVK.L	21
828	gi|29436540	Creatine kinase, brain b [*Danio rerio*]	41	42900/ 48100	5.42/ 6.3	1	K.TFLMWVNEEDHLR.V	3

835	gi|31322099	Creatine kinase muscle isoform 2 [*Chaenocephalus aceratus*]	547	42700/ 48100	6.47/ 6.35	5	R.GTGGVDTASVGGVFDISNADR.L	15
836	gi|359390897	Muscle-type creatine kinase [*Sebastes inemis*]	562	42900/ 48100	6.23/ 6.5	4	R.LGSSEVEQVQLVVDGVK.L	19
848	gi|61218043	RecName: Full=Actin, alpha skeletal muscle	78	42286/ 40200	5.23/ 5.7	2	K.SYELPDGQVITIGNER.F	7
891	gi|348504638	Predicted: 14 kDa phosphohistidine phosphatase-like [*Oreochomis niloticus*]	85	16100/ 10500	9.5/ 6.0	1	K.IPDVEIDPEGTFK.Y	9
905	gi|335955228	Tropomyosin [*Epinephelus bruneus*]	265	21800/ 41500	4.61/ 4.65	4	R.KLVIIEGDLER.T	20
915	gi|295792268	Tropomyosin [*Epinephelus coioides*]	386	32700/ 40800	4.54/ 4.7	5	K.KATDAEGDVASLNR.R	14
916	gi|29570808	Fast muscle-specific myosin heavy chain, partial [*Danio rerio*]	85	95006/ 37047	5.22/ 5.1	10	R.QLEEKEALVSQLTR.G	19
934	gi|768908418	PREDICTED: pyruvate dehydrogenase (acetyl-transferring) kinase isozyme 2, mitochondrial-like [*Takifugu rubripes*]	58	46757/ 31896	6.46/ 5.0	6	K.NAALASAPK.H	15
954	gi|197631853	Capping protein (actin filament) muscle Z-line beta [*Salmo salar*]	275	31000/ 35200	5.25/ 5.4	7	K.ELSQVLTQR.Q	24
983	gi|94469901	Fast/white muscle troponin T larval isoform [*Sparus aurata*]	208	27300/ 33500	10.04 /4.85	5	K.IPDGEKVDFDDIQK.K	21
996	gi|1174688032	Neuron navigator 1 isoform X7 [*Oryzias latipes*]	65	187943 /26723	8.14/ 4.8	18	-.MSSSGLENVSK.D	18
1000	gi|6653228	Skeletal alpha-actin [*Sparus aurata*]	125	42158/ 30206	5.28/ 5.6	3	K.AGFAGDDAPR.A	38
1003	gi|6653228	Skeletal alpha-actin [S*parus aurata*]	185	42185/ 26006	5.28/ 5.5	7	K.AGFAGDDAPR.A	54
1009	gi|831322033	PREDICTED: signal-induced proliferation-associated 1-like protein 1 [*Clupea harengus*]	64	182495 /30206	6.84/ 5.8	9	K.RPPADHTVGGSIPATDEFYTR.H	10

1024	gi|6653228	Skeletal alpha-actin [*Sparus aurata*]	126	42185/ 24628	5.28/ 5.2	3	K.AGFAGDDAPR.A	38
1046	gi|6686379	Apolipoprotein A-I	382	29600/ 28900	5.07/ 4.9	7	R.AVNQLDDPQYAEFK.T	28
1054	gi|617416779	PREDICTED: myosin light chain 1/3, skeletal muscle isoform [*Poecilia formosa*]	222	20822/ 23007	4.62/ 4.7	3	R.VFDKEGNGTVMGAELR.I	26
1065	gi|7678732	Myosin light chain 1 [*Pennahia argentata*]	331	20700/ 25300	4.47/ 4.7	5	R.VFDKEGNGTVMGAELR.I	30
1067	gi|7678732	Myosin light chain 1 [*Pennahia argentata*]	341	20700/ 25300	4.47/ 4.65	5	K.AGFEDYVEGLR.V	29
1092	gi|159137835	Peroxiredoxin 2 [*Thunnus maccoyii*]	248	21800/ 22600	6.53/ 5.9	4	R.DYGVLKEDDGIAYR.G	24
1096	gi|617416779	PREDICTED: myosin light chain 1/3, skeletal muscle isoform [*Poecilia formosa*]	178	20822/ 19276	4.62/ 4.8	3	K.IEFSADQIDDYR.E	30
1099	gi|348500116	Predicted: lactoylglutathione lyase-like [*Oreochromis niloticus*]	201	20300/ 18200	4.93/ 4.9	3	R.FSLFFLGYEDKK.E	13
1114	gi|742141995	PREDICTED: ellis-van Creveld syndrome protein isoform X1 [*Esox lucius*]	55	111583 /16823	6.38/ 4.6	9	R.ENHPSDCVSNSKK.G	12
1117	gi|974087902	PREDICTED: protein FAM184A-like isoform X3 [*Cyprinodon variegatus*]	79	136670 /16062	5.63/ 4.8	9	K.NDEHEEEIESLK.E	9
1149	gi|542213490	PREDICTED: tropomyosin alpha-1 chain isoform X4 [*Oreochromis niloticus*]	67	32534/ 14682	4.76/ 4.8	4	K.LDKENALDR.A	26
1151	gi|326535727	Myosin light chain 2 [*epinephelus coioides*]	441	19100/ 15700	4.56/ 4.6	6	K.NICYVITHGEEKEE.	44
1152	gi|432926489	Predicted: eukaryotic translation initiation factor 5A-1-like isoform 1 [*Oryzias latipes*]	123	17200/ 16500	4.97/ 5.3	2	K.VNLVGIDIFTNK.K	8

1155	gi|6653228	Skeletal alpha-actin [*Sparus aurata*]	141	42185/ 14288	5.28/ 5.4	5	R.AVFPSIVGRPR.H	28
1161	gi|6653228	Skeletal alpha-actin [S*parus aurata*]	133	42185/ 13716	5.28/ 5.9	4	R.GYSFVTTAER.E	23
1167	gi|410902987	Predicted: myosin regulatory light chain 2 [*Takifugu rubripes*]	464	16800/ 14900	4.26/ 4.65	5	K.DDLRDVLASMGQLNVK.N	48
1174	gi|47217809	Unnamed protein product [*Tetraodon nigroviridis*]	438	16500/ 14500	4.2/ 4.8	6	K.LKGADPEDVILSAFK.V	41
1175	gi| 5852836	Fast skeletal myosin light chain 3 [*Sparus aurata*]	644	17000/ 15200	4.2/ 4.35	7	K.EVDALQKGTYDDYVEGLR.V	42
1176	gi|410902987	Predicted: myosin regulatory light chain 2 [*Takifugu rubripes*]	559	16800/ 14500	4.26/ 4.7	6	K.LKGADPEDVILSAFK.V	52
1191	gi|432889657	Predicted: creatine kinase M-type-like [*Oryzias latipes*]	457	42700/ 13300	6.34/ 6.4	4	R.LGSSEVDQVQLVVDGVK.L	20
1212	gi|5852836	Fast skeletal myosin light chain 3 [*Sparus aurata*]	119	16976/ 11032	4.36/ 4.4	2	K.EAFGLFDR.V	61
1223	gi|327358389	Heart type fatty acid binding protein, partial [*Oryzias melastigma*]	98	12600/ 11900	5.32/ 5.1	2	K.LGEEFDETTADDRK.V	21
1245	gi|261825915	Parvalbumin [*Sparus aurata*]	384	11500/ 9300	4.66/ 4.75	4	M.PFAGLTDADVAAALDGCK.D	43
1246	gi|48476449	Parvalbumin-like protein [*Sparus aurata*]	551	11500/ 9100	4.4/ 4.4	4	K.AFLAAGDSDGDGKIGVDEFAALVK .A	46
1255	gi|348502437	Parvalbumin alpha-like [*Oreochromis niloticus*]	237	11500/ 8700	4.47/ 4.6	4	K.LFLQNFSASAR.A	26
1259	gi|50953783	Parvalbumin 2 [*Kryptolebias marmoratus*]	446	11400/ 8700	4.49/ 4.7	5	K.SGFIEEDELKLFLQNFSASAR.A	28
1262	gi|6729202	Myosin regulatory light chain [*Cyprinus carpio*]	95	18998/ 8995	4.72/ 4.9	1	K.EAFTIIDQNRDGIISK.D	18
1264	gi|5852838	Myosin light chain 2 [*Sparus aurata*]	50	19180/ 8635	4.62/ 4.7	1	K.EAFTIIDQNR.D	11

Chapter 4.2

Effect of EDTA enriched diets on farmed fish allergenicity and muscle quality; a proteomics approach

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Effect of EDTA enriched diets on farmed fish allergenicity and muscle quality; a proteomics approach

Abstract

Fish is one of the most common elicitors of food-allergic reactions worldwide. These reactions are triggered by the calcium-binding muscle protein β-parvalbumin, which was shown to have reduced Immunoglobulin E (IgE)-binding capacity upon calcium depletion. This work aimed to reduce gilthead seabream allergenicity using diets supplemented with a calcium chelator. Three experimental feeds were tested, differing in ethylenediaminetetraacetic acid (EDTA) supplementation, and its effects on muscle and parvalbumin's IgE-reactivity were analyzed. Chromatographic determination of EDTA showed no accumulation in the muscle and sensory results demonstrated that the lowest concentration did not affect fish quality as edible fish. Proteomics revealed one protein related to muscle contraction with significantly different relative abundance. Immunoblot assays performed with fish-allergic patients sera indicated a 50% reduction in IgE-reactivity upon EDTA presence. These preliminary results provide the basis for the further development of a non-GMO approach to modulate fish allergenicity and improve safety of aquaculture fish.

Keywords: Fish allergens; Parvalbumin; Gilthead seabream; IgE-reactivity; Fish nutrition

4.2.1 Introduction

World's population has been rapidly increasing in the past decades, along with a rising demand for fish as a valuable and healthy food source in human consumption, driving a considerable growth of fish aquaculture production (Rodrigues et al., 2012). By 2016, half of the world fishery production was yet covered by aquaculture livestock (Herrero et al., 2017).

Food allergies are steadily on the rise and it is estimated that nowadays they affect up to 3% of adults and up to 8% of children (Tordesillas et al., 2017). Fish is among the eight most common elicitors of food allergies, the so-called 'Big-8' of food allergens. Clinical reactions manifest rapidly after fish ingestion affecting single or several organs and leading to clinical symptoms ranging from mild to severe anaphylaxis (Sharp and Lopata, 2014).

Among other allergens such as enolase and aldolase (Kuehn et al., 2013), β-parvalbumin (PV) is considered to be the major fish allergen responsible for most of the IgE-mediated reactions (Kuehn et al., 2014, Sharp and Lopata, 2014). PV is a small (molecular weight 10-12 kDa), acidic (isoelectric point between pH 4 and 5), calcium-binding muscle protein. Its remarkable stability to thermal, chemical and proteolytic denaturation seems to contribute to its allergenic potency, which is not altered during the gastrointestinal processing (Sánchez et al., 2016). Fish PV is an EF-hand protein, characterized by presenting two functional EF-hand domains (motifs of helix-loop-helix structures) capable of chelating one divalent metal ion each, with high binding affinity for calcium (Arif, 2009). Fish may express multiple PV isoforms differing by their allergenicity, an observation that is not well understood so far (Kuehn et al., 2011). In teleost (bony) fish, IgE cross-reactivity among PVs of different species is highly common, due to the high amino acid and structural identity between the homologs, explaining the high clinical cross-reactions to various fish in allergic patients (Stephen et al., 2017). Monosensitivity to specific fish species or single parvalbumin isoforms has also been reported (Kuehn et al., 2011). To date, the only effective clinical management of fish-allergic patients relies on complete avoidance of any fish-containing food (Kuehn et al., 2014). However, patients might also be exposed accidentally to the offending food. Other common strategies to induce immunological tolerance, like allergen-specific immunotherapies, which relies on the administration of allergenic extracts, are complicated and may induce side reactions or sensitization to new allergens present in the crude extract (Reisacher and Davison, 2017). Hence, the need of new developments to overcome this prevalent allergy is urgent.

Several studies showed a reduction in the PV's IgE-binding capacity upon calcium depletion. This was explained by a global-folding rearrangement of the protein structure, affecting major epitope regions. Studies proved that calcium-binding is essential to maintain the parvalbumin stable structure and its IgE-reactivity (Kobayashi et al., 2016a, Permyakov et al., 2008). Swoboda *et al.* successfully produced hypoallergenic mutant allergens from carp PV (Cyp c 1), with point mutations in the two functional calcium-binding sites and clearly reduced the molecule's IgE-reactivity (Swoboda et al., 2013). Tomura *et al.* was able to reduce Pacific mackerel PV IgE-reactivity by substituting specific amino acid residues in the calcium-binding sites (Tomura et al., 2008). Moraes *et al.* and Kumeta *et al.* performed important studies regarding the 3D-structure, dynamics and stability of cod (Gad m 1) and Pacific

mackerel (Sco j 1) parvalbumins, respectively, targeting on the various IgE-binding modes of these allergens (Kumeta et al., 2017, Moraes et al., 2014).

Although, no research work has yet been done, to our knowledge, using targeted aquaculture to modulate fish allergenicity, a new approach targeting the β-parvalbumin conformation was introduced in this work. Based on the previously mentioned findings, a calcium-chelating agent was used to supplement fish feeds, in different concentrations, aiming to induce this calcium-free form of PV. Gilthead seabream (*Sparus aurata*) was the chosen species for this work, since it is one of the most valuable fish species for the aquaculture industry, being widely cultured in the Mediterranean region. Additionally, it constitutes an interesting animal model for research, with high availability and commercial value. This work aimed to reduce the allergenicity of farmed gilthead seabream (*Sparus aurata*) by inducing a calcium-depleted PV through EDTA-supplemented diets. The effect of EDTA over the fish muscle proteome will be addressed and quality as edible fish after this supplementation will be monitored. EDTA's effectiveness in depleting calcium ions from PV will be evaluated through IgE-immunoassays with fish-allergic patients' sera, since specific IgE-antibodies are an indicator of immediate hypersensitivity responses in humans.

4.2.2 Material and methods

4.2.2.1 Fish rearing conditions and sampling procedure

In this experiment, carried out at the Ramalhete Research Station (CCMAR, University of Algarve, Faro, Portugal), three hundred specimens of gilthead seabream with initial body weights of 208.0 ± 2.95 g (mean ± standard deviation) were equally distributed by twelve 500 L conical plastic tanks (25 fish per tank) and reared from June to September 2015. Fish were fed twice a day by hand, *ad libitum*, and tanks were supplied with natural flow-through seawater from Ria Formosa Lagoon, and kept under natural temperature and photoperiod, with artificial aeration (dissolved oxygen above 5 $mg.L^{-1}$), salinity (37 ± 0.39 ‰), and an initial rearing density around 10 kg/m^3 (25 fish per tank). Fish were supplied by a commercial fish farm.

Four different diets were tested: control (CTRL), without EDTA supplementation in the commercial feed, EDTA 3% (EDTA3), EDTA 5% (EDTA5) and EDTA 8% (EDTA8). Experimental conditions were settled in triplicate, with randomly sorted tank numbers. A zootechnical

characterization performed during fish rearing, showed that fish from dietary treatments EDTA5 and EDTA8 did not grow and were not commercially suitable. Thus, samples from these treatments were not considered for further analyses (see Supplementary Material Table S4.2.1).

After 98 days of trial, five random fish from each tank were anesthetized with tricaine methanesulfonate (MS-222; Sigma Aldrich, St. Louis, Missouri, USA) for blood collection. Blood samples of approximately 2 ml were collected from the caudal vein with a heparinized syringe and centrifuged at 2,000 xg for 20 min. Plasma samples were taken and stored at -80°C until posterior analyses. For proteomic analysis, the same fish were then slaughtered with an overdose of anesthetic and muscle samples were collected, immediately frozen in liquid nitrogen, and stored at -80°C. For biochemical measurements, seven random fish from each tank were slaughtered with an overdose of the same anesthetic and stored in polystyrene boxes with ice during the analyses (72 h). Five random fish from each tank were killed, by immersion in water and ice, for sensory analysis with a human panel (EFSA, 2009). This killing method was exceptionally applied in this case, since fish were used for human consumption, according to the Portuguese legislation nº113/2013 – 2013-08-07 and the Council Regulation (EC) nº1099/2009 of 24th September 2009 on the protection of animals at the time of killing.

All fish were weighed and heighted. Prior to slaughter, fish were starved for 48 h to clean the entire digestive tract.

This project was approved by the ORBEA Animal Welfare Committee of CCMAR and the Portuguese National Authority for the Animal Health (DGAV) with the permit no. 0421/000/000/2019 in February 15th 2019. The experiment described was conducted in accordance with the Guidelines of the European Union Council (Directive 2010/63/EU) and the Portuguese legislation for the use of laboratory animals, and under a “Group-1” license (permit number 0420/000/000-n.99- 09/11/2009) from the Veterinary Medicine Directorate, the competent Portuguese authority for the protection of animals, Ministry of Agriculture, Rural Development and Fisheries, Portugal.

4.2.2.2 Cortisol measurement

Plasma cortisol was measured (*n* = 10) using a commercial Cortisol ELISA kit RE52061

(IBL International, Hamburg, Germany), following the manufacturer's instructions. The plate was read at 450 and 620 nm along with a prepared standard curve on a microplate reader Biotek Synergy 4 Hybrid Technology™ (Biotek Instruments Inc, Winooski, USA).

4.2.2.3 Biochemical and quality characterization of fish muscle

Muscle pH measurements were performed (*n* = 9), using a waterproof pH spear for food testing (Oakton® Instruments, Nijkerk, Netherlands), in the dorsal muscle, at 0, 1, 2, 4, 6, 8, 24, 48 and 72 h *post-mortem*, approximately 1-2 cm apart.

At the same *post-mortem* periods, *rigor mortis* index was assessed (*n* = 12) as previously described (Matos et al., 2010).

Instrumental texture measurements were performed (*n* = 14) using a texturemeter analyzer (TA.XTplus Texture Analyzer, Stable Micro Systems, Surrey, UK), at room temperature, with raw fish muscle portions (approximately 4 x 2.7 x 1.5 (height) cm). Compression test was performed with a flat-ended cylindrical metal probe P75, with 75 mm diameter, equipped with a load cell of 30 kg (approximately 24 h after death). Muscle portions were compressed by 40 % of their height at a speed of 2 mm/s. Two compressions were performed (texture profile analysis – TPA) on each sample giving the texture parameters hardness, cohesiveness, adhesiveness, springiness and chewiness.

Sensory analysis was conducted in an acclimatized test room, equipped with individual booths, and several attributes were analyzed by a trained panel (*n* = 10 panelists; 7 women, aged within the range 32 – 53 years old). Fish samples (identical muscle portions to the ones used in texture measurements) were steamed, without spices or salt addition, for 7 min, at 98°C, in a steam oven (Rational Combi-master CM6). Samples were coded and presented in white dishes under normal white light to the panelists who rated the intensity of several attributes/descriptors, such as odor, flavor, appearance and texture, on a five points scale (0 – absent; 1 – slight; 2 – moderate; 3 – intense; 4 – strong). A description of attributes and terminology used were discussed with the panelists. Results were reported as the average of scores. Texture and sensory analyses were performed at Instituto Português do Mar e da Atmosfera (IPMA), Lisbon, Portugal.

4.2.2.4 High-performance liquid chromatography (HPLC) determination of EDTA

EDTA accumulation in the muscle was assessed by reversed-phase-high-performance-liquid-chromatography-diode-array-detection (RP-HPLC-DAD). Approximately 0.5 g of muscle samples from CTRL and EDTA3 conditions (*n* = 15) were freeze-dried, added 0.2 ml of ferric chloride solution and diluted with 4 ml of Milli-Q water. Sample solutions were homogenized with an Ultra-Turrax IKA T8 (IKA-WERG, Germany), shaked for 5 min and heated in an oven at 70°C for 30 min. Following, centrifugation was performed at 1300 xg for 15 min and the supernatant filtered with PVDF filters (polyvinylidene-difluoride, 0.22 µm) prior to injection. EDTA determination was performed as previously described (Chiumiento et al., 2015), with minor changes. Briefly, data were acquired using an AZURA® HPLC system (KNAUER Advanced Scientific Instruments GmbH, Berlin, Germany) equipped with a manual sampler with a 50 µl sample loop, a multi-solvent delivery system, a thermostatic column oven and a diode array detector (UV detector 2600). Flow rate was set to 1.2 ml/min with a running time of 5 min for EDTA standard solutions, and 10 min for sample solutions. The column used was a Phenomenex Kinetex C18 (Phenomenex, Torrance, USA), 150 x 4.6 mm, particle size 2.6 µm and the injection volume was 20 µl. The limit of detection (LOD) and limit of quantification (LOQ) were set at 5.5 and 25 ppm, respectively. Data is presented in mean ± SD (ppm).

4.2.2.5 Muscle proteome analysis

4.2.2.5.1 Protein extraction

For protein extraction, 100 mg of muscle tissue from each sample (two samples from each tank, randomly picked) was dissolved in 500 µl of DIGE buffer (7M urea, 2M thiourea, 4% CHAPS, 30 mM Tris pH 8.5) as extraction buffer, along with 5 µl of protease inhibitor cocktail (Sigma Aldrich, St. Louis, Missouri, USA) and 2 µl of EDTA 250 mM. Tissues were homogenized with an Ultra-Turrax IKA T8 (IKA-WERG, Germany) for 5 cycles of 10 sec, followed by an incubation period on ice for 1h, and 5 pulses of sonication with ultrasounds. After centrifugation at 13,000 g, 4°C for 20 min, supernatant was collected and total protein content measured with Bradford assay using BioRad Quick Start Bradford Dye Reagent 1X (Bio-Rad Laboratories, Hercules, California, USA) and bovine serum albumin (BSA) as standard, BioRad Bovine Serum Albumin Standard Set (Bio-Rad Laboratories, Hercules, California, USA).

4.2.2.5.2 Protein labeling

Samples' pH was checked with a pH-indicator paper, Sigma-P4536 (Sigma Aldrich, St. Louis, Missouri, USA) and adjusted to 8.5 using 0.1 M NaOH. For DIGE minimal labeling, 50 µg of protein from each sample was labeled, as described by the manufacturer, using CyDye™ DIGE Fluor minimal labeling kit 5 nmol (GE Healthcare, Little Chalfont, UK), with 400 pmol fluorescent amine reactive cyanine dyes freshly dissolved in anhydrous dimethylformamide (DMF). Labeling was performed on ice for 30 min, in the dark, and the reaction quenched with 1 mM of lysine for 10 min. Six samples per experimental condition were labeled with Cy3 and six with Cy5 in order to reduce impact of label difference, while an internal standard consisting of a pool of all samples, with equal amounts, was labeled with Cy2. Samples were randomly sorted in order to reduce variable confounding, due to inherent dye-dependent bias.

4.2.2.5.3 Protein separation by 2D-E

Labeled proteins from each experimental condition were mixed with the internal standard and rehydration buffer (8 M urea, 2% CHAPS, 50 mM DTT, 0.001% bromophenol blue, 0.5% Bio-lyte 3/10 ampholyte (Bio-Rad Laboratories, Hercules, California, USA) to complete 450 µl. Passive rehydration was conducted for 15 h on 24 cm Immobiline™ Drystrips (GE Healthcare, Little Chalfont, UK) with linear pH 3-7, on an IPG Box (GE Healthcare, Little Chalfont, UK). Rehydration was followed by isoelectric focusing (IEF), performed in 5 steps: 500 V gradient 1 h, 500 V step-n-hold 1 h, 1,000 V gradient 1 h, 8,000 V gradient 3 h and 8,000 V step-n-hold for a total of 60,000 Vhr using Ettan IPGphor at 20°C (GE Healthcare, Little Chalfont, UK). Prior to second dimension, strips were reduced and alkylated with 6 ml of equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol and 2% SDS) with 1% (w/v) ditiotreitol (DTT) or 2.5% (w/v) iodoacetamide (IAA) respectively for 15 min each, in constant agitation. Strips were then loaded onto 12.5% Tris-HCl SDS-PAGE gels and ran in an Ettan DALT*six* Large Vertical System (GE Healthcare, Little Chalfont, UK) at 10 mA/gel for 1 h followed by 60 mA/gel until the bromophenol blue line reaches the end of the gel, using a standard Tris-Glycine-SDS running buffer. Two rounds were performed, six gels each.

4.2.2.5.4 Gel image acquisition and analysis

Gels were scanned on a Typhoon laser scanner 9400 (GE Healthcare, Little Chalfont, UK) using three laser emission filters (520 BP40 for Cy2, 580 BP30 for Cy3 and 670 BP30 for

Cy5) at 100 µm resolution, with emission and excitation wavelengths recommended by the manufacturer. Gel images were analyzed using SameSpots software (Totallab, Newcastle, UK). Analysis includes background subtraction, filtering, spot detection, spot matching, normalization and statistical analysis. Statistical significance was assessed using Student's T-test ($p < 0.05$) and average fold-ratio (ratio > 1.0). Resultant protein spots with statistically different intensities were considered for further manual excision and identification by mass spectrometry (MS).

4.2.2.5.5 Protein identification

Protein spots with statistically different intensities were excised, reduced with DTT and alkylated with IAA, followed by digestion with trypsin. After stopping the digestion with trifluoroacetic acid, the tryptic peptides were desalted, concentrated, co-crystallized with a matrix on a MALDI plate and analyzed with a MALDI-TOF/TOF-MS Ultra fleXtreme (Bruker Corporation, Billerica, Massachusetts, USA). Automatic spectra were obtained with software Flex control™ vs3.4 (Bruker Corporation, Billerica, Massachusetts, USA), externally calibrated, and analyzed with Flex analysis™ vs3.4 (Bruker Corporation, Billerica, Massachusetts, USA). After conversion to MS and MS/MS peak lists, search was performed on SwissProt database restricted to Actinopterygii taxonomies with 100 ppm of mass error tolerance in MS and MS/MS precursor, and 0.3 Da tolerance on MS/MS fragments. A second search was made with the same parameters on NCBI database. Proteins were identified by peptide mass fingerprinting (PMF) and peptide fragment fingerprinting (PFF) on a Mascot server vs2.2.06 (Matrix Science Ltd, London, UK). Protein identification was performed in GIGA Research Centre, University of Liège, Liège, Belgium, as an external service.

4.2.2.6 IgE-reactivity to PV with fish-allergic patients' sera

4.2.2.6.1 Patient sera samples

Twenty (12 male, 8 female; mean age 21.5 years) patients with fish allergy were recruited at the Centre Hospitalier de Luxembourg (CHL). The study was approved by the National Committee for Medical Research Ethics in Luxembourg (Ref. 201307/04) and all participants signed the informed consent. Inclusion criteria for study participation were a clinical history of an IgE-mediated codfish allergy, a positive skin prick test with cod extract

and a positive specific IgE-titer to cod extract (mean titer 11.2 kU_A/L) as detected by ImmunoCAP (>0.35 kUA/L positive; Phadia-ThermoFisher, Uppsala, Sweden).

4.2.2.6.2 Protein extraction

For total protein content extraction, 500 mg of muscle tissue were mixed with 1 ml of single detergent lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl) including a protease inhibitor (Complete tablets EDTA-free Easypack, Roche, Basel, Switzerland) and 1 steal bead of 5 mm diameter, and the tubes placed on a Tissue Lyser II (Qiagen, Hilden, Germany) following manufacturer's instructions with minor changes, as described below. Samples were homogenized with two cycles of 10 min at 25 Hz, followed by a 1 h period of incubation at 4°C on an orbital shaker, and then centrifuged at 20,000 xg for 10 min. The supernatant was collected and the total protein content quantified using the Bradford method, with BSA as standard protein.

Extracts from each sample were separated on a 7 cm SDS-PAGE gel (15%) for 40 min at 200 V followed by Coomassie blue staining (Pierce, Erembodegem, Belgium), to check and compare protein expression patterns and the quality of the extracts.

4.2.2.6.3 1D-PAGE IgE immunodetection

IgE immunoblots using patient sera were performed as described before (Kuehn et al., 2016) (Kuehn et al., 2016). Briefly, two pools of muscle samples from CTRL and EDTA3 were prepared and 450 µg of the protein extracts were loaded on 7 cm preparative SDS-PAGE gels (15%), 2 preparative gels for each fish, and separated in two steps: 100 V for 20 min followed by 200 V for 55 min. Resultant gels were blotted onto 0.22 µm PVDF membranes (Millipore, Burlington, Massachusetts, EUA) for 1 h at 14 V, and blocked in buffer containing 3% BSA overnight at 4°C. Membranes were cut using an Accutran Strip Cutter (Inotech, Nabburg, Germany) and each immunoblot stripe incubated with 700 µl of patient serum (1:3 diluted in blocking buffer containing 3% BSA) overnight, at room temperature and constant agitation. A mix of two in-house antibodies (1:10,000; rabbit pAb against cod/salmon PV; rabbit pAb against herring/mackerel PV) was used as positive control for PV-detection (C). Following, immunoblot stripes were washed 4 times for 5 min each with Tris-buffered saline/Tween-20 (TBS-T) buffer. Each patient stripe was incubated with 1 ml of anti- human IgE Southern Biotech 9160-04-alkaline phosphatase (AP) antibody (1:10,000) for 2 h at room temperature

with constant agitation. Control strip was incubated with a mix of anti-mouse IgG A2429-AP (1:10,000) and anti-rabbit IgG Sigma A3812-AP (1:10,000). Alkaline phosphatase buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) was used for the revelation with addition of AP substrate solution (nitro-blue tetrazolium, NBT: 5- bromo-4-chloro-3'-indolyphosphate, BCIP) (Promega, Madison, WI) to perform a total volume of 1 ml. The colorimetric reaction was stopped by washing with TBS-T buffer.

4.2.2.7 Statistical analysis

To assess normality of distributions, a Shapiro-Wilk test was applied, while homoscedasticity assumption was checked by Levene's test. Cortisol data were primarily subjected to Grubb's test for outliers' detection. Statistical significance in plasma cortisol levels was assessed using Student's T-test. *Rigor mortis* data were previously transformed by arcsine square root. Differences in pH, *rigor*, and texture, between conditions, were assessed using Student's T-test, if homogeneity of variances was verified. In case any assumption was violated, data was subjected to a non-parametric Mann-Whitney U test for statistical significance. Results are presented in mean ± standard deviation (S.D.). Statistical analyses were performed using SPSS Statistics 25 software (IBM Analytics, Armonk, New York, USA) for MacOSX. Data regarding sensory analyses was subjected to the non-parametric Mann-Whitney U test using STATISTICA analytics 8.0 (StatSoft) for Windows. A significance level of $\alpha = 0.05$ was used in all tests performed.

4.2.3 Results and discussion

4.2.3.1 Zootechnical characterization and the EDTA effect on gilthead seabream growth performance

A negative effect of high EDTA concentrations was observed over the fish growth performance and feed efficiency. Growth and daily feed intake tended to be lower the higher the EDTA supplementation. Fish raised in tanks of EDTA8 condition were recorded to have the lowest growth rate and the highest mortalities. A one-way analysis of variances (ANOVA) performed to the final body weight showed significant differences between all experimental conditions ($p < 0.05$) (See Supplementary Material Table S4.2.1). Samples from dietary treatments EDTA5 and EDTA8 were not considered for the variables in this study, once growth

performance showed that fish from these conditions were not commercially suitable and was not possible to ensure that the results obtained were induced by EDTA supplementation and not a consequence of the observed low feed intake. However, EDTA supplementation did not affect fish growth rates when applied in low concentrations (EDTA3). Values from fish specific growth rate (SGR) belonging to CTRL and EDTA3 experimental conditions correspond to previous nutritional studies with gilthead seabream (unpublished results), presenting similar initial body weights and reared under the same conditions of temperature and salinity (De Francesco et al., 2007, Fountoulaki et al., 2009) (De Francesco et al., 2007; Fountoulaki et al., 2009). Contrarily, fish from EDTA5 and EDTA8 conditions showed low and negative growth rates, respectively, which is supported by the feed conversion ratio (FCR) obtained. FCR is a major indicator of feed efficiency in fish farming and related research. Its values are lower, the higher the weight gain obtained with certain feed. FCR values for CTRL and EDTA3 fish correspond to previously reported for this species (De Francesco et al., 2007, Fountoulaki et al., 2009). Low palatability, digestibility and unpleasant odor might be in the origin of the low feed intake and negative weight gain observed. Further studies and improvements must be performed as the addition of an attractive ingredient or EDTA microencapsulation might favor the diet acceptability by the fish.

4.2.3.2 Plasma cortisol quantification and the physiological response to stress

No significant differences (p = 0.788) were found in plasma cortisol between both conditions (5.18 ± 4.85 and 5.78 ± 4.09 ng/ml in CTRL and EDTA3, respectively), suggesting that EDTA level included in the diet had no impact over the plasma cortisol concentration (Figure 4.2.1A). A high standard deviation was verified due to an enormous biological variability. Minimizing stress conditions and preserving fish welfare is nowadays one of the main concerns in aquaculture, since growth performance and health status are strongly associated with the stress status of the animal. Several stressors have been extensively reported in aquaculture: *pre-* and *post-mortem* handling practices, high stocking densities, presence of diseases, among others (Alves et al., 2010). Results of the current study show that gilthead seabream exposed to EDTA did not experienced stress, as indicated in plasma cortisol levels in comparison with the basal levels of control fish. No literature regarding the effect of EDTA on cortisol release and fish welfare was found. Plasma cortisol concentration levels

measured in fish from both treatments are according to the reference levels for this species (Alves et al., 2010, Yildiz, 2009). However, both groups registered a high individual variability most likely due to individual differences in stress responses.

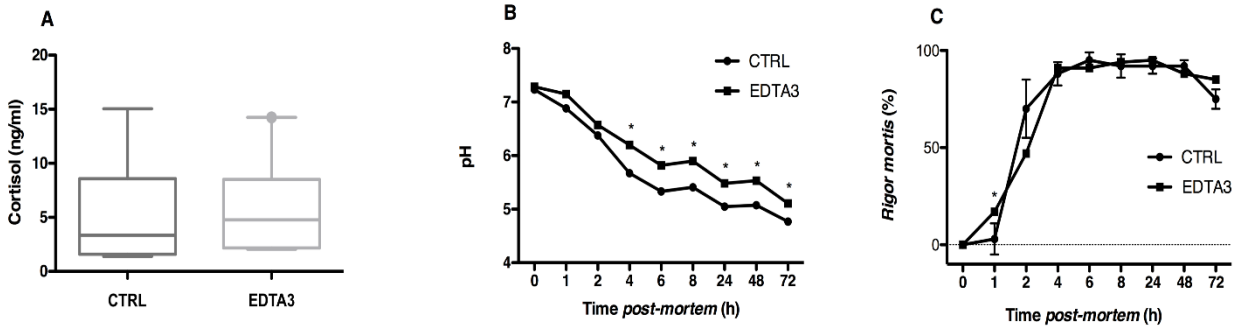


Fig. 4.2.1 A - Plasma cortisol levels in blood samples from gilthead seabream (*Sparus aurata*) submitted to control and EDTA supplemented feeds (*n* = 10). The box includes observations from 25th to the 75th percentiles; the horizontal line within the box represents the median value. Lines outside the box represent the 10th and 90th percentiles. No significant differences were found among conditions ($p > 0.05$). B - *Post-mortem* changes in muscle pH of gilthead seabream (*Sparus aurata*) stored in ice for 72 h. Data points are the mean of n = 9 for each sampling time. *$P < 0.05$ vs. control at 4, 6, 8, 24, 48 and 72 h *post-mortem.* C - Development of *rigor mortis* of gilthead seabream (*Sparus aurata*) stored in ice for 72 h. Data points are the mean of n = 12 for each sampling time. *$P < 0.05$ vs. control at 1 h *post-mortem*.

4.2.3.3 Biochemical and muscle quality characterization

Flesh quality of the fish product was determined in this study by several physical-chemical parameters. Several authors have reported the properties of EDTA as a shelf-life extender in fish, added at the storage step, but only a few have studied its effect on other quality attributes and in particular on the organoleptic properties of fish flesh (Ghaly et al., 2010). Muscle pH declined between T0 and T72, ranging from pH 7.2 and 7.3 after slaughtering to 4.8 and 5.1, in fish from CTRL and EDTA3 groups, respectively (Figure 4.2.1B). Significant differences between both conditions were found at 4, 6, 8, 24, 48 and 72 h *post-mortem* ($p < 0.05$). Results from the present study followed the same pH trends as previous studies on the same species. The muscle pH registered immediately after slaughtering are concordant with the values reported by Bagni et al. (2007), who registered an initial muscle pH ranging from 7.0 to 7.3 in gilthead seabream 30 months aged. Nevertheless, pH values registered at 48 h *post-mortem,* 5.1 and 5.5, for CTRL and EDTA3 respectively, are below the reported by Matos et al. (2013) and Silva et al. (2012b), at the same time. The values 4.8 and

5.1, for CTRL and EDTA3, respectively, at the last measuring time, are below the so-called ultimate pH considered around 5.5, suggesting a total depletion of glycolytic energy reserves. Lower pH levels indicate higher levels of glycogen in the muscle immediately after slaughtering (Toldrá, 2010).

The onset of *rigor mortis* (Figure 4.2.1C) occurred 1 h *post-mortem* in both groups, when muscle pH registered 6.9 and 7.1 for CTRL and EDTA3 groups, respectively. Maximum *rigor* strength was reached at 6 h *post-mortem* in both groups, with 95 ± 3%, and 96 ± 4% in CTRL and EDTA3, respectively, which is in accordance with Matos et al. (2013). Significant differences were observed between both conditions at 1 h *post-mortem* ($p < 0.05$). The process of *rigor mortis* occurs when ATP levels in muscle decrease. When reaching low levels, actin and myosin bind together to form the actomyosin complex inducing stiffness of the fish body. Contrarily, resolution of *rigor mortis* makes fish muscle less rigid, losing its elasticity (Delbarre-Ladrat et al., 2006). In this study, the measurement at 72 h was performed since rigor was still on at 48 h *post-mortem*. The onset of *rigor mortis* started 1 h after slaughtering, which is consistent with previous studies (Venugopal, 2002). The non-resolution of this state after 72 h is not consistent with the 36 h reported for seabass stored at 0°C (Delbarre-Ladrat et al., 2006).

Significant differences observed in muscle pH and *rigor mortis* between both conditions can possibly be explained by a delayed tenderization of the fish muscle. Tenderization is the process fish muscle undergoes within hours after death, responsible for the softening of the muscle through the degradation of key structures in the muscle sarcomere (Delbarre-Ladrat et al., 2006). This can be due to a low availability of calcium ions caused by the presence of EDTA. The onset of *rigor mortis* along with the pH drop, leads to an increase in sarcoplasmic calcium ions content, from 0.1-0.8 µM to about 0.2 mM, which will induce the activation of calcium-dependent proteases (e.g. collagenases, calpains and cathepsins). This increased protease activity is responsible for the destruction of Z-disks and the actomyosin complex, causing softening of the flesh and the resolution of *rigor mortis* (Salem et al., 2004). Calcium also induces Ca^{2+}-dependent phosphorylase kinase, which is responsible for the regulation of glycolysis, by activating the glycogen phosphorylase. This enzyme catalyzes glycogen molecules consequently lowering the pH (Berg et al., 2002). Results observed in this study for the pH and *rigor* measures might suggest an EDTA effect: a possible low availability of free calcium ions due to the presence of EDTA, leads to a non-

activation/delayed activation of the calcium-dependent proteins, consequently slowing the rate of pH drop (Ayala et al., 2010, Delbarre-Ladrat et al., 2006) (see Supplementary Material Fig. S4.2.1).

EDTA supplementation affected significantly the instrumental texture of raw gilthead seabream muscle (Table 4.2.1). Five textural parameters were evaluated, whereas 3 demonstrated significant differences between both conditions, being these adhesiveness (p = 0.029), springiness (p = 0.018) and chewiness (p = 0.017). Fish muscle texture is one of the most important quality parameters and has a higher impact on consumers' acceptance (Delbarre-Ladrat et al., 2006). Instrumental texture of gilthead seabream muscle suggests a possible EDTA effect, since 3/5 textural parameters revealed significant differences between conditions (p < 0.05). Values obtained for hardness, in both conditions, are practically 2-fold higher than the reported for seabream, with similar body weights, stored in ice (Ayala et al., 2010). The ones registered for springiness are about 3-fold lower, while the registered for cohesiveness and adhesiveness are consistent. The same relation is observed with other studies (Ayala et al., 2011).

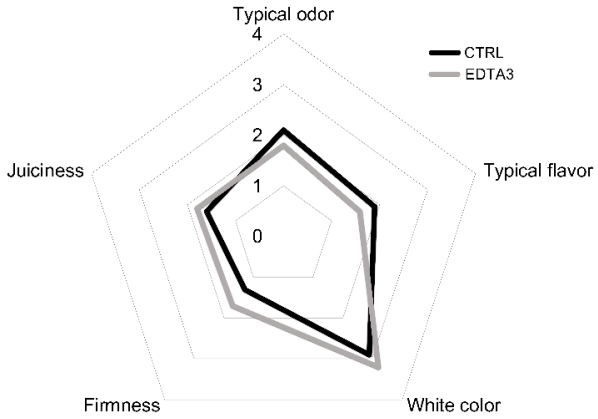
Table 4.2.1. Instrumental texture parameters of gilthead seabream (*Sparus aurata*) muscle after 24 h storage in ice.

Texture (n = 14)	**Experimental treatment**	
	CTRL	EDTA3
Hardness (N)	53.93 ± 9.39	56.91 ± 11.51
Adhesiveness	-0.58 ± 0.13[a]	-0.47 ± 0.11[b]
Springiness	0.64 ± 0.03[a]	0.60 ± 0.04[b]
Cohesiveness	0.40 ± 0.04	0.40 ± 0.05
Chewiness	13.67 ± 2.09[a]	13.48 ± 2.72[b]

Values are means ± standard deviations (n = 14). In each row values with different letters are significantly different (p <0.05).

Five descriptors were selected during the sensory evaluation. The terms "typical odor" and "typical flavor" were used to describe the attributes odor and flavor, the color "white" was selected for appearance, while "firmness" and "juiciness" were the descriptors employed for texture. The panelists found no differences between the organoleptic properties of fish fed commercial and EDTA supplemented diets (Figure 4.2.2). The typical odor and flavor of the fish were present in only 70% and 90% of the samples belonging to EDTA3 group and two members of the panel indicated that the white color of EDTA3 fish flesh was not the

characteristic white but instead, very intense, “almost milky”. Half of the panel did not perceive different odors, flavors or colors on CTRL samples, contrarily to 30% in EDTA3 samples. No significant differences were found between the sensorial attributes of both conditions ($p > 0.05$).



Intensity scale: 0 – Absent, 1 – slight, 2 – moderate, 3 – intense, 4 – very intense

Fig. 4.2.2. Sensory profile of gilthead seabream (*Sparus aurata*) muscle from CTRL (black line) and EDTA3 (grey line) experimental conditions. Scores of the attributes are given as absent-very intense. No significant differences were found between treatments ($p > 0.05$).

4.2.3.4 EDTA accumulation in fish muscle

In order to ensure that EDTA was not retained in the fish muscle in toxic quantities and was safe for human consumption, the concentration was determined in fish muscle samples from CTRL and EDTA3 groups. Values were below the LOD and LOQ in the control samples; the average registered for the muscle samples of fish fed with diets supplemented with 3% EDTA was 66.6 ± 1.95 ppm (Supplementary Material Table S4.2.2). These levels are below the maximum concentrations allowed in fish products for human consumption in the EU (120 ppm) (INERIS, 2012).

4.2.3.5 IgE immunoblots

Comparative tests of IgE-reactivity showed differences between CTRL (Figure 4.2.3A) and EDTA3 samples (Figure 4.2.3B). A total of 17 patients’ sera resulted in a band in the molecular weight corresponding to PV (ca. 10 kDa), testing positive for IgE-reactivity to gilthead seabream PV in CTRL samples, whereas only 8 had detectable IgE-binding signals for

PV in EDTA3 samples (indicated by a black star in figure 4.2.3). A positive control using a mix of anti-PV antibodies (C) confirmed the presence and identity of the allergen, in both conditions, at the molecular weight around 10-12 kDa, which is also in concordance with the theoretical Mw of gilthead seabream PV (UniprotKB accession number: D0VB96). Parvalbumin-negative patients (3 out of 20 patients) showed other visible bands (ca. 21, 31 or 45 kDa) (data not shown), suggesting the presence of IgE antibodies to other fish allergen proteins (e.g. enolase from gilthead seabream presents a Mw of 28 kDa). Sequencing of these bands would have to be performed to confirm protein identity. No IgE-reactivity to the EDTA3 samples was found for the last 9 patients. Results suggest that PV epitope regions were affected by the EDTA-supplemented diet, possibly originated by calcium depletion, which is known to result in lower IgE-reactivity (Kubota et al., 2016, Tomura et al., 2008). Further analysis of the calcium-binding status of PVs from control and EDTA3-fish will be required to confirm this hypothesis. In a follow-up study, an IgE-ELISA with the purified allergen would have to be performed to corroborate the reduction in the IgE-binding capacity upon EDTA presence. Additionally, patient's sera with clinical reactivity to gilthead seabream would improve the results.

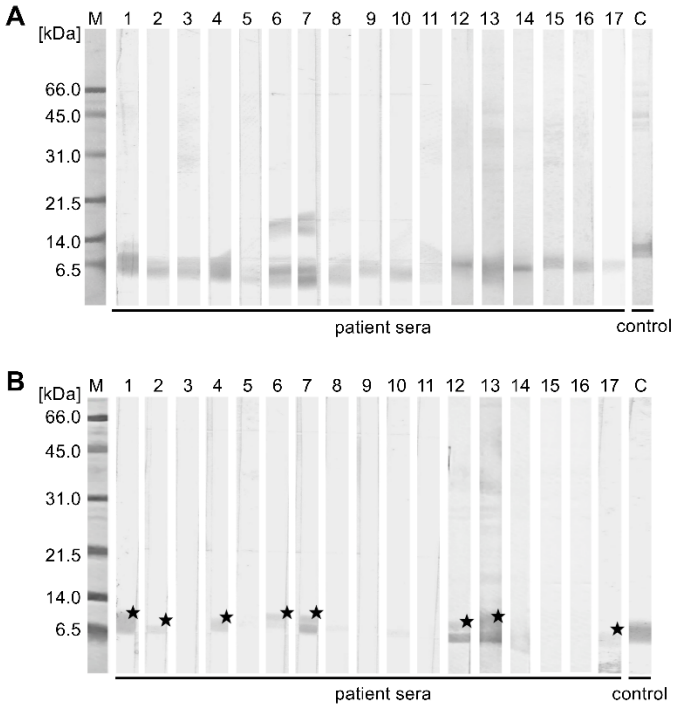


Fig.4.2.3. IgE-reactivity of single patient sera (1-20) to gilthead sea bream (*Sparus aurata*) PV from control (A) and EDTA3 (B) experimental conditions. The black star indicates positive IgE-binding signals. Control antibodies (C) were used to detect PV. M, marker

4.2.3.6 Effect of EDTA over the gilthead seabream muscle proteome

Total protein content of gilthead sea bream muscle revealed very clear 2D gels, where a statistical analysis to protein spots' intensity identified 2 spots as significantly different between the experimental conditions ($p < 0.05$). After spots' excision and MS analysis, identification for 1 protein spot was obtained, which was then classified according to their cell function as belonging to the contractile apparatus. Figure 4.2.4 shows a representative 2D gel of the muscle proteome from gilthead seabream, with the identified spot circled. This spot, identified by PMF in a MALDI-TOF/TOF MS as tropomyosin, registered a theoretical Mw of 18 kDa, a pI of 5, and the higher normalized spot volume in fish fed with EDTA 3% enriched diets. Myosin molecules, composed of two heavy and four light chains subunits, constitute the myosin filaments in muscle cells, whose heads, located on the surface of the filament, interact with actins for muscle contraction. Tropomyosins, found in the fish fast skeletal muscle, form a family of actin-binding proteins, responsible for covering the myosin binding sites on actin filaments. In the presence of calcium ions, tropomyosins uncover the myosin-binding sites, by binding to the troponin complex. The myosin heads attach to the thin filaments of actin, sliding horizontally and originating muscle contraction (Cooper and Hausman, 2007). The up-regulation of this protein can be explained by the reduced proteolysis of the myofibrillar proteins, during the tenderization process, due to the presence of EDTA, as explained above.

Parvalbumin was identified in spot Ex85 (data not shown) with a calculated pI matching the theoretical one and a calculated Mw of 8403 Da, 3 kDa lower than the theoretical one. PV's expression was not significantly different between conditions ($p > 0.05$). EDTA was used to induce a rearrangement of the allergen structure, a calcium-depleted hypoallergenic conformation, rather than reducing its expression. Comparative proteomics showed no significant differences in parvalbumin's spot intensity in fish muscles from different EDTA-conditions. Its location in the representative 2D gel of the muscle proteome is circled in dashed line, obtained in previous identifications (data not shown). Mw (~12kDa) and pI (~5) are consistent with the theoretical values, obtained through the protein accession number.

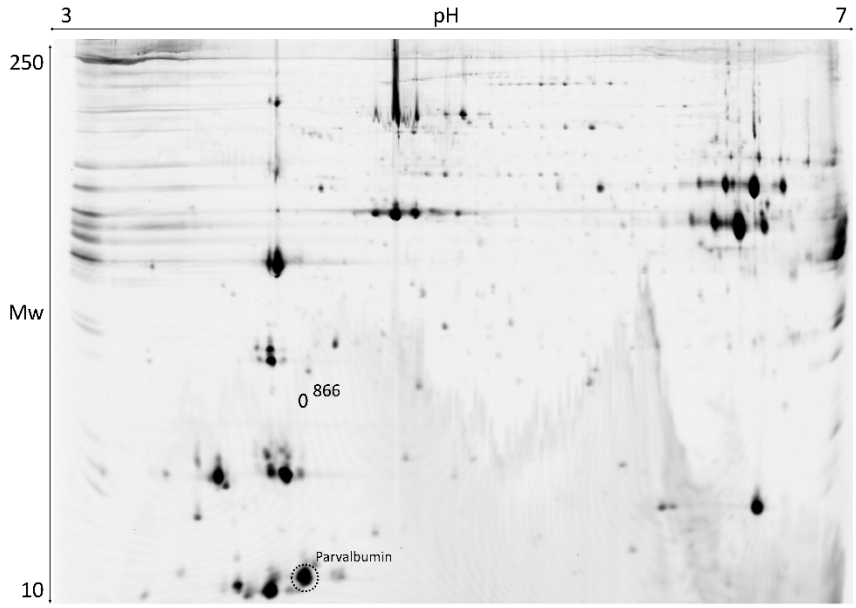


Fig. 4.2.4. Representative 2D gel of gilthead sea bream (*Sparus aurata*) muscle on a 12.5% polyacrylamide 2D gel. Circled spots, representing significantly different expressed spots (p<0.05) between different conditions, were sequenced and identified (Table 4.2.2).

4.2.4 Conclusions

Conclusively, this preliminary study revealed a novel, non-GMO approach and a promising strategy to achieve potential hypoallergenic fish, using targeted aquaculture techniques. IgE-tests with fish-allergic patients sera showed that EDTA3-fish has decreased the biological IgE-reactivity, without impact on fish growth performance, muscle proteome and organoleptic properties. Conformational PV studies (e.g., circular dichroism analysis, IgE test by ELISA using purified PVs from control and EDTA3-fish), will be required to better understand the correlation of IgE-reactivity and calcium-binding. *In vitro* digestibility tests, more specifically, a resistance to pepsin digestion test with the calcium-free-parvalbumin fill further shed light on the allergenic potency of this protein. Furthermore, in order to optimize and confirm the allergenicity reduction, complementary analysis on a fish allergy animal model, skin-prick tests, basophil histamine release assays and at the very end double-blind placebo-controlled food challenge (DBPCFC) in human would be required. Additionally, comparative proteomics demonstrated that 3% concentrations of EDTA modulated the expression of a single protein showing that EDTA at this concentration was able to reduce the IgE-reactivity without affecting the fish muscle metabolism.

Table 4.2.2. Differently expressed ($p < 0.05$) muscle proteins between gilthead seabream (*Sparus aurata*) submitted to control and EDTA 3% supplemented diets, identified by MALDI-TOF/TOF MS after separation by 2D-DIGE. Differences in expression are indicated in the "fold change" column.

Spot n°	**Identified protein (species)**	**NCBI accession number**	**Score[1]**	**Mw T/C[2]**	**pI T/C[3]**	**Sequence coverage (%)[4]**	**Fold change (Highest volume)[5]**	**FDR[6]**	**N° of matched peptides**	**Best peptide match sequence**	**Protein function**
Contractile apparatus											
866	PREDICTED: tropomyosin alpha-1 chain isoform X1 [*Astyanax mexicanus]*	gi|597755671	80	32774/ 18255	4.69/ 4.9	35	-1.52 (EDTA3)	0.0447	6	K.LDKENALDR.A	Calcium-dependent regulation of muscle contraction

[1]Score – a non-probabilistic protein score obtained from the ions score

[2]Theoretical (T) and calculated (C) molecular weight (Mw)

[3] Theoretical (T) and calculated (C) isoelectric point (pI)

[4]Sequence coverage (%) -percentage of protein covered by matched peptides

[5]Fold change (Highest volume) - Significant changes in protein abundance across conditions

[6]FDR – False discovery rate

[7]Nº of matched peptides –Nº of peptides matched to entry with significant *E*-value < 0.05

To our knowledge, this was the first work reported regarding the use of supplemented diets to induce a calcium-free PV fish and achieve hypoallergenicity, however, this preliminary experiment can be the origin of a wide range of future works. In the future, it can improve the knowledge regarding the effect of EDTA on fish allergenicity modulation and contribute to the development of an allergen-specific therapy and consequent reduction of a worldwide prevalent allergy.

4.2.5 Acknowledgments

Tanja Scheuermann, Thorsten Graf and Dominique Revets, from Luxembourg Institute of Health, for the technical support and scientific guidance. This work has been financially supported by project ALLYFISH (Ref. 16-02-01-FMP-0014-ALLYFISH: Desenvolvimento de um peixe de aquacultura com reduzido teor alergénico – Mar 2020) and received Portuguese national funds from FCT - Foundation for Science and Technology through project UID/Multi/04326/2019. Cláudia Raposo acknowledge financial support through a research grant within the project ALLYFISH (Ref. 16-02-01-FMP-0014-ALLYFISH: Desenvolvimento de um peixe de aquacultura com reduzido teor alergénico – Mar 2020). Denise Schrama acknowledge financial support through a research grant within the project ALLYFISH (Ref. 16-02-01-FMP-0014-ALLYFISH: Desenvolvimento de um peixe de aquacultura com reduzido teor alergénico – Mar 2020).

4.2.6 Supplementary Material

Table S4.2.1 Growth performance and feed utilization of gilthead seabream (*Sparus aurata*) reared for 98 days under different feeding conditions. Values are mean ± SD (n = 75).

Specification	Conditions			
	Control	EDTA 3%	EDTA 5%	EDTA 8%
Initial Body Weight – IBW (g)	208.0 ± 4.0	209.3 ± 2.0	208.0 ± 4.0	206.7 ± 2.3
Final Body Weight – FBW (g)	396.1 ± 8.0^{a}	314.6 ± 18.0^{b}	257.6 ± 12.2^{c}	202.4 ± 13.0^{d}
Body weight gain (%IBW d^{-1})	0.9 ± 0.0	0.5 ± 0.0	0.23 ± 0.05	-0.03 ± 0.06
Specific Growth Rate – SGR (% d^{-1})	0.7 ± 0.0	0.4 ± 0.0	0.22 ± 0.03	-0.02 ± 0.06
Feed Conversion Ratio – FCR (g/g)	1.7 ± 0.0	2.3 ± 0.0	3.67 ± 0.66	-1.20 ± 8.74
Feed Efficiency – FE (g/g)	0.6 ± 0.0	0.4 ± 0.0	0.28 ± 0.06	-0.09 ± 0.19
Voluntary Feed Intake – VFI (%IBW d^{-1})	1.5 ± 0.0	1.1 ± 0.0	0.81 ± 0.06	0.33 ± 0.02

Table S4.2.2 EDTA accumulation in the muscle of gilthead sea bream (*Sparus aurata*), per sample. Values are in ppm (n = 15).

Conditions	Tanks	Samples				
		Fish 1	Fish 2	Fish 3	Fish 4	Fish 5
CTRL	Tank 1	< LQ	<LD	<LQ	<LD	<LD
	Tank 6	< LD	<LD	<LD	<LQ	<LQ
	Tank 12	< LQ	<LQ	<LQ	<LQ	<LQ
EDTA 3%	Tank 2	69.9	66.2	68.9	61.5	69.9
	Tank 5	63.9	63.4	66.0	67.1	70.6
	Tank 11	64.5	66.6	65.2	67.5	68.1

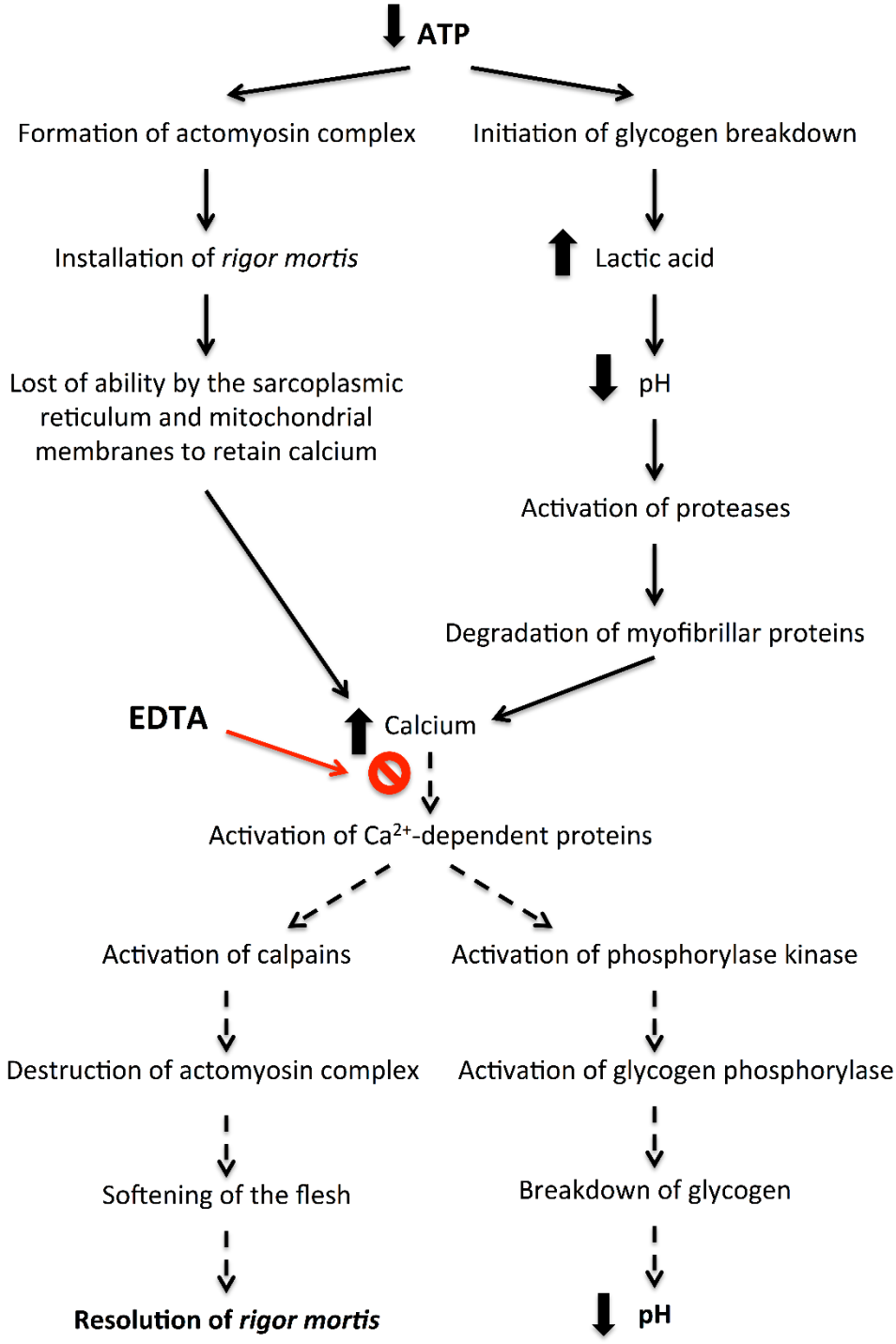


Fig. S4.2.1. Sequence of *post-mortem* events fish muscle undergoes immediately after death, responsible for flesh softening, and dissolution of rigor mortis. In the presence of EDTA, the concentration of free calcium ions might be reduced causing a delaying/suppression of the further steps (dashed arrows).

Chapter 4.3

Effect of Creatine and EDTA supplemented diets on European seabass (*Dicentrarchus labrax*) allergenicity, fish muscle quality and omics fingerprint

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Effect of Creatine and EDTA supplemented diets on European seabass (*Dicentrarchus labrax*) allergenicity, fish muscle quality and omics fingerprint

Abstract

The relatively easy access to fish worldwide, alongside the increase of aquaculture production contributes to increased fish consumption which result in higher prevalence of respective allergies. Allergies to fish constitute a significant concern worldwide. β-parvalbumin is the main elicitor for IgE-mediated reactions. Creatine, involved in the muscle energy metabolism, and ethylenediamine tetraacetic acid (EDTA), a calcium chelator, are potential molecules to modulate parvalbumin. The purpose of this study was to test creatine (2, 5 and 8%) and EDTA (1.5, 3 and 4.5%) supplementation in fish diets to modulate β-parvalbumin expression and structure and its allergenicity in farmed European seabass (*Dicentrarchus labrax)* while assessing its effects on the end-product quality. Fish welfare and muscle quality parameters were evaluated by plasma metabolites, *rigor mortis*, muscle pH and sensory and texture analysis. Proteomics was used to assess alterations in muscle proteome profile and metabolic fingerprinting by Fourier transform infrared spectroscopy was used to assess the liver metabolic profile. In addition, IgE-reactivity to parvalbumin was analyzed using fish allergic patient sera. Metabolic fingerprinting of liver tissue revealed no major alterations in infrared spectra with creatine supplementation, while with EDTA, only absorption bands characteristic of lipids were altered. Comparative proteomics showed up regulation of (tropo)myosin and phosphoglycerate mutase 2 with Creatine supplementation. In the case of EDTA proteomics showed up regulation of proteins involved in cellular and ion homeostasis. Allergenicity seems not to be modulated with creatine or EDTA supplementation as no decreased expression levels were found and IgE-binding reactivity showed no quantitative differences.

Keywords: Allergenicity, creatine, EDTA, European seabass, muscle quality

4.3.1 Introduction

The aquaculture industry is currently the fastest-growing animal production sector worldwide. The continuous grow of the population, the quota regulations restraining fisheries and the rising demand for fish makes this industry of great importance to alleviate pressure over the natural environment and increase access to high quality protein (Kroodsma et al., 2018, FAO, 2020). However, the dynamics of the aquaculture global value chain around the emergent sustainable intensification of the sector carries hand in hand needs in the food security governance. Within this framework, farmed fish are required to be monitored to safeguard its welfare, which is related with increased food safety and (nutritional) quality (Macagnano et al., 2005, Silva et al., 2012a). Divergent (or synergic) analytical methods for assessing plasma metabolites (cortisol, glucose and lactate), muscle texture, *rigor mortis* or pH are often used in the endeavor of determining flesh quality (Wang et al., 2015a). Indirect approaches, such as tailoring fish feeds e.g., fish feed formulas targeting the species nutritional requirements are used within the same scope. Nutritional studies are continuously trying to supplement specific ingredients to feeds to secure higher flesh quality (Silva et al., 2012b, Wang et al., 2015a, Schrama et al., 2018). Nevertheless, these studies have been more focused on improvements that are ultimately linked with production efficiency and industry sustainability (Roques et al., 2020) e.g., improving growth performance, enhancing the animals immunity, or replacing the expensive fish meal/fish oil by more sustainable protein sources (Cerqueira et al., 2020b, Farinha et al., 2021). One other gap in nutritional research is dietary supplementation in modifying (specific) proteins present in tissues like liver, muscle and plasma with functional purposes (Jobling, 2016).

Fish, as mentioned before, is highly consumed worldwide, but its ingestion has been reported to cause an adverse response of the immune system in sensitized individuals resulting mostly in an IgE-mediated allergic reaction (Klueber et al., 2019). Allergic sensitization studies, by skin and serum testing, show a prevalence of up to 2.9% in the general population (Klueber et al., 2019). The major fish allergen is known to be β-parvalbumin (Kuehn et al., 2010), a small (12 kDa), acidic (pH 3.5-4.5), stable and calcium-binding protein (Van Do et al., 2005). β-Parvalbumin is significantly present in the white muscle of fish. In muscle tissue, creatine plays an important role in the energy expenditure of the biological system such as muscle contraction/relaxation. In vertebrates, the amino acids

arginine and glycine are required for the synthesis of creatine, which is regulated by L-arginine:glycine amidinotransferase (AGAT, also known as GATM) and guanidinoacetate-N-methyltransferase (GAMT) enzymes. Through them, creatine is reversibly converted to phosphocreatine (PCr) producing creatinine as a by-product. When more energy is demanded, adenosine triphosphate (ATP) is produced by the donation of a phosphate group from PCr to adenosine diphosphate (ADP) (Borchel et al., 2019). Although the metabolism of supplemented creatine in fish is not fully understood, a study was performed using rainbow trout (*Oncorhynchus mykiss*) (Borchel et al., 2014), with the aim of studying the differences between mammals and fish, showing that creatine seems to be synthesized in the muscle. Later, authors confirmed that creatine is mainly produced in fish muscle by the high expression of GATM in this tissue (Borchel et al., 2019). A previous study with Creatine supplementation in diets for murines showed that the expression levels of Ca^{2+}-ATPase increased and that the parvalbumin expression decreased (Gallo et al., 2008). EDTA is known as a mineral and metal chelator, and is used in humans for medical purposes (Garg et al., 2016), in pharmacy or as a food additive (Heindorff et al., 1983). The metabolism of EDTA in fish is unknown, nevertheless some studies were performed using this chemical compound as feed supplement in gilthead seabream (*Sparus aurata*) (Raposo de Magalhães et al., 2020b) and Nile tilapia (*Oreochromis niloticus*) (Abdel-Tawwab et al., 2017). In case of EDTA several studies showed that, due to its ability to chelate calcium, the IgE-binding capacity decreases, resulting from a rearrangement of the parvalbumins structure, affecting major epitope regions (Kobayashi et al., 2016a). Therefore, Creatine and EDTA were studied for allergenicity modulation in fish (Schrama et al., 2018, Raposo de Magalhães et al., 2020b). Although creatine supplementation (up to 8%) in gilthead seabream showed no differences in IgE-reactivity amongst testes fish allergic patients (Schrama et al., 2018), supplementation with EDTA (3%) did show a reduction of this IgE-reactivity in this species (Raposo de Magalhães et al., 2020b).

Following the positive results of the use of EDTA in modulating fish allergenicity in gilthead seabream (Raposo de Magalhães et al., 2020b) and those from (Gallo et al., 2008) that showed a decrease in parvalbumin expression in murine upon creatine supplementation in their diets, we present in this work a detailed study of the effect of creatine (2, 5 and 8%) and EDTA (1.5, 3 and 4.5%) supplemented diets on European seabass, *Dicentrarchus labrax,* L. allergenicity, fish muscle quality and omics fingerprint.

Metabolic fingerprinting was analyzed by Fourier transform infrared (FT-IR) spectroscopy to reveal metabolic modifications in the fish liver as a result of diet supplementation (de Magalhães et al., 2020). This relatively simple and fast technique requires a small amount of sample and is used in several fish related studies with the advantage to specify a comprehensive view on fish metabolism (de Magalhães et al., 2020, Silva et al., 2014a).

Proteomics is a cutting-edge technology, valuable to divergent fields of aquaculture, including products for quality analysis and food allergens studies (Rodrigues et al., 2012, Silva et al., 2014a, Hoffmann-Sommergruber, 2016, Schrama et al., 2018, Raposo de Magalhães et al., 2020b). Biological modifications related to the ingestion of specific foods are highly investigated using proteomics. With this approach any unsuspected nutritional effects might be identified (Pedreschi et al., 2010, Rodrigues et al., 2018). Knowing that allergens are species-specific, proteomics may narrow avoidance strategies against fish consumption and increase individual allergy specificity.

4.3.2 Material and methods

4.3.2.1 Fish and rearing conditions

Two independent trials using European seabass (obtained from a commercial fish farm, Maresa, Mariscos de Estero S.A, Huelva, Spain) were performed at the Ramalhete experimental station (CCMAR/University of Algarve, Portugal) from April to July. Fish were reared in 500 L flat-bottom tanks, in an indoor flow-through system. Fish with an initial body weight of 186 ± 0.83 g were allocated in 12 tanks at an initial density of 7.4 $kg.m^{-3}$ (20 fish per tank) for the Creatine trial and 174 ± 1.29 g, at an initial density of 7.6 $kg.m^{-3}$ (22 fish per tank) for the EDTA trial. Fish were fed twice a day by hand (at 10h00 and 16h00), *ad libitum,* and kept under natural temperature (20.5 ± 2.53°C and 20.3 ± 2.14 °C for the creatine and EDTA trial respectively), dissolved oxygen maintained above 5 $mg.L^{-1}$, and salinity was 35.0 ± 0.6 psu. Fish were exposed to natural photoperiod conditions. Three fish from the initial stock in each trial were sampled for proximate composition determination. Trials were performed for 91 and 98 days, for the Creatine and EDTA trial, respectively.

Four different diets were tested, namely - for the Creatine trial, Control (Ctrl, without creatine supplementation), 2, 5 and 8% of creatine supplementation, which will be referred

as Creat2, Creat5 and Creat8 - for the EDTA trial, Control (Ctrl, without EDTA supplementation), 1.5, 3.0 and 4.5% of EDTA supplementation which will be referred as EDTA1.5, EDTA3 and EDTA4.5. Each dietary treatment was tested in triplicate tanks, randomly sorted. Diets were formulated to be similar to the commercial feed used for European seabass juveniles (SPAROS, Lda., Olhão, Portugal). A previously published study by our group in which EDTA-supplemented diets were tested in gilthead seabream, showed that this compound was not highly accepted by fish (Raposo de Magalhães et al., 2020b). Therefore, in this EDTA trial, an attractant (5% fish hydrolysate, CPSP90 from Sopropêche, France) has been added to the feed. The production procedures follow the methods described earlier (Schrama et al., 2018) with slight modifications. Creatine or EDTA were mixed with the raw ingredients and incorporated in the oil fraction until homogeneity in different concentrations according to each target formulation and applied by vacuum coating in a Pegasus vacuum mixer (PG-10VCLAB, DINNISEN, The Netherlands).

4.3.2.2 Sample collection and analytical procedures

At the end of the trials (after 91 and 98 days for the Creatine and EDTA trial, respectively), a total of 18 fish per tank were sampled (6 fish were anaesthetized with tricaine methanesulfonate (MS-222, Sigma Aldrich) for blood collection from the caudal vein, using heparinized 1ml syringes, only three of these six fish were lethally anaesthetized with MS-222 for liver and muscle (right dorsal) sampling and another 12 fish were sampled in ice slurry for fish quality measurements) (Supplementary material Fig. S4.3.1). Blood centrifugation was performed at 2000 xg for 20 min for plasma separation. Liver samples were weighed for hepatosomatic index (HSI) determinations. All samples were frozen in liquid nitrogen and stored at -80°C till further analysis. In case of fish quality measurements fish were used as follows: Three fish for muscle pH determinations, another 3 fish for *rigor mortis* determinations and the remaining 6 fish for instrumental texture measurements and sensory analysis. After *rigor mortis* analysis fish were frozen for proximate composition analysis. All sampled fish were weighed and measured. The remaining fish in the tanks were bulk weighed.

4.3.2.3 Growth indices

Zootechnical details like initial and final body weight (IBW and FWB, respectively), weight gain, daily growth coefficient (DGC), feed conversion ratio (FCR) and feed efficiency (FE) were determined using the following formulas adapted from (Glencross et al., 2007).

Weight gain, calculated as WG (%IBW) = 100 x (FBW - IBW) x IBW^{-1}

Daily growth coefficient, calculated as DGC (g day^{-1}) = 100 x ($FBW^{1/3}$–$IBW^{1/3}$) x t^{-1}

Feed conversion ratio, calculated as FCR = FC x (BMf−BMi)$^{-1}$

Feed efficiency, calculated as FE = (BMf−BMi) x FC^{-1}

Where FBW and IBW are the final and initial fish body weight, respectively, t are total days of the trial, BMf and BMi are the final and initial biomass, respectively and FC is the feed consumption.

4.3.2.4 Plasma metabolites

Plasma cortisol (n=18) was determined as described earlier (Schrama et al., 2018) using a commercially available ELISA kit (IBL International) following the manufacturer's instructions. Briefly, samples were diluted 2x or 5x (Creatine or EDTA trial, respectively) in standard A and analyzed in triplicate using a 96 well-microplate. Optical density was measured at 450 nm. Plasma glucose (n=18) and lactate (n=18) levels were assessed using commercial colorimetric kits (Spinreact, Girona, Spain), following manufacturer's instructions. Plasma samples were used directly without dilution and analyzed in triplicate using a 96-well microplate, optical density was measured at 505 nm in both cases.

4.3.2.5 Analytical methods

Proximate composition analysis of whole fish was determined as follows. Before analysis, pooled (per tank) whole-body fish were finely ground. Dry matter was determined by drying the samples at 105°C for 24 h and ash content by combustion in a muffle furnace at 550°C for 6 h. Freeze-dried samples were analyzed for crude protein (nitrogen x 6.25) content using Vario EL III elemental analyzer (Elementar). Crude fat was analyzed by a gravimetric

method using petroleum ether (40-60 °C) as extraction solvent on a Soxtec™ 2055 (Foss, Höganäs, Sweden) and gross energy by a calibrated adiabatic calorimetric pump using benzoic acid on a Werke C2000 (IKA, Staufen, Germany). Phosphorous content (following the AFNOR V 04-406 norm) was tested using the vanado-molybdate reagent, by digestion at 230°C in a Kjeldatherm block digestion unit, followed by digestion at 75°C in a water bath and absorbance determination at 820 nm.

4.3.2.6 Quality characterization of fish muscle

Characterization of fish muscle quality was performed as described in (Raposo de Magalhães et al., 2020b, Schrama et al., 2018) with slight modifications. Muscle pH measurements (n=9) and *rigor mortis* index (n=9) were performed at 0, 1, 2, 4, 6, 8, 24, 48 and 72h after death (HAD). Fish were kept on ice and handled carefully to avoid secondary effects on the *rigor* state's development. Texture measurements (n=18 per treatment) were performed on a TA.XTplus texture analyzer (Stable Micro Systems, Surrey, UK) equipped with a load cell of 30 kg using the double compression method (texture profile analysis – TPA) with a cylindrical metal probe P75 which compressed the raw fillet cubes (40% of their height) at a constant speed of 2 $mm.s^{-1}$. Sensory analysis was performed on steamed fillets (100 °C, 6 minutes, steam oven Rational Combi-master CM6) using twelve trained panelists (age within the range 28 – 60 years old) and a five-points intensity scale (0 – absent; 1 – slight; 2 – moderate; 3 – intense; 4 -strong). Coded samples were presented to the sensory panel in a balanced randomized order. Texture and sensory analysis were performed at Instituto Português do Mar e da Atmosfera (IPMA), Lisbon, Portugal.

4.3.2.7 Metabolic fingerprinting by solid phase transmission Fourier transform infrared (FT-IR) spectroscopy

The FT-IR spectra of the fish liver samples (n=9 per treatment) were acquired for all conditions using the protocol described by (Silva et al., 2014a). Briefly, lyophilized samples were mixed with potassium bromide (KBr) powder (Sigma Aldrich, St.Louis, MO, USA) until homogenous in a 1:3 ratio. Samples were placed in an evacuated die (13 mm diameter) to obtain pellets for transmission IR spectroscopy. Two individual FT-IR spectra per biological sample were acquired using a "Tensor" FT-IR equipment (Bruker Optik GmbH, Ettlingen, Germany) coupled to the OPUS analysis software (Bruker GmbH). Each spectrum was

obtained covering the middle-infrared wavenumber range of 400-4000 cm $^{-1}$, at a resolution of 4 cm $^{-1}$, averaged for 32 scans. Transmittance spectra generated were exported for further analysis. The spectral pre-processing were performed as described before (de Magalhães et al., 2020).

4.3.2.8 Protein extraction and CyDye labeling

Proteins from muscle samples (n=6 per treatment) were extracted as described in (Schrama et al., 2018) with some slight modifications. Briefly, samples were homogenized, using DIGE buffer (7 M urea, 2 M thiourea, 4% CHAPs, 30 mM Tris, pH8.5, 1 mM EDTA and 1% v/v protease inhibitor) and a 5 mm steal bead, in a tissue lyser (VWR International, Radnor, USA) for 2 cycles of 30 sec at 25 Hz. Samples were centrifuged at 13,000 g for 15 min at 4°C to pellet insoluble material. Quantification of the supernatant was performed using Quick Start™ Bradford Protein Assay with bovine albumin as standard (Bio-Rad, Hercules, USA).

For DIGE minimal labelling, 50 µg of proteins from each sample were labelled with 400 pmol of fluorescent amine reactive cyanine dyes, previously resuspended in anhydrous dimethylformamide (DMF). Three samples per dietary treatment were labelled with Cy3 and 3 with Cy5 to prevent eventual label difference, while an internal standard was labelled with Cy2 containing the same protein amount of all samples.

4.3.2.9 Two-dimensional electrophoresis, gel image acquisition and analysis

Labelled proteins were separated according to the protocol described in (Schrama et al., 2018) with some slight modifications. For each strip, 50 µg of protein from each dietary treatment were mixed with internal standard and rehydration buffer. Isoelectric focusing was performed on 24 cm Immobiline™ Drystrip with a pH 3-10 nonlinear gradient (GE Healthcare, Chicago, USA). Before the second dimension, proteins were reduced and alkylated for 15 min each with dithiothreitol (DTT) and iodoacetamide, respectively. Proteins were separated according to their molecular weight on 12.5% polyacrylamide gels. Protein profile images were obtained by scanning the gels on a Typhoon laser scanner 9400 (GE Healthcare). The final gel images were analyzed using SameSpots™ Software v4.6.1.218 (TotalLab, Newcastle, UK; http.//totallbal.com/samespots), including background subtraction (average normalized volume <=10,000 and spot area <=500, simultaneously), filtering, spot detection, spot matching, normalization and statistical analysis. Spots showing volume ratios with significant

difference ($p<0.05$, One-way ANOVA on normalized log_2-transformed spot volumes, followed by $q<0.05$ – Tukey's *post-hoc* Test) were manually excised from SYPRO® Ruby-stained (Invitrogen) 2D gels.

4.3.2.10 Protein identification by mass spectrometry (MS)

Protein identification by MS was performed at the Luxembourg Institute of Science and Technology (LIST) as described in (Raposo de Magalhães et al., 2020a). Briefly, a wash step of 20 min with 50 mM ammonium bicarbonate solution in 50% (v/v) methanol followed by dehydration with 75% acetonitrile (ACN) was performed twice. Proteins were digested with 8 µl of 5 ng/µl trypsin in 20 mM ammonium bicarbonate for 6 h at 37°C. Peptides were extracted using a 0.1% trifluoroacetic acid (TFA) solution in 50% ACN and spotted with 7 mg/ml α-cyano-4-hydroxycinnamic acid in 50% ACN/0.1% TFA. MALDI TOF/TOF analysis was performed with a TOF/TOF™ 5800 (AB SCIEX, Redwood City, CA, USA) mass spectrometer in MS and MS/MS mode. For each spot, the 10 most intense peaks of the MS spectrum were selected for MS/MS acquisition. Database interrogation was carried out with ProteinPilot v4.5 (AB Sciex) on an in-house Mascot server version 2.6.1 (Matrix Science Ltd., London, UK). Mass lists were searched against NCBInr database restricted to the taxonomy "other Actinopterygii" (tax ID 7898 excluding 31,033 and 7955) with the following parameters: maximum 2 missed cleavages by trypsin, peptide mass tolerance ± 100 ppm, fragment mass tolerance set to 0.5 Da, carbamidomethylation of cysteine selected as fixed modification and tryptophan dioxidation, histidine, tryptophan and methionine oxidation, and tryptophan to kynurenine as variable modifications. Protein hits not satisfying a significance threshold ($P < 0.05$ and a total ion score > 102) were further searched against vertebrate EST (expressed sequence tags) database also restricted to the taxonomy "other Actinopterygii

4.3.2.11 Proteomics data analysis

The theoretical isoelectric points (pI) and molecular weight (mW) were calculated using the ProtParam Tool (http://us.expasy.org/tools/protparam.html) using the one-letter amino-acid sequences code. Normalized log_2-transformed spot volumes were imported into R v4.0.3 for MacOSX (https://www.r-project.org). Volcano plots to elucidate the number of differential proteins between the supplemented diets and the corresponding control group were obtained with the EnhancedVolcano function from package *EnhancedVolcano (Blighe et*

al., 2021). Principal component analysis (PCA) analysis was performed on the log2-transformed normalized spot volumes obtained from Samespots, with autoscaling. Hierarchical clustering (HCL) analysis was achieved with the heatmap.2 function from package *gplots* (Warnes et al., 2015). Heatmaps were generated by comparing Z-scores of differential proteins (One-way ANOVA, $p<0.05$) on row-wise scaled data, while the hierarchical clustering of samples and protein spots was performed taking the Euclidean distance as metric distance and the average linkage as agglomeration method. Functional annotation, including gene ontology (GO) classification analysis, on the basis of biological process, was performed using the OmicsBox software v1.4.11 (BioBam Bioinformatics SL, Valencia, Spain; https//www.biobam.com/omicsbox). The FASTA sequences of the identified proteins were used as input and blasted using the NCBI blast+ via CloudBlast with *Danio rerio* and *Dicentrarchus labrax* as taxonomy filters. The top 10 blast hits for each query protein, with an E-value less than 1.0E-3 were retrieved. Mapping and annotation were performed based on the Blast2GO annotation methodology (Conesa et al., 2005), using the default settings. The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) web-based tool v11.0 (https://string-db.org/) was used to screen for potential protein-protein interactions (PPI) and perform a Kyoto Encyclopedia of Gene and Genomes (KEGG) pathway enrichment analysis of orthologues in *Danio rerio* (FDR, $q < 0.05$). Protein nodes showing no interactions were excluded from further analysis. Network visualization and topological analysis were performed on Cytoscape v3.8.2 (http://www.cytoscape.org/). Important hub proteins were screened and ranked by the degree values of each node in the network.

4.3.2.12 IgE-reactivity to parvalbumin

Proteins from fish muscle were extracted with a lysis buffer (50 mM Tris-HCl, pH 8 and 150 mM NaCl, with freshly added 1% Triton X-100) using the same protocol as described previously (Kuehn et al., 2013). IgE-reactivity was detected using a sandwich ELISA format using NUNC Maxisorp 96 well plates. Coating was performed overnight by adding 5 µg/ml protein per well. Unsaturated surface-binding sites were covered with blocking buffer containing 3% BSA. Patients' sera were diluted (dilutions vary between sera, 20x-100x) in the same blocking buffer. Detection antibody was a biotinylated anti-human IgE (Southern Biotech, Birmingham, Alabama, USA) diluted 1:1000. As final step to accomplish the sandwich ELISA, Streptavidin-alkaline-phosphatase (BD Biosciences, San Jose, California, USA) was

applied for 30 min and revealed with the substrate p-Nitrophenyl Phosphate (pNPP, Sigma Aldrich) in a continuous mode at 405 nm. Demographics and clinical characteristics of the allergic study cohort are given in Supplementary Material (Table S4.3.1). In parallel, an IgE-calibration curve was applied with the readout in kUA/L. As negative control serum was replaced by buffer and as positive control a polyclonal rabbit anti-European seabass was used 1:10000 (Eurogentec, Liege, Belgium). Sera used for the present study were collected in the frame of a study of fish allergy approved by the National Committee for Medical Research Ethics in Luxembourg (Ref. 201307/04).

4.3.2.13 High-performance liquid chromatography (HPLC) determination of EDTA

EDTA accumulation in EDTA trial's muscle sample was assessed as described in (Raposo de Magalhães et al., 2020b). Briefly, muscle samples from all treatments (n=9) were homogenized with a ferric chloride solution and heated for 30 min at 70°C. Samples were filtered before injection into an AZURA® HPLC system, equipped with a 50 µl sample loop, a thermostatic column oven and a diode array detector. A C18 Phenomenex Kinetex column, 150 x 4.6 mm, particle size 2.6 µm was used with a flow rate of 0.8 ml/min and a running time of 13 min for sample solutions.

4.3.2.14 Ethical approval

The experiments in this project were approved by the Portuguese National Authority for the Animal Health (DGAV) with permit no.003894, following guidelines for fish welfare established in Council Directive 2010/63/EU and Portuguese legislation for the use of laboratory animals, permit number 0420/00/000-n.9909/11/2009.

4.3.2.15 Statistical analysis

Statistical analysis was performed using R v4.0.3 (R Core Team, 2013) for MacOSX. Significant differences in plasma and muscle parameters were assessed by a one-way analysis of variance (one-way ANOVA) on log10-transformed data, in case of cortisol, glucose and lactate and weight gain data; in the case of HSI, *rigor mortis* and proximate composition analysis, data were previously transformed by arcsine square root. In case of FBW, DGC, FCR, FE, texture and sensory analysis data differences were assessed by one-way ANOVA without

transformations. Before multiple comparison by post-hoc Tukey HSD, residuals' normality was checked with a Shapiro-Wilk test and homoscedasticity with a Levene's test. In case the normality assumption was not verified a Kruskal-Wallis followed by a Dunn's test were used, and in the case of homoscedasticity assumption, a Welch's ANOVA followed by a Games-Howell test were used. Significant difference was considered when $p<0.05$. All results are shown as mean ± standard deviation.

4.3.3 Results and discussion

4.3.3.1 Zootechnical analysis

After 91 days for the Creatine trial fish show a similar growth with a final body weight of 339 ± 5.05 g (one-way ANOVA, p=0.68). For the EDTA trial, after 98 days fish showed a final body weight of 354 ± 13.36 g without any significant differences among treatments (one-way ANOVA, p=0.11). No mortalities were registered for both trials (Table 4.3.1). Specific growth details including initial and final body weight (IBW and FBW, respectively), weight gain, daily growth coefficient (DGC), feed conversion ratio (FCR) and feed efficiency (FE) for each trial are also shown in Table 4.3.1. Fish fed the EDTA4.5 diet presented a higher FCR than CTRL fish, nevertheless the EDTA supplementation had no impact on fish final body weight.

Fish growth was not affected by creatine and EDTA supplementation. These results are, to some extent, in accordance with previous studies which examined the performance of red drum (*Sciaenops ocellatus*) with an IBW of 8.9 ± 1.8 g at moderate salinity for 5 weeks, where creatine supplementation (up to 4%) did not affect fish growth (Burns and Gatlin, 2019). Nevertheless, in the case of red drum salinity and water temperatures are two of the main factors for optimal growth conditions as creatine supplementation results in increased fish growth when salinity is kept low and temperatures are high (Burns and Gatlin, 2019, Stites et al., 2020).

Moreover, hepatosomatic index (HSI, Fig.4.3.1) showed no significant differences among treatments in the EDTA trial (one-way ANOVA, p=0.057). Nevertheless, significant differences were shown in the Creatine trial between Creat2 and Creat8 (one-way ANOVA, followed by post-hoc Tukey, p=0.03). Creat2 fish showed a higher HSI suggesting a slight modification in liver composition. However, these results were unexpected as HSI increases with a 2% supplementation and suddenly drops back to control levels with increased creatine

concentration. Our results are contrary to those shown in red drum, where no differences in HSI were observed when creatine supplementation was 2% (Stites et al., 2020). However HSI increases linearly with supplementations of 0.5, 1, 1.5, 2 and 4% (Burns and Gatlin, 2019). Also gilthead seabream results showed no differences in HSI with creatine supplementation up to 8% (Schrama et al., 2018).

Table 4.3.1 Growth performance parameters of European seabass from Creatine and EDTA trials.

Diet	IBW (g fish^{-1})	FBW (g fish $^{-1}$)	Weight Gain (%IBW)	DGC (g day^{-1})	FCR	FE
CTRL	186 ± 1	340 ± 12	82.64 ± 6.78	1.39 ± 0.09	1.63 ± 0.08	0.62 ± 0.03
Creat2	183 ± 5	338 ± 13	84.57 ± 12.21	1.41 ± 0.15	1.73 ± 0.15	0.58 ± 0.05
Creat5	186 ± 2	345 ± 10	85.70 ± 3.97	1.44 ± 0.06	1.67 ± 0.11	0.60 ± 0.04
Creat8	189 ± 6	333 ± 14	76.14 ± 7.41	1.31 ± 0.10	1.83 ± 0.14	0.55 ± 0.04
CTRL	175 ± 2	367 ± 12	110.24 ± 6.09	1.60 ± 0.07	1.50 ± 0.09*	0.67± 0.04*
EDTA1.5	174 ± 1	363 ± 15	108.35 ± 9.46	1.58 ± 0.11	1.64 ± 0.03	0.61 ± 0.01
EDTA3	174 ± 1	345 ± 16	98.93 ± 9.93	1.47 ± 0.12	1.64 ± 0.05	0.61± 0.02
EDTA4.5	175 ± 2	339 ± 13	94.09 ± 5.55	1.41 ± 0.07	1.67 ± 0.05*	0.60 ± 0.02*

Table with initial body weight (IBW), final body weight (FBW), weight gain per day, daily growth coefficient (DGC), feed conversion rate (FCR) and feed efficiency (FE) calculated per treatment (n = 60 or n = 66, for the Creatine and EDTA trial, respectively) at the end of the trial (91 or 98 days).
* Represents significant differences by one-way ANOVA followed by post-hoc Tukey ($p<0.05$)

4.3.3.2 Plasma metabolites

Stress metabolites were measured in the plasma of European seabass. This biological fluid indicates the physiological condition of fish. Cortisol is used as a primary stress response indicator, where lactate and glucose are used as secondary indicators. A high biological variability resulted in several outlying data points (Fig.4.3.2). These different responses might be due to behavioral aspects, as previously reported by (Samaras and Pavlidis, 2018, Carbonara et al., 2019, Cerqueira et al., 2020a). Cortisol levels on both trials showed significant differences (one-way ANOVA, $p=4.8e^{-6}$ for the creatine trial and Kruskal-Wallis, $p=4.3e^{-5}$ for the EDTA trial).

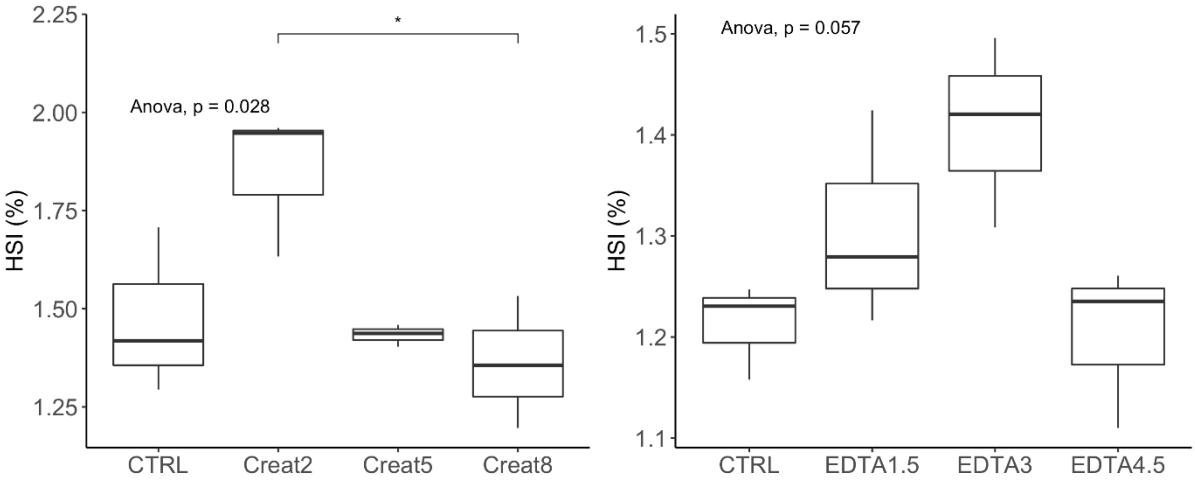

Fig. 4.3.1 Box plots of the fish hepatosomatic index (HSI) distribution. Plots showing the distributions of fish hepatosomatic index estimated from individual measurements (n=9 per treatment) at the end of each trial. Results are shown by quartiles (from Q1 to Q3) and the horizontal line in each box represents the median value. Lines outside the boxes represent the minimum and maximum. Significant differences are shown by asterisk (*) (one-way ANOVA, post-hoc Tukey, $p<0.05$).

For the Creatine trial, CTRL showed significant lower cortisol levels than the supplemented diets, and Creat2 showed significant lower levels than Creat5. As for the EDTA trial, CTRL showed significant lower cortisol levels than EDTA3, and EDTA4.5 showed significant lower levels than EDTA3 and EDTA1.5. European seabass is known to have a highly reactive cortisol response (Samaras et al., 2016). Control basal values shown in the creatine trial are similar to those observed at pre-stress conditions (Samaras et al., 2016). In the case of the EDTA trial, the control levels are similar to the ones obtained in stress predictability studies (Cerqueira et al., 2020a). Plasma glucose levels showed no significant differences in both trials, nevertheless, significant differences were found in plasma lactate concentrations in the Creatine trial (one-way ANOVA, $p=0.03$), specifically between Creat2 and Creat8 and between Creat5 and Creat8, being higher in Creat8. Secondary stress levels in plasma glucose and lactate were not following the expected pattern of the primary stress levels in cortisol. As explained previously (Raposo de Magalhães et al., 2020a) cortisol levels increase rapidly after an acute stressor, returning usually to basal levels within 24 h. Glucose and lactate levels should be increasing as a result of the primary response. As this is not shown in our experiment, we believe that the significant differences in cortisol are a result of the sampling procedure and not because of the diet supplementation. A suggestive explanation is the

activation of the hypothalamic-pituitary-interrenal (HPI)-axis which leads to a hypo response of cortisol (Rotllant et al., 2000, Bernier and Peter, 2001).

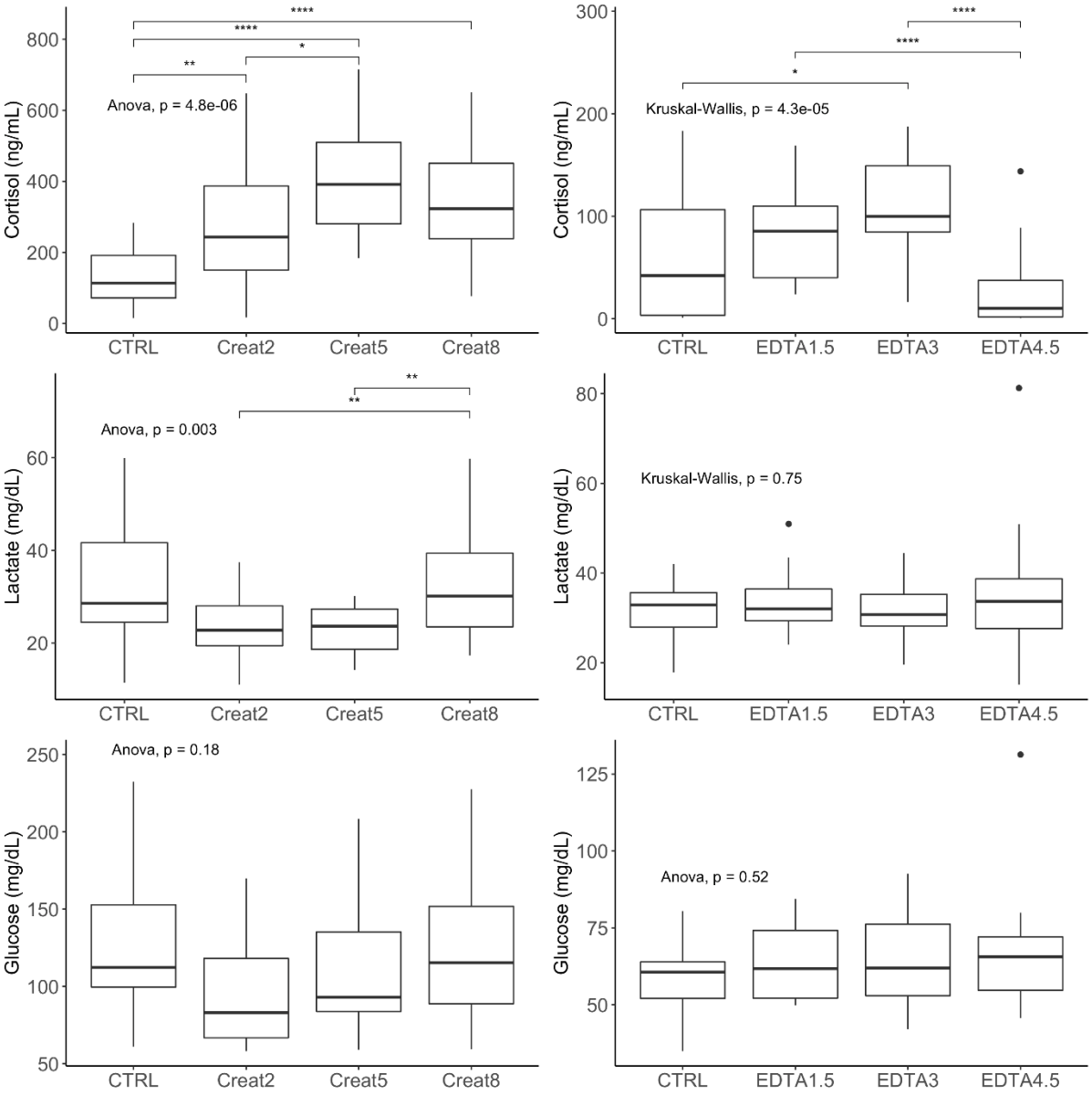

Fig. 4.3.2 Boxplot of cortisol (ng/ml), lactate (mg/dL) and glucose (mg/dL) concentration in plasma of European seabass at the end of each trial (n=18). Results are shown by quartiles (from Q1 to Q3) and the horizontal line in each box shows the median. Lines outside the boxes represent the minimum and maximum. Significant difference is shown by asterisks (*) (one-way ANOVA, followed by post-Hoc Tukey, $p<0.05$ or Kruskal-Wallis, followed by *post-Hoc* Dunn, $p<0.05$).

4.3.3.3 Analytical data

The whole fish proximate composition showed that dry matter, crude protein, crude fat, phosphorus and gross energy were not affected by the supplemented diets in each trial (Table 4.3.2). Gross energy (kJ g^{-1} DM) showed slightly higher values in Creat2 which seems to be in line with the HSI results, although with whole fish the results were not significant with whole fish. Creatine supplementation has been studied, with different aims, in different fish species like gilthead seabream (Schrama et al., 2018), rainbow trout (McFarlane et al., 2001) and red drum (Burns and Gatlin, 2019, Stites et al., 2020), but only with red drum proximate composition was determined. No significant differences were found regarding moisture, protein and lipids in red drum with 4% creatine supplementation (Burns and Gatlin, 2019).

4.3.3.4 Fish quality analysis

For fish quality, muscle pH and *rigor mortis* index were measured during the first 72 h *post-mortem*. Initial muscle pH values were similar to those reported in European seabass (Samaras et al., 2016). In both trials, muscle pH decreased with time (Fig.4.3.3), showing significant differences between the creatine treatments at 4 HAD. These decreasing values in muscle pH were similar to those reported by (Nathanailides et al., 2011), who registered an initial muscle pH of 6.8 and a final pH of 6.6 for European seabass with 171 g. In our case, values started off higher (7.5 for the creatine and 7.0 for the EDTA trial), but the final values were in accordance to (Nathanailides et al., 2011). This decrease might be due to the *post-mortem* increase of lactic acid, due to the degradation of glycogen present in the muscle (Matos et al., 2013). Also, energy reserves at the time of death influence fish muscle pH levels, controlling the decrease rate of pH (Raposo de Magalhães et al., 2020a).

In the case of the *rigor* index fish reached almost a full index 24 h *post-mortem* with no significant differences between treatments at any time point, for the creatine trial. Regarding the EDTA trial, significant differences are shown at 1 HAD and 48 HAD (Fig. 4.3.3). Also, in *rigor mortis,* energy levels and muscle pH influence the time for total stiffness of the fish. Fish muscle suffers *post-mortem* modifications in its myofibrillar structure and metabolic pathways, which are demonstrated by a decrease in pH, an increase of calcium and proteolysis of tropomyosin (Delbarre-Ladrat et al., 2006). When energy reserves and pH are low, the *rigor* onset reaches total stiffness, which was observed one day after death in both trials.

Table 4.3.2 Proximate composition of whole fish at the beginning and at the end of each trial

Proximate composition	Initial	CTRL	Creat2	Creat5	Creat8	Initial	CTRL	EDTA1.5	EDTA3	EDTA 4.5
Dry matter (DM, %)	35.36	35.73 ± 0.46	37.29 ± 1.74	35.44 ± 1.91	36.03 ± 2.01	37.33	38.35 ± 1.00	38.25 ± 2.19	37.84 ± 1.19	38.63 ± 3.17
Crude protein (% DM)	52.89	45.20 ± 4.49	46.68 ± 1.82	48.35 ± 3.36	50.35 ± 2.98	49.93	46.12 ± 4.96	47.18 ± 5.59	45.90 ± 0.42	46.97 ± 5.47
Crude fat (% DM)	36.79	38.21 ± 1.78	42.16 ± 4.21	40.26 ± 3.23	39.60 ± 2.30	39.58	43.97 ± 1.10	43.86 ± 2.57	43.51 ± 1.77	42.04 ± 3.51
Phosphorus (%)	1.55	1.53 ± 0.08	1.37 ± 0.17	1.44 ± 0.23	1.56 ± 0.10	1.24	1.12 ± 0.13	1.12 ± 0.14	1.12 ± 0.11	1.18 ± 0.27
Gross Energy (kJ g^{-1} DM)	26.23	26.52 ± 0.40	27.08 ± 0.84	27.01 ± 0.61	26.58 ± 0.33	26.62	26.78 ± 0.14	26.78 ± 0.71	27.02 ± 0.35	26.59 ± 1.38

No significant differences were observed between treatments in either trial (one-way ANOVA, $p>0.05$).

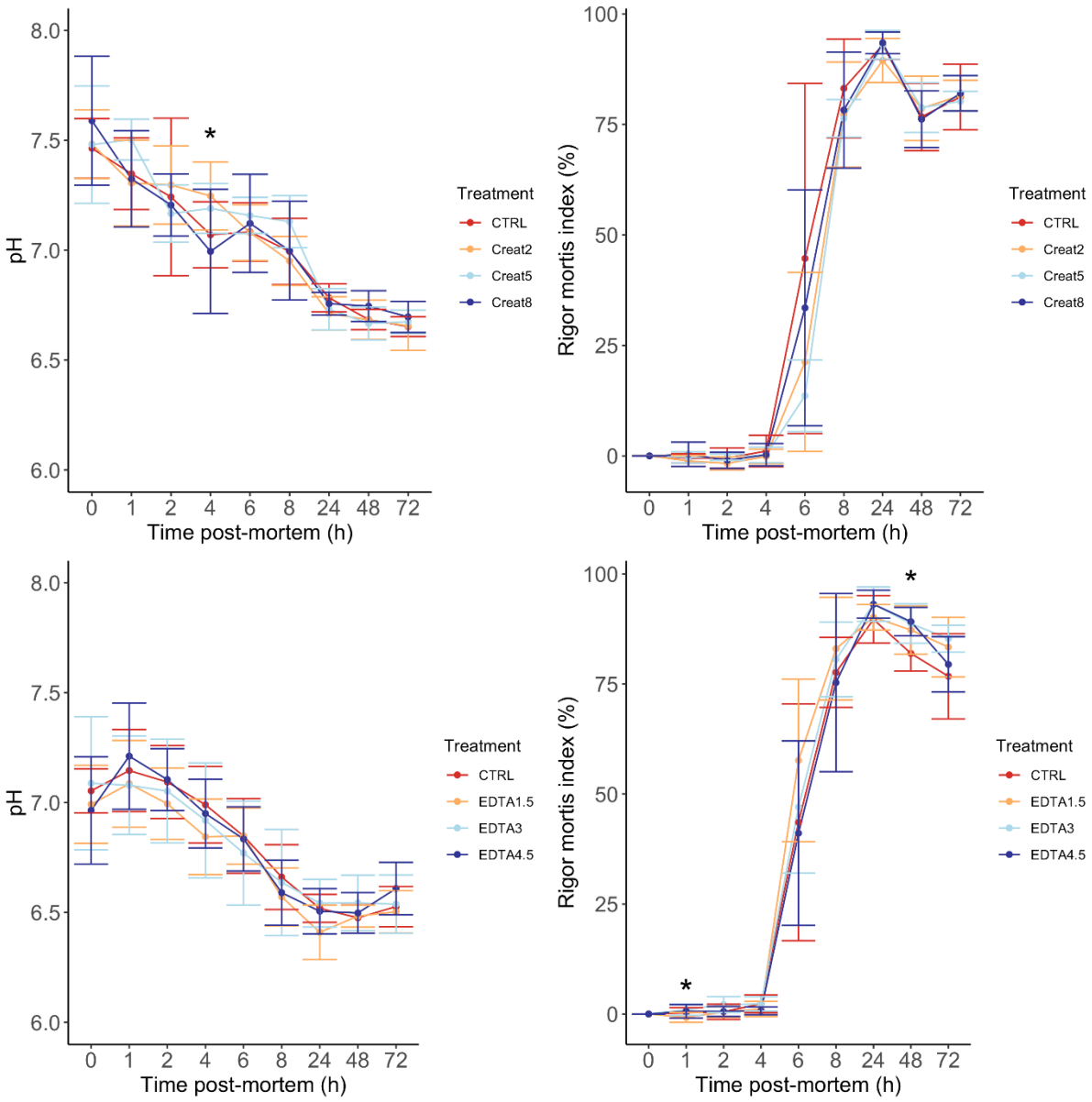

Fig. 4.3.3 *Post-mortem* changes in muscle pH and *rigor mortis* of European seabass fed supplemented diets. Data points are the mean ± SD of n=9 fish for each sampling time. Means labelled * are significantly different at p<0.05 (one-way ANOVA, followed by *post-hoc* Tukey).

Supplementations with creatine and EDTA should not change the quality and edibility of fish and therefore a sensory analysis was performed. For the Creatine trial, some variability in the sensory perception, regarding taste and texture of European seabass fillets was observed. Nevertheless, significant differences in these attributes among the four treatments were not found (Fig.4.3.4). The typical taste (close to intense in the case of CTRL) as well as the texture (firmness and succulence) were both scored as moderate. The sensory panel did not perceive uncharacteristic taste in any treatment. In the case of the EDTA trial, significant

differences in the typical taste or texture were also not found (Fig. 4.3.4). The sensory panel rated the fish from all treatments with typical moderate taste

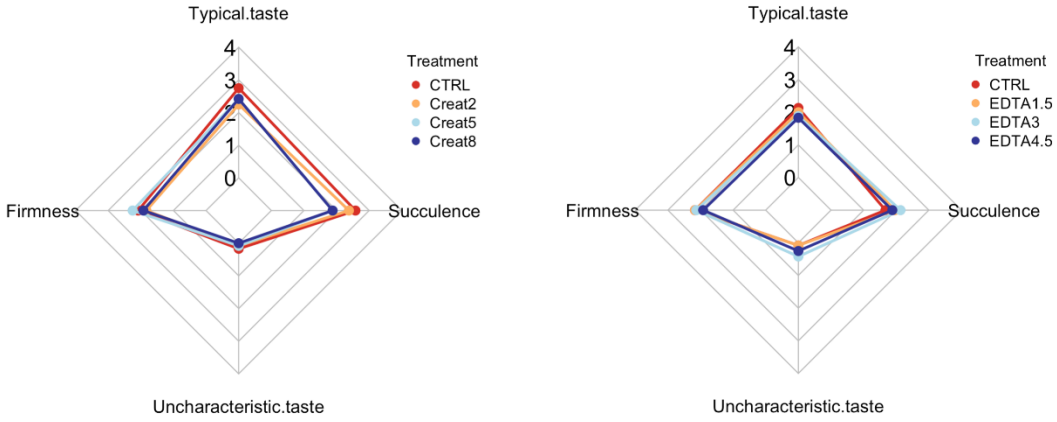

Fig. 4.3.4 Effect of the different feeds on the sensory profile of European seabass (steamed fillets). Results are the mean values (n=12, 0.0≤ SD≤ 1.2). Intensity scale: 0 – absent; 1 – slight; 2 – moderate; 3 – intense; 4 – strong. No significant differences were observed between treatments ($p>0.05$) (one-way ANOVA).

Regarding the texture properties, an important muscle quality parameter, determined mechanically (hardness, adhesiveness, springiness, cohesiveness and chewiness) no significant differences between treatments in all trials (Table 4.3.3). For the Creatine trial, these results are in accordance with those obtained in gilthead seabream, where also no differences were observed in texture parameters (Schrama et al., 2018). Nevertheless, our EDTA trial results showed no differences, contrary to 3% EDTA supplementation in gilthead seabream diets, where significant differences were observed in adhesiveness, springiness and chewiness (Raposo de Magalhães et al., 2020b). No other literature was found, describing the effect of creatine and EDTA supplementation in fish feed in texture parameters. These findings are in agreement with the proximate composition results, where no differences were found neither in protein nor in fat content, factors that influence the texture of fish fillets (Grigorakis, 2007).

Table 4.3.3 Texture parameters, determined on a texture analyzer, of European seabass muscle at the end of each trial.

Parameters	CTRL	Creat2	Creat5	Creat8	CTRL	EDTA1.5	EDTA3	EDTA4.5
Hardness (N)	50.4 ± 16.4	47.2 ± 12.0	49.1 ± 16.1	46.9 ± 12.1	33.7 ± 12.0	32.4 ± 9.8	36.8 ± 9.0	36.3 ± 9.4
Adhesiveness (g.sec)	-0.16 ± 0.06	-0.17 ± 0.06	-0.17 ± 0.04	-0.16 ± 0.06	-0.20 ± 0.09	-0.18 ± 0.05	-0.19 ± 0.10	-0.18 ± 0.06
Springiness	0.65 ± 0.05	0.65 ± 0.04	0.66 ± 0.05	0.64 ± 0.06	0.69 ± 0.08	0.64 ± 0.06	0.67 ± 0.04	0.65 ± 0.04
Cohesiveness	0.46 ± 0.05	0.45 ± 0.07	0.45 ± 0.06	0.46 ± 0.07	0.44 ± 0.08	0.44 ± 0.05	0.41 ± 0.05	0.42 ± 0.05
Chewiness	14.9 ± 5.0	13.7 ± 3.9	13.9 ± 3.9	13.2 ± 2.6	9.4 ± 2.4	9.0 ± 3.0	10.0 ± 2.1	9.7 ± 2.2

Table 4.3.4 Assignment of spectral bands to molecular vibrations of functional groups and biochemical compounds (Adapted from (de Magalhães et al., 2020)).

Band	Wavenumber (cm^{-1})	Vibrational modes and functional groups	Main biochemical compounds	Other biochemical compounds
1	3295-3285	N-H stretching of amides (Amide A), O-H stretching of polysaccharides	Proteins	Carbohydrates
2	3008	Olefinic =C-H stretching	Unsaturated fatty acids	Aromatics
3	2925	CH_3, CH_2 asymmetric stretching	Saturated lipids	Proteins, carbohydrates, nucleic acids
4	2854	CH_3, CH_2 symmetric stretching	Saturated lipids	Proteins, carbohydrates, nucleic acids

5	1745	C=O stretching of esters and aldehydes	Triglycerides, cholesterol esters	Lipids, phospholipids
6	1655	C=O stretching of amides (Amide I), C=C stretching of unsaturated hydrocarbons	Proteins	Unsaturated fatty acids
7	1545	N-H bending and C-N stretching of amides (Amide II), C=C stretching of aromatic hydrocarbons	Proteins	Aromatics
8	1462	CH_2 symmetric and asymmetric bending	Lipids	Proteins
9-10	1415-1377	COO^- symmetric stretching, olefinic C-H bending, P=O stretching in phosphates	Amino acids, fatty acids	Other carboxylates, alcohols, aromatic aminoacids
11	1237	PO_2^- asymmetric stretching	Nucleic acids	Phospholipids
12	1160	CO-O-C asymmetric stretching of esters and glycogen. =C-H bending in aromatics	Phospholipids and carbohydrates	Aromatics, cholesterol esters
13-14	1095-1006	C-O stretching of glycogen, PO_2^- symmetric stretching	Carbohydrates and nucleic acids	Phospholipids

4.3.3.5 Metabolic fingerprinting

FT-IR spectra of European seabass liver from the different feeding trials showed a typical intricate metabolic pattern. Only the spectral region between 3600-950 cm^{-1} was used during analysis as both the spectra head and end showed excessive noise. A total of 14 bands assigned to specific vibrational modes, functional groups and biochemical compounds were described in both trials (Table 4.3.4). After univariate statistical analysis of the individual spectral features (wavenumbers) using ANOVA followed by the *post-hoc* Dunnett's test, some significant differences were found in lipid-assigned bands for the EDTA trial (Fig. 4.3.5). These differences were detected in the spectral regions around 2925, 2854, 1745, 1462 and 1160 cm^{-1} (corresponding to the absorption bands 3, 4, 5, 8 and 12, respectively). More specifically, bands 3 and 8 differ significantly in EDTA3 and EDTA4.5 groups, when compared to CTRL. For bands 4 and 5 the significant differences were found between EDTA4.5 and CTRL. No differences were found in the creatine trial.

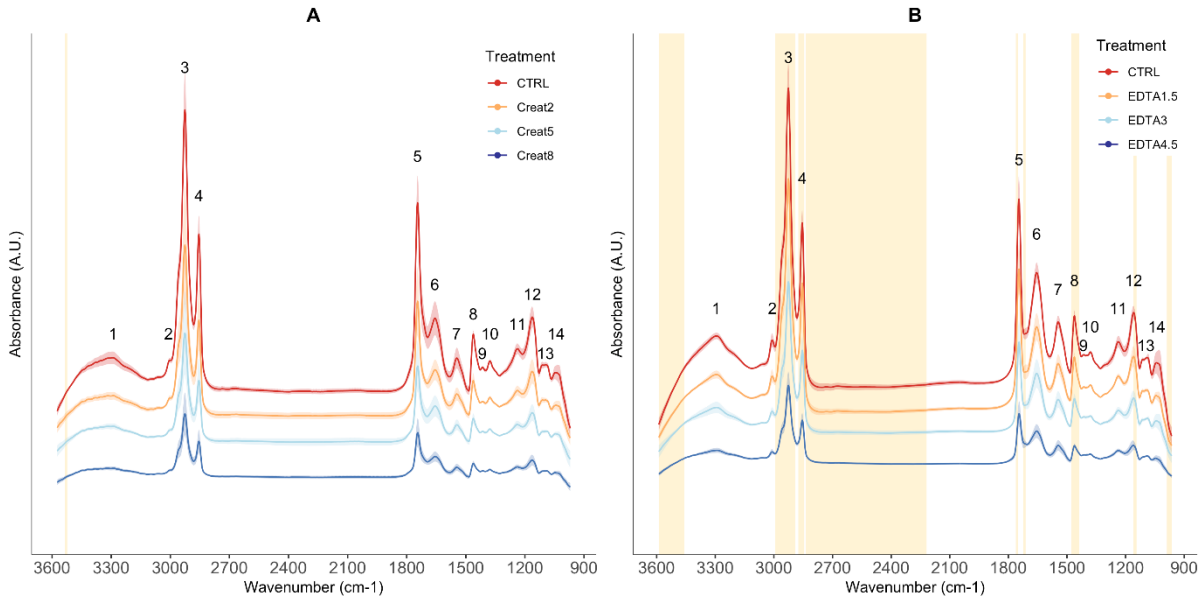

Fig. 4.3.5 Hepatic Fourier transformed infrared (FT-IR) spectra of European seabass (*Dicentrarchus labrax*) liver submitted to different feeding conditions ((A) – Creatine trial, (B) – EDTA trial). FT-IR spectra for each treatment are shown as absorbance values (in arbitrary units (A.U.)) of 8 averaged spectra (solid) ± SD (shaded ribbon). Mean spectra were offset along the absorbance axis for easier readability. Numbers indicate the bands assigned to biomolecules, listed in Table 4. Colored areas represent the wavenumbers with significant differences (One-way ANOVA, *post-Hoc* Dunnett's test $p<0.05$) between treatments and control.

4.3.3.6 Comparative proteomics

Proteomic analysis performed with 2D-DIGE showed a total of 485 spots detected in the muscle gel images corresponding to the Creatine trial and 805 spots for the EDTA trial. Volcano plots in Fig. 4.3.6 show the fold changes of all spots obtained in each trial. Results indicate that Creatine and EDTA supplementation in fish feed do not alter various proteins in muscle tissue, which is in accordance with our objective of targeting specific proteins only. As was shown by FT-IR in liver, no bands assigned to proteins were significantly different, confirming that proteins are not highly affected by the supplementation.

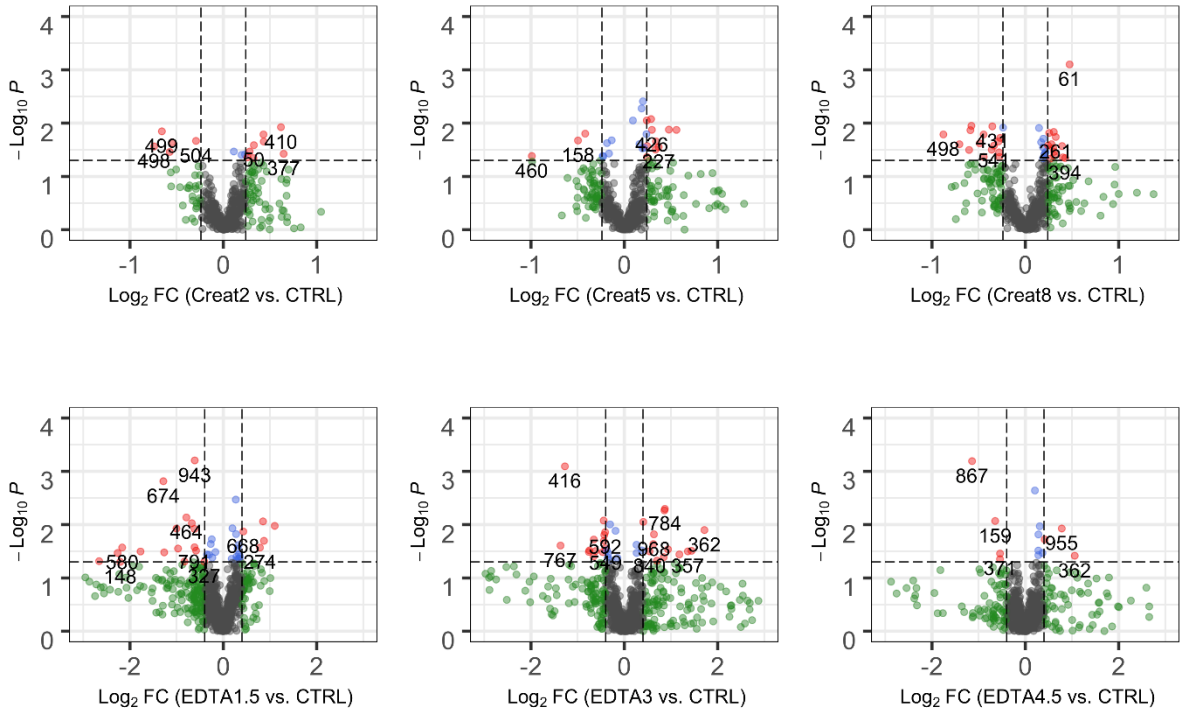

Fig. 4.3.6 Volcano plots of all muscle proteins detected by DIGE analysis on the Creatine and EDTA trials samples. Each point represents the difference in abundance (fold-change) between treatments and CTRL fish plotted against the level of statistical significance. Dotted vertical lines represent the established fold variation (FC > 1.18 and FC > 1.32, in case of Creatine and EDTA, respectively), while dotted horizontal lines represent the significance level of p<0.05 (t-student).

From the detected spots in both trials, four spots in the creatine trial and eight spots in the EDTA trial were significantly different in intensity ($p<0.05$ and $q<0.05$) and were manually picked for MS identification (Fig.4.3.7 and table 4.3.5). Differences in experimental groups are shown only versus CTRL and not in between treatments.

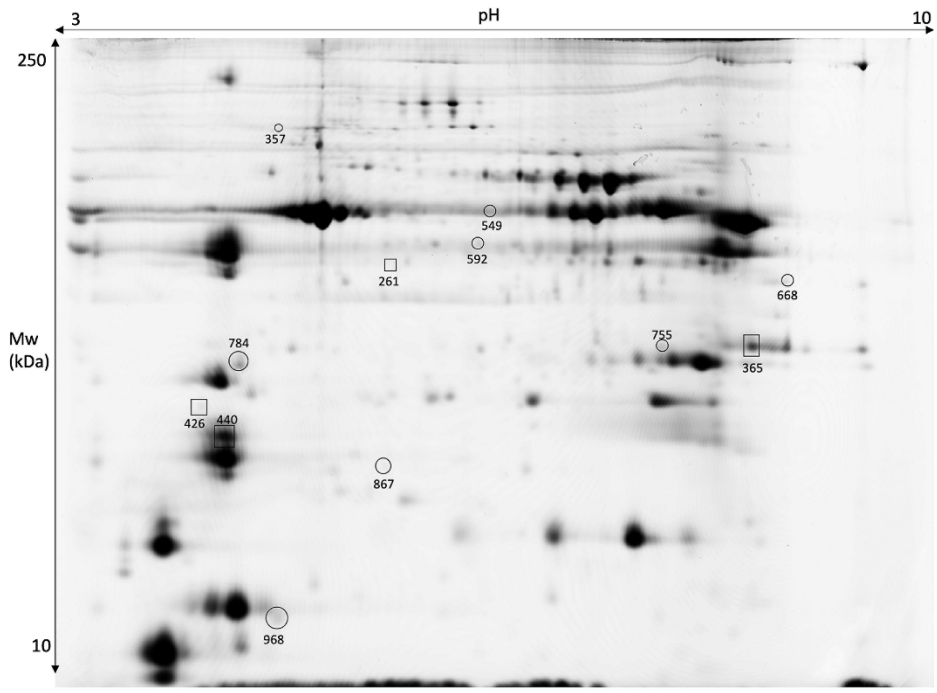

Fig. 4.3.7 Representative 2D-DIGE gel of European seabass (*Dicentrarchus labrax*) muscle on a 12.5% polyacrylamide gel in a pH range of 3-10 NL. Squared (creatine trial) and circled (EDTA trial) spots, with significantly different abundances (One-way ANOVA, *post-hoc* Tukey, $p<0.05$), were sequenced and identified by MALDI-TOF/TOF MS (Table 5).

For the Creatine trial, tropomyosin was up regulated in Creat8. Tropomyosin interacts with actin, regulating muscle contraction (Silva et al., 2011, Ochiai and Ozawa, 2020). Phosphoglycerate mutase (spot 365) and myosin light chain (spot 426) were up regulated in Creat5, although the myosin light chain was also down regulated in Creat8 (spot 440). The up- and down-regulation of myosin might be due to the existence of multiple isoforms (de Vareilles et al., 2012). In a study performed on rainbow trout, it was shown that creatine synthesis occurs mainly in the muscle instead of transporting it between tissues, therefore creatine supplementation in fish feed does not seem to influence the amount in the muscle (Borchel et al., 2014). These findings are, to some extent, confirmed by our results, where it seems that energetic pathways - due to higher concentrations of creatine - were not explicitly altered, as seen by a mixed expression of myosin. Gene ontology (GO) determination of biological process, molecular function and cellular component of all identified proteins are shown in the Supplementary Material (Table S4.3.1). No differential abundance of the parvalbumin protein was observed in the Creatine trial. Modulation of this protein has been

reported in murine species (Gallo et al., 2008), where oral supplementation of creatine lowered parvalbumin expression. It seems that creatine supplementation in fish diets, in species like European seabass and gilthead seabream (Schrama et al., 2018) does not modulate the major fish allergens expression. As explained before, in theory, an increasing capacity for high-energy phosphate shuttling lowers fish's allergenicity by modulating the parvalbumin expression. This was not the case in our experiment, which agrees with the results obtained in gross energy where no differences were found between the conditions, showing that no modulation would have been achieved.

For the EDTA trial, a warm temperature acclimation protein was up regulated in EDTA3. This protein showed high similarities with hemopexin (Diaz-Rosales et al., 2014), confirmed by a BLAST search against *Danio rerio*, which is known to be involved in temperature acclimation and immune response (Diaz-Rosales et al., 2014). As temperature did not vary over the trial, this result suggests that this protein was involved in the enhancement of the fish's immune response. Two proteins identified as apolipoproteins (spots 784 and 968), were both up regulated in EDTA3. Besides its role in the lipid metabolism, apolipoprotein is also involved in antimicrobial activity, as shown in studies with carp (*Cyprinus carpio*) and rainbow trout (*Onchorhynchus mykiss)* (Villarroel et al., 2007). These results suggest that EDTA supplementation affects fish immune response. Beta-enolase, creatine kinase, myosin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), related to the energy metabolism (Schrama et al., 2018), were identified in spots 549, 755, 867, and 668, respectively. These proteins were down regulated in EDTA3 (spots 549 and 755), EDTA4.5 (spot 867) and EDTA1.5 (spot 668). Spot592, identified as a partial parvalbumin, was found to be down regulated in the EDTA3 group. As shown in the 2D gel representation (Fig.4.3.7), this spot is located in a high molecular weight area (52 kDa) and seems to be part of another non-identified protein.

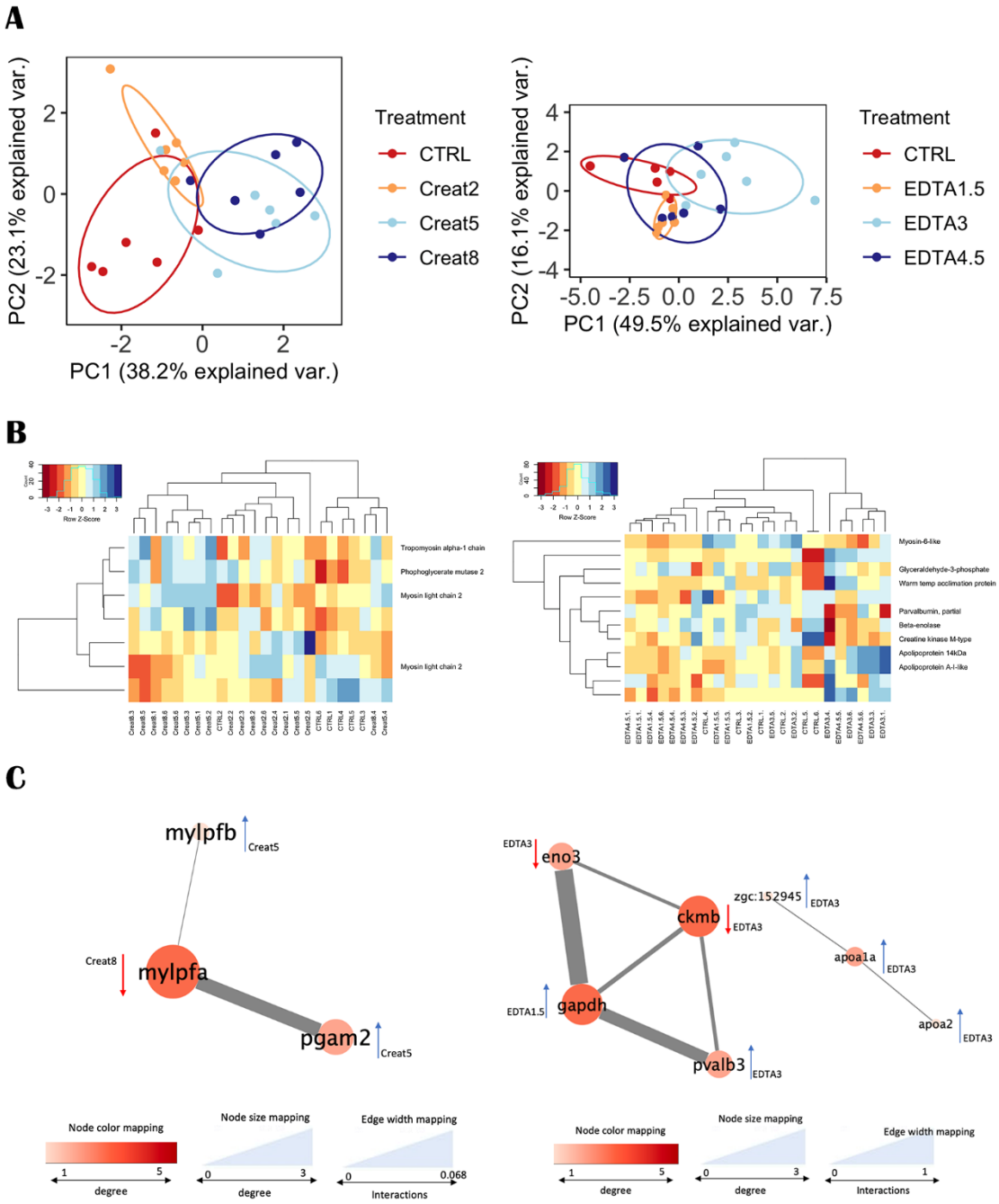

Fig. 4.3.8 A - Principal component (PCA), B- Hierarchical clustering (HCA) analyses performed with the normalized spots volumes of the identified proteins and C- Protein-protein interaction network generated with the identified proteins in the muscle samples of European seabass in the Creatine and EDTA trial. For HCA, each column corresponds to a biological replicate (fish). Cell color indicates the normalized Z-scores of the spot volumes. For the interaction network, nodes represent proteins and edges the experimental determined interactions between them. Red arrows represent down-regulated proteins with supplemented diets and blue arrows represent up-regulated proteins with supplemented diets.

Principal component (PCA) and hierarchical clustering (HCA) analysis were performed with the normalized spots volumes of the significant different proteins to check for clusters of observations (Fig. 4.3.8A and 4.3.8B). PCA results showed that no main clusters are formed in both trials. More specifically, in the Creatine trial's score plot, the Creat8 group was more distant from CTRL, where Creat2 and Creat5 show some overlap. In the EDTA trial, the EDTA3 and EDTA4.5 groups overlapped with CTRL, where EDTA1.5 showed a small cluster in the EDTA4.5 group. These results were consistent with other findings showing that used supplementations did not have a major impact on protein differentiation. The HCA in both trials showed, in each case, two main groups of observations, but when we looked at the specific conditions established at the bottom there was a mix of some biological replicates which was also shown in the corresponding PCA. The identified proteins were blasted against *Danio rerio* to search for orthologues and used for the protein-protein interaction (PPI) analysis. A PPI network was generated on the STRING website revealing 3 nodes/proteins (1 protein had no interaction and is not shown) for the Creatine trial. Two different interactions/networks, one with 4 nodes/proteins and another with 3 nodes/proteins (1 protein had no interaction and is not shown), were generated for the EDTA trial. Further analysis were performed on Cytoscape, where node color and size were determined by degree and edge width was determined by experimentally demonstrated interactions (Fig.4.3.8C).

4.3.3.7 IgE-reactivity

As mentioned before, supplementation with EDTA would induce the apo-form of parvalbumin, a structural rearrangement blocking the functional epitopes of the protein, becoming less allergenic. Therefore, we analyzed the IgE-reactivity of crude muscle protein extracts from the EDTA trial using a sandwich IgE-ELISA. A total of 6 fish allergic patients' sera were used (Supplementary Material Table S4.3.2). The obtained results showed no significant differences in the IgE-reactivity to parvalbumin from muscle samples of the EDTA-fed fish (Table 4.3.6). As aforementioned, EDTA supplementation was studied previously in gilthead seabream (Raposo de Magalhães et al., 2020b) showing to decrease IgE-reactivity, however, in this study, it seems that European seabass allergenicity was not affected by the experimental diets. Further analysis needs to be performed to better understand the allergenic potential of European seabass.

Table 4.3.5. Identification of European seabass (*Dicentrarchus labrax*) muscle proteins. Identified protein name with *Danio rerio* homolog for STRING annotations, Score, Mw T/C (Molecular weight, theoretical/calculated), pI T/C (Isoelectric point theoretical/calculated), number of matched peptides, fold change (Log2) treatment vs CTRL, *q*-value (one -way ANOVA, followed by *post-hoc* Tukey).

Spot nº	NCBI accession number	Identified protein [*Species*], and *Danio rerio* homolog preferred name (for STRING annotation)	Protein/ion Score	Mw T/C	pI T/C	Number of matched peptides	Fold change (Log_2)	*q*-value
261	XP_022046522.1	Tropomyosin alpha-1 chain isoform X1 [*Acanthochromis polyacanthus*], TPM1	272/187	32721/39572	4.69/5.6	5	0.33 (Creat8)	0.035
	AFN10643.1	Myosin light chain 2 polypeptide [*Oryzias javanicus*], mylpfa	173/173	19140/39572	4.69/5.6	3	0.33 (Creat8)	0.035
357	DAA12504.1	TPA_exp: warm temperature acclimation protein 65-2 [Dicentrarchus labrax], zgc:152945	359/339	49262/82207	5.47/5.0	6	1.36 (EDTA3)	0.026
365	XP_010730548.1	PREDICTED: Phosphoglycerate mutase 2 [*Larimichthys crocea*], pgam2	283/242	28764/30481	8.31/8.2	3	0.24 (Creat5)	0.024
426	CBN81401.1	Myosin light chain 2 [*Dicentrarchus labrax*], mylpfb	869/756	19333/24523	4.58/4.7	9	0.30 (Creat5)	0.036
440	BAA95137.1	Myosin light chain 2 [*Trachurus trachurus*], mylpfa	1030/920	19136/21996	4.68/4.9	9	-0.45 (Creat8)	0.021
549	XP_029281999.1	Beta-enolase [Cottoperca gobio], eno3	471/425	47709/59396	6.11/5.8	7	-0.40 (EDTA3)	0.028
592	ACP30426.1	Parvalbumin, partial [Hypomesus transpacificus], pvalb3	371/350	11661/52581	4.43/5.8	5	-0.41 (EDTA3)	0.018
668	XP_030639690.1	Glyceraldehyde-3-phosphate dehydrogenase [Chanos chanos], gapdh	198/155	36297/44694	7.83/8.5	5	0.43 (EDTA1.5)	0.040

755	XP_017291772.1	Creatine kinase M-type [Kryptolebias marmoratus], ckmb	618/548	42847/35023	6.22/6.8	6	-0.44 (EDTA3)	0.030
784	XP_035536036.1	After BLAST: Apolipoprotein A-I-like [Morone saxatilis], apoa1	566/412	26350/32292	5.22/4.9	6	0.87 (EDTA3)	0.006
867	XP_035534285.1	After BLAST: Myosin-6-like [Morone saxatilis], myh6	281/261	27039/21511	5.63/5.6	5	-1.14 (EDTA4.5)	0.022
968	QCP69302.1	Apolipoprotein 14kDa [Lateolabrax maculatus], apoa2	191/178	15753/11695	5.68/5.0	3	0.64 (EDTA3)	0.018

Table 4.3.6 IgE-reactivities (kUA/L) of 6 fish allergic patients against European seabass muscle proteins from the EDTA trial. Data are represented as mean ± SD

Treatment	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
CTRL	212.8 ± 30.2	108.3 ± 3	24.2 ± 0.4	172.0 ± 4.1	341.6 ± 5.1	14.7 ± 0.2
EDTA1.5	155.0 ± 20.4	105.9 ± 2.7	24.3 ± 0.1	159.2 ± 17.7	310.6 ± 10.0	14.7 ± 0.5
EDTA3	183.9 ± 9	102.5 ± 20.8	23.1 ± 0.8	150.0 ± 11.1	354.4 ± 4.9	14.5 ± 0.3
EDTA4.5	179.9 ± 2.5	124.3 ± 4.1	22.9 ± 0.6	154.1 ± 11.1	363.2 ± 31.8	12.7 ± 1.6
p-value*	0.326	0.555	0.265	0.63	0.282	0.389

4.3.3.8 HPLC

Concentrations of EDTA in food are considered safe for human consumption if below 120 ppm (INERIS, 2012). Therefore, EDTA concentrations in fish muscle were determined by HPLC. The highest obtained concentration was 101.35 ± 3.27 ppm (in EDTA3), and therefore below the established limit in the EU. This has also been shown in a trial performed with gilthead seabream where values are below the threshold concentration (Raposo de Magalhães et al., 2020b).

4.3.4 Conclusion

Creatine (up to 8%) and EDTA (up to 4.5%) supplementation in fish feed preserved fish quality as edible fish. Some primary stress response was shown in terms of higher cortisol levels with supplemented diets, but to draw more objective conclusions regarding welfare, other parameters must be analyzed. Nevertheless, growth performance data suggests that creatine and EDTA supplementation do not compromise welfare and higher cortisol levels might indicate the activation of the HPI system's activation due to continuous muscle relaxation/contraction bioenergetics. Parvalbumin expression was shown not to be modulated by creatine supplementation. Similarly, IgE-reactivity performed with muscle samples of the EDTA trial shows no modification of allergenicity. Nevertheless, proteomics showed some differences in the comparative proteome for creatine and EDTA dietary supplemented fish diets over control diets, but overall does not seem to influence important metabolic pathways. A similar conclusion can also be drawn by the metabolic screening of liver tissue, where supplementation with EDTA appears to have a small influence on fish liver metabolic fingerprint. Even though omics analysis shows no modulation of the allergenicity of European seabass, both proteomics and metabolomics were able to detect some changes in the comparative proteome and metabolic fingerprint respectively, demonstrating their high sensibility and importance in nutritional studies, even when no differences are detected by other techniques. Although our main objective of modulating fish allergenicity with diet supplementation was not achieved, we believe that our research in this field brings new relevant information regarding fish allergy modulation and provides useful data for future investigation in this subject.

4.3.5 Acknowledgments

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4.3.6 Supplementary Material

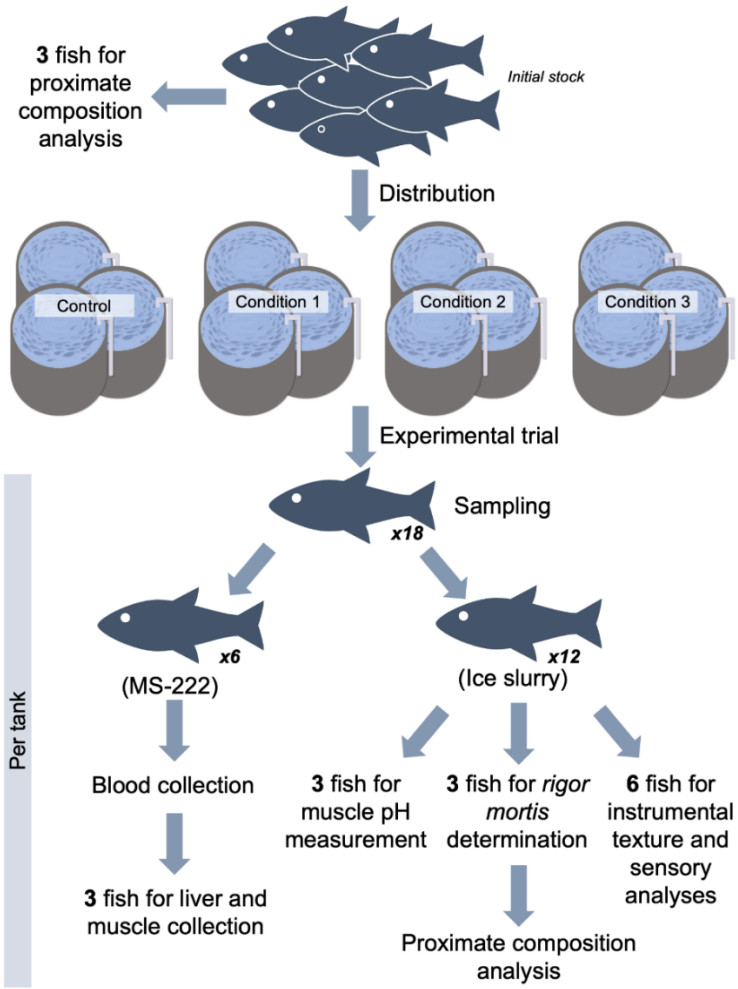

Fig. S4.3.1. Illustration of the sampling procedure for both creatine and EDTA trials

Table S4.3.1 Major Gene Ontology (GO) determinations for each identified protein from the Creatine and EDTA trial. N.A. – not attributed

Spot nº	NCBI accession number	Identified protein [*Species*]	GO Biological process	GO Molecular function	GO Cellular component
261	XP_022046522.1	Tropomyosin alpha-1 chain isoform X1 [*Acanthochromis polyacanthus*]	Muscle contraction (GO:6936); Actin filament organization (GO:7015); Skeletal myofibril assembly (GO:14866);	Protein homodimerization activity (GO:42803); Protein heterodimerization activity (GO:46982); Actin filament binding (GO:51015)	Cytoplasm (GO:5737); Actin filament (GO:5884)
	AFN10643.1	Myosin light chain 2 polypeptide [*Oryzias javanicus*]	N.A.	Calcium ion binding (GO:5509)	N.A.
357	DAA12504.1	TPA_exp: warm temperature acclimation protein 65-2 [Dicentrarchus labrax],	Cellular iron ion homeostasis (GO:6879); Heme transport (GO:15886); Heme metabolic process (GO:42168);	Heme transmembrane transporter activity (GO:15232); Metal ion binding (GO:46872)	Extracellular space (GO:5615)
365	XP_010730548.1	PREDICTED: Phosphoglycerate mutase 2 [*Larimichthys crocea*],	Glycolytic process (GO:6096); Myoblast fusion (GO:7520)	(Bi)phosphoglycerate activity (GO:4082, GO:4619)	N.A.
426	CBN81401.1	Myosin light chain 2 [*Dicentrarchus labrax*],	N.A.	Calcium ion binding (GO:5509)	N.A.
440	BAA95137.1	Myosin light chain 2 [*Trachurus trachurus*],	N.A.	Calcium ion binding (GO:5509)	N.A.
549	XP_029281999.1	Beta-enolase [Cottoperca gobio]	Glycolytic process (GO: 6096)	Magnesium ion binding (GO: 287); Phosphopyruvate hydratase activity (GO:4634)	Phosphopyruvate hydratase complex (GO:15)
592	ACP30426.1	Parvalbumin, partial [Hypomesus transpacificus]	N.A.	Calcium ion binding (GO:5509)	Cytoplasm (GO:5737)
668	XP_030639690.1	Glyceraldehyde-3-phosphate dehydrogenase [Chanos chanos]	Glycolytic process (GO:6096); Oxidation-reduction process (GO:55114); Glucose metabolic process (GO:6006); Protein stabilization (GO: 50821)	Peptidyl-cysteine S-nitrosylase activity (GO:35605)	Cytosol (GO:5829)

755	XP_017291772.1	Creatine kinase M-type [Kryptolebias marmoratus]	Phosphorylation (GO:16310); Phosphocreatine biosynthetic process (GO:46314)	Creatine kinase activity (GO:4111); ATP binding (GO:5524)	Extracellular space (GO:5615)
784	XP_035536036.1	After BLAST: Apolipoprotein A-I-like [Morone saxatilis]	Lipoprotein metabolic process (GO:42157); Positive regulation of lipid metabolic process (GO:45834); Lipid homeostasis (GO:55088)	Lipid binding (GO:8289)	High-density lipoprotein particle (GO:34364)
867	XP_035534285.1	After BLAST: Myosin-6-like [Morone saxatilis]	Skeletal muscle thin filament assembly (GO:30240); Slow-twitch skeletal muscle fiber contraction (GO:31444); Striated muscle myosin thick filament assembly (GO:71688)	Motor activity (GO:3774); ATP binding (GO:5524); Actin filament binding (GO:51015)	Myosin complex (GO:16459)
968	QCP69302.1	Apolipoprotein 14kDa [Lateolabrax maculatus]	Nuclear division (GO:280)	N.A.	N.A.

Table S2. Demographics and clinical characteristics of the allergic study cohort

Patient No.	**Gender/age**	**Cod**	**Salmon**	**Tuna**	**Gad m 1**	**Sal s 1**	**Thu a 1**	**Cyp c 1**	**Clinical history of fish allergy**
	[yrs]	[kU_A/L]							
1	M/12	100.0	100.0	ND	36.7	26.3	39.0	39.9	A, AE, AP
2	M/14	17.0	16.9	ND	39.2	38.0	42.1	40.1	AE
3	M/20	19.8	33.1	ND	36.0	22.7	35.0	27.6	A, AE, U
4	M/16	3.1	1.8	ND	38,8	41.2	41.9	41.0	AP, V
5	F/15	22.0	13.5	ND	41.9	41.8	43.5	42.3	AP, AE, N, V
6	F/14	37.5	23.6	ND	13.5	37.5	29.7	27.3	AE

ND, not determined; A, Astma; AE, angioedema; AP, abdominal pain; N, nausea; U, urticarial; V, vomiting

Chapter 5

General discussion

The main objective of the present thesis was to investigate the possibility of producing a low allergenic gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*), two important commercial Southern Europe aquaculture species. To pursue this end, different strategies were followed, namely by:

i. modulating the main fish allergen parvalbumin through the use of different additives in fish diets,
ii. analyzing this allergen stability after digestion and processing methods, and
iii. understand if allergen modulation changes flesh quality parameters that may affect its consumption.

Fish-allergic consumers need to be able to determine if a specific food may be consumed without triggering an allergic reaction (Kalic et al., 2022, Schrama et al., 2022a), and therefore a study was performed to understand consumer's willingness to pay for a low allergenic premium fish. In this general discussion, specific results of each chapter will be highlighted.

5.1 Parvalbumins characterization and stability to digestion and processing

The main fish muscle allergen, parvalbumin, is known to be relatively stable against thermal, chemical and proteolytic degradation (Costa et al., 2022). First, both parvalbumin structures from gilthead seabream and European seabass were obtained using circular dichroism (CD). This spectrophotometric analysis determines the secondary structures of proteins when used in the far ultraviolet (UV) region. Parvalbumin structures of gilthead seabream and European seabass were analyzed in **Chapter 2.** A higher content of α-helices than β-sheets was observed for both parvalbumin structures when analyzed at room temperature (20°C). This was also reported for the structures of Baltic cod and common carp parvalbumins (Bugajska-Schretter et al., 2000, De Jongh et al., 2011). Additionally, at boiling temperature parvalbumin presents a random conformation. However, contrary to what was observed in Baltic cod and common carp parvalbumin, the main allergen from gilthead seabream and European seabass seems unable to refold completely when cooling back to room temperature (Bugajska-Schretter et al., 2000, De Jongh et al., 2011). This was observed due to a shift of zero ellipticity to lower wavelengths, which demonstrates the loss of some α-helices. A possible explanation for this might be the a molten globule state where, proteins

like parvalbumin, with over 100 amino acids, show an intermediate folding state prior to completely returning to their native state (Dobson, 2004, Radford, 2000). As shown in a pressure-temperature phase diagram of Atlantic cod parvalbumin, the molten globule state is a partially unfolded protein without aggregation (Somkuti et al., 2012).

To better characterize the stability of parvalbumin from gilthead seabream and European seabass, several food processing methods were selected. As stated in the introduction of this PhD thesis, food allergies are triggered upon ingestion or contact (e.g., by inhalation or skin) of allergens. To cause an allergy by ingestion, parts of the allergens have the ability to resist gastrointestinal digestion, where food proteins are subjected to acidic environments and proteolytic enzymes like pepsin and trypsin. These conditions might result in protein unfolding and/or breakdown into peptides. However, this breakdown does not necessarily mean that parvalbumins allergenicity decreases, as B cells or IgE antibodies might still recognize (parts of) the allergen (Aalberse and Crameri, 2011). We showed that the detectability of parvalbumin during the gastric phase decreased, followed by a further decline during the intestinal phase, resulting in parvalbumin not being detected in European seabass after 240 min. The harsh conditions of the gastrointestinal tract could change the conformation or destroy the structure of parvalbumin, resulting in less or no binding of the antibodies to the allergen. Protein detection by SDS-PAGE also showed the degradation of parvalbumin into smaller fragments. However, *in vitro* conditions might be slightly different from *in vivo*, where consumption of different foods result in diverse pH values (Minekus et al., 2014). Indeed, pH fluctuates throughout a meal digestion, starting with a fasted pH below 2 and increasing up to 5 and above after food intake (Minekus et al., 2014). To mimic as best as possible the gastric conditions, a static value of pH 3 was used in our analysis. A review on gastrointestinal digestion showed the resistance or formation of stable fragments of several food allergens, against the total digestion of non-allergens within seconds (Moreno, 2007). However, some pepsin sensitive allergens like α-lactalbumin showed low structural stability, but proteolytic fragments have the potential to trigger an allergic reaction (Costa et al., 2022). This might also be the case with the digested fragments of parvalbumin, but since we did not analyze IgE-reactivity, only speculations about its allergenicity can be made. It is stated that fragments should have at least 15 amino acids each, and two IgE binding sites or epitopes should be available for an allergic reaction (Huby et al., 2000). Allergic reactions might still occur with IgE-binding to the fragments, which shall be analyzed in the near future. Another

factor influencing allergenicity is the relative abundance of the allergen present in a specific food (Mills et al., 2004). Parvalbumin is highly abundant in fish muscle, which is speculated to survive digestion and cause allergy to the consumer.

Further, in **Chapter 2,** analysis of parvalbumins stability after thermal and non-thermal methods, showed that detectability decreased, especially with the addition of 5% NaCl. Salting is one of the oldest preservation methods for fish and several concentrations (in combination with drying) might be used depending on the desired conservation time (Brás and Costa, 2010, Nguyen et al., 2010). The relatively low concentration of the used brine solution (5%) changed parvalbumins structure resulting in a low recognition of the antibody. Likewise, steaming and autoclaving (121°C, 1.1 bar pressure) decreased the detectability of parvalbumin. Moreover, 12 months freezing at -20 °C showed that the stability of parvalbumin was affected in almost all processing techniques in both species, except for raw and boiled samples of European seabass. SDS-PAGE confirmed the stability of parvalbumin in boiled samples. For salting, steaming and autoclaving, parvalbumin was still detected by Coomassie but with less intensity. Therefore, it seems that these processing techniques can denature the allergen in various ways. Modifications in the structure might occur by unfolding, aggregation and cross-linking, which influence allergenicity (Rahaman et al., 2016). Noteworthy, processing techniques can either reduce, increase or not affect allergen content/allergenicity as conformational epitopes might be hidden or exposed, by aggregation or unfolding, respectively, while the sequential epitope remains intact (Rahaman et al., 2016). A recent study on seafood products showed that extensive washing, thermal processes, and inclusion of additives alter the conformation and aggregation of parvalbumin. The reduction of the content of this allergen also showed less IgE-reactivity (Pérez-Tavarez et al., 2021). Another study performed with several fish species, showed that parvalbumin content was reduced in Atlantic salmon and increased in tuna after heating (Saptarshi et al., 2014). In the case of cross-linking and related to other food ingredients, a Maillard reaction is known to influence sequential epitopes and modifying allergenicity, given the non-enzymatic glycation process to reduce sugars and amino acids (Gou et al., 2022). In seafood, studies on allergenicity after a Maillard reaction showed a reduction in glycated Alaska Pollock parvalbumin and an increase in IgE-binding of codfish parvalbumin after glycation (Zhang et al., 2021, De Jongh et al., 2013). With the determination of parvalbumins detectability after heat, steam, salt, and freezing, further research on gilthead seabream and European seabass

allergenicity should be performed to understand if fish-allergic consumers show any reduced IgE-reactivity to processed fish.

5.2 Consumer's acceptance of low allergenic premium fish

Evaluating consumers' awareness and receptivity to a specific modified aquaculture fish is crucial for its development. Two approaches were used to understand consumers' acceptance, one based on a telephone interview in Portugal and the second based on a sensorial evaluation of the produced fish.

Data were collected by a questionnaire performed with Portuguese adults (above 18 years) and eligible answers were obtained from 25.8% of the contacted individuals, as described in **Chapter 3**. The objective was to determine the consumers' willingness to pay a higher price for low allergenic fish. Results showed that 36% of fish consumers have the intention to purchase fish with low allergenic potential. Data also showed that, on average, consumers were willing to pay 5% more to acquire these fish. Our findings are in accordance with studies showing consumers' willingness to pay more for sustainable fish or acceptance of farmed fish as a functional food (Zander and Feucht, 2018, Ramalho Ribeiro et al., 2019). In the latter, Portuguese consumers' willingness to purchase fortified seabream revealed a relative high interest (55% of respondents). However, it is also true that this interest decrease (35%) when prices are higher (Ramalho Ribeiro et al., 2019). Further, on an institutional level, specifically, hotels, schools and elderly houses, Pereira et al. (2017) evaluated the potential commercialization for fortified seabream. Results showed that almost half (45.8%) of the respondents are willing to buy this fish product. Besides this, on average, Portuguese consumers were willing to pay 7.8% more than the current price (Pereira et al., 2017). However, it should be noted that consumers' choice of functional foods may be influenced by price (Annunziata and Vecchio, 2013). Fish welfare is another factor of importance in aquaculture. An evaluation of consumers showed that 48% were willing to pay a quarter extra for welfare rainbow trout (Solgaard and Yang, 2011). Besides the importance of product pricing, other factors like health image/information and taste were important for consumers to try functional foods (Baker et al., 2022). This PhD thesis showed hereby that a future production of low allergenic fish has a positive economic potential on the Portuguese market and beyond. Therefore, this newly developed product with high quality and value contributes to more dietary options for fish consumers.

The main objective of fish diet supplementation with specific additives is improving fish performance such as growth and welfare, including health and immunity. It might also be used with specific proposals such as the modulation of target proteins. Fish consumers are aware of the sensory properties of different species and receptiveness of this new fish product is essential for its development. Therefore, any negative effect of additives in the quality of the fish flesh should be avoided. Classifications like, uncharacteristic and typical taste, firmness and succulence are common traits used during a sensorial evaluation performed by a trained panel. These classifications were also given to gilthead seabream and European seabass fed with creatine or EDTA supplemented diets. The evaluation by a panel was performed in random order and no differences in organoleptic properties were found, due to the supplementation of creatine or EDTA in gilthead seabream **(Chapter 4.1 and 4.2)** or European seabass **(Chapter 4.3)** diets, with respect to control groups. Related publications with these additives in fish diets are scarce (Wuertz and Reiser, 2022) but a study performed with pork meat showed no differences in juiciness with 0.2% creatine supplementation (Bahelka et al., 2020). With no significant effects of these additives on fish sensorial attributes, consumers' expectations regarding the quality of this product were not affected.

Another important element analyzed in these fish was the eventual muscle accumulation of creatine and EDTA. Detected levels in muscle were below legal accepted values. Therefore, when linking the analysis about consumer's willingness to pay, the sensory attributes and levels of detection in muscle, the premium fish products show a positive prospective for aquaculture production. Fish nutritional values combined with an eventual lower allergenic potential are valuable characteristics for the importance of this product in the aquaculture fish market.

5.3 Diet supplementation and fish quality

The quality of fish is mainly evaluated by its freshness, which can be determined by whole fish or fillets texture, appearance and by the aforementioned sensory properties (Cheng et al., 2014). Several intrinsic and extrinsic factors like different post mortem stages, protein degradation and ATP decomposition, influence the quality of fish (Cheng et al., 2014). It is known that feeding diets with specific ingredients (additives in this case), are one of the physical factors that might influence the fish quality, besides e.g. species, age and size or chemical factors like protein content (Cheng et al., 2014). Determination of muscle texture

gives more insight into fish quality using indicators obtained by instrumental determination of e.g. firmness, hardness and chewiness (Olafsdottir et al., 2004). In this study, texture analysis was performed to determine if diet supplementation affects the quality of fish fillets. Instrumental texture of gilthead seabream muscle showed no differences with creatine supplementation **(Chapter 4.1)**, on the contrary, it suggests a possible EDTA effect since 3/5 textural parameters (adhesiveness, springiness and cohesiveness) revealed significant differences between conditions ($p < 0.05$) **(Chapter 4.2)**. Nevertheless, the vital factor which affects the acceptability of fish fillets, hardness, did not show any significant differences (Hu et al., 2019). No differences were found for both additives (creatine and EDTA) in European seabass **(Chapters 4.3)**. In fish, these studies are lacking but a study performed with Pacific white shrimp (*Litopenaeus vannamei*) showed higher muscle hardness and chewiness than the control group with a creatine supplementation of 0.8% (Cheng et al., 2021).

Two other important indicators for fish quality (determined on whole fish) are muscle pH and the *rigor mortis* state of fish during the first post-mortem days. Due to the increase of lactic acid in fish muscle, pH values decline after slaughter, normally measured up to 72 h post-mortem. This results in an increase in protein denaturation and, consequently a decrease in texture (Skjold et al., 2020). The state of developing *rigor* depends mainly on the degradation of ATP, which usually takes up to a day after slaughter. ATP catabolites affect the taste, firstly in a positive manner but followed by a negative effect, and after the resolution of *rigor mortis* quality decreases with the deterioration of texture and increase of odor (Poli et al., 2005). Highly stressed fish show a faster onset of *rigor mortis* due to the faster degradation of ATP (Borderías and Sánchez-Alonso, 2011). As shown in **Chapter 4.1** pH values showed a constant variation, with initial pH lower than expected, nevertheless this could not be attributed to stress as indicated by lower cortisol levels in fish fed supplemented diets. In **Chapters 4.2 and 4.3**, pH values decreased over the 72 h post-mortem as expected with no major differences between treatments in the case of European seabass. For gilthead seabream fed EDTA supplemented diets, pH values ended lower than reported elsewhere (Matos et al., 2013, Silva et al., 2012b), suggesting a total depletion of glycolytic energy reserves. The *rigor mortis* onset was faster in gilthead seabream than in European seabass, which seems to be related to lower pH values in the first post-mortem hours. Significant differences in muscle pH and *rigor mortis* in gilthead seabream fed with EDTA supplemented diets might be explained due to low availability of calcium ions. This might lead to a non-

activation/delayed activation of calcium-dependent proteins which, consequently slows the rate of pH drop (Ayala et al., 2010, Delbarre-Ladrat et al., 2006). With these results, we could show that creatine supplementation in fish feed did not affect flesh quality, as no major differences were observed in muscle texture, pH and *rigor mortis* compared to control groups. This was also the case for European seabass fed with EDTA. On the contrary, for gilthead seabream some differences were found in texture and pH and *rigor mortis.* More research is needed to confirm fish quality in this case, although it is not likely to affect overall quality as no differences were found in cortisol, hardness, nor the sensory analysis.

Combining all the results about consumer perception, cortisol, muscle quality, pH, and *rigor mortis*, we showed the positive potential of these Mediterranean species on the fish market without major differences in quality. Therefore, consumer's expectations about the health benefits of fish consumption are maintained, contributing to the growth and sustainability of the aquaculture businesses.

5.4 Gilthead seabream and European seabass allergenicity

To analyze the allergenic potential of the two Mediterranean species, gilthead seabream and European seabass fed with supplemented diets, two different techniques were used in this PhD thesis. For creatine or EDTA supplementation, parvalbumin was detected using proteomics (2D-DIGE) or immunoblots/IgE ELISA, respectively. It is known that Ca^{2+} is essential for several physiological functions, such as muscle relaxation/contraction. With creatine supplementation, the pool of creatine phosphate will produce more ATP, which is needed for the SERCA pump to transport Ca^{2+} into the SR. With the decrease of cytosolic Ca^{2+} there is less need for buffering by parvalbumin, eventually downregulating its expression/concentration. It is reported that upon a lower concentration of parvalbumin, calcium homeostasis is regulated by mitochondria (Butera et al., 2021). However, **Chapters 4.1 and 4.3** showed no differences in parvalbumin expression between the different creatine supplementations and control. Supplementing up to 8% of creatine to fish diets did not modulate gilthead seabream or European seabass' allergenic potential. A study performed on rainbow trout (*Oncorhynchus mykiss*) demonstrated that creatine contents in fish muscle are higher than in mammals, which might be explained due to their differences in mobility (Borchel et al., 2014). It also seems that fish muscle produces its own creatine; therefore, it does not need an intake of creatine (Borchel et al., 2014) to keep up with its normal needs.

However, a swimming performance study in rainbow trout showed higher endurance with creatine supplementation (McFarlane et al., 2001). Although our results did not show any differences in glycogen reserves, proteomic results did show some minor differences in proteins involved in the energy metabolism (creatine kinase and myosin). Therefore, supplementation with creatine slightly influences fish' energy homeostasis, but does not affect main allergens, like parvalbumin. Besides, it is important to note that this supplementation did not affect the final body weight of fish between treatments. Despite related literature being scarce, studies performed on red drum (*Sciaenops ocellatus*) and hybrid striped bass (*Morone saxatilis x M. chrysops*) showed an increase in growth, with supplementations up to 4% creatine, when salinity is kept low or brackish, respectively (Burns and Gatlin, 2019, Burns and Gatlin III, 2022).

In **Chapter 4.3,** no differences were found in IgE-reactivities of fish-allergic patients when using EDTA supplementation in European seabass, showing therefore no reduction in the allergenic potential. On the contrary, a significant reduction in IgE-reactivity was found when feeding EDTA supplemented diets to gilthead seabream (**Chapter 4.2**). In this case, 9 out of the 17 positive patients showed no IgE-reactivity to 3% EDTA samples. This showed a promising potential for reducing this species allergenicity, as IgE of more than 50% of the patients had no or less reaction with parvalbumin of gilthead seabream fed 3% EDTA supplemented diet. It seems that parvalbumins epitopes were affected by a conformational change, possibly due to calcium depletion, which results in lower recognition of IgE. Hereby, it should be noted that supplementation with EDTA affect fish growth. In the case of gilthead seabream, supplementation was limited to 3%. In the case of European seabass, a tendency of increasing FCR was shown until 4.5% (even with 5% fish hydrolysate added as an attractant). A proteomic analysis was also performed to understand if EDTA supplementation influences fish muscle proteome. However, in this case it was not expected that parvalbumin would show any difference in expression, as EDTA is a Ca^{2+} chelator and therefore should only modify the conformation of calcium-binding proteins instead of its expression.

With the promising results in lowering allergenic potential, fish growth needs to be improved to keep aquaculture production sustainable. Previous studies with tilapia (*Oreochromis niloticus* and *Sarotherodon galilaeus*) showed an increase in body weight when supplemented with up to 2% EDTA (Abdel-Tawwab et al., 2017). The mechanism of EDTA in fish is still unclear and therefore we can only speculate on the reason for such a growth

difference between species. It might be due to differences in the digestion and absorption of these different fish species. More details need to be investigated to understand the lower growth performance of the two Mediterranean species used in this PhD thesis.

5.5 Proteomics and allergenicity

As a final highlight of the present thesis a short explanation of proteomics and its use in the aquaculture industry will show the possibilities of this technique involving food safety and allergenicity.

Proteomics is a high-throughput technology used in modern food science to understand biological processes, regardless of the organism, by identifying, quantifying and looking into its proteome. Detection of fish products in foods is one of the main areas related to the safety of food products where the applications of proteomics methodologies can have a significant impact (Farinha et al., 2022). Besides this, proteomics has been used as a monitoring tool in farmed fish to understand the biological mechanisms related to quality changes, with the scope of presenting better quality farmed fish to increase its acceptance by consumers (Rodrigues et al., 2017). Moreover, several studies demonstrated that proteomics plays an important role in understanding the effects of feed supplementation on fish metabolism (Schrama et al., 2017, Richard et al., 2016, de Vareilles et al., 2012). Taking this in mind, proteomics was used in this thesis research to understand if fortified feeds change the main fish allergen. Proteomics established methodologies allow for highly accurate, sensitive, and rapid detection and identification of seafood allergens, leveraging the consumption of fish by allergic consumers. Results showed that proteomics (2D-DIGE) was able to accurately separate parvalbumin in muscle samples from gilthead seabream and European seabass, allowing us to analyze its expression in experimental groups versus control groups. Further studies with mass spectrometry methodology (e.g LC-MS) can even deepen our knowledge regarding how impactful feed supplements can have on fish allergens.

Chapter 6

Conclusions and future perspectives

6.1 Conclusions

Worldwide allergic reactions to fish occur mainly (70-95% of cases) against an allergenic protein called parvalbumin. With the supplementation of creatine and EDTA to fish diets, we aimed to modulate parvalbumin to obtain a farmed fish with lower allergenic potential as an end product. Fish-allergic consumers would benefit from the possibility of purchasing low allergenic farmed fish.

The objectives of the work pointed out in the beginning of this chapter were attained:

i. Characterization and stability of parvalbumin was performed. The secondary structure of parvalbumin from gilthead seabream and European seabass, presents mainly α-helices and some β-sheets. Both structures could not refold totally when cooling down after heating which seems to be explained due to the presence of a molten globule state. When in the presence of Ca^{2+}, this relative stable allergen seems to be susceptible to gastrointestinal digestion, where enzymes like pepsin (1U/µg of protein) cause degradation of the parvalbumin structure. Consequently, parvalbumins detectability decreased when using sandwich ELISA. Processing techniques like high temperature combined with pressure and salting were able to alter the conformation of this important fish allergen, resulting in lower detectability of the antibody. At this point we cannot draw firm conclusions about allergenicity as no IgE reactivity was analyzed.

ii. Consumer's willingness to pay for premium products was surveyed. A marketing study performed to investigate the selling potential of low allergenic fish showed that consumers have an increased willingness to pay 5% more for low allergenic fish. This positive outcome shows the potential for the Portuguese and worldwide aquaculture market to produce a newly developed premium product with added value.

iii. The quality of gilthead seabream and European seabass fed with supplemented diets, and the effect of this nutritional strategy on allergenicity was assessed. To produce a low allergenic gilthead seabream and European seabass, creatine or EDTA were added as supplements into their diets. Sensorial attributes were not affected by the inclusion of these additives.

Additionally, no accumulation of over legally established values for these compounds was found in fish muscle. The creatine metabolism in fish is not fully understood, therefore we can only speculate about the reason why the supplementation with this organic compound does not decrease parvalbumins expression. One reason is that fish already contains higher levels of creatine than mammals. Consequently, the higher levels of ATP needed by the SERCA pump to decrease cytosolic Ca^{2+} do not necessarily mean that parvalbumin deactivates its function and reduces its concentration. The calcium chelator EDTA seems to have a promising potential for producing a low allergenic gilthead seabream, as it showed a reduction in IgE-reactivity in more than 50% of the tested fish-allergic consumers. This encouraging result showed that it is possible to modulate parvalbumins structure to decrease its allergenicity. Nevertheless, more research is still required as this additive affects fish growth performance, in order not to jeopardize the aquaculture industry.

Overall, this thesis is a first part of a long way to understand how to decrease fish allergenicity and potentially help millions of consumers to have access to high quality protein, which may be important to less developed countries populations. Nevertheless, we showed that parvalbumins structure is susceptible to gastrointestinal digestion and processing methods, like salting, canning, and freezing. Diet additives, like creatine and EDTA have no negative effects on fish quality, except for gilthead seabream with EDTA (which deserves to be further addressed). Parvalbumins structure is important for allergenicity, and without functional epitopes, due to the calcium chelator EDTA, a lower allergenic structure of this protein was achieved in gilthead seabream. More research with creatine is necessary to understand its potential in fish allergenicity. Besides this, it seems that results are species specific and therefore more research is needed in the context of different aquaculture commercial fish species.

6.2 Future perspectives and limitations

The worldwide population keeps growing with a perspective of reaching 9.3 billion in 2050. Therefore, there is a need for 60 % more food to keep up with this increase. Also, with more population, eventually more people will present a kind of allergy. To complement the work presented in this PhD thesis and produce a low allergenic fish in industrial manners more research needs to be accomplished.

As the creatine metabolism is not fully understood in fishes, it would be beneficial to study the different creatine-related genes in gilthead seabream and European seabass to deep in our understanding on how to use this valuable additive in fish diets. With this, it would be possible to understand if creatine supplementation changes fish metabolism and if this will lead to more ATP in fish muscle, which is necessary for calcium homeostasis. Furthermore, different calcium chelator agents, might modify parvalbumins structure without compromising fish performance, especially growth. Besides this, different incorporation percentages of creatine or (different) calcium chelators might modulate parvalbumins expression or conformation, respectively. However, growing trials should be performed priorly analyzing fish growth performance. Calcium is fundamental for the healthy development of bone tissue in fish, besides its involvement in muscle contraction. Therefore, to prevent low levels of calcium, in future trials free calcium should also be measured. Even a combination of different additives (after analyzing their compatibility), would possibly modulate parvalbumin to decrease its allergenic potential. As additives need to be incorporated into the control diets, production limitations might be encountered. High costs or difficult diet incorporations might limit the development of new fish feeds. Development for new feed production technology would be therefore key to enhance these bottlenecks.

Processing methods, such as autoclaving, salting or freezing showed to decrease parvalbumins detectability. Therefore, the next step would be to analyze IgE-reactivity of fish-allergic patients using techniques like immunoblots and/or IgE-ELISA. If positive results are shown in these steps, clinical trials could be performed by skin prick tests to analyze allergic reactions *in vivo*. Besides this, a basophil activation test (BAT), measuring the degree of degranulation by flow cytometry after contact with an allergen, would give insight into the reactions of allergic patients or control. The processing methods in this thesis were only performed with control fish, therefore, it would be interesting to combine the new feeding

trials with specific additives with thermal and non-thermal processing afterwards. After these steps being covered, we speculate to see a lower allergenic potential of gilthead seabream and European seabass.

Besides additives, digestion, and processing methods, there are ongoing studies with immunotherapy. Although not yet available in clinical trials, research on immunization with hypoallergenic fish allergens could be a promising tool to prevent allergic reactions in sensitized individuals.

The research conducted in this dissertation was planned and performed in a relative "small scale", and to fully understand how to modulate fish allergens, specifically parvalbumin, parallel research should be scaled up to other species, with other additives, assessing the IgE reactivity, and testing other processing methods.

Implications for aquaculture

This dissertation describes how to potentially decrease fish allergenicity. The outcomes of these research suggest that we are closer to provide the industry with new tools to offer allergic consumers the possibility of eating fish. A foreseen increase in fish allergic consumers will therefore benefit from our baseline tools and procedures to provide all people with premium quality fish. Despite slightly higher costs of producing these fish or the increased costs of processing these fish species, consumers are willing to pay more. Nevertheless, regardless of the steps to enhance these processes, they are harmless to fish and consumers. This will increase fish farming sustainability, the safety for consumers and may well increase the welfare of the farmed fish, with the use of fortified diets. Besides, as stated at the beginning of this section, humanity relies even more on aquaculture with the growing population, given its undeniable role in global food security and nutrition. However, the increasing demand for healthy and less expensive protein puts more pressure to increase aquaculture production, and like other industries, such growth must not come at the cost of aquatic ecosystem health, animal welfare and people's health. Fish farming practices needs, therefore, to adapt to cope with this demand, being advantageous to introduce new farming practices to reduce the impact of intensification.

Chapter 7

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