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UNIVERSIDADE DO ALGARVE

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Master in Aquaculture and Fisheries

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DECLARAÇÃO DE AUTORIA DE TRABALHO

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

Universidade do Algarve, 30 de Setembro de 2016

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Abstract

A continuously increase of the world's population has been registered over the last decades, along with an augment of fish consumption worldwide. Fish is one of the most common elicitors of food-allergies. The panallergen in fish, β-parvalbumin (β-PV), is responsible for the initiation of immunoglobulin-E (IgE)-mediated allergic reactions. PV is a calcium-binding muscle protein, highly stable and IgE cross-reactive. Conserved IgE epitopes are located in the calcium-binding domains where a reduced IgE-binding capacity was found upon calcium depletion. The present work aims to reduce farmed fish allergenicity by inducing this potentially less allergenic PV (apo-PV), through specific fish diets supplemented with Ethylenediaminetetraacetic acid (EDTA), a calcium chelator used as tool to deplete calcium-ions from PV. Gilthead sea bream (*Sparus aurata*) was reared under four different conditions differing in the supplementation of EDTA. After 98 days of trial, fish were slaughtered and muscle and plasma samples were taken. A biochemical characterization was performed, along with a proteomic analysis. Fish extracts were prepared from fish muscles and proteins were separated by two dimensional difference gel electrophoresis (2D-DIGE) followed by spot identification using matrix-assisted laser desorption ionization – time of flight/ time of flight mass spectrometry (MALDI-TOF/TOF). Fish allergenicity was evaluated by skin-prick tests (SPT) and immunoblot assays with sera from fish-allergic patients. Low concentrations of EDTA showed to have a positive impact in reducing allergenicity without affecting fish growth performance and organoleptic properties. Comparative proteomics showed an effect of the diets with 8% of EDTA in the expression of several proteins with a variety of functions. As expected, parvalbumin's expression was unchanged. SPTs and immunoblot assays revealed a slightly reduction of the allergenicity. This preliminary experiment shows that an EDTA supplemented diet might be a promisor tool to modulate fish allergenicity and it is a valuable contribution to improve safety of aquaculture fish.

Keywords: EDTA, parvalbumin, fish allergy, proteomics, IgE-reactivity

Resumo

Um crescimento contínuo da população tem sido registado ao longo das últimas décadas, acompanhado de um aumento do consumo de peixe a nível mundial. Este aumento do consumo de peixe, associado à promoção de hábitos alimentares que incita o consumo deste produto alimentar, e à diminuição dos *stocks* de peixes selvagens nos oceanos levou a uma maior procura de peixes de aquacultura. No entanto, o peixe é um dos principais instigadores de alergias alimentares e estudos que englobem as medidas de criação de peixe em aquacultura para modulação deste potencial alergénico são escassos ou praticamente inexistentes. As alergias alimentares são um problema em crescimento na sociedade atual e uma elevada prevalência das alergias a peixe tem sido registada principalmente em países costeiros e países onde o consumo de peixe é elevado. O principal alergéneo no peixe, designado por β-parvalbumina (β-PV), é responsável por desencadear reações alérgicas mediadas por anticorpos imunoglobulina-E (IgE). A PV é uma proteína do músculo, pequena (Mw ~12 kDa), acídica (pI ~ 6), que participa na contração e relaxamento muscular. Pertence à família designada por *EF-hand*, caracterizada por apresentar dois domínios *EF-hand* capazes de se ligarem a iões de cálcio. Assim, esta proteína é capaz de se ligar a dois iões cálcio ao mesmo tempo. A quantidade de PV varia também consoante o tipo de músculo e quantidades mais elevadas desta proteína têm sido associadas a uma maior rapidez de relaxamento muscular. É uma proteína altamente estável a desnaturação química, térmica, e proteolítica, e que apresenta reatividade cruzada com os anticorpos IgE. Esta reatividade cruzada é responsável pelo facto de grande parte dos pacientes alérgicos a peixes serem alérgicos a mais do que uma espécie diferente de peixe. Ou seja, estes domínios de ligação podem apresentar uma elevada homologia entre espécies. Na PV estes domínios proteicos onde se ligam os iões de cálcio apresentam epítopos, zonas onde se ligam os anticorpos IgE, que contêm regiões conservadas de aminoácidos. A capacidade de ligação dos IgE aos epítopos é reduzida aquando da libertação dos iões de cálcio da proteína uma vez que uma diferente conformação é induzida. Assim, o presente trabalho visa reduzir a alergenicidade do peixe de aquacultura induzindo uma forma da PV potencialmente menos alergénica (apo-PV), através de dietas suplementadas com ácido etilenodiaminotratraacético (EDTA). Este quelante de cálcio foi usado neste trabalho como uma ferramenta para diminuir a disponibilidade de cálcio no músculo ou mesmo para retirar os iões de cálcio da PV uma vez que foi provado já em diversos estudos, que o EDTA é capaz de imitar a PV na

maioria das suas tarefas de contração/ relaxamento muscular. Doze grupos de douradas (*Sparus aurata*), constituídos por 25 peixes cada, foram submetidos a quatro condições de crescimento (três tanques para cada condição) diferindo na concentração de EDTA presente na dieta: controlo (0% de EDTA), EDTA3%, EDTA5% e EDTA8%. Após 98 dias de ensaio, os peixes foram abatidos em água e gelo e retiradas amostras de músculo e plasma. Foi realizada uma caracterização bioquímica do músculo do peixe onde foram avaliados os seguintes parâmetros como medidas de qualidade e bem-estar: pH, *rigor mortis*, capacidade de retenção de água do músculo (WHC), textura e análise sensorial. Foi feita também uma quantificação dos níveis sanguíneos de cortisol nos peixes de modo a poder avaliar-se o estado de stress a que os animais estavam submetidos. Seguiu-se uma quantificação da PV através do método de imunodetecção ELISA. Este método foi validado com uma análise proteómica uma vez que os métodos ELISA disponíveis no mercado apresentam anticorpos contra a parvalbumina do bacalhau e, tendo em conta a alta reatividade cruzada que esta proteína apresenta, os resultados podiam não ser significativos. Na análise proteómica foram preparados extratos de músculo de peixes das quatro condições diferentes, seguindo-se uma separação das proteínas através de uma electroforese diferencial em gel a 2 dimensões (2D-DIGE). Nesta técnica as proteínas são separadas consoante o seu peso molecular e ponto isoelétrico e marcadas com flurocromos o que permite a comparação de proteínas pertencentes a diferentes amostras no mesmo gel. As proteínas musculares detectadas como diferencialmente expressas foram retiradas do gel, submetidas a digestão por tripsina e identificadas usando a técnica de espectrometria de massa designada por ionização por dessorção a laser assistida por matriz – tempo de voo/tempo de voo (MALDI-TOF/TOF). A alergenicidade dos peixes foi avaliada através de testes cutâneos (SPTs) e técnicas de imunodeteção utilizando soro de pacientes alérgicos a peixe. Baixas concentrações de EDTA mostraram um impacto positivo na redução da alergenicidade sem efeito sobre o crescimento dos peixes ou propriedades organolépticas o que demonstra possivelmente que uma diferente conformação, menos alergénica da PV foi induzida. Estas baixas concentrações de EDTA tiveram também um ligeiro efeito positivo sobre o pH e a capacidade de retenção de água do músculo possivelmente devido ao facto do EDTA diminuir a disponibilidade de cálcio e assim as protéases dependentes de iões cálcio não serem ativadas. A quantificação do cortisol revelou um estado de stress ligeiramente elevado que pode dever-se a diversas causas como as técnicas de manuseamento. A análise proteómica revelou um ligeiro efeito das

dietas enriquecidas com 8% de EDTA sobre a expressão de determinadas proteínas com funções conhecidas como contração muscular, metabolismo de energia, estrutura celular e reguladores de transcrição de DNA e tradução de mRNA. Tal como era esperado, a expressão da PV manteve-se inalterada, o que foi comprovado pela técnica ELISA assim como pela análise proteómica. Os SPTs com pacientes alérgicos a peixe revelaram uma ligeira redução da alergenicidade para peixes submetidos a 3% de concentração de EDTA. Os testes de imunodeteção realizados com soro de pacientes alérgicos a peixe mostraram igualmente uma ligeira redução do potencial alergénico. Este teste preliminar, apesar de requerer estudos complementares, mostra que dietas suplementadas com EDTA são uma medida promissora para modular o potencial alergénico em peixes sendo uma contribuição importante para a segurança alimentar em peixes de aquacultura.

Palavras-chave: EDTA, parvalbumina, alergia a peixe, proteómica, reacção de IgEs

Abbreviations

µl - Microliter

µm – Micrometer

2-DE – Two-dimensional electrophoresis

2D-DIGE – Two-dimensional difference gel electrophoresis

3D – Three dimension

AA – Amino acid

Acetyl-coA – acetyl-coenzyme A

ANOVA – Analysis of variance

Apo-PV – apo-parvalbumin

ATP – Adenosine triphosphate

BLAST – Basic Local Alignment Search Tool

CHAPS – 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate

CHL – Centre Hospitalier de Luxembourg

Cm – Centimeter

Ctrl – Control

Cy2 – bright, green-fluorescent dye that can be excited using the 492 nm laser line

Cy3 – bright, orange-fluorescent dye that can be excited using the 532 nm laser line

Cy5 – bright, far-red-fluorescent dye that can be excited with 633 nm or 647 nm laser lines

DMF – Dimethylformamide

DNA – Deoxirribonucleic acid

DTT – DL-Dithiothreitol

EAACI – European Academy of Allergy and Clinical Immunology

EDTA – Ethylenediaminetetraacetic acid

EDTA3 - Ethylenediaminetetraacetic acid 3 %

EDTA5 - Ethylenediaminetetraacetic acid 5%

EDTA8 - Ethylenediaminetetraacetic acid 8%

EGTA - ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

ELISA – Enzyme-linked immunosorbent assay

ESI-Ion Trap – Electrospray ionization-Ion trap

EU – European Union

FAO – Food and Agriculture Organization

FBW – Final body weight

FCR – Feed conversion ratio

FE – Feed efficiency

FI – Feed intake

g - Grams

GE – General electronics

h - Hour

HCl – Hydrogen chloride

His73 – Histidine 73

HRP – Horseradish peroxidase

HSP – Heat shock proteins

IBW – Initial body weight

IEF – Isoelectric focusing

IgE – Immunoglobulin E

IgG – Immunoglobulin G

IPMA – Instituto Português do Mar e da Atmosfera

IUIS – International Union of Immunological Societies

kDa – Kilo daltons

LIH – Luxembourg Institute of Health

M - Molar

mA/gel – Milliamps per gel

MALDI-TOF/TOF – Matrix-assisted laser desorption/ionization-time of flight

min - Minutes

mM – Milimolar

mRNA – Messenger ribonucleic acid

MS – Mass spectrometry

Mw – Molecular weight

NaOH – Sodium hydroxide

nmol – Nanomole

PDH – Pyruvate dehydrogenase

PDHC – Pyruvate dehydrogenase complex

PDHK2 – Pyruvate dehydrogenase kinase, isoform 2

PEA – Plano Estratégico para a Aquicultura portuguesa

PFF – Peptide fragment fingerprinting

pI – Isoelectric point

PMF – Peptide mass fingerprinting

Pmol - Picomole

POP – Plano Operacional das Pescas

PTMs – Post-translational modifications

PUFA – Poly unsaturated fatty acids

PV - Parvalbumin

RAST – Radioalergosorbent test

RB – Rehydration buffer

S.D. – Standard deviation

SDS – Sodium dodecyl sulfate

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SGR – Specific growth rate

SIPA1L1 – Signal-induced proliferation-associated 1 like protein 1

SPT – Skin-prick test

SR – Sarcoplasmic reticulum

TMA - Trimethylaminuria

TMAO – Trimethylamine N-oxide

TMB – 3,3',5,5'-Tetramethylbenzidine

TnC – Troponin C

TnI – Troponin I

TnT – Troponin T

Tris-HCl – Tris(Hydroxymethyl)aminomethane hydrochloride buffer

UK – United Kingdom

V - Volts

v/v – Volume per volume

Vhr – Volume per hour

VFI – Voluntary feed intake

w/v – Weight per volume

Wdf – Weight of dead fish

WG – Weight gain

WGt – Total weight gain

WHC – Water holding capacity

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1. State of art

1.1. Aims of the work

The main goal of this work is to verify the effect of EDTA on the allergenic potential of farmed gilthead sea bream (*Sparus aurata*) through six major scientific objectives: 1 – evaluate the effect of EDTA enriched diets (with different concentrations of EDTA) on the activity, structure and expression of the fish β-parvalbumin; 2 – realize a proteomic characterization in order to assess the effect of EDTA on the muscle proteome and identify the differentially expressed proteins; 3- perform a biochemical characterization to verify the effect of EDTA supplementation on the organoleptic properties of the fish muscle; 4 – perform a zootechnical characterization in order to assess the effect of EDTA enriched diets on the fish behaviour and growth performance; 5 – evaluate the allergenic potential of gilthead sea bream fed with EDTA enriched diets through SPTs and finally; 6 – assess the effect of EDTA on the IgE-reactivity of fish-allergic patients' sera through immunodetection assays.

1.2. State of the aquaculture worldwide

The continuous growth of the world's global population, which is expected to reach 9.6 billion people in 2050 coupled with the challenge of feeding the world while trying to maintain its natural resources for future generations, leads to an increased food demand, comprising fish. Fish is an extremely nutritious food product and a valuable source of essential AAs, polyunsaturated fatty acids (PUFA) and lipid-soluble vitamins, currently consumed as never before (Kuehn *et al.* 2014; Lopata & Lehrer 2009; Tsabouri *et al.* 2012). In 2010, fish represented already 16.7% of the world's population's intake of animal protein (FAO 2014). Consequently, this increased consumption of fish from a few decades on, led to a worldwide decline in wild fish stocks and thus to a decreasing in global capture fisheries (Figure 1.1) (Kime *et al*. 2001; Rodrigues *et al.* 2012). Aquaculture plays here a significant role, emerging as a solution to meet this high fish demand, providing already almost half of all fish for human consumption (Figure 1.1). In 2012, farmed fish contributed already with 42.2% of the total 158 million tonnes of fish produced, including for non-food purposes (FAO 2014). Aquaculture is also considered, nowadays, the food-producing sector in fastest expansion (Rodrigues *et al.* 2012).

China leads by far the world's aquaculture production with estimated values of 43.5 million tonnes of food fish in 2013, of the 70.5 million tonnes registered for the global aquaculture production in the same year (FAO 2014).

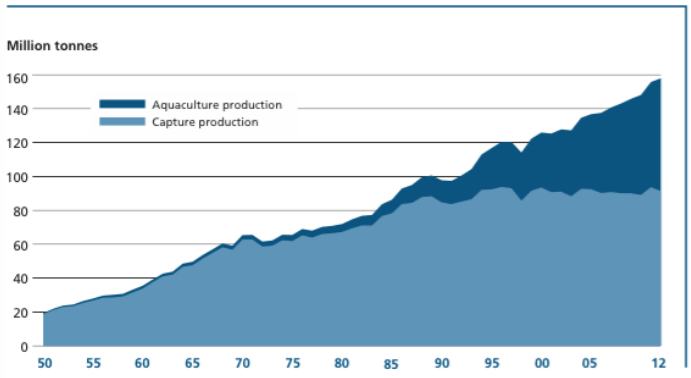


Figure 1.1. World's fish production by the fisheries and aquaculture industry, in million tones (Adapted from: FAO 2014)

Contrarily to the overall growth of the aquaculture production, the aquaculture output in some European countries has been fallen in the last years, contributing to a global decreasing in farmed fish production by the European continent (Figure 1.2). Among these countries are Spain, France and Italy. The main cause for this decline is believed to be the availability of imported fish with lower production costs, leading to a fall, especially in finfish and molluscs production by the mentioned European countries. In 2012, the farmed fish production in Europe contributed with only 4.32% for the world total production being thus the continent with the lowest average annual growth rate in the period of 2000-2012, reaching only 2.9% (FAO 2014).

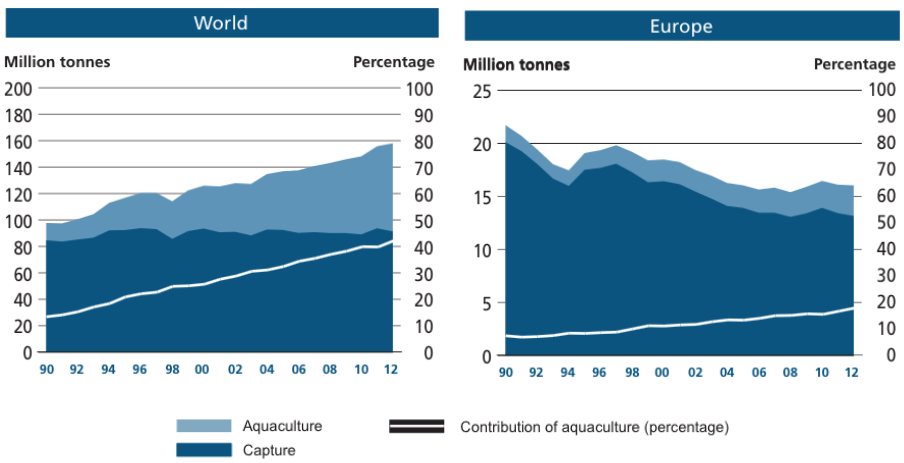


Figure 1.2. World and Europe's fish production by the fisheries and aquaculture industry, in million tones, and the contribution of the aquaculture industry for these numbers (Adapted from: FAO 2014)

Portugal offers a great geomorphological advantage for the aquaculture sector since it presents 1187 km of coastline and a huge diversity of species favourable for farming and with great market value. However, it is a very exposed coast to harsh weather conditions making offshore aquaculture an enormous challenge. Great part of the Portuguese aquaculture enterprises and farms are concentrated in the Algarve coast, south of Portugal, with bivalves' production predominating the output (POP 2007-2013; PEA 2014-2020).

Before the 90's, the Portuguese aquaculture was uniquely focused on bivalves, mainly clams of the species *Ruditapes decussatus* and rainbow trout. Only on this decade, marine species like sea bream and sea bass, and recently turbot and sole, started to be produced and commercialized (PEA 2014-2020).

Farmed fish production increased from 4457 tonnes in the early 90's to 10317 tonnes in 2012, with turbot production representing approximately 4400 tonnes of this amount. Algarve production represents here 5465 tonnes of the national aquaculture production in the same year. Although the increased volume of farmed fish verified in the last decade, this aquaculture production is not sufficient to balance the negative deficit of the fisheries sector, mainly because Portugal is one of the countries in the world with the highest fish consumption, reaching 56,7 kg per capita/year originating high volumes of importation (Figure 1.3) (PEA 2014-2020).

In an European Union (EU) context, Portugal represented in 2011 only 2% of the global aquaculture production (PEA 2014-2020).

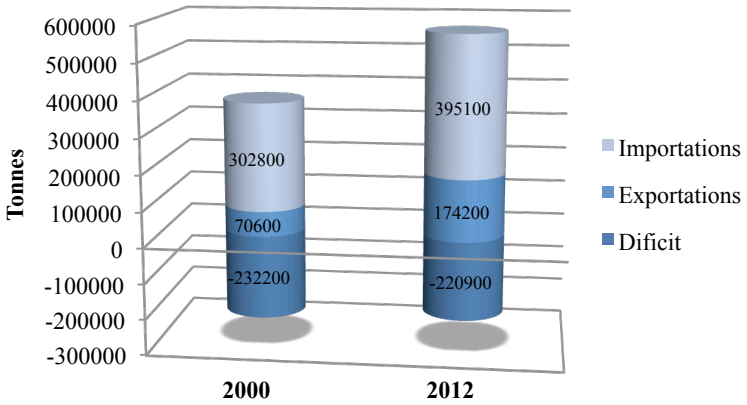


Figure 1.3. External deficit of fishery products in Portugal, in million tonnes. The aquaculture production is not sufficient to make this balance positive since Portugal is a country with high fish consumption per capita (Adapted from: PEA 2014-2020).

1.3. Fish quality and welfare

Farmed fish attempt to become the major source of dietary protein in our society (Kime *et al.* 2001) and this competition increases the need to store and transport food between places making food preservation a very important step to maintain the nutritional value, texture, flavour, and the highest quality of the fish product (Ghaly *et al.* 2010; Kolbe *et al.* 2006; Schiavone *et al.* 2008).

The shelf life of a food product consists of the maximum length of time a given product is fit for human consumption, which means that, in the case of fish, it corresponds to the time from when it is taken from the water until is no longer fit to eat (Doyle 1989). Thus, it is of great importance for the aquaculture industry to improve and extend the shelf life of seafood.

After harvesting, a seafood product passes through several stages of spoilage originating multiple compounds and the breakdown of others, responsible for changing odour, flavour and texture of the meat: *rigor mortis*, dissolution of *rigor mortis*, enzymatic autolysis, oxidation and microbial growth (Ghaly *et al.* 2010; Kolbe *et al.* 2006; Sigholt *et al.* 1997). One of the most noticeable events in the post-mortem changes is the *rigor mortis* (Figure 1.4) (Dunajski 1979), which corresponds to the process through which fish loses its flexibility due to stiffening of fish muscles after a few hours of its death (Adebowale *et al.* 2008). Immediately after death, muscle continues to hydrolyse ATP however at a slower rate, since the breakdown of glycogen into lactic acid maintains the levels of this molecule. Once the glycogen stores deplete, the ATP levels falls to zero and the myosin heads strongly attach to the thin filaments, or actin filaments, of the muscle myofibrils (Figure 1.5.) resulting in a rigid and stiff muscle (Offer *et al.* 1989). Multiple factors affect the rate and resolution of *rigor mortis* like the species, temperature, handling procedures, size and physical condition of the fish (Huss 1995). Enzymatic autolysis is mainly characterized by a reduction in textural quality, production of hypoxanthine by autolytic enzymes, production of formaldehyde by the degradation of Trimethylamine *N*-oxide (TMAO) originating Trimethylamine (TMA) and a pH drop, result of glycolysis, which breaks down glycogen originating lactic acid. Oxidation involves lipid oxidation, which is the major cause of deterioration for fish species presenting high fat content being responsible for the common off-flavour known as rancidity. In the last step of deterioration, microbial present in fish microflora and others tend to proliferate producing amines like putrescine,

histamine and cadaverine, ammonia, indole and acids resulting also in an unpleasant flavour and odour (Ghaly *et al.* 2010).

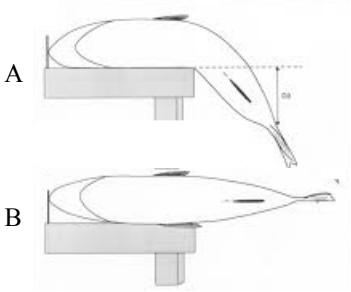


Figure 1.4. Evolution of *rigor mortis*. A- Pre-*rigor mortis*; B – *rigor mortis.* (Adapted from: Diouf & Rioux 1999)

Multiple factors affect freshness and quality of the fish flesh, like for example, chemical composition, storage temperature, ante-mortem handling, treatments after capture, and others (Dunajski 1979; Sigholt *et al.* 1997). Handling procedures, for example, increases the stress levels of the fish, causing an increased muscle activity and the release of cortisol, consequently reducing tissue water content (Sigholt *et al.* 1997). Fish muscle consists of 80% of fat and water, and, depending on the flesh properties and handling procedures, the muscle may lose or gain water, which for the market is very important since fish is sold by weight (Ofstad *et al.* 1996). Water holding capacity (WHC) of the muscle decreases with time in dead fish since *post-mortem* fish muscle sarcolemma, the plasma membrane surrounding the sarcoplasm, or the cytoplasm of a muscle fiber (Figure 1.5) (Martini *et al.* 2012), separates from the myofibrils and no longer traps water in between. WHC is therefore strongly influenced by structural changes in proteins, which are also strongly influenced by temperature, ionic strength and pH (Nordic Council of Ministers 1995; Ofstad *et al.* 1996). Evaluating the WHC will thus indicate the state of denaturation of the muscle proteins.

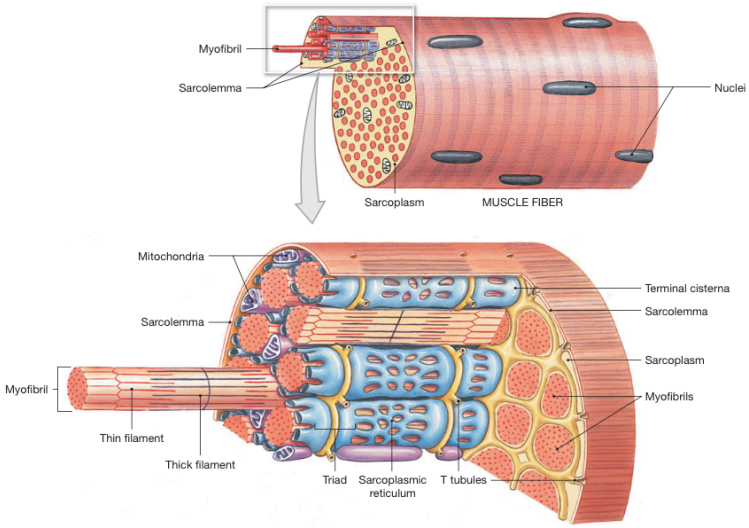


Figure 1.5. Internal organization of a skeletal muscle fiber (Adapted from: Martini *et al.* 2012)

Fish welfare is mainly evaluated through the stress status of the fish and the resultant stress physiological responses (Barton 2002). This is of great interest for the aquaculture industry since growth and reproductive performances, health status and diseases susceptibility are strictly related to stress conditions (Alves *et al.* 2010). As mentioned above, handling procedures can increase stress levels, but other stressors like chemical and perceived stressors can also evoke physiological responses (Figure 1.6), which are a mechanism of the fish to maintain its normal homeostatic state. These are grouped as primary, secondary and tertiary responses. Primary responses involve the release of corticosteroid and catecholamine hormones into circulation. Cortisol is the main corticosteroid present in actinopterygian fish and, therefore, its circulating level is commonly used as an indicator of the stress level of the fish. Secondary responses include metabolic changes and expression of heat-shock proteins (HSPs). Finally, tertiary responses include changes in growth and behaviour, for example (Barton 2002).

Based on the changes occurring in fish during its process of quality loss and in previous studies it is possible to affirm that it is of great importance for the market to evaluate fish shelf life through tests like muscle pH, WHC, *rigor mortis*, flesh firmness, sensory analysis among others (Aman 1983; Özogul *et al.* 2005; Özogul *et al.* 2006; Özyurt *et al.* 2009).

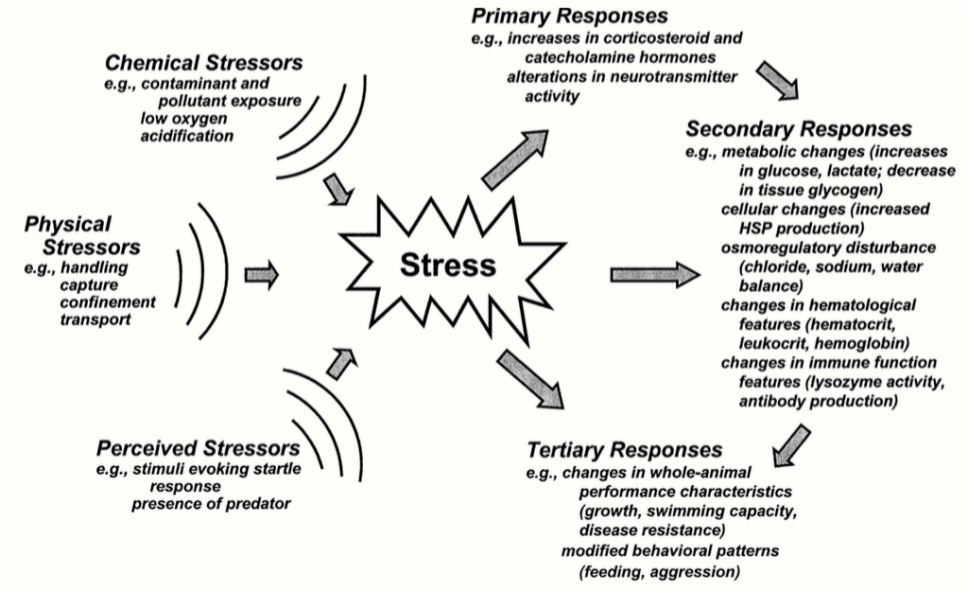


Figure 1.6. Different types of stressors able to induce different physiological responses to stress in fish, grouped as primary, secondary and tertiary responses. Some responses can evoke others, like the grey arrows indicate (Adapted from: Barton 2002)

1.4. Food allergies

Producing fish under strict controlled conditions and the possibility of manipulate an ideal product are two of the greatest advantages of the aquaculture against fisheries to fulfil the population's huge and rising demand for fish. However, as well as several food sources, fish, being these wild or farmed, is one of the most common elicitors of food allergies. This increased consumption of fish has resulted in more frequent reports of these adverse reactions leading to the need of understanding the reasons for the persistence of this allergy (Tsabouri *et al.* 2012). Previous studies on how farming practices can help modulating fish allergenicity are little or practically inexistent.

Food allergies have become, in the last several decades, an important public health concern with a continuously increasing prevalence, without any cure known (Burks *et al.* 2012; Kulis *et al.* 2015), and it's now considered a worldwide problem in westernized nations (Sampson 2004). Food allergy can be defined simply as an adverse health effect derived from an immune response directed toward food (Burks *et al.* 2012; Flanagan 2014; Kulis *et al.* 2015). More than 170 foods have already been reported as potentially allergenic and among these, considered the most common ones, are peanuts, tree nuts, eggs, milk, fish, crustacean shellfish, wheat and soy (Burks *et al.* 2012; Flanagan 2014). Food allergies can appear in childhood and outgrow, persist through adulthood, named *de*

novo sensitization, or appear only in adulthood (Burks *et al.* 2012). Allergies to milk, soy, egg and wheat, normally resolve around school age in children, contrarily to peanut and seafood allergies that are usually permanent (Longo *et al.* 2013; Sicherer & Sampson 2006; Valenta *et al.* 2015).

Food allergenicity is mediated by specific proteins, named food allergens able to trigger specific immunologic reactions, which are initiated by allergen-specific immune cells (Burks *et al.* 2012; Flanagan 2014; Kulis *et al.* 2015). Reactions can occur when ingesting food, either raw or cooked, considered class 1 food allergy, or simply by inhaling the food allergens, which are called aeroallergens, recognized in this case as class 2 food allergy (Burks *et al.* 2012; Sicherer & Sampson 2006). The major group of food allergens are the class 1 allergens characterized by water-soluble glycoproteins, relatively small (normally with 10-70 kDa) and very stable to thermal, acidic and proteolytic denaturation. Includes proteins like the caseins from milk, ovomucoid from eggs, and β -parvalbumins from fish. Bet v 1 from birch pollen is an example of a class 2 allergen that is able to induce sensitization through the respiratory route. A food may also have numerous homologous allergens sharing cross-reactivity between them, capable of inducing different immunological responses. Cooking food, like heating it, may reduce or enhance their allergenicity, depending on the allergen structure (Sicherer & Sampson 2006).

Adverse reactions to food are classified based on the pathogenetic mechanism (Figure 1.7). Toxic food reactions are a result of small contaminants, named toxins that can be naturally present in food or incorporated by food processing, for example. These molecules have an effect on all individuals who eat contaminated food. Contrarily to it, non-toxic food reactions occur with food that is not tolerated by only a few individuals. It can be sub-divided into immunological responses (food allergy) and non-immunological responses (food intolerance). Food intolerance can also be sub-divided into enzymatic and pharmacological food intolerance. First one includes lactose intolerance, the most common case, characterized by a deficiency on the β-galactosidase levels, the enzyme responsible for the hydrolysis of lactose. The second one is caused by vasoactive amines present in foods, like histamine and dopamine (Ortolani & Pastorello 2006). Among the so-called food allergies it is possible to distinguish two kinds of responses: IgE-mediated (e.g. milk, eggs and fish) and non-IgE mediated ones (e.g. gluten intolerance) (Flanagan 2014; Kulis *et al.* 2015; Ortolani & Pastorello 2006). The most common reaction, result of a non-IgE mediated food allergy, is the increase of IgG antibodies levels on the blood circulation

(Ortolani & Pastorello 2006). According to Valenta *et al.* (2015), the most common type among food allergies are the IgE-mediated ones, affecting approximately 1-3% of the world's population.

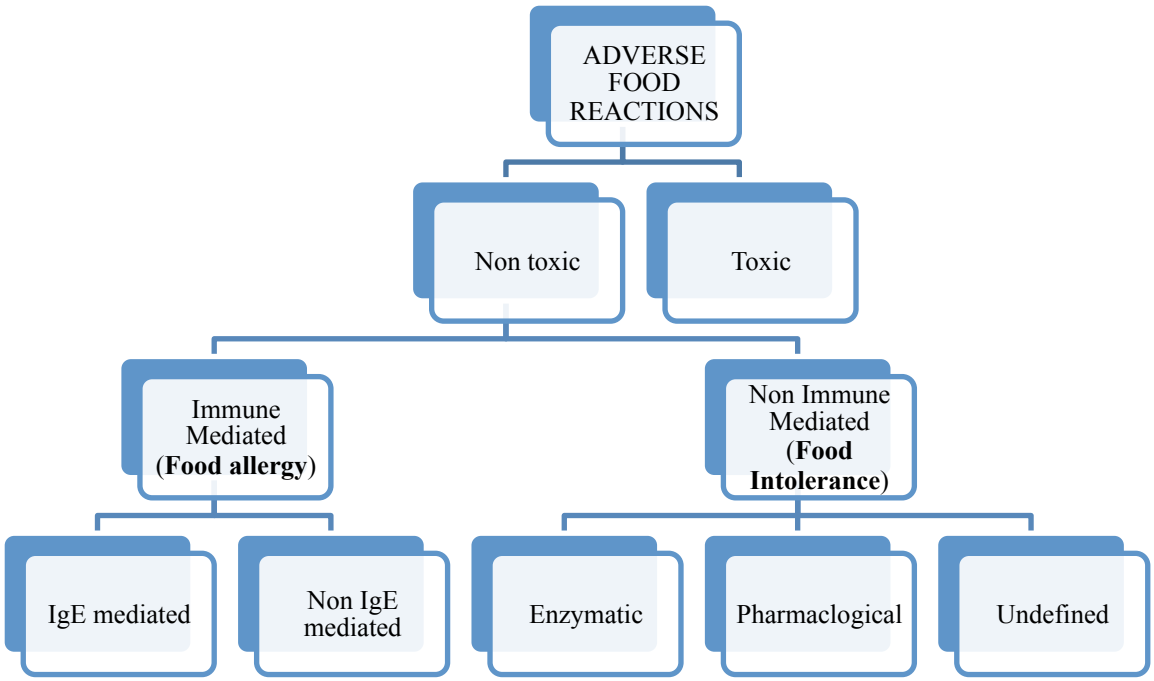


Figure 1.7. Adverse food reactions according to the European Academy of Allergy and Clinical Immunology (EAACI) classification

1.5. IgE-mediated food allergies

The term "IgE" refers to Immunoglobulin E, a type of antibody found only in mammals. When a food allergen-specific IgE enters the blood circulation (Figure 1.8) and is recognized by a B cell, it will produce antibodies IgE against it and, upon further exposure of any allergenic protein of the same kind, these antibodies are induced and bind onto high-affinity IgE FcεRI receptors present on mast cells, basophils and cells of the mucosa and skin (Flanagan 2014; Kulis *et al.* 2015). The activation of these cells requires cross-linking of IgE receptors with the allergen, thus, more than two IgE molecules need to be bound to one single allergen (Matsuo *et al.* 2015). This will cause degranulation and release of chemical mediators, such as histamine, responsible for causing the characteristic symptoms of allergy (Flanagan 2014; Kulis *et al.* 2015). An immediate reaction takes place a few minutes after contact with the allergen followed by a late-phase response that begins

4-6 hours after the contact and continues for several days (Ortolani & Pastorello 2006). This is the chain of reactions that happens with fish allergens.

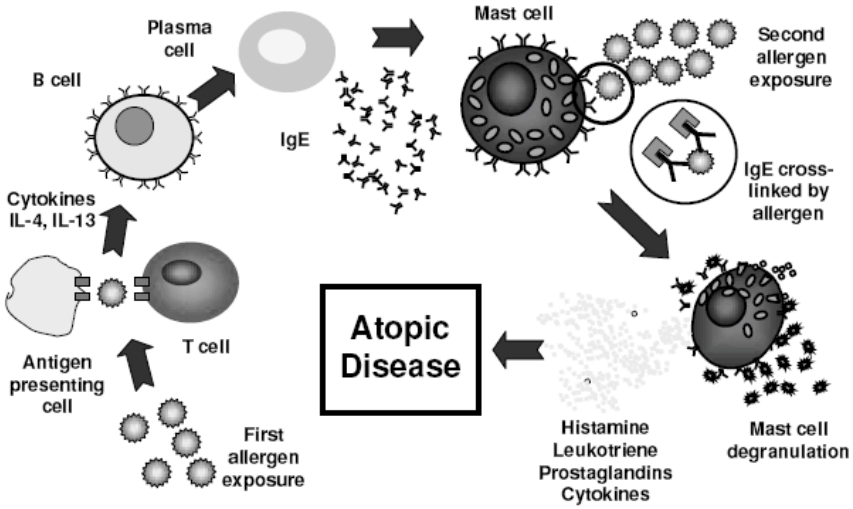


Figure 1.8. Typical immunological response caused by a food allergen. A food allergen, like the ones present in fish, initiates an IgE-mediated reaction. After the first exposure to the allergen, the recently produced IgE antibodies bind to the mast cells. Upon second exposure, the allergens bind to the sensitized cell inducing it to produce histamine and other mediators responsible for the allergy symptoms (Adapted from DePestel *et al.* 2008).

IgE antibodies bind to specific recognition sites in allergens, called IgE-binding epitopes (Figure 1.9), responsible for the allergenicity of the allergen. These can be divided into linear (sequential) and conformational (discontinuous) epitopes. First ones are characterized by continuous amino acid (AA) sequences, while the second ones by spatially adjacent AAs, distantly located in the AA primary sequence of the allergen. Improving the knowledge about the epitope structures of an allergen can contribute to a more accurate diagnosis and possibly for the development of hypoallergenic foods. Some epitopes have already been well mapped and characterized, like the ones from allergen molecules belonging to chicken eggs, cow's milk, wheat and shrimp. Studies on shrimp allergens are the most developed among seafood allergies and tropomyosin is the main allergen present (Table 1.1). Crustacean-allergic patients can show cross-reactivity among different types of shellfish since tropomyosins among crustaceans share around 98% of homology (Matsuo *et al.* 2015). Cross-reactivity is therefore characterized by the phenomenon when IgE antibodies recognize similar epitopes in an allergen to the ones they were originally directed at in another allergen (Ortolani & Pastorello 2006). This phenomenon can be caused when allergens share at least 50-70% of sequence identity and

it was already observed among various fish species and edible frog species (Ma *et al.* 2008). Table 1.1 shows an example of similarity between epitopes from two different isoforms of tropomyosin.

Both sequential and conformational epitopes are evidently responsible for allergic reactions, however, people presenting IgE antibodies against sequential epitopes react to food in any form, cooked or raw, while people with IgE antibodies against conformational epitopes are able to tolerate small amounts of cooked or hydrolysed food, since this processing induces structural changes in the proteins and conformational epitopes are destroyed (Sampson 2004).

Table 1.1. Shrimp most common allergens and their correspondent IgE-binding epitopes

Protein name	IUIS name	MW (kDa)	AA length	Accession no.	IgE-binding epitopes (AA number)	Ref
Tropomyosin from *Penaeus aztecus*	Pen a 1	37	284	AAZ76743 (Pen a 1.0101)	1-36, 37-63, 61-81, 82-105, 115-150, 142-162, 157-183, 190-210, 246-284	(Ayuso *et al.* 2002)
Tropomyosin from *Litopenaeus vannamei*	Lit v 1	37	284	ACB38288 (Lit v 1.0101)	43-57, 85-105, 133-148, 187-202, 247-284	(Ayuso *et al.* 2010)

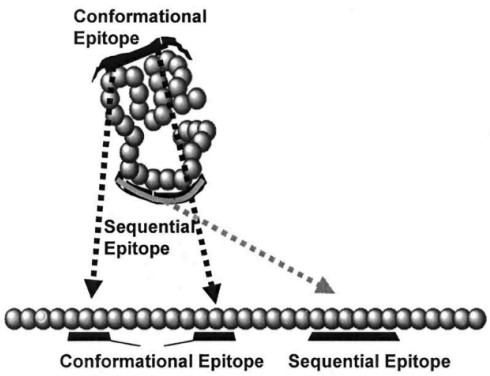


Figure 1.9. Conformational epitopes, contrarily to sequential epitopes are destroyed when structural changes in the protein occur during food processing (Adapted from: Sampson 2004).

Multiple *in vivo* and *in vitro* assays can be performed to assess the IgE-reactivity of food allergens (Velickovic & Gavrovic-Jankulovic 2014). In *in vivo* detection the most common tests are the SPTs that basically consists of puncturing the skin with a small food sample (Griesmeier 2009). The test is applied on the forearm and, after 15-20min, if the

person is sensitized it will develop a red swelling were the allergen was applied (Heinzerling *et al.* 2013). This is mainly used as a first test to confirm or refute allergic reactions since it's not very specific, especially for fish allergy, because patients seem to demonstrate monosensitivity to specific species (Sharp & Lopata 2014). A positive skin-prick test merely indicates that the patient has been sensitized to a particular food (Lee & Burks 2006). One of the advantages of this method is that multiple different allergens can be tested simultaneously since the consequential reaction appears right of the area of the SPT (Heinzerling *et al.* 2013). *In vitro* tests include the measurement of serum-specific IgE using several types of quantitative immunoassays like for example radioalergosorbent test (RAST) and enzyme-linked immunosorbent assay (ELISA), or immunoblots performed with sera from fish-allergic patients.

1.6. Fish allergies

As stated above, the increased production and consumption of fish have led to constant reports of adverse health problems, and, in western countries like Portugal and Spain where the consumption of seafood is high, coastal countries, and countries with high number of fish-processing industries, it is one of the most common food allergens (Kuehn *et al.* 2014; Tsabouri *et al.* 2012). Fish are capable of inducing IgE-mediated allergic reactions and prevalence rates vary considerably according to the region. In general seafood allergy affects up to 5% of all children and 2% of all adults (Sharp & Lopata 2014). However the actual prevalence of fish allergies is still not well established but it is estimated that corresponds approximately between 0.2-2.29% of the general population (Kuehn *et al.* 2014; Sharp & Lopata 2014). Fish allergy can be manifested in a variety of symptoms, from mild to severe reactions, comprising urticaria, allergic contact dermatitis, diarrhea or anaphylaxis (Van Do *et al.* 2005).

Cross-reactivity among fish species has been repeatedly reported and seems to be even more frequent between closely related fish (Kuehn *et al.* 2014). The study of Van Do *et al.* (2005) describes the cross-reactivity of allergens from 9 commonly consumed fish and stated that cod, salmon, pollack, herring and wolf-fish contained the most potent cross-reacting fish allergens (parvalbumins in this case), whereas halibut, flounder, tuna and mackerel were the least allergenic. This also explains why allergic people exhibit the same symptoms upon contact with different fish species (Swoboda *et al.* 2002). Contrarily to

these evidences few fish-allergic patients are able to tolerate certain fish species, such as tuna. Monosensitivity to a single fish has also been reported for sole, swordfish, pangasius, tuna and more recently for salmon and salmonid fish (Kuehn *et al.* 2014, Ma *et al.* 2008).

1.7. Fish parvalbumins

Several fish allergens have been identified during the last decades: enolases, aldolases, gelatin, vitellogenin and parvalbumins. Multiple studies have identified parvalbumins as the major cross-reactive allergens in multiple fish species and these have been so far, the most well characterized fish allergens (Bugajska-Schretter *et al.* 2000; Kuehn *et al.* 2014; Lopata & Lehrer 2009). The first analysis performed to fish allergens was in Baltic cod (*Gadus callarias*), identifying Gad c 1, a parvalbumin, as the major codfish allergen (Elsayed & Bennich 1975). Thus, this is usually used as reference to which other fish allergens are related (Van Do *et al.* 2005). The allergenicity of parvalbumins has been studied in the last few years in different fish species and by 2012, the allergome database (www.allergome.org) had already 218 allergenic isoforms of fish parvalbumins inserted (Velickovic & Gavrovic-Jankulovic 2014).

Parvalbumins are low molecular-weight proteins (10-12 kDa) belonging to a group of acidic (pI = 4.0-5.2), water-soluble, Ca^{2+}-transporting muscle proteins responsible for promoting relaxation in the fast-twitch muscle fibres (Bugajska-Schretter *et al.* 2000, Lee *et al.* 2012; Lopata & Lehrer 2009; Van Do *et al.* 2005; Velickovic & Gavrovic-Jankulovic 2014). According to Coughlin *et al.* (2007), higher amounts of parvalbumin are associated with faster rates of relaxation. The contraction-relaxation cycle of vertebrate muscles is promoted by the exchange of Ca^{2+} between the sarcoplasmic reticulum (SR) and the myofibrils, while relaxation is achieved by the return of Ca^{2+} from myofibrils back to the SR. In fast-twitch muscle fibres, parvalbumins facilitate the muscle relaxation by carrying the calcium from troponin-C (TnC), a subunit of the regulatory protein troponin, back to the SR (Figure 1.10) (Arif 2009). Bony fish muscle is composed by two types of tissues, the light and the dark muscle differing in their function and composition (Kuehn *et al.* 2014). Kobayashi *et al.* (2016) affirms that parvalbumin content varies by muscle parts, presenting the highest levels in white muscle and the lowest in dark muscle. Tuna is therefore considered less allergenic since its flesh is mainly composed by dark muscle of low parvalbumins' expression (Lim *et al.* 2005). Physical and chemical effects of food

processing techniques may also alter the fish allergenicity through parvalbumin degradation or oligomerization, which causes a decrease or an increase in the number of IgE-binding epitopes (Sletten *et al.* 2010).

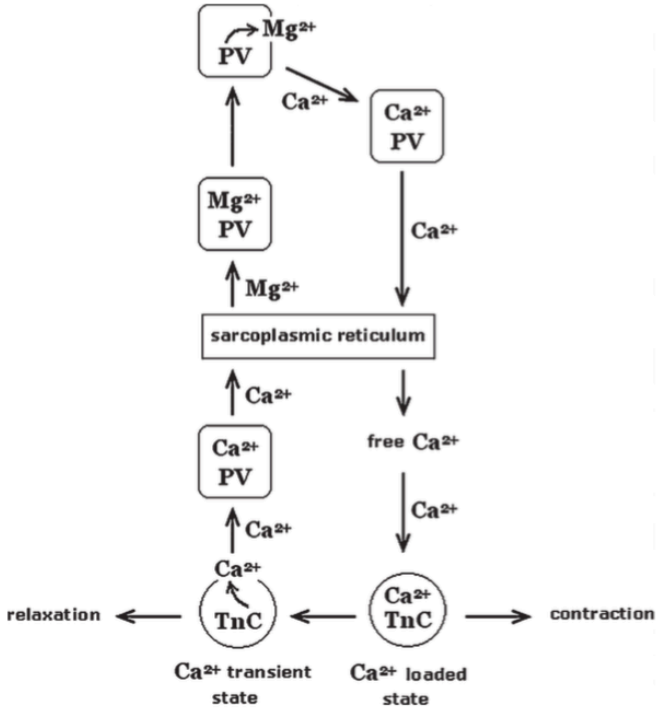


Figure 1.10. Role of parvalbumin in the promotion of fish muscle relaxation: the protein is responsible for shuttling calcium ions from the TnC to the SR (Adapted from: Arif 2009)

Parvalbumins are included on a family of proteins named EF-hand proteins. The three-dimensional (3D) structure of fish parvalbumin reveals six α-helices named A, B, C, D, E and F arranged in three homologous domains, AB, CD and EF, consisting each one of two helices interspaced by one loop (Figure 1.11). This makes the three EF-hand motifs, characteristic of this family of proteins, capable of chelating one divalent metal ion each, like calcium and magnesium (Arif 2009; Elsayed & Apold 1983; Kuehn *et al.* 2014). Only two of these motifs are functional, the CD and de EF domains, and able to bind to the metal ions (Bugajska-Schretter *et al.* 1999). The first motif, AB, the non-functional one, forms a cap covering the hydrophobic surface of the pair of functional domains (Swoboda *et al.* 2002).

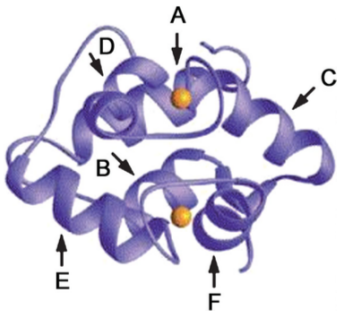


Figure 1.11. Representation of the three-dimensional helix-loop-helix structure of carp parvalbumin. The capital letters indicate the six α-helices and the yellow spheres represent the calcium ions (Adapted from: Arif 2009)

Parvalbumins can be sub-divided into two distinct phylogenetic lineages, the alpha (α) and the beta (β), which differ in their isoelectric point ($pI_\alpha > 5.0$, $pI_\beta < 4.5$), molecular weight and in other characteristics of the primary structure of the protein (Bugajska-Schretter *et al.* 2000; Kuehn *et al.* 2014; Lopata & Lehrer 2009; Van Do *et al.* 2005). They also differ in their affinity for metal ions: β-parvalbumin has 16% greater affinity for Mg^{2+} but an about 200% greater affinity for Ca^{2+} compared to its α homologous (Arif 2009). In amphibian tissues, both parvalbumins subtypes have been detected whereas in mammalian and avian muscles only α-parvalbumin was reported (Kuehn *et al.* 2014). In bony fish muscle it is the β-parvalbumin that can be found, contrarily to cartilaginous fish muscle that expresses exclusively α-parvalbumin (Goodman *et al.* 1979). However, only fish β-parvalbumins were considered so far a cross-reactive food allergen, presenting stability to thermal, chemical and proteolytic denaturation (Bugajska-Schretter *et al.* 2000; Hilger *et al.* 2004; Lopata & Lehrer 2009; Van Do *et al.* 2005). The same fish usually expresses two or more different β-parvalbumin isoforms, differing in the amino acid sequence and in their allergenicity, as in the case of salmon (β1 and β2), which is related to the IgE-binding epitopes (Kuehn *et al.* 2014; Velickovic & Gavrovic-Jankulovic 2014). Salmon isoforms β1 and β2 have more than 64% identity. This influences the reaction of one fish-allergic patient, who can react more to one isoform than to another, explaining the challenge of diagnosing a fish allergy (Velickovic & Gavrovic-Jankulovic 2014).

Several studies show a global rearrangement of the protein upon the depletion of calcium from the functional sites, promoting its apoform, named apo-parvalbumin. Helices A, B and F remain practically unchanged in their position, but C, D and E helices undergo substantial reorientation (Henzl & Tanner 2007). This apo-parvalbumin, which is calcium-

depleted, presents a substantial reduction of IgE binding from fish-allergic patients suggesting thus a reduction of the allergenic potential of the protein under this form (Bugajska-Schretter *et al.* 2000; Hamada *et al.* 2003; Kuehn *et al.* 2014; Swoboda *et al.* 2007; Perez-Gordo *et al.* 2013). Bugajska-Schretter *et al.* (1998), also observed a reduction of IgE antibodies bound to parvalbumin after Ca^{2+} depletion. This suggests that the IgE-binding epitopes of the fish β-parvalbumins are of the conformational type.

Additionally, studies based on the molecular dynamics and structure of the parvalbumin, suggest that the functional EF-hand loops do not exhibit the same binding properties when separated from their protein environment (Cates *et al.* 2002). As mentioned above, parvalbumin can bind to two Ca^{2+}, with high affinity, but it can also bind to Mg^{2+} with moderate affinity (Allouche *et al.* 1999; Johnson *et al.* 1999). Allouche *et al.* (1999) stated that, in the presence of Mg^{2+} in the environment, the affinity of Ca^{2+} to both binding sites is lower than if there was no competitor. Johnson *et al.* (1999) also affirms that apo-parvalbumin can rapidly bind to Ca^{2+}, contrarily to Mg-parvalbumin which is not able until Mg^{2+} dissociates. Thus, this emphasizes that a change in the environment surrounding the β-parvalbumin, like for example, in order to increase competition for the binding sites, or for the calcium, will promote its apoform, a potentially less allergenic form of parvalbumin.

1.8. Gilthead sea bream (*Sparus aurata*): biology, production and allergenic potential

Gilthead sea bream, *Sparus aurata* (Linnaeus, 1758) (Figure 1.12), is a high valuable fish for aquaculture industry, widely cultured in the Mediterranean region and constitutes an interesting animal model for research (Calduch-Giner *et al.* 2013).

Figure 1.12. Photography of a gilthead sea bream specimen (Adapted from: FAO 2005-2016)

Belonging to the Sparidae family it can reach up to 70 cm long and weight 10 kg. It is commonly found throughout the Mediterranean sea and along the Eastern Atlantic

coasts, from the United Kingdom to the Canary Islands (Fischer *et al*. 1987). It can live in marine waters as well as in the brackish waters of coastal lagoons. It commonly inhabits rocky or sandy bottoms, but can be also seen in seagrass beds. During the spawning period, between October and December, adults migrate into deeper waters (until 150 m depth). It is a hermaphrodite species, maturing for the first and second year of life as a male and then throughout the second or third year as a female. It feeds on molluscs, crustaceans and small fish (Fischer *et al*. 1987; Pavlidis & Mylonas 2011).

Sea bream was successfully reproduced for the first time in captivity in the 1980s and since then intensive rearing systems (sea cages) started to be developed, becoming this species, one of the main products of European aquaculture, with Greece leading the production (Figure 1.13). Nowadays, most of the sea bream production comes from juveniles already produced in hatcheries. This species reaches commercial size usually after one and a half years (Pavlidis & Mylonas 2011).

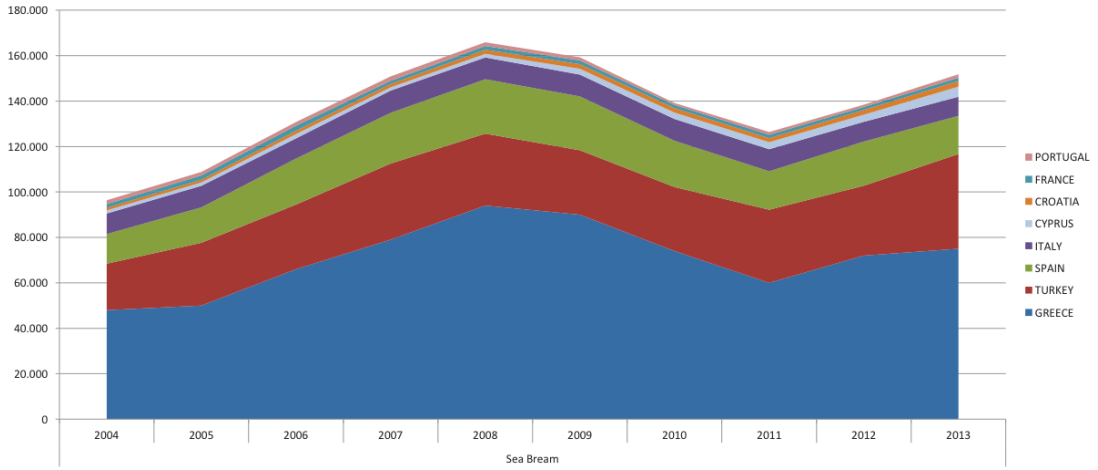


Figure 1.13. Gilthead sea bream production (in tons) by the main producer countries of this fish species (Adapted from: FEAP 2014)

Skeletal muscle is the largest organ system in fish representing, in commercially sized gilthead sea bream, around 34.3-48% of the total body weight (Grigorakis & Alexis 2005; Testi *et al.* 2006). Several types of proteins, with several different functions, encompassing myosin, transferrin, enolase, tropomyosin, parvalbumin, among others, constitute fish muscle tissue (Addis *et al.* 2010). Muscle parvalbumins' content, as referred above, can be assessed in order to evaluate the fish allergenic potential and thus, an ELISA assay performed prior to this experiment, aiming to compare the parvalbumin concentration in the muscle of different fish species, identified gilthead sea bream as the

second most allergenic species, either in farmed or wild fish (Figure 1.14). Giving this data, along with the high commercial value of this species and the high availability of it, gilthead sea bream was the species selected for the present study.

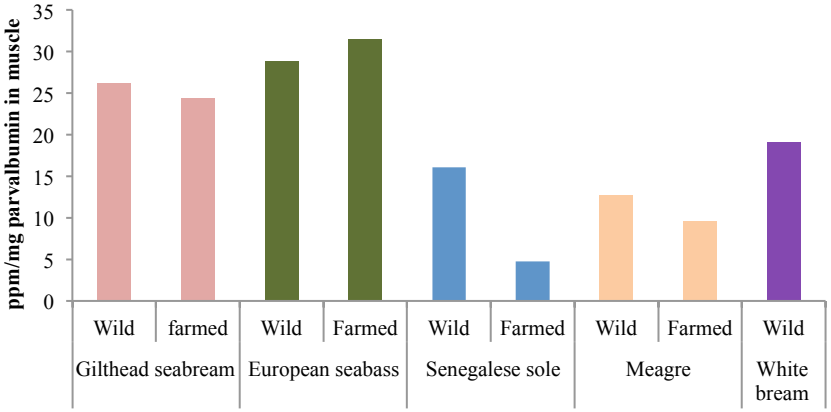


Figure 1.14. Comparison of parvalbumin concentration, in ppm/mg, between the muscle tissue of farmed and wild fish, of different fish species

1.9. EDTA as a competitor to β-parvalbumin

Based on these studies, it's possible to affirm that the most effective way of changing the ionic environment of a fish muscle tissue and thus try to promote the β-parvalbumin apoform, is through a formulated diet. This is how aquaculture and its possibility of controlling fish farming conditions appear here as an important tool to try to modulate the allergenicity of cultivated aquatic species.

EDTA is a compound with numerous utilities that can chelate Ca^{2+} and Mg^{2+}, although with a higher affinity to calcium. Upon complex formation, meaning the binding of the metal ion, both nitrogen ions and four carboxylate oxygen ions bind to it and curl up to envelop the metal ion (Figure 1.15) acquiring, the chelator, an increased rigidity (Moeschler *et al.* 1980). EDTA was found to be a promisor compound to induce the potentially less allergenic form of parvalbumin, the apo-parvalbumin, since Johnson *et al.* (1999) discovered that intracellular EDTA mimics the major fish allergen in the promotion of muscle relaxation. Johnson *et al.* (1999) proved that EDTA can be used as an "artificial" parvalbumin since it can assume practically all its tasks, like for example, its Ca^{2+} and Mg^{2+} dissociation rates are identical and its behaviour in sequestering Ca^{2+} while in Mg-bind form is practically the same. Alpod & Elsayed 1979 (1983), in studies with Gad c 1, reported a decrease of approximately 30% of IgE bound to the allergen, devoid of Ca^{2+}, in

direct RAST, when the uncoupling of the two calcium ions was induced by EDTA. With this experiment the authors proved that these ions are crucial to maintain the native tertiary structure of the protein. Huber & Pette (1996) investigated the protective effect of Ca^{2+} and Mg^{2+} against proteolytic denaturation (tryptic cleavage) using rabbit and rat parvalbumins as subtract and proved that the proteins were stable in the presence of the divalent ions but rapidly digested in the presence of EDTA. In studies of the thermodynamic metal binding parameters of carp parvalbumin, Moeschler *et al.* 1980, used EDTA for the preparation of Ca^{2+}-free parvalbumin. Thus, EDTA can be assumed as a competitor to parvalbumin for the calcium ions contrarily to magnesium ions that compete for the ion-binding sites, promoting its apoform and consequently diminishing the allergenic potential of farmed fish. However, the allergenicity of the β-parvalbumin under these changes and even the allergenicity of EDTA have never been evaluated.

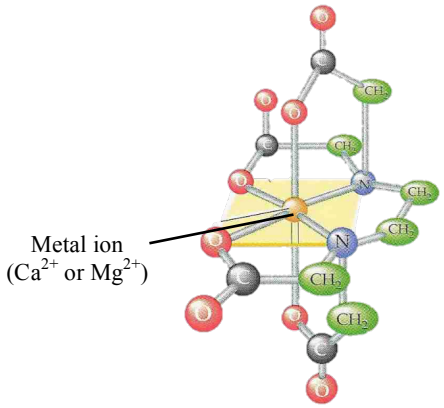


Figure 1.15. Schematization of the EDTA structure bound to a metal ion (Adapted from: Andreea 2013)

1.10. β-Parvalbumins detection: immunochemical methods vs proteomics

Nowadays, the most used methods for the direct detection of the main fish allergen are the immunochemical methods, like western blotting or enzyme-linked immunosorbent assay (ELISA) (Chen *et al.* 2006; Nakamura & Teshima 2013; Velickovic & Gavrovic-Jankulovic 2014). ELISA test (Figure 1.16) uses a microplate coated with antibodies specific for the target antigen (allergen), called capture antibodies. The samples are added to the wells and the antigens bind to the capture antibodies. A detection antibody linked to an enzyme is subsequently added, which also binds to the same antigen. Additionally, an enzymatic substrate (e.g. TMB) is added to produce a visible signal (e.g. change in colour)

and facilitate the quantification of the allergen (Fæste & Plassen 2008). Westernblot can also be used to validate proteomic results and assess the IgE-reactivity of fish-allergic patients' sera to a certain allergen.

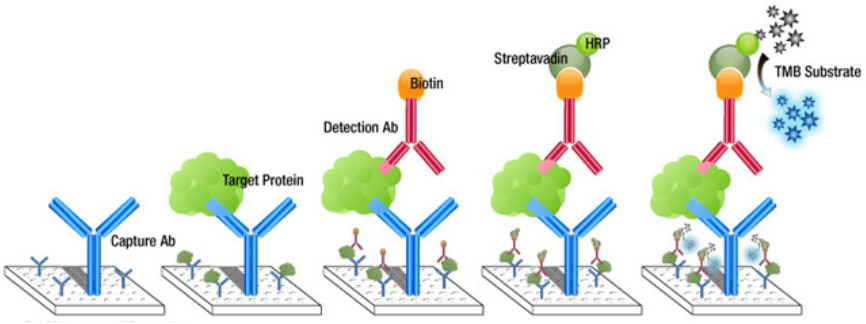


Figure 1.16. Main steps of the ELISA assay (Adapted from Epitomics 2008)

However, these techniques have some limiting factors requiring thus an alternative method presenting more reproducibility, sensitivity and specificity (Carrera *et al.* 2012). The most important limiting factor is the lack of an universal antibody or a combination of antibodies capable of covering all the β-parvalbumin isoforms since the only commercialized ELISA kits for fish allergen quantification, for example, are predefined exclusively with antibodies against Gad c 1, the main allergen of Atlantic cod. Cross-reactivity of parvalbumins will allow these proteins to bind to this antibodies but the method does not guarantee to be quantitative amongst other fish species. Thus, high-throughput approaches are mandatory to screen a large number of proteins. Proteomics can give important information about changes in protein activity and expression (post-translation changes), once the proteome of an individual can be modified by its physiological status, and studies at the genome and transcriptome level do not allow to observe this changes (Barbosa *et al.* 2012; Rodrigues *et al.* 2012). The proteome can be defined as the entire set of expressed proteins in a sample (cells, tissue, organism) at a given time under specific conditions (Kvasnička 2003; Peng 2013). Given the limitations referred above, proteomic methods can thus provide a good alternative tool to ensure and validate the parvalbumin quantification through immunochemical methods.

Since these technologies started to be applied in aquaculture, an increased understanding of the aquatic organisms biology was verified resulting in a high productivity of a better quality product. Proteomics in aquaculture can be applied in a variety of fields like, welfare, establishing a breeding programme, modulate a desire

nutrition plan for a fish stock or evaluate the immunological state of a fish for allergy prevention (Rodrigues *et al.* 2012). Characterizing the muscle proteome can gather more knowledge about many aspects of fish aquaculture as the physiology, growth, food safety, seafood authentication and quality, traceability and shelf-life (Addis *et al.* 2010; Mazzeo & Siciliano 2016). Recently, proteomic techniques started to become a powerful tool in allergen analysis, coining the term "allergenomics" and allowing to study the presence, composition and nature of food allergens (Nakamura & Teshima 2013; Piras *et al.* 2016; Velickovic & Gavrovic-Jankulovic 2014).

A common proteomic workflow (Figure 1.17) includes three main steps: 1) sample preparation; 2) protein separation and quantification; and 3) protein identification and characterization. After the first step, a proteomic analysis can follow two different approaches: a gel-based or a gel-free analysis (Rodrigues *et al.* 2012). These two different pathways can also be named top-down and bottom-up strategies (Barbosa *et al.* 2012). Sample preparation usually consists in the protein extraction using an appropriate solvent (Rodrigues *et al.* 2012), making this step a very important part for the further analysis, since a bad extraction provides consequently bad electrophoretic results and a loss of important proteins and information (Barbosa *et al.* 2012). This step can be improved by sample fractionation, simplifying the protein extract, which can be performed by a chromatography, electrophoresis, ultra centrifugation and others. The protein separation and quantification is the step that differs the gel-based and gel-free designations from the top-down and bottom-up ones. After sample preparation, the extract can be digested and the peptides used for further analysis (bottom-up strategy) or proceed with the proteins intact (top-down strategy). It is also possible to classify the two different further pathways by the technique chosen to separate and quantify the proteins: a gel-based analysis, as the name indicates, consists on a two-dimensional gel electrophoresis (2-DE) and a gel-free analysis on a mass spectrometry (MS) technique (Rodrigues *et al.* 2012). However, the top-down strategy is usually more used than the bottom-up one (Armirotti & Damonte 2010) since in the bottom-up fragments can be easily lost during separation (Barbosa *et al.* 2012).

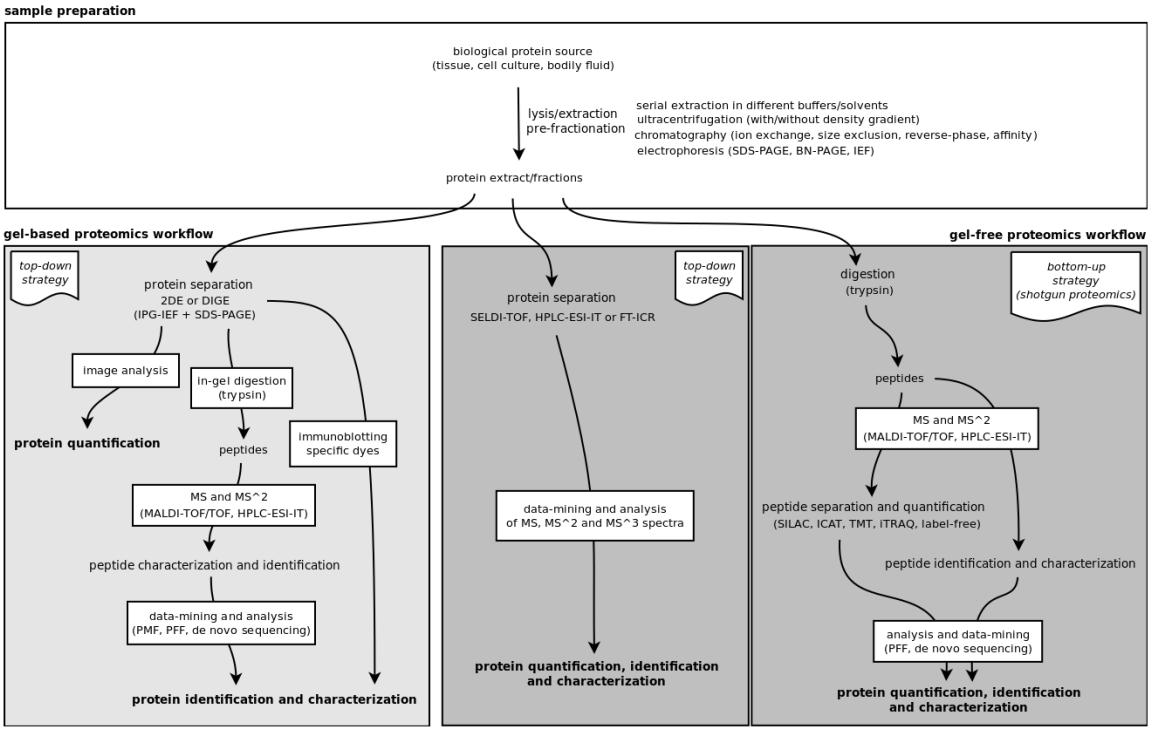


Figure 1.17. Common proteomic workflow for aquaculture studies. Whatever the strategy used in a proteomic study, both of the approaches, top-down and bottom-up have two main features in common: sample preparation and protein identification and characterization, being this last one, the aim of the workflow (Adapted from Rodrigues *et al.* 2012).

A 2-DE allows the simultaneous separation of thousands of proteins using a denaturing environment (Beckett 2012) and it consists of two steps: the separation of the proteins by their isoelectric point (pI) on a Isoelectric focusing (IEF), followed by an electrophoresis SDS-PAGE, which separates the proteins, in a perpendicular direction of the IEF, meaning, by their molecular weight (Barbosa *et al.* 2012). Recently, a breakthrough in 2-DE appeared, a technique named 2D-DIGE (Figure 1.18) that has been increasing its popularity among aquaculture species proteomic studies (Barbosa *et al.* 2012; Ünlü *et al.* 1997). It consists of a 2-DE but with an important step prior to it, the tagging of the proteins with different dyes. This not only allows running multiple samples in a single gel but it also provides a common reference channel across all gels of an experiment, reducing thus variation between gels and the need of presenting technical replicates (Barbosa *et al.* 2012; Rodrigues *et al.* 2012). The fluorescent dyes used for labelling in this technique are specially modified cyanine dyes matching for Mw and charge and two approaches can be performed: minimal labelling, consisting of the binding of the dye to a limited number of lysine residues, and saturation labelling, where dyes bind

to all the cysteine residues. The minimal dyes (Cy™2, Cy3, Cy5), the most used ones, are small molecules (approximately 450 Da) carrying a positive charge (+1) and only a single molecule binds per protein with no overall change of the protein pI since the dye's charge corresponds to the lysine charge (Beckett 2012).

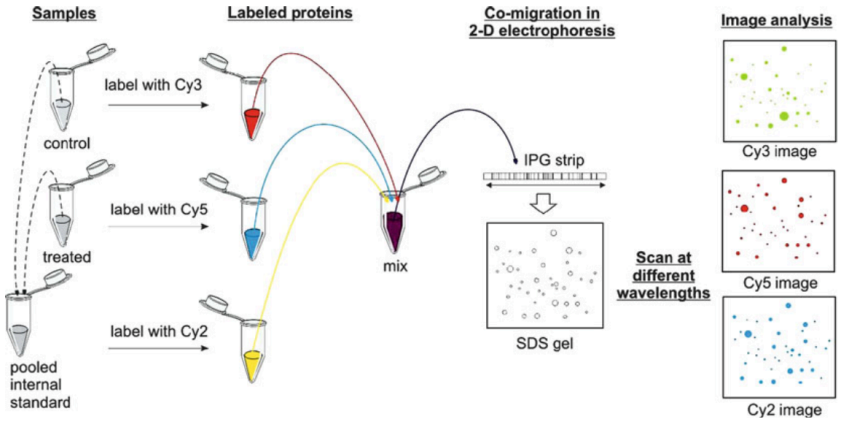


Figure 1.18. Workflow of a 2D-DIGE using minimal labelling technique. The sample proteins are labelled with the dyes Cy3 and Cy5 and the Cy2 is used as internal standard consisting of a pool of all the proteins. The different resultant images are associated with the fluorescence correspondent to each CyDye (Adapted from: Posch 2010)

The last step of the proteomic workflow, the protein identification and characterization, is a common task in both pathways and the aim of the workflow. Usually it's achieved by MS or tandem MS (MS/MS), using instruments like the electrospray ionization ion trap (ESI-Ion Trap), MALDI-TOF/TOF, among others (Barbosa *et al.* 2012; Rodrigues *et al.* 2012). MS consists of producing charged molecular species in vacuum and separate them by magnetic and electric fields based on their mass to charge (m/z) ratio allowing the characterization of proteins to its AA level. Every mass spectrometer has three basic components: an ion source, a mass analyser and an ion detector. The first component is responsible for producing gas phase ions of the molecules, usually by the addiction of protons (H^+), being MALDI one of the most widely used methods of soft-ionization. The ions are subsequently accelerated through an electric field and separated according to their m/z in the mass analyser, or previously selected by a specific m/z, followed by fragmentation by MS/MS. Finally, the ions reach the detector that is connected to a computer originating a MS profile (Barbosa *et al.* 2012). The principle of MALDI ionization is the co-crystallization of analytes, peptides resultant from digestion with trypsin (Suckau *et al.* 2003), with an organic, light-absorbing matrix (e.g. alfa-cyano-

4-hydroxy cinnamic acid or sinapinic acid), on a MALDI plate, that is activated by a laser, ionizing thus the analyte. Usually MALDI ionizers are coupled with TOF analysers capable of determining the mass of intact biopolymers (Susnea *et al.* 2013).

The final identification can be assessed by peptide mass fingerprint (PMF) in a MS if the organisms being analysed have their genome fully sequenced, or if they have not, by peptide fragment fingerprinting (PFF), or *de novo* sequencing, through tandem MS (Rodrigues *et al.* 2012). Usually, through a nucleotide sequence is possible to infer the corresponding primary structure of a protein, however many organisms still present their genome to be completely sequenced, as in the case of most fish species, leading to a lack of entries in protein databases for these organisms and the major problem for the proteomic studies in aquaculture (Carrera *et al.* 2010; Ciereszko *et al.* 2016; Rodrigues *et al.* 2012). Only by 2014, the first fully sequenced genome of a salmonid (*Oncorhynchus mykiss*) was published (Berthelot *et al.* 2014). Therefore, information about a protein sequence corresponding to these organisms can only be achieved by *de novo* sequencing, via MS/MS (Carrera *et al.* 2010). MS/MS can also be simply used to improve protein identification when PMFs do not provide sufficient information about a protein. The principle of MS/MS, contrarily to a simple MS, is the selection of a precursor ion and its fragmentation into pieces that are posteriorly mass analysed. The MALDI-TOF/TOF mass spectrometer (Figure 1.19) is a highly used instrument for tandem MS. It is characterized by two TOF mass analysers, each equipped with an ion source that allows acceleration and focusing of the ions released from the MALDI ionizer. TOF1 allows for the acceleration, selection of precursor ions and its fragmentation. These ions continue to the source of TOF2 where they are accelerated and mass analysed (Suckau *et al.* 2003). All this process allows to obtain fragmentation spectra that can be assigned to putative peptide sequences using computer protein databases like MASCOT or SEQUEST (Carrera *et al.* 2010). In November 2011, the database UniProtKB presented already a total of 163 fish β-parvalbumins belonging to the Teleostei group sequenced, obtained by MS/MS (Carrera *et al.* 2012).

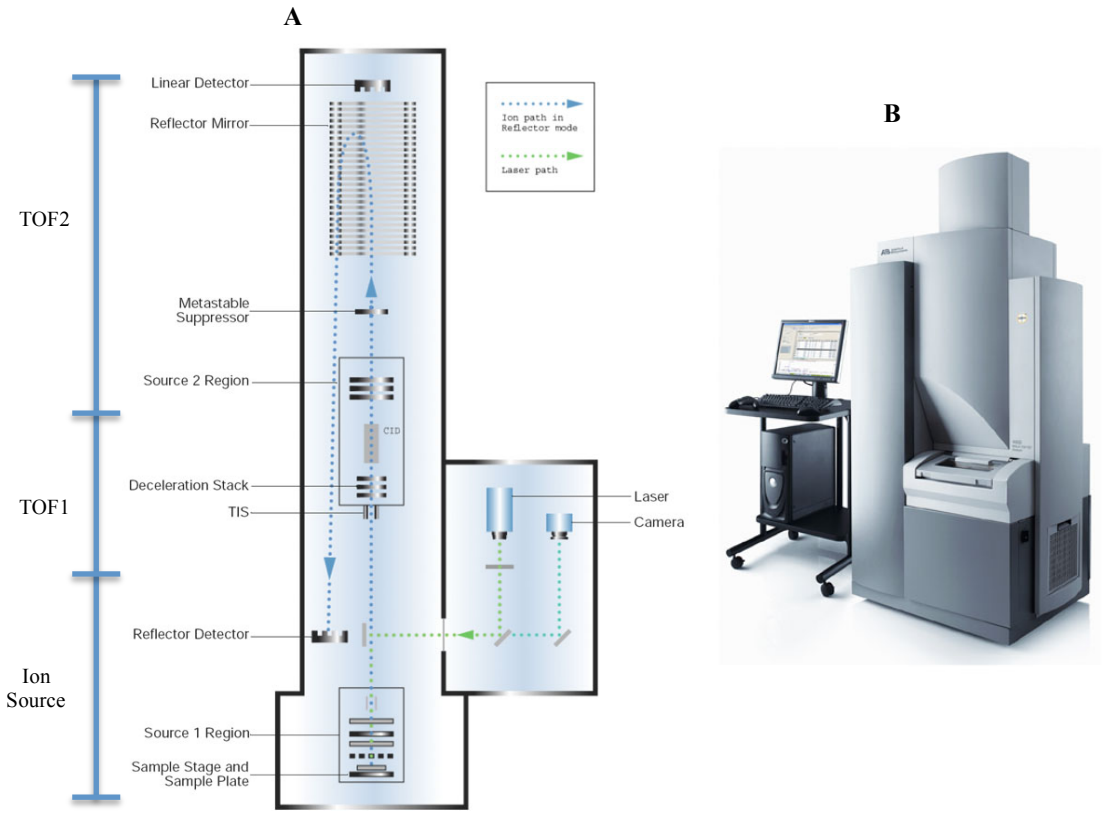


Figure 1.19. A – Internal working of a MALDI-TOF/TOF mass spectrometer; B – Example of a MALDI-TOF/TOF instrument used for MS/MS. The system is able to identify gel separated proteins by determining accurate masses of tryptic peptides through the fragmentation of a molecular ion of interest, in the collision-induced dissociation (CID), selected in the timed ion selection (TIS) (Adapted from: Applied Biosystems)

2. Materials and Methods

2.1. Fish and rearing conditions

In the present experiment, twelve groups of 25 gilthead sea bream each, with initial body weights of 208.0 ± 2.95 g, were reared for three months (from June 2015 to September 2015), under different feeding conditions, in 500L conical plastic tanks (Figure 2.4) with natural flow-through seawater, at the Ramalhete experimental station of the University of Algarve, Faro, Portugal. Four different rearing conditions were settled with an intake of EDTA in the commercial feed used: control (no intake of EDTA), EDTA 3%, EDTA 5% and EDTA 8%. Three tanks were used in each condition and the number of the tanks randomly sorted (Figure 2.4).

Fish were fed twice a day by hand, *ad libitum*, and kept under natural temperature and photoperiod, with artificial aeration (dissolved oxygen above 5 $mg.L^{-1}$), salinity (37 ± 0.39 ‰) and a rearing density of about 10 $kg\ m^{-3}$.

Figure 2.1. Tanks 1 and 11, belonging to the conditions Ctrl and EDTA3, respectively, at Ramalhete experimental station

2.2. Sampling

After 98 days of trial, five random fish from each tank were anaesthetized with 2-phenoxyethanol (Sigma Aldrich), weighted and samples of blood were taken. Approximately 2 ml of blood were withdrawn using heparinized syringes and the plasma collected and stored at -80ºC till posterior analysis. Fish were then sacrificed with an overdose of anesthetic, weighted, and muscle samples were taken. These samples were

frozen in liquid nitrogen and stored at -80°C until subsequent analysis (Figure 2.4). The five fish samples from each tank were identified by number of tank and number of fish as 1.1 (Tank 1, fish 1), 1.2. (Tank 1, fish 2) and so on.

Prior to slaughter, fish were starved for 48 hours to clean the entire digestive tract.

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" licence (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.

2.3. Zootechnical characterization

In order to verify the effect of EDTA on fish growth performance and feed consumption, a zootechnical characterization was performed along with the rearing trial. Several parameters like weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), feed efficiency (FE) and voluntary feed intake (VFI) were calculated as follows:

$$WG\ (\%IBW\ d^{-1}) = WG_t * 100/\ Biom_i * t$$

$$SGR\ (\%\ d^{-1}) = 100 * (\ln FBW - \ln IBW)/\ t$$

$$FCR\ (g/g) = FI\ /\ WG_t$$

$$FE\ (g/g) = WG_t\ /\ FI$$

$$VFI\ (\%IBW\ d^{-1}) = (FI/\ Biom_i)/\ t$$

Being:

$Biom_i$ – Initial biomass (g)

t – time (98 days)

WG_t – total weight gain ($WG_t = Biom_f - Biom_i + W_{df}$) (g)

$Biom_f$ – Final biomass

W_{df} – dead fish weight

FI – feed intake (g)

2.4. Biochemical characterization of fish muscle

Five parameters were analysed to characterize biochemically the fish muscle: pH, *rigor mortis*, WHC, texture (flesh firmness) and sensorial analysis (Figure 2.4). Fish were sacrificed by immersion in water and ice for 30min and stored in polystyrene boxes filled with ice during all the subsequent analysis. All fish were weighted and heighted.

2.4.1. pH

Three individuals per tank were used for the muscle pH measure at 0, 1, 2, 4, 6, 8, 24, 48 and 72h *post-mortem*, using a waterproof pH Spear for food testing (Oakton® Instruments).

2.4.2. Rigor mortis

Four individuals per tank were used for the *rigor mortis* analysis of the fish muscle at the same *post-mortem* periods as the pH analysis. *Rigor mortis* was assessed by measuring the caudal fin angle of the fish.

2.4.3. WHC

A muscle sample was collected from each of the three specimens used for the pH measure, at 0, 6 and 24h *post-mortem*. Duplicates were used in this case. WHC analysis started by weighing one filter paper (P1) per sample. Two portions of approximately 1 g each were weighted from each (Pm) muscle sample collected at the mentioned times, and putted on the filter papers previously placed on proper tubes (identified with the number of the sample, and the replicate indication, e.g. T1F1A (tank 1, fish 1, replicate A)). These were centrifuged at 1000 g for 10 min at room temperature. Damp filter papers were weighted (P2) and placed on a drying oven at 100ºC for 2 days. Dried filter papers (P3) were again weighted. Calculations were performed using the following equations:

$$\% \text{ liquid loss} = [(P2-P1)/Pm]*100$$

$$\% \text{ fat loss} = [(P3-P1)/Pm]*100$$

$$\text{WHC } (\%) = 100 - [(P2-P3)/Pm]*100$$

2.4.4. Texture

Texture analysis was performed in Instituto Português do Mar e da Atmosfera (IPMA) facilities, Lisbon, Portugal. Equal portions of the dorsal muscle of each specimen were chopped with known measures. Textural parameters were measured in raw flesh by compression using a probe P75 with 75 mm diameter coupled on a TA.XT*plus* Texture Analyser instrument to perform this test with a speed of 2 mm/sec. Test conditions involved two compressions that were directly analysed on a computer software giving seven texture indicators that were determined from each curve: hardness (peak force of the first compression), cohesiveness (ratio of positive force area during the second compression compared to that during the first compression), adhesiveness (negative force area under the baseline between the two compressions), springiness (height that the sample recovers during the time that elapses between the two compressions), gumminess (hardness multiplied by cohesiveness), chewiness (hardness multiplied by cohesiveness multiplied by springiness) and resilience (measurement of how a sample recovers from deformation in relation to the speed and force applied). All measures were performed at room temperature.

2.4.5. Sensorial analysis

Fish were sampled for sensory analysis in Instituto Português do Mar e da Atmosfera (IPMA) facilities, Lisbon, Portugal. To ensure the current analysis was a blind trial, numeric codes were attributed to the samples. A muscle portion was placed on an aluminium sheet, with no spices, and properly closed in order not to touch the upper part of the sample. Fish samples were steamed for 7 min at 98ºC in a steam oven (Rational Combi-master CM6). A trained panel (n=10) evaluated multiple parameters related to smell, appearance, texture and flavour using a linear scale to measure the intensity of the attribute: 0 – absent; 1 – light; 2 – intermediate; 3 – intense; 4 – very intense.

2.4.6. Statistical analysis

All data from pH, *rigor mortis*, WHC and texture analysis were subjected to a one-way analysis of variances (ANOVA). If the differences were significant between conditions, means were subsequently analysed by a post-hoc Tukey test (*p*

≤ 0.05), a multiple range test. All data regarding sensorial analysis were subjected to the non-parametric Mann-Whitney test ($p < 0.05$). All statistical analyses were performed using STATISTICA analytics software (StatSoft) for Windows. Results are presented in mean ± standard deviation (S.D.).

2.5. Parvalbumin quantification – ELISA method

A fish-check ELISA kit (Fish-Check ELISA Bio-Check (UK)) was used to perform the parvalbumin quantification (Figure 2.4). Muscle samples previously collected and frozen at -80°C were used to perform an ELISA allergen quantification method. Test portions of 350 mg were weighted on an analytical balance. Protein extraction was performed with an extraction buffer furnished in the kit, according to the manufacturer instructions, after homogenization by chopping. The solution with the muscle was mixed by vortex and allowed to settle down for 30 min. Supernatant was carefully collected and diluted (1:5). Samples were incubated in an antibody-coated 96-well microplate, furnished by the kit, and washed several times. Furthermore, a solution containing a HRP enzyme-labelled 2nd antibody was pipetted into the wells followed by washing steps to remove the excess. A chromogenic substrate, TMB, was then added to the wells developing yellow color. Afterward the plate was read in a microplate reader at 450 nm. A prepared standard curve was ran with the assay. All data were subjected to a one-way analysis of variances (ANOVA). If the differences were significant between conditions, means were subsequently analysed by a post-hoc Tukey test ($p \leq 0.05$), a multiple range test. All statistical analyses were performed using STATISTICA analytics software (StatSoft). Results are presented in mean ± standard deviation (S.D.).

2.6. Cortisol quantification – ELISA method

For the quantitative determination of plasma cortisol levels a Cortisol ELISA kit (IBL International) was used practically following the same protocol described above for the parvalbumin quantification. Cortisol levels were measured using the plasma samples previously collected from the five random fish picked from each tank (Figure 2.4). This cortisol measure was performed aiming to determine primary stress levels (welfare) in the farmed fish. All data were subjected to a one-way analysis of variances (ANOVA). If the

differences were significant between conditions, means were subsequently analysed by a post-hoc Tukey test ($p \leq 0.05$), a multiple range test. All statistical analyses were performed using STATISTICA analytics software (StatSoft). Results are presented in mean ± standard deviation (S.D.).

2.7. Proteomic analysis

2.7.1. Protein extraction

To perform the protein extraction, the frozen muscle samples collected from each individual in the sampling process were used. Two samples from each tank were randomly picked. From each of these samples, a portion of 100 mg, approximately, was taken and 500 µl DIGE buffer (7M urea, 2M thiourea, 4% CHAPS, 30 mM Tris pH 8.5) used as extraction buffer, along with 5 µl of protease inhibitor cocktail (Sigma Aldrich) and 2 µl of EDTA 250 mM was added. Tissues were homogenized with an ultraturrax (IKA) for 5 cycles of 10 seconds, followed by an incubation period of 1h in ice. Once this incubation period is over, 5 pulses of sonication with ultrasounds were performed. After a centrifugation step at 13000 g, 4ºC for 20 minutes, the supernatant was collected. Total protein content was performed with a 96-well microplate using the Bradford assay (BioRad QuickStart Bradford Dye Reagent 1X) and bovine serum albumin as standard (BioRad Bovine Serum Albumin Standard Set).

2.7.2. Protein labelling for 2D-DIGE

Each sample was diluted with DIGE buffer in order to obtain 50 µg of protein from each one. The pH of the samples was checked using a pH-indicator paper (Sigma, P4536) and adjusted to 8.5 using 0.1 M NaOH. Proteins were minimally labeled, as described by the manufacturer (5 nmol labeling kit, GE Healthcare), with 400 pmol fluorescent amine reactive cyanine dyes freshly dissolved in anhydrous dimethylformamide (DMF). The labeling process was performed with the samples on ice for 30 minutes, kept in the dark, and the reaction quenched with 1 mM of lysine for 10 min. A total of twenty-four samples were labeled: six samples per condition were labeled with Cy3 and six with Cy5 in order to reduce impact of label differences, while an internal standard consisting of a

pool of proteins from all samples, with equal amounts, was labeled with Cy2. Samples were randomly sorted in order to reduce variable confounding, due to the inherent dye-dependent bias.

2.7.3. Protein separation by 2D electrophoresis

After protein labelling, the first step of the separation, named isoelectric focusing (IEF), was performed. The process started by the rehydration of the strips that were loaded with the labelled proteins from each dietary treatment, plus the internal standard (labelled with Cy2) and a rehydration buffer, RB (8 M urea, 2% CHAPS, 50 mM DTT, 0.001% bromophenol blue, 0.5% Bio-lyte 3/10 ampholyte (Bio-Rad))) in order to complete a volume of 450 µl. The passive rehydration process was conducted for 15 hours on 24 cm Immobiline™ DryStrips (GE Healthcare) with linear pH 3-7, on an IPG box (GE Healthcare). This rehydration process was followed by the IEF, which was performed in 5 steps: 500 V gradient 1h, 500 V stp-n-hold 1h, 1000 V gradient 1h, 8000V gradient 3h and 8000 V step-n-hold for a total of 60.000 Vhr using Ettan IPGphor at 20 ºC (GE Healthcare). Prior to the second dimension, strips passed through reduction and alkylation steps that were performed using 6 ml of an equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol and 2% SDS) with 1% (w/v) 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 at 10 mA/gel for 1h followed by 60 mA/gel. This process of separation finishes when 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.

2.7.4. Gel image acquisition, analysis and statistics

Gels were scanned using a Typhoon laser scanner 9400 (GE Healthcare) and three laser emission filters (520 BP40 for Cy2, 580 BP30 for Cy3 and 670 BP30 for Cy5) at 100 µm resolution, one for each dye, with the emission and excitation wavelengths recommended by the manufacturer. Gel images were analysed using the computer software SameSpots (Totallab, UK). This analysis includes background subtraction, filtering, spot detection, spot matching and

normalization. Statistical significance was assessed using Student's T-test ($p < 0.05$) and average fold-ratio (ratio > 1.0).

Protein spots identified by the software as differentially expressed were considered for further manual excision from the 2-DE gels and subsequent identification by MS.

2.7.5. Protein identification

The proteins analysed by the software as differentially expressed were identified by MALDI-TOF/TOF MS in collaboration with the GIGA Research Centre from the University of Liège, Liège, Belgium. Protein spots were reduced with DTT and alkylized with IAA, followed by a digestion step with trypsin. This last reaction was interrupted using trifluoroacetic acid. The tryptic peptides resultant from the digestion were desalted, concentrated and further cocrystallized with a matrix on a MALDI plate and analysed using a MALDI-TOF/TOF mass spectrometer. Spectra obtained were externally calibrated and further analysed and converted to MS and MS/MS peak lists using a computer software, which also removed peaks of common MS contaminants (e.g. human keratin and trypsin autolysis products). Proteins were subsequently identified by PMF via the MASCOT MS/MS Ion database search program using the Matrix Science webserver (http://www.matrixscience.com/).

2.8. Skin-prick tests

Skin-prick tests (SPT) were performed in fish-allergic patients in order to access the allergenic potential of the farmed gilthead sea bream reared under control conditions in comparison to the fish reared with EDTA supplemented diets (Figure 2.4). Only muscle fish samples from control condition and EDTA3 condition were used. These tests were performed using the muscle samples collected in task 2.2 and conducted in the National Unit of Immunology and Allergology of the Centre Hospitalier de Luxembourg (CHL) under the supervision of Dr^{a} Martine Morisset. Patients were children under the age of 16 years old with a proved clinical record of fish allergies. The test consisted of gently pricking the skin on the forearm, using a small plastic probe or a needle, with a small portion of the sample containing the allergen, to allow it to enter just below the surface. Results appeared after approximately 30 min: positive results were indicated by a red

wheal. Comparison of the wheal's diameter in each case was used to obtain information about their allergenic potential.

2.9. IgE-reactivity to PV with fish-allergic patients' sera - 1D-PAGE immunodetection

These, and the analysis described in 2.10, were performed in the Department of Infection and Immunity, Luxembourg Institute of Health (LIH) in collaboration with the National Unit of Immunology and Allergology of the Centre Hospitalier de Luxembourg (CHL), in Luxembourg.

2.9.1. Protein extraction

Gilthead sea bream, submitted to two different rearing conditions, control and EDTA3, were used to compare IgE-reactivity patterns to fish allergic patients in order to evaluate the EDTA's effect on the IgE-binding. Total protein content was extracted as described below.

From each fish, 500 mg of muscle tissue were added to 1 steal bead with 5 mm of diameter and 1 ml of Single detergent lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl) with added protease inhibitor (Complete tablets EDTA-free Easypack, Roche), and the tubes placed on a Tissue Lyser II method from Qiagen (Hilden, Germany) following the manufacturer's protocol, with minor changes as described below. Two cycles of 5 min each were performed at 25 Hz, followed by a 1h period of incubation at 4ºC on an orbital shaker to ensure homogenization of the suspension. Samples were then centrifuged at 20 000 xg for 10 min, the supernatant collected and the total protein content quantified using the Bradford method, having BSA as standard protein.

In order to check the quality of the samples, extracts of 25 and 50 µg from each fish were separated on a 7 cm SDS-PAGE gel (15%) for 2h at 100V followed by Coomassie blue staining.

2.9.2. Evaluation of sera's IgE-reactivity from fish-allergic patients

Sera, previously collected from fish-allergic patients in the CHL and from the LIH sera-stock, were tested for IgE-reactivity performing line blots as described below (Figure 2.2). 450 µg of protein extracts were taken from the samples extracted in 2.9.1, loaded on 7 cm preparative SDS-PAGE gels (15%) – 2 preparative gels for each fish - and separated for 2h at 100V. All gels were then blotted onto 0.22 um PVDF membranes for 1h at 14V, which were then blocked in 3% BSA overnight at 4ºC. Membranes were cut with the aid of a Accutran Strip Cutter (Inotech) and each line was incubated with 1 ml of patient serum (1:10) for 3h at 37ºC with constant agitation. Two in-house antibodies (1:10.000; rabbit pAb against cod/salmon parvalbumin; rabbit pAb against herring/mackerel parvalbumin) and two commercial antibodies (1:10.000; mouse pAb against carp parvalbumin; rabbit pAb against rat parvalbumin) were used as positive controls. Following, line blots were washed for 3 times for 5 min each with Tris-buffered saline/Tween-20 (TBS-T) buffer. Each patient line was incubated with 1 ml of anti-human IgE-alkaline phosphatase (AP) antibody (1:10.000) for 1h at room temperature with constant agitation. Control strips were incubated with an anti-mouse IgG-AP (1:10.000) or an anti-rabbit IgG-AP (1:10.000). For the revelation alkaline phosphatase buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) was used and the AP substrate solution (nitro-blue tetrazolium, NBT: 5-bromo-4-chloro-3'-indolyphosphate, BCIP) added in a total volume of 1 ml. The colorimetric reaction was interrupted with TBS-T buffer.

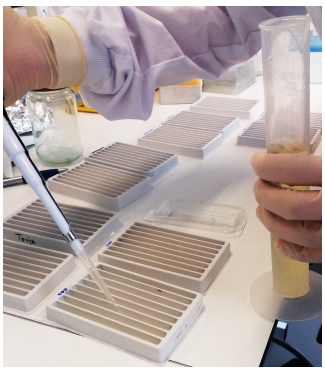


Figure 2.2. Immunodetection procedure. For each fish, sera from 14 patients were tested for IgE-reactivity

2.9.3. Clean-up of protein extracts

Fish extracts from 2.9.1 were subjected to a clean-up procedure in order to reduce the fat content using a chloroform-methanol precipitation protocol described as follows. Methanol (400 µl) was mixed with 800 µg of protein and shaken thoroughly. Chloroform (100 µl) was added to the mix (shaking for 10 sec) and 300 µl of ultrapure water were added. The samples were centrifuged for 2 min at 15000 rpm to settle the proteins at the interface of the sample. Supernatant were carefully discarded and 300 µl of methanol were added, followed by centrifugation for 2 min at 15000 rpm. After supernatants' removal, pellets were dried at room temperature and subsequently dissolved in sample buffer (30 mM Tris, 7 M urea, pH 8.5). Protein quantification was performed using the Bradford method having BSA as standard.

2.9.4. Protein labeling

Proteins were labeled using the Refraction 2D labeling kit from Dyeagnostics (Halle, Germany) following the manufacturer protocol. Fifty µg of protein were minimally labeled with 1 µl of G-Dye, followed by an incubation period of 30 min on ice, in the dark. Gilthead sea bream extracts, control and EDTA3, were labeled with G-Dye 200 and G-dye 300. The labeling reaction was quenched with the addition of 1 µl of labeling stop solution for 10 min on ice. The quality of the labeling was evaluated performing a separation by SDS-PAGE (15%), which ran for 30 min at 100V and after for 50 min at 200V, followed by laser scanning using a fluorescence-imaging mode (Typhoon; GE Healthcare, Freiburg, Germany).

2.9.5. Protein separation by 2D gels – multiplexing

In one gel, 30 µg of labeled gilthead sea bream control and 30 µg of labeled gilthead sea bream EDTA3 extracts were mixed. Volumes of the sample mixes were adjusted to 200 µl with rehydration buffer (8M urea, 1% CHAPS, 13 mM DTT, 0.5% Serva HPE IPG 3-10 strip buffer).

In the first dimension of the 2D electrophoresis, the isoelectric focusing, Serva IPG bluestrips (11 cm) with linear pH 3-10 were used. Passive rehydration of the strips was performed on the IPG box (GE Healthcare) followed by isoelectric

focusing on a 3100 Offgel Fractionator (Agilent Technologies) with the program described as follows: stp-n-hold for 1h at 150V, followed by stp-n-hold for 1h at 300V, gradient up to 1000V for 1h, up to 6000V for 2h and a stp-n-hold for 2.5h at 6000V (current/strip: 70 µA). Gel strips were afterwards scanned by the Typhoon laser scanner in order to revise the quality of this step.

The second dimension separation started with the equilibration of the strips with DTT and IAA for 15 min each diluted in equilibration buffer (SERVA electrophoresis). Cooler contact fluid (Serva) was applied on the flatbed plate, before the adding of the 2D HPE double gels NF 10-15% polyacrylamide (Serva), to help maintaining the system at 15ºC, along with the circulatory refrigerator bath (HPE cooling unit, Serva). Following, the electrode paper wicks were prepared with anode and cathode solutions, and placed in contact with the precast gels. The strips and the Amersham rainbow full-range marker (GE Healthcare) were applied to the proper slots in the double gel. The following running protocol was then applied to the gel: step 1 - 30 min at 100V, step 2 - 30 min at 200V, step 3 - 10 min at 300V and step 4 – 2h40min at 1000V (or until the running buffer reached the end of the gel) (HPE BlueHorizon flatbed electrophoresis system; Serva, Heidelberg, Germany) (Figure 2.3). After 70 min of run, meaning, after step 3, the strips were taken off the gel and scanned on the Typhoon laser scanner in order to confirm the migration of the proteins from the strips into the double gel. As well, completed 2D gels were documented using the laser scanner at 50 µm.

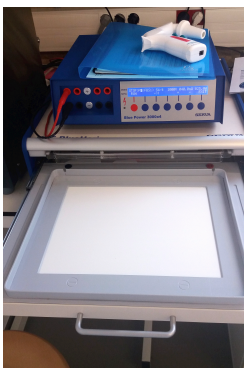


Figure 2.3. Serva HPE Blue Horizon flatbed electrophoresis system with the plate, where the gels are placed, and the power supply

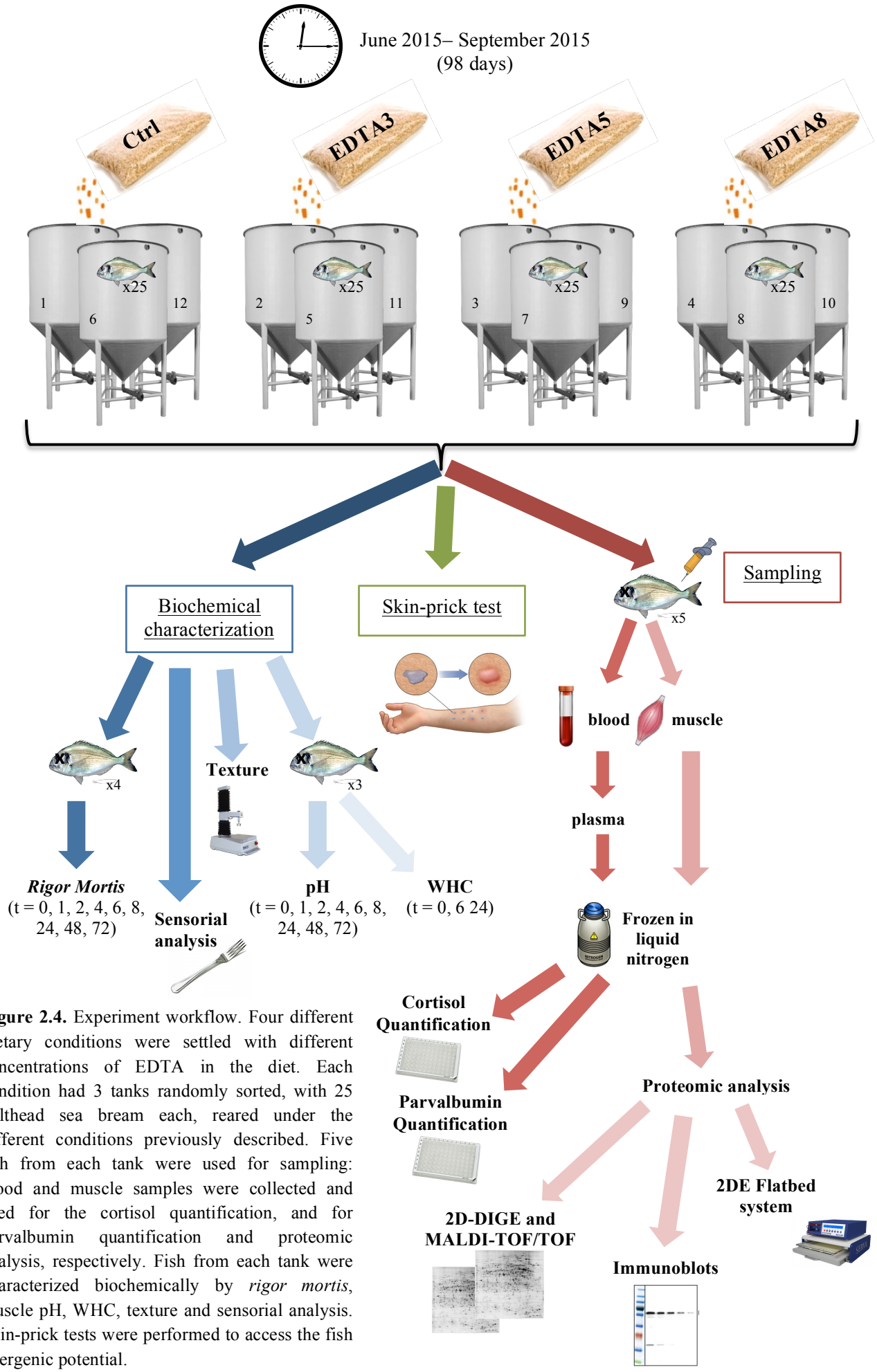


Figure 2.4. Experiment workflow. Four different dietary conditions were settled with different concentrations of EDTA in the diet. Each condition had 3 tanks randomly sorted, with 25 Gilthead sea bream each, reared under the different conditions previously described. Five fish from each tank were used for sampling: blood and muscle samples were collected and used for the cortisol quantification, and for parvalbumin quantification and proteomic analysis, respectively. Fish from each tank were characterized biochemically by *rigor mortis*, muscle pH, WHC, texture and sensorial analysis. Skin-prick tests were performed to access the fish allergenic potential.

3. Results

3.1. Zootechnical characterization

During the 98 days of trial, survival ranged from 76% in EDTA8 to 93% in control, with the condition EDTA8% registering the lowest value, 5 mortalities. Simultaneously, control group registered 2 dead fish during the trial and EDTA3 and EDTA5 groups registered 3 dead fish each. The growth was faster and the feed efficiency was higher in gilthead sea bream fed with control diets and diets supplemented with 3% EDTA. Simultaneously, the daily feed intake, measured as percent of initial body weight tended to be lower the higher the concentration of EDTA supplementation was. Overall growth performance and feed utilization of the fish is reported in Table 3.1. Figure 3.1 shows clearly a remarkable higher growth of the control group comparing with the groups from the other conditions.

Samples from groups EDTA8 and EDTA5 were chosen not to be considered in further analysis of biochemical characterization, SPTs, immunoblot assays and multiplexing gels since growth performance data show that fish from these conditions are not commercially suitable.

Table 3.1. Growth performance and feed utilization of gilthead sea bream (*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	314.6 ± 18.0	257.6 ± 12.2	202.4 ± 13.0
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

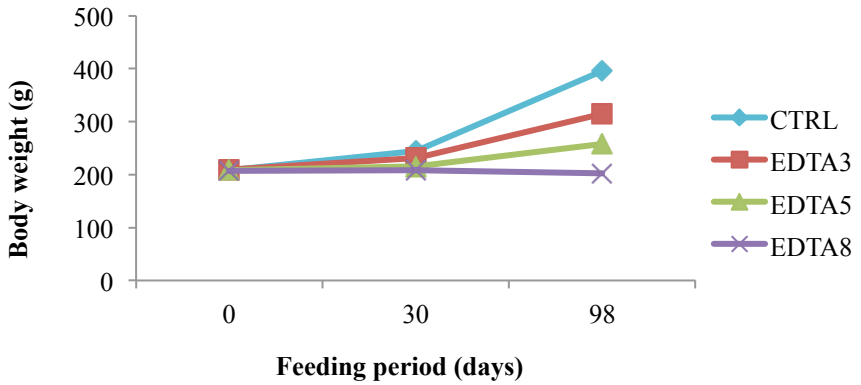


Figure 3.1. Growth of gilthead sea bream (*Sparus aurata*) from the 4 different feeding conditions reared over 98 days. Data points represent the mean of n = 75.

3.2. ELISA assays – parvalbumin and cortisol quantification

The results shown in figure 3.2 show that the muscle concentration of PV and the subsequent binding of antibodies to it in ELISA was unaffected by higher concentrations of EDTA. Parvalbumin levels in the muscle of gilthead sea bream were not significantly different ($p \leq 0.05$) between the groups of the four different feeding conditions.

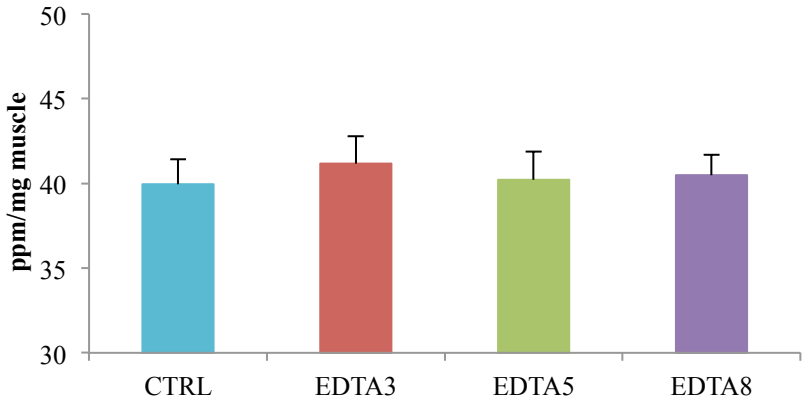


Figure 3.2. Parvalbumin levels determined by ELISA assay in muscle samples from gilthead sea bream (*Sparus aurata*) submitted to the different diet conditions described: control, EDTA3, EDTA5 and EDTA8. Data bars are presented as mean ± SD (n = 12) for each condition. No significant differences were found between conditions ($p \leq 0.05$).

Besides the extremely low feed efficiency (Table 3.1) registered in the tanks from condition EDTA8, the comparison of plasma cortisol levels of replicate tanks subjected to the different feeding conditions indicated no significant differences ($p \leq 0.05$). EDTA8

groups had mean cortisol concentration two-fold lower than that of the control groups (Figure 3.3).

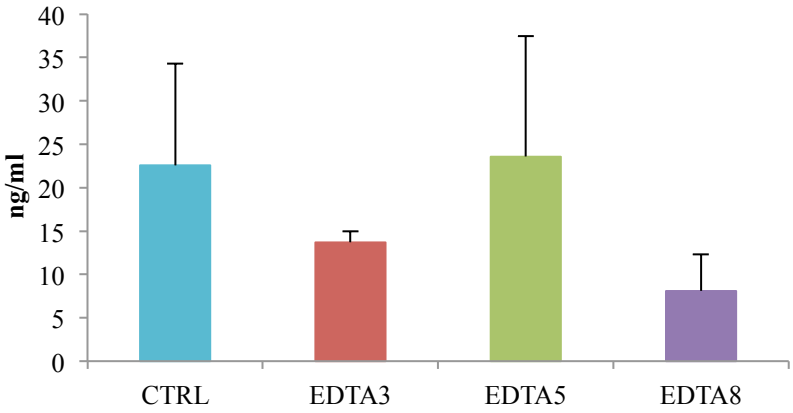


Figure 3.3. Cortisol levels determined by ELISA assay in blood samples from gilthead sea bream (*Sparus aurata*) submitted to the different diet conditions described: control, EDTA3, EDTA5 and EDTA8. Data bars are presented as mean ± SD (n = 12) for each condition. No significant differences were found between conditions ($p \leq 0.05$).

3.3. pH

Gilthead sea bream muscle pH changed during the 72h it remained stored in ice. The pH values ranged from 7.23 and 7.28 after slaughtering to 4.75 and 5.11 at 72h *post-mortem*, in fish from control groups and fish from EDTA3 groups, respectively (Figure 3.4). In the same figure in can be noticed that at 4, 6, 8, 24, 48 and 72h *post-mortem*, pH values differ significantly between the two conditions ($p \leq 0.05$).

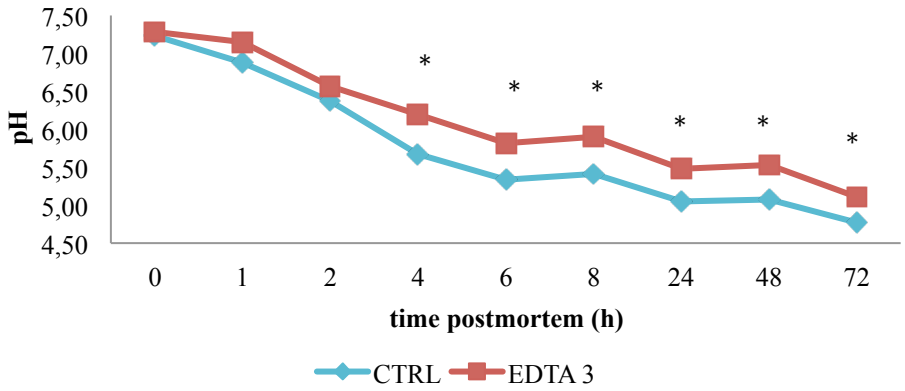


Figure 3.4 *Post-mortem* changes in pH in the muscle of gilthead sea bream (*Sparus aurata*) stored in ice during 72h. Data points are the mean of n = 9 for each sampling time. *$P \leq 0.05$ vs. control at 4, 6, 8, 24, 48 and 72h *post-mortem*.

3.4. *Rigor mortis*

Figure 3.5 shows that for both groups of gilthead sea bream, control and EDTA3, the onset of *rigor mortis* occurred after around 1h, when the muscle pH was about 6.88 and 7.14 for fish from control and EDTA3 groups, respectively. Maximum *rigor* strength was registered, for both groups at 48h *post-mortem*, and it was higher in fish from condition EDTA3 (92 ± 2%, comparing to 90 ± 3% from control condition). *Rigor mortis* started to slightly dissolute after 72h. Figure also presents significantly different values ($p \leq 0.05$) between conditions at 1h *post-mortem*.

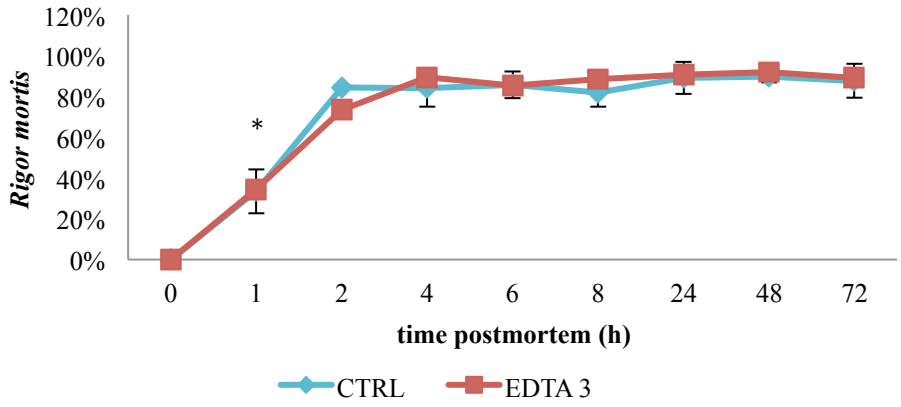


Figure 3.5. Development of *rigor mortis* during ice storage of gilthead sea bream (*Sparus aurata*). Data points are the mean of n = 12 for each sampling time. *$P \leq 0.05$ vs. control at 1h *post-mortem*.

3.5. WHC

In general, the process of liquid (Figure 3.6 - A) and fat loss (Figure 3.6 - B) that gilthead sea bream muscle has undergone after slaughtering is consistent with the changes in WHC (Figure 3.6 - C). Liquid loss increased from 18.84 ± 2.06 and 19.64 ± 2.11% at time 0 *post-mortem* to 21.44 ± 1.64 and 21.99 ± 2.96% after 24h, while fat loss increased from 2.54 ± 0.59 and 2.33 ± 0.75% immediately after slaughtering to 3.07 ± 1.09 and 3.91 ± 1.19% at 24h *post-mortem*, in fish from control and EDTA3 groups respectively. The rate of liquid and fat loss was higher after 6h *post-mortem* when the WHC of the muscle started to decrease. Figure 3.6 also shows that significant differences ($p \leq 0.05$) between the two conditions were found for the fat loss at 6h *post-mortem*.

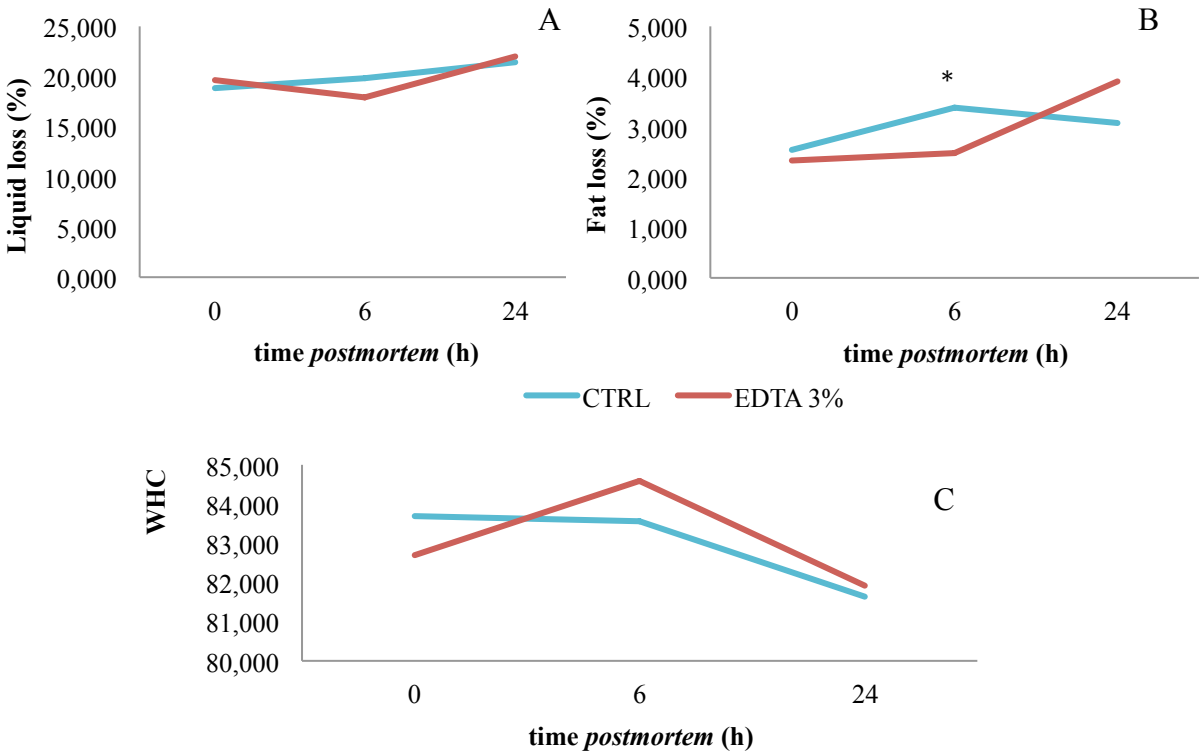


Figure 3.6. *Post-mortem* changes in liquid loss (A), fat loss (B) and WHC (C) in gilthead sea bream (*Sparus aurata*) muscle during 24h ice storage. Data are the mean of n = 9 for each sampling time. *$P \leq 0.05$ vs. control at 6h *post-mortem* in fat loss.

3.6. Texture and sensorial analysis

Figure 3.7 shows changes on textural parameters of gilthead sea bream muscle between the groups from the two conditions, control and EDTA3, however, statistical differences ($p \leq 0.05$) were found exclusively in the springiness and cohesiveness parameters.

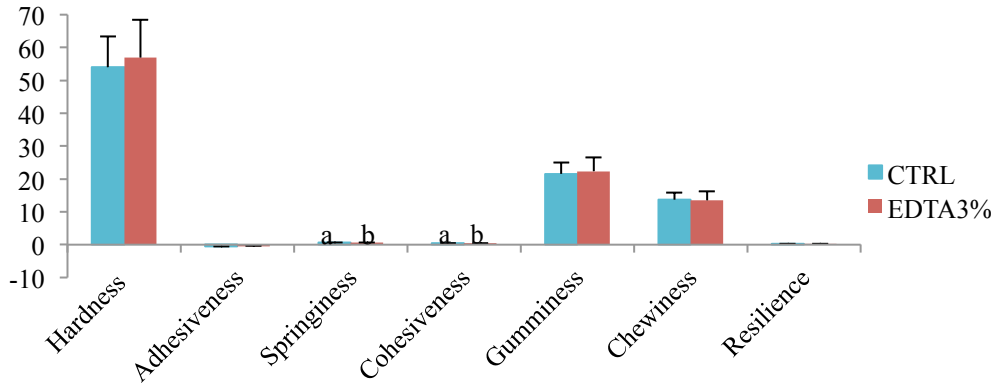
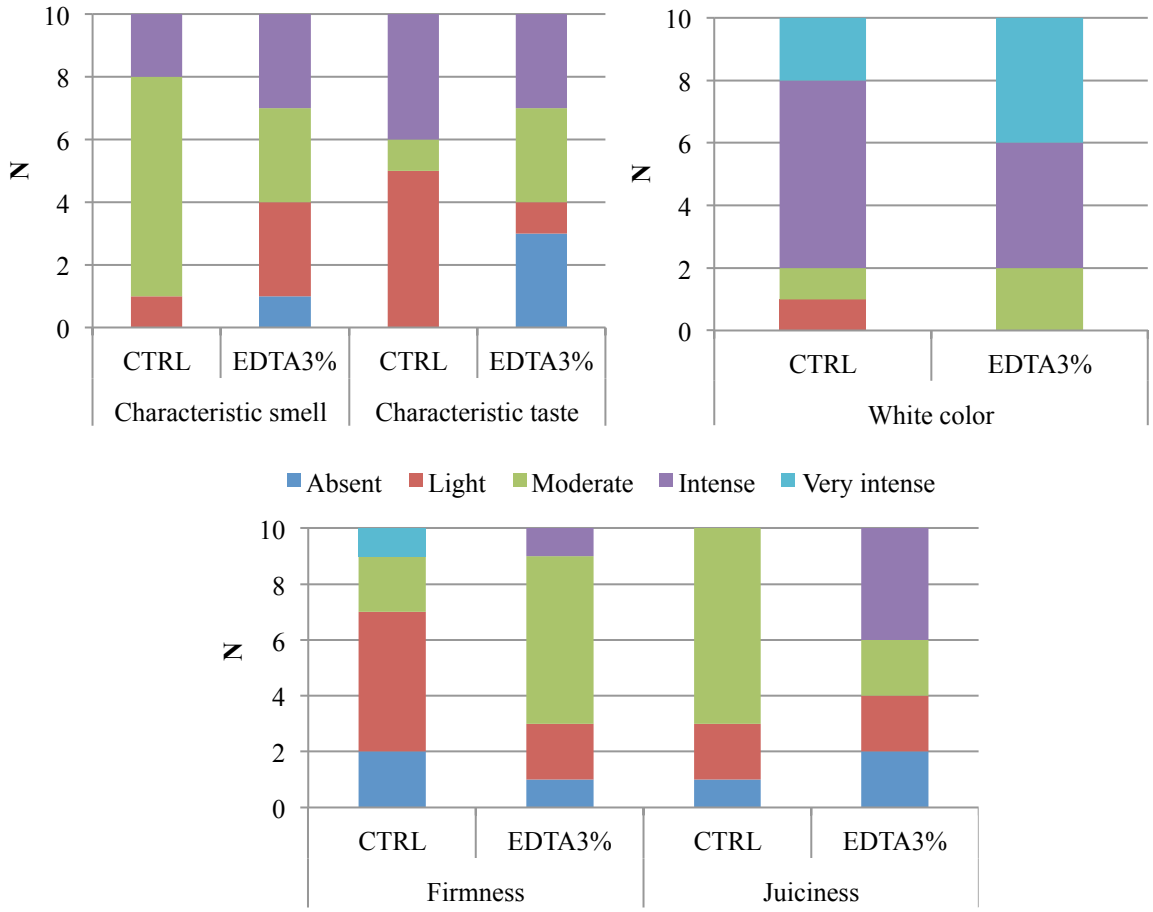


Figure 3.7. Textural parameters of gilthead sea bream (*Sparus aurata*) muscle after *post-mortem* storage on ice. Data bars are presented as mean ± SD (n = 14) for each parameter. Different letters indicate significant differences among conditions ($p \leq 0.05$)

Diet supplement with EDTA3 slightly affects the organoleptic properties of the fish although the taste panel (n =10) detected no statistical differences ($p \leq 0.05$) in any of the sensory attributes between the two groups of gilthead sea bream (Figure 3.8). Figure 3.8 also shows that 100% of the taste panel states that the characteristic flavor of gilthead sea bream was present in fish belonging to control groups, against only 70% when referring to fish from EDTA3 groups 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". Fifty-percent of the panel did not detect different smells, flavors or colors for the meat belonging to the fish from control groups (Figure 3.8 – A) while regarding the fish from EDTA3 groups (Figure 3.8 – B), only 30% of the panel did not detect other different characteristics.



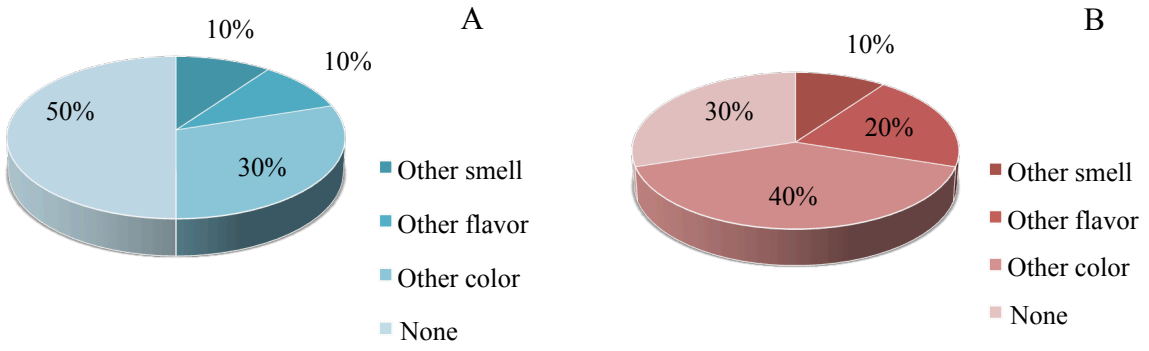


Figure 3.8. Sensory analysis performed on gilthead sea bream (*Sparus aurata*) fed with the control diet and with the EDTA3% for 98 days. Scores of the attributes are given as absent-very intense. No significant differences were detected between the fish from the two conditions ($p \leq 0.05$). A – CTRL, B – EDTA3

3.7. Skin-prick tests

Preliminary skin-prick tests (Table 3.2) show stronger skin reactions to gilthead sea bream fed with control diet in patients 1 and 2, and equal skin reactions between gilthead sea bream fed with control and EDTA3 diets for patients 3-5. Due to the difficult accessibility of organizing patients and ethical acceptance, once these tests are performed *in vivo* with fish-allergic patients, these trials are still being performed.

Table 3.2. Scoring of the skin-prick tests performed in fish-allergic patients concerning the red skin wheal's diameter

Patients	Control	EDTA3
1	+++	+
2	+++	++
3	+++	+++
4	++	++
5	+++	+++

3.8. Immunoblot

Figure 3.9 shows a suitable extraction method for the species in study. Separation on SDS-PAGE (15%) of new extracted proteins from control and EDTA3 samples revealed a similar distribution of Mw ranging from 6 to 70kDa (Figure 3.10). Two parvalbumin-like bands can be distinguished in fish from both conditions (ca. 10kD).

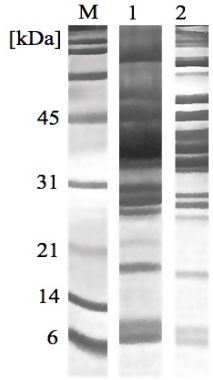


Figure 3.9. Coomassie-stained 15% SDS-PAGE gels of gilthead sea bream from control condition (1) and gilthead sea bream from EDTA3% condition (2). M, marker

Comparative tests of IgE-reactivity to proteins from gilthead sea bream belonging to control and EDTA3 groups by immunoblot assays using sera from 14 fish-allergic patients, revealed a parvalbumin double band in gilthead sea bream from EDTA3 group, while gilthead sea bream from control group presented a single band (ca. 10kDa). Figure 3.10 show a stronger reaction of IgE to parvalbumin, comparing with other visible bands (ca. 21, 31 or 45 kDa) that might represent other fish allergen proteins and/or parvalbumins oligomers. A total of 5 patients out of 14 reacted to parvalbumin in gilthead sea bream control (Figure 3.10 – A), and 3/14 to gilthead sea bream from EDTA3 group (Figure 3.10 – B). Positive controls using anti-parvalbumin antibodies confirmed the presence of this allergen in both fishes

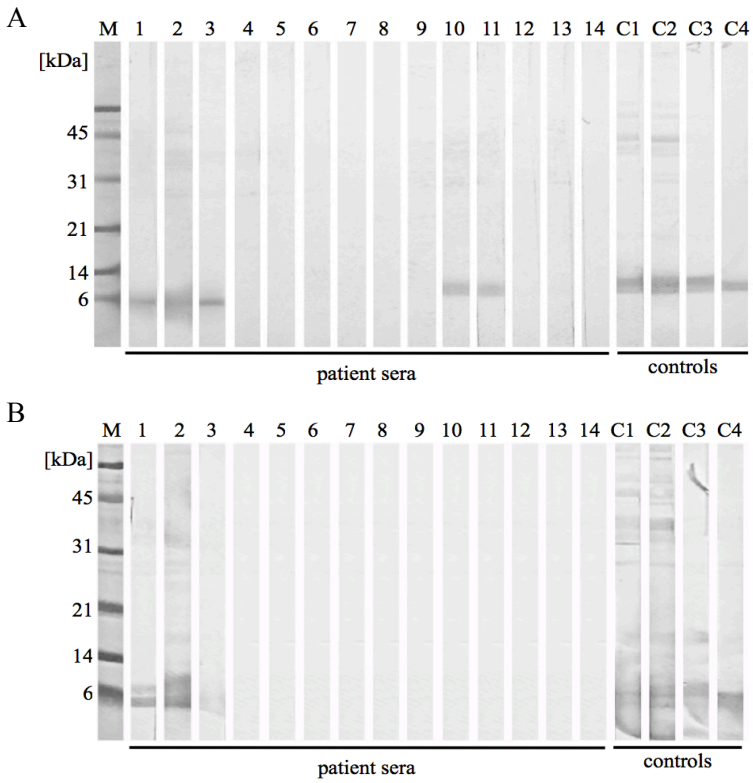


Figure 3.10. IgE-reactivity of single patient sera (1-14) to gilthead sea bream from control condition (A) and gilthead sea bream from EDTA3% condition (B). Control antibodies (C1-4) were used to detect parvalbumin. M, marker

3.9. Multiplex 2D-gel

The new extracted proteins from gilthead sea bream fed with control and EDTA3 diets were separated on a multiplex 2D gel (Figure 3.11) and labeled with different dyes to assess the different proteomic profiles from the fish muscle belonging to the two conditions and check the effect of EDTA on the muscle proteome. Parvalbumin is located on the Mw between 7 and 13 kDa and pI around 6, according to figure 3.12. Three different proteins were overexpressed on the gilthead sea bream EDTA3, one of these corresponding to parvalbumin.

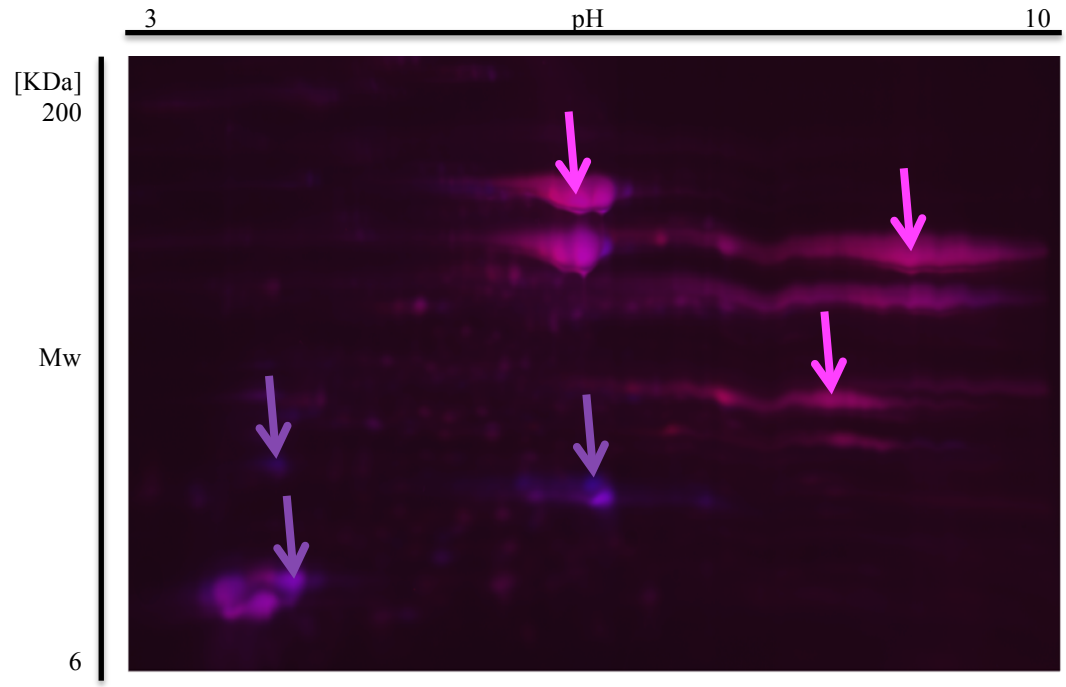


Figure 3.11. Gilthead sea bream (*Sparus aurata*) muscle proteome on a 2D Multiplex gel showing differences in expression of certain proteins between the control and EDTA3 conditions. Pink arrows, overlapping of control and EDTA3 gilthead sea bream; Purple arrows, EDTA3 gilthead sea bream

3.10. Proteomic analysis

A proteomic analysis was performed to study the effect of EDTA over the muscle proteome of the fish. The isolation and separation of all protein content of the gilthead sea bream muscle samples revealed 24 very clear 2D gels where, further analysis with proper computer software to the spot intensities indicated 79 protein spots as significantly different ($p \leq 0.05$) among the four conditions. From these 79 spots, 1 was differently expressed between conditions EDTA5/EDTA8, 2 between CTRL/EDTA3, 3 between CTRL/EDTA5, 13 between EDTA3/EDTA8 and 60 between CTRL/EDTA8. Since the effect of the diet with 3% of EDTA over the muscle proteome, in comparison with the control, was practically inexistent the proteomic analysis comprised also samples from EDTA5 and EDTA8 groups. However, an effect of EDTA over the proteins' expression was only registered with the EDTA8, in comparison with the control. Subsequently analysis of mass spectrometry identified 14 proteins and peptide fragments from the differently expressed proteins, which were classified according to their cell function. A representative 2D gel of muscle from gilthead sea bream is shown on figure 3.12. Theoretical Mw of the identified proteins ranged from 19 to 183 kDa with pI range

between 4 and 9. Out of the 14 identified protein spots, 12 were differently expressed between control samples and EDTA8 samples (Table 3.3), where 7 of those presented highest volumes on EDTA8 samples, 1 was differently expressed between EDTA3 and EDTA8 (Table 3.4) where the highest volume was again registered on the EDTA8 sample, and finally the last one was differently expressed between the control and EDTA3 (Table 3.5), registering the highest protein volume in the EDTA3 sample. According to cell function, 6 out of the 12 identified proteins were classified as belonging to the contractile apparatus, 2 as nucleoplasm proteins, 4 as cell structure proteins, 1 as mitochondrial matrix protein and 1 with unknown function.

Parvalbumin was identified in the 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 value. The PV expression was not significantly different between conditions.

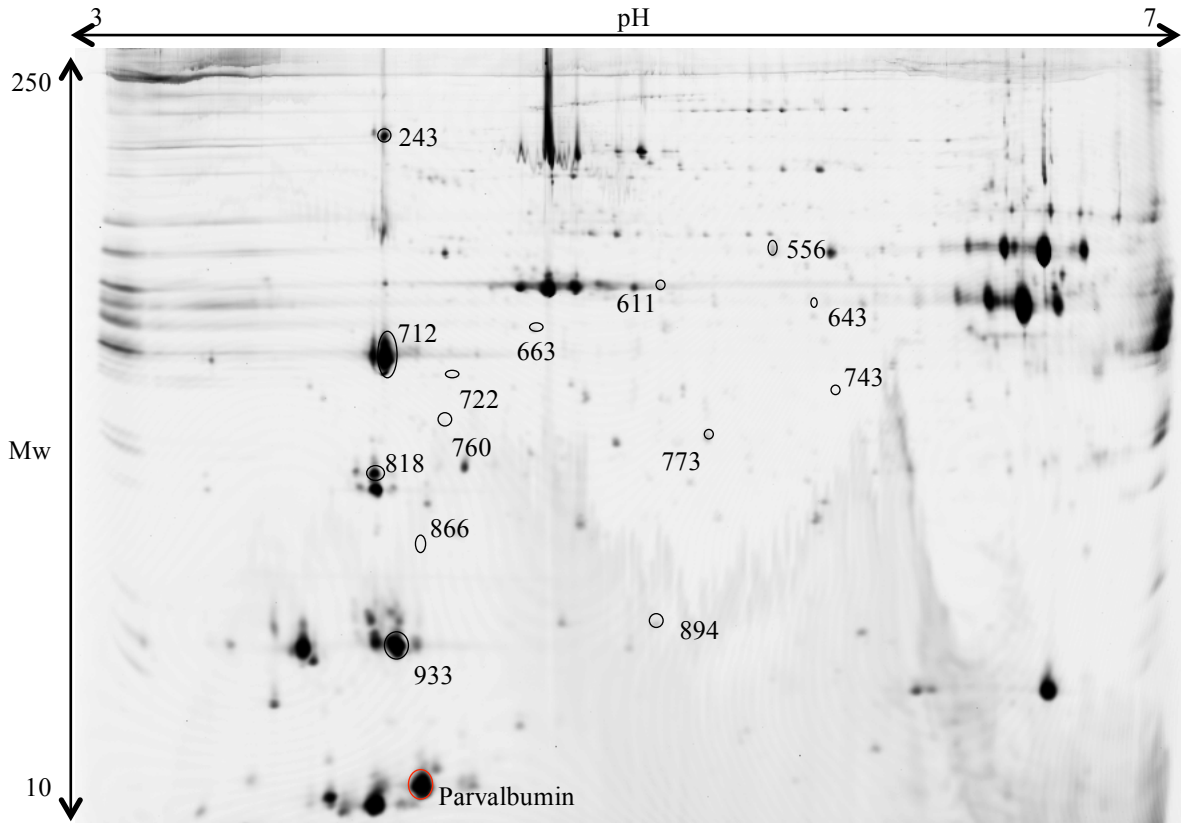


Figure 3.12. Representative 2D gel of muscle from gilthead sea bream (*Sparus aurata*) on a 12.5% polyacrylamide 2D gel. Circled spots, representing significantly different expressed proteins between the different conditions, were sequenced and identified (Tables 3.3, 3.4 and 3.5).

Table 3.3. – Differently expressed ($p \leq 0.05$) proteins between gilthead sea bream (*Sparus aurata*) from control and EDTA8 conditions identified by MALDI-TOF/TOF MS after separation by 2D-DIGE. Negative fold changes are down expressed in control. Positive fold changes are up expressed in control.

Spot nº	Identified protein	NCBI accession number	Score	Mw T/C*	pI T/C**	Sequence coverage (%)	Fold changes	Nº of peptides	Best peptide sequence	Protein Function
Contractile apparatus										
556	PREDICTED: myosin heavy chain, fast skeletal muscle-like, partial [*Takifugu rubripes*]	gi|410932121	89	118546/47981	5.16/5.6	27	-1.47	14	K.KDIDDLELTLAK.V	Muscle contraction
663	fast muscle-specific myosin heavy chain, partial [*Danio rerio*]	gi|29570808	85	95006/37047	5.22/5.1	19	1.48	10	R.QLEEKEALVSQLTR.G	Muscle contraction
712	slow myotomal muscle tropomyosin, partial [*Salmo trutta*]	gi|3063940	76	32661/33681	4.71/4.7	8	-1.19	2	R.IQLVEEELDRAQER.L	Muscle contraction
818	PREDICTED: myosin light chain 1/3, skeletal muscle isoform [*Poecilia formosa*]	gi|617416779	222	20822/23007	4.62/4.7	26	-1.45	3	R.VFDKEGNGTVMGAELR.I	Muscle contraction
933	myosin light chain 2 [*Sparus aurata*]	gi|5852838	125	19180/13168	4.62/4.8	21	-1.22	3	K.EFLEELLTTQCDR.F	Muscle contraction
Nucleoplasm proteins										
611	PREDICTED: homeobox protein cut-like 1 isoform X1 [*Sinocyclocheilus grahami*]	gi|1020396140	66	163214/42449	5.79/5.5	7	-1.25	10	R.QDETEQSRK.K	Transcription regulation
760	PREDICTED: la-related protein 1-like isoform X1 [*Neolamprologus brichardi*]	gi|583979549	72	125778/27461	8.90/5.0	7	-1.59	7	K.WVPLMIEVK.S	Translation regulation
Cell structure proteins										
643	RecName: Full=Actin, alpha skeletal muscle	gi|1351868	78	42286/40200	5.23/5.7	7	1.19	2	K.SYELPDGQVITIGNER.F	Muscle contraction

743	PREDICTED: signal-induced proliferation-associated 1-like protein 1 [*Clupea harengus*]	gi|831322033	64	182495/30206	6.84/5.8	10	1.35	9	K.RPPADHTVGGSIPATDEFYTR.H	Actin cytoskeleton reorganization
773	skeletal alpha-actin [*Sparus aurata*]	gi|6653228	185	42185/26006	5.28/5.5	54	1.46	7	K.AGFAGDDAPR.A	Muscle contraction
Mitochondrial matrix proteins										
722	PREDICTED: pyruvate dehydrogenase (acetyl-transferring) kinase isozyme 2, mitochondrial-like [*Takifugu rubripes*]	gi|768908418	58	46757/31896	6.46/5.0	15	1.58	6	K.NAALASAPK.H	Catalytic activity, enzyme regulation
Other proteins										
243	PREDICTED: uncharacterized protein LOC100701294 [*Oreochromis niloticus*] (by blast insulin-like growth factor I [*Sparus aurata*])	gi|908436892	72	38341/77645	8.89/4.7	37	-1.35	8	M.ILYVTNNKDCLFVCLFR.T	Unknown

*Theoretical (T) and calculated (C) molecular weight (Mw)

** Theoretical (T) and calculated (C) isoelectric point (pI)

Table 3.4 – Differently expressed ($p \leq 0.05$) proteins between gilthead sea bream (*Sparus aurata*) from control and EDTA3 conditions identified by MALDI-TOF/TOF MS after separation by 2D-DIGE. Negative fold changes are down expressed in control. Positive fold changes are up expressed in control.

Spot nº	Identified protein	NCBI accession number	Score	Mw T/C*	pI T/C**	Sequence coverage (%)	Fold changes	Nº of peptides	Best peptide 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	6	K.LDKENALDR.A	Calcium-dependent regulation of muscle contraction

*Theoretical (T) and calculated (C) molecular weight (Mw)
** Theoretical (T) and calculated (C) isoelectric point (pI)

Table 3.5. – Differently expressed ($p \leq 0.05$) proteins between gilthead sea bream (*Sparus aurata*) from EDTA3 and EDTA8 conditions identified by MALDI-TOF/TOF MS after separation by 2D-DIGE. Negative fold changes are down expressed in control. Positive fold changes are up expressed in control.

Spot nº	Identified protein	NCBI accession number	Score	Mw T/C*	pI T/C**	Sequence coverage (%)	Fold changes	Nº of peptides	Best peptide sequence	Protein Function
Cell structure proteins										
894	skeletal alpha-actin [*Sparus aurata*]	gi|6653228	141	42185/14288	5.28/5.4	28	-1.62	5	R.AVFPSIVGRPR.H	Muscle contraction

*Theoretical (T) and calculated (C) molecular weight (Mw)
** Theoretical (T) and calculated (C) isoelectric point (pI)

4. Discussion

This study aimed to reduce farmed fish allergenicity by inducing the apo-PV, a potentially less allergenic form of PV through EDTA supplemented diets, including verifying the effect of this compound on the growth performance, feed utilization and organoleptic properties of gilthead sea bream (*Sparus aurata*), a teleost widely distributed in the Mediterranean and the Atlantic seas with high commercial value and of great interest in aquaculture.

This was the first reported study in aquaculture field where EDTA was used for dietary supplementation in order to produce a hypoallergenic fish product. Little research work is done using aquaculture rearing techniques to modulate fish allergenicity, even being fish one of the most common food allergy elicitors. *Post-mortem* processing of fish products are more common treatments to reduce fish allergenic potential than treatments during the rearing phase, like the ones demonstrated by Bernhisel-Broadbent *et al.* 1992 with canned tuna fish and salmon or by Chatterjee *et al*. 2006 with boiled and fried muscle extracts of four different fish species.

The zootechnical characterization of the fish performed during the 98 days trial shows that the growth rates of gilthead sea bream fed with EDTA3 diets were unaffected by EDTA supplementation (Table 3.1). The overall growth remained higher for the groups CTRL and EDTA3 (Figure 3.1). Values from fish SGR belonging to these groups (0.7 ± 0.0 and 0.4 ± 0.0 %/day) correspond to the ones reported by Fountoulaki *et al.* 2009 and De Francesco *et al.* 2007 for gilthead sea bream with similar initial body weights, reared under the same conditions of temperature and salinity. This also shows the good condition of the fish and the quality of the feeding procedure used in the present experiment. Gilthead sea bream fed with diets supplemented with 5 and 8% of EDTA presented low and negative growth rates, respectively, strongly suggesting a negative influence of this compound on the daily consumption of these diets. This statement can be supported by the values of the FCR obtained for the mentioned conditions. FCR is a major indicator of feed efficiency in fish farming and in the field of research. The lower the FCR, the higher the weight gain obtained with the feed, meaning that, in this case, a greater quantity of EDTA5 diet (3.67) was required for a unit weight gain of fish whereas control diet (1.7) was required in lower quantity. Thus, values obtained for fish from control and EDTA3 groups correspond to those reported by previous studies with gilthead sea bream, contrarily to those from groups EDTA5 and EDTA8 (De Francesco *et al.* 2007; Dimitroglou *et al*.

2010; Fountoulaki *et al.* 2009). Samples from group EDTA8 presented a totally negative feed efficiency considering the FCR value (-1.02). The value obtained can be explained by the number of mortalities occurred during the feeding period (6) which resulted in a negative weight gain. Low palatability, digestibility and unpleasant odor might be the most plausible factors for a poor feed intake. Previous studies related to toxicity, absorption, metabolism and excretion of EDTA were extensively reported and the majority stated no alarming results obtained for none of the parameters until 5% concentration (Lanigan & Yamarik 2002). Contrarily, one study made by Kawamata *et al.* 1980 that reported several mortalities (number not shown) of rats fed with 5% disodium EDTA and others with marked suppression of body weight gain. Most of these studies also reported that EDTA is almost entirely secreted in the urine and feces (Wynn *et al.* 1970; Yang & Chan 1964; McCay *et al.* 1994). Lanigan & Yamarik 2002 also established the lowest dose able to cause a toxic effect in animals as 750 mg/kg/day.

Flesh quality and shelf life of the fish product was determined in this study by several physical-chemical parameters and revealed that in gilthead sea bream, muscle tissue degradation begins very early after death, resembling findings made by Ayala *et al*. 2010 in sea bream, and in other aquaculture reared species like sea bass (Ayala *et al*. 2005) and cod (Ofstad *et al*. 1996).

Within hours *post-mortem*, ATP levels in fish muscle decrease leading to the breakdown of glycogen molecules. Consequently pH values drop significantly due to the onset of lactic fermentation. These values were measured in fish from control and EDTA3 groups at 0, 1, 2, 4, 6, 8, 24, 48 and 72h *post-mortem* (Figure 3.4). Immediately after slaughtering, the pH values were similar to what was reported by Silva *et al.* 2012 with gilthead sea bream stored in ice for 48h (7.3 for control fish), and by Bagni *et al*. 2007 who registered an initial pH muscle ranging from 7.0 to 7.3 in gilthead sea bream 30 months aged. Results from the present study followed the same pattern of pH changes as the former studies. Nevertheless, the values registered were lower than the reported by Simat *et al*. 2012 for gilthead sea bream stored in ice for 20 days (6.15 for the farmed fish). Presented variations amongst the values of pH after slaughtering possibly mirror different farming conditions, season, diets and stress levels during the catch. However, no information about the rearing conditions of the last study was available to make comparisons in the two cases and confirm this statement. Love 1992 and Sigholt *et al*. 1997 also explained and proved that fish size strongly influence the pH equilibration, with

larger fish tending to equilibrate after death to a lower pH. Farmed fish from Simat *et al*. (2012) study presented a mean body weight of 400 g, being larger than fish from present study. At 48h *post-mortem*, pH values registered for control and EDTA3 fish muscle, 5.08 and 5.53, respectively, are below the reported by Silva *et al.* (2012) at the same time. The last sampling time registered pH values of 4.77 and 5.11, for fish fed with control and EDTA3 diets, respectively, which are slightly below the so-called ultimate pH, considered about 5.5, suggesting a total depletion of glycolytic energy reserves (Toldrá 2010). Lower pH levels can possibly be due to higher levels of glycogen in the muscle after slaughtering (Love 1980; Toldrá 2010). The rate of pH drop was slower in fish belonging to EDTA3 group comparing to fish belonging to control group and significant differences ($p \leq 0.05$) were observed between conditions at 4, 6, 8, 24, 48 72h *post-mortem*.

Rigor mortis was measured in gilthead sea bream fed with control and EDTA3 diets, as a degree of quality and welfare, at 0, 1, 2, 4, 6, 8, 24, 48 and 72h *post-mortem* (Figure 3.5). The measurement at 72h was performed since rigor was still on at 48h *post-mortem*. This process is initiated when ATP levels in muscle decrease, and when reaching low values, actin and myosin bind together to form the actomyosin complex causing stiffness of the fish body (Delbarre-Ladrat *et al*. 2006). In the present study, the onset of *rigor mortis* started at 1h after slaughtering which is consistent with previous studies reported by Watanabe & Turner 1993, however, after 72h (last measuring time), its dissolution was still not initiated, which does not correspond to Delbarre-Ladrat *et al*. 2006 stating that this condition usually lasts maximum for a day and a half in sea bass stored at 0 ºC. Nevertheless, Roth *et al.* 2006 also reported a resolution of *rigor mortis* in Atlantic salmon only after 72h on control fish. The dissolution of *rigor mortis* makes fish muscle less rigid, loosing its elasticity. In general, from 8 to 48h *post-mortem*, the maximum rigor values were observed, ranging between 82% and 90% in fish from control group, with the peak of rigor strength at 48h (90%), and between 88% and 92% in fish from EDTA3 group, with a peak at the same time. These observations are similar to the reported by Jerret & Holland 1998 with Chinook salmon. Significant differences between conditions were registered exclusively at 1h *postmortem*.

The results obtained for the pH and *rigor mortis* in this study, more precisely the different rates of pH drop between the two conditions and the extent of *rigor mortis* longer than reported in previous studies, can possibly be explained by the delayed tenderization of the fish muscle caused by a low availability of calcium ions, which can be due to the

presence of EDTA. Tenderization (Figure 4.1) is the process fish muscle undergoes within hours after death and continues during *post-mortem* storage (Ando *et al.* 1991) responsible for the softening of the flesh through the degradation of key structures in muscle sarcomere (Delbarre-Ladrat *et al*. 2006). Fish muscle is mainly composed by myofibrillar proteins accounting to 60-80% of the total protein content and characterized by a remarkable organization of the contractile proteins into striated myofibrils, repeated in units and arranged in series, the sarcomeres (Figure 4.2). The onset of *rigor mortis* leads to an increase in sarcoplasmic calcium ions content, from 0.1-0.8 μM to about 0.2 mM, result of the release of these ions by the sarcoplasmic reticulum and the mitochondria once they loose their ability of accumulation. Parallel to this process, the pH drop caused by the degradation of the glycogen reserves leads to the activation of several acidic proteases once they reach their pI (between 5.8 and 7.4). These proteases are responsible for alterations/degradation of proteins and/or structures, especially myofribrillar proteins, to which several ions are usually bound, like calcium, likewise increasing the concentration of free calcium ions. These free calcium ions will induce the activation of calcium-dependent proteases, like collagenases, calpains and cathepsins, being calpains, proteins belonging to the EF hand family, considered the main responsible proteases by muscle softening. Calpains can also be activated by exogenous calcium (Salem *et al*. 2004). This increased protease activity is responsible for the destruction of the Z-disks, the actomyosin complex and the connective tissue leading to the complete myofibril destructuration. This cascade of events causes softening of flesh and the consequently resolution of *rigor mortis.* Calcium also induces Ca^{2+}-dependent phosphorylase kinase, a protein responsible for the regulation of glycolysis, activating the glycogen phosphorylase, which catalyzes glycogen molecules consequently lowering the pH (Berg *et al.* 2002). In the presence of EDTA, the content of free calcium ions might be reduced. This low availability of calcium leads to potentially non-activation of the calcium-dependent proteins consequently delaying the resolution of *rigor mortis* and slowing the rate of glycogen breakdown and pH drop (Ayala *et al.* 2010; Berg *et al.* 2002, Delbarre-Ladrat *et al*. 2006; Prates 2002; Roth *et al.* 2006,). This hypothesis can be supported by some important findings in previous reported studies. Astier *et al.* (1991), realized that the content of tropomyosin increased in the presence of 5 mM of ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), a chelating agent related to EDTA, and it was observed in lower quantities in extracts with 5 mM Ca^{2+}. Kubota *et al.* (2001) showed that EDTA suppressed tenderization of flounder

muscle. The fact that fish initiated *rigor mortis* at pH values of 6.88 and 7.14 in control and EDTA3, respectively, also reinforces the described theory. Values from control fish are consistent with data reported by Erikson & Misimi (2008) in studies with Atlantic salmon, which are in turn lower than those observed in fish from EDTA3 groups. Several tests would be required to corroborate this hypothesis like the quantification of EDTA retained in the fish muscle, measurements of calcium concentration and evaluation of flesh softening.

Water and lipids constitute 80% of the fish muscle (Ofstad *et al*. 1996), thus, WHC has been reported to be a good indicator for fish quality evaluation showing a strong relation with muscle tenderness, *rigor mortis*, texture and pH (Offer *et al.* 1989). In the current study, WHC (Figure 3.6) increased from 82.7 ± 1.5 to 84.6 ± 2.0 in fish muscle from EDTA3 group in the first 6h of ice storage and then a decline was registered from 6 to 24h *post-mortem*, with the lowest value at 81.9 ± 2.4. Fish muscle from control groups registered a continuous decrease, from 83.7 ± 1.9 to 81.6 ± 2.2 with a faster rate from 6 to 24h *post-mortem*. These measures observed in control fish are lower than the reported by Ocaño-Higuera *et al.* (2009) with cazon fish (*Mustelus lunulatus*) stored in ice for 18 days, nevertheless, higher than the results obtained by Campus *et al.* (2010) with farmed gilthead sea bream presenting similar body weights and stored in ice for 24h. Alterations of texture and WHC in fish muscle under cold storage have been associated with changes in charges and structure of the myofibrillar proteins (Haard 1992; Mackie 1993). These results suggest that the properties of myofibrillar proteins were affected by the fish lost of freshness changes but not significantly different between conditions ($p > 0.05$). Therefore, EDTA does not significantly affect liquid loss by the muscle, although literature regarding the effect of this compound on the WHC of the muscle is scarce or practically inexistent. Offer *et al*. (1989) stated that rigor contractions due to the *rigor mortis* onset cause shrinkage of the myofibrils, and results from the present study are consistent with this effect. The higher liquid loss and fat loss and the lower WHC at 24h correspond to the higher rigor strength registered (from 24 - 48h *post-mortem*). As described before, when pH drops closer to the pI of the myofibrils, actin and myosin, their lateral shrinkage reduces the capillary forces and thus the liquid-holding capacity. The increasing registered in the WHC from fish muscle fed with EDTA3 diet from 0 to 6h *postmortem* and the constantly higher values registered, comparing to the ones from control, can be explained by Fennema 1990,

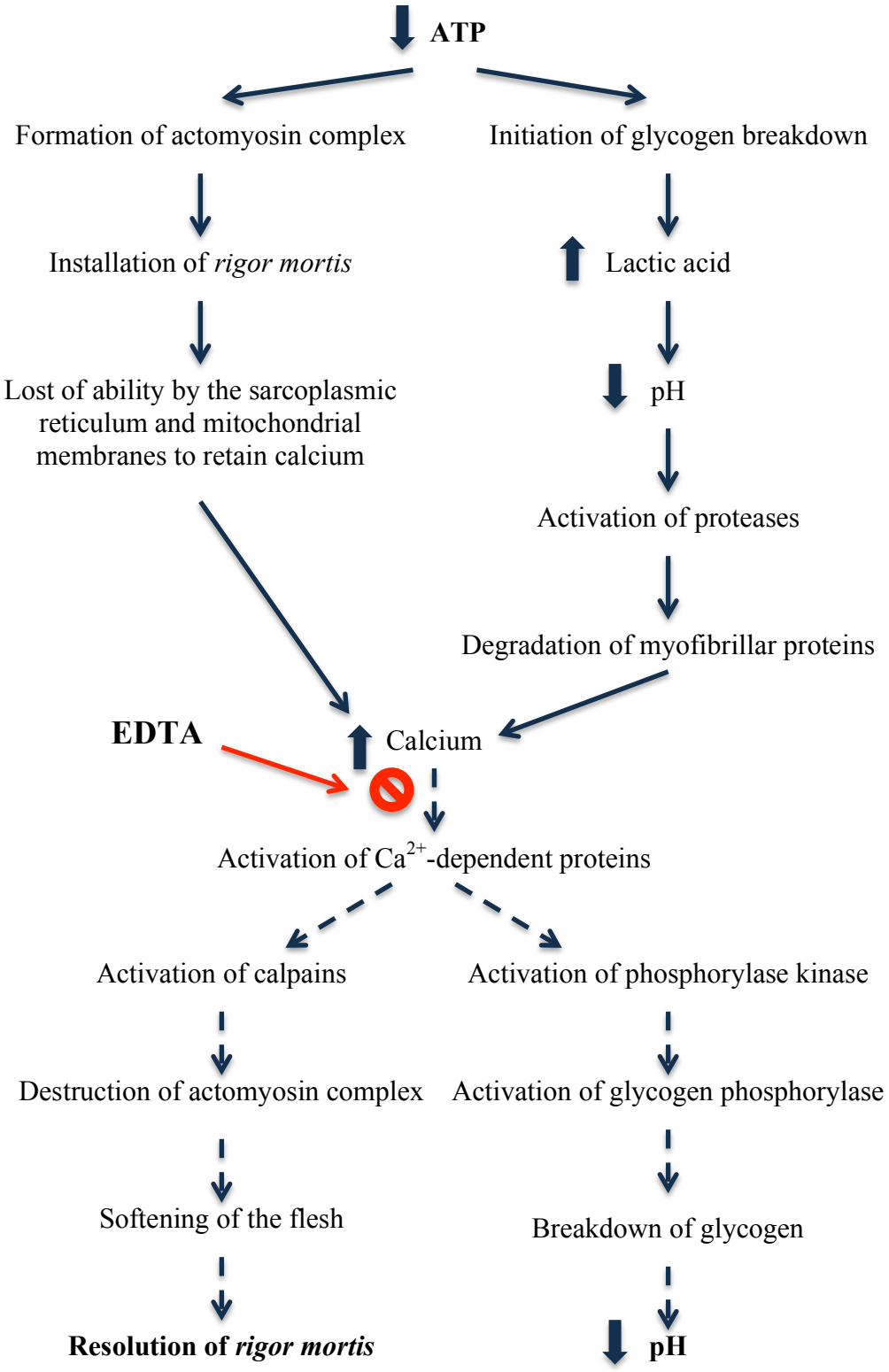


Figure 4.1. Sequence of *post-mortem* events fish muscle undergoes immediately after slaughtering process, responsible for flesh softening, named tenderization, and dissolution of *rigor mortis*. In the presence of EDTA, the concentration of free calcium ions is reduced and the further steps (dashed arrows) are delayed or suppressed.

stating that one factor affecting negatively the WHC of muscle tissue is the presence of divalent cations, especially calcium and magnesium. Therefore, the presence of EDTA might positively affect the WHC, since the free calcium content is possibly reduced. Offer & Trinick (1983) also state that at a greater pH, swelling filaments of the sarcomere is induced improving WHC. A faster decline in muscle pH has a more detrimental effect on WHC.

The texture of fish muscle is one of the most important freshness quality attributes and probably the one that has higher impact on consumers' satisfactoriness (Chèret *et al*. 2006). Texture is commonly measured and presented through several mechanical properties as hardness/firmness, gumminess, resilience, cohesiveness, springiness, adhesiveness and chewiness parameters that were evaluated in the current study by instrumental methods. Results reveal higher texture values for EDTA3 groups (excepting only in chewiness, which are practically equal), although no significant differences between conditions were obtained in most of the parameters with exceptions to springiness and cohesiveness ($p > 0.05$) (Figure 3.7). Springiness is the measure of how easy a product physically springs back after the first compression deformation. Cohesiveness demonstrates how well a product withstands a second deformation in comparison with the sample behavior to the first deformation (Kaewmanee *et al.* 2015). This possibly suggests a slight effect of EDTA on fish muscle texture. This parameter of freshness in strongly associated with WHC; when a loss of fluid and reduction of water-holding capacity occurs fish muscle usually becomes tougher, which can also be explained by the process schematized in figure 4.1. Values obtained for hardness (CTRL: 53.9 ± 9.4; EDTA3: 56.9 ± 11.5) and gumminess (CTRL: 21.6 ± 3.4; EDTA3: 22.4 ± 4.1) are practically 2-fold higher than the reported by Ayala *et al.* (2010), the registered for cohesiveness (CTRL: 0.4 ± 0.04; EDTA3: 0.4 ± 0.05) and adhesiveness (CTRL: -0.6 ± 0.13; EDTA3: -0.5 ± 0.11) are very similar, the ones of springiness (CTRL: 0.6 ± 0.03; EDTA3: 0.6 ± 0.04) are about 3-fold lower and finally the ones measured for chewiness (CTRL: 13.7 ± 2.1; EDTA3: 13.5 ± 2.8) are consistent only with the ones reported at stage "5-days *postmortem*", all comparing to the same study previously mentioned, performed with sea bream presenting similar body weights and stored in ice. The exactly same relation is observed between the results from the present study and the data reported by Ayala *et al.* (2011). These results go according with the studies made by Ayala *et al*. (2010), who found a negative correlation between the proteolysis of the cytoskeletal components and the entire muscle tissue

degradation, with the loss of hardness and other textural parameters in fish muscle. Therefore, the process that fish muscle undergoes in the presence of EDTA, described in figure 4.1 could explain the differences observed. Structural and ultrastructural studies with muscle samples by light microscopy in order to calculate percentages of fibre-to-fibre detachments and intrafibrillar breaks would be needed to confirm this hypothesis. Kaewmanee *et al.* (2015) also found out that higher hardness, springiness and cohesiveness values are related with higher lipid contents in fish.

Sensory taste panels showed minor changes between control fish flesh and EDTA3 fish flesh, although with no statistical differences in any of the sensory attributes (Figure 3.8). These results suggest that EDTA has no obvious effect on the organoleptic properties of the fish. Nevertheless, fish from EDTA3 group was considered juicier, which may result from the higher WHC registered. Juiciness is characterized by the impression of moisture running out of the meat while it's bitten (Zayas 1991). Grigorakis *et al.* (2003) also states that loosely bound water contribute to the juiciness impression. Several authors have reported the properties of EDTA as a shelf life-extender in fish, repressing lipid oxidation and growing of certain bacterial species, but little have studied the properties of this chelator on the organoleptic properties (Ghaly *et al.* 2010; Kelleher *et al.* 1992).

One of the main challenges for the aquaculture fish producers is to minimize stress conditions and preserve fish welfare since growth and reproductive performances and health status are strictly related to the stress status of the animal (Conte 2004). Pre- and *post-mortem* handling practices, confinement and crowding have been extensively reported as the major stressors in fish (Arends *et al.* 1999; Barton *et al*. 2005) In the current study, the stress condition in gilthead sea bream subjected to the different feeding conditions were evaluated through measurement of cortisol levels in the bloodstream using plasma samples on an ELISA assay (Figure 3.3). Results show that gilthead sea bream experienced stress although not affected by the EDTA supplementation since no statistical differences were found between conditions. Quantification revealed higher levels of cortisol in plasma samples belonging to control and EDTA5 fish, registering 22.6 ± 11.7 and 23.6 ± 13.9 ng/ml, respectively. However, these groups registered a high individual variability most likely due to the individual differences in stress responses (Barton 2002). Fish belonging to EDTA3 group registered 13.7 ± 1.3 ng/ml and EDTA8 fish, with the lowest value, 8.1 ± 4.2 ng/ml. Plasma cortisol concentrations in control fish are 10-fold higher than the baseline values reported in previous studies with gilthead sea bream (Alves *et al*. 2010;

Yildiz 2009). No literature regarding the effect of EDTA on cortisol release and fish stress status was found. Despite the extremely low feed intake and feed efficiency obtained, fish from EDTA8 group presented the lowest levels of plasma cortisol, suggesting a possible adaptation to the stress conditions, one of the most common responses to stress. Besides the poor growth performance of EDTA8 group it was also the one registering the highest number of mortalities, reducing thus the density in the tanks, although possibly not significant for statistical analysis.

An ELISA assay was also used to perform a preliminary quantification of the parvalbumin and, as expected, EDTA had no effect over the quantity of parvalbumin in the muscle, since the aim of this chelator was to induce a different conformation of this allergen, the so-called apo-parvalbumin, and not to reduce its concentration (Figure 3.2). No significant differences were found among conditions. Values obtained for parvalbumin concentration are consistent with the reported by Shibahara *et al.* 2013 in the sandwich ELISA performed with several species like silver salmon, pacific cod and rainbow trout. Parvalbumin contents greatly vary among fish species (Kuehn *et al*. 2010). Fæste & Plassen (2008) tested the cross-reactivity of different fish species in the cod parvalbumin ELISA and the accuracy of the test, and found that for gilthead sea bream, 98% of the protein was recovered.

Six samples from each condition, two from each tank, were randomly picked for proteomic analysis to compare different protein expression levels among conditions and the effect of EDTA on the muscle proteome from gilthead sea bream. Proteins were separated according to their Mw and pI on a 2D-gel. Differences between spot intensities of the four conditions were tested by a one-way ANOVA ($p \leq 0.05$) and 79 spots were detected as differentially expressed. Out of these, 14 were identified (Figure 3.12) using the Mascot search engine (http://www.matrixscience.com) and all of them presented sequence homology with proteins from the database (Table 3.3, 3.4 and 3.5). The identified proteins present different cell functions, being these, muscle contraction, energy metabolism, regulation of DNA transcription and mRNA translation processes, and one with unknown function. Variation registered between calculated and theoretical Mw and/or pI values is due to post-translation modifications (PTMs) and protein degradation (proteolysis) indicating potentially fragments or isoforms of the parent protein.

In the present study, one spot (nº 722) was identified as a protein involved in energy metabolism, the isoform 2 of the pyruvate dehydrogenase kinase (PDHK2). This

protein is a component of the pyruvate dehydrogenase complex (PDHC) and it's responsible for the phosphorylation and subsequently inactivation of the pyruvate dehydrogenase (PDH). This inhibition of the PDH activity interrupts the formation of acetyl-coenzyme A (acetyl-CoA) from pyruvate regulating, thus, the glucose metabolism. In human, PDHK2 is the major isoform responsible for the regulation of PDHC (Gudi *et al*. 1995; Sugden *et al*. 1998). Studies reported that this protein's activity is enhanced under stress situations, especially as a response to starvation (Fullen & Randle 1984; Sugden *et al*. 1998). This found is consistent with studies made by Moyle & Cech (1996) who stated that fish subjected to stressing conditions tend to mobilize energetic reserves, activating gluconeogenesis in order to ensure a continuous supply of glucose into the muscles. This statement can be supported by results obtained by Alves *et al*. (2010) where an abundance reduction of PDH in stressed fish was reported, result of an adaptation to promote glucose production. This low concentration of PDH can be due to an increased concentration of PDHK, which is inactivating it. In the current study, PDHK is differently expressed between control and EDTA8 conditions, with higher expression levels in the control samples, which corresponds to the high levels of plasma cortisol registered for the control group. Measurements of glucose levels could possibly corroborate this hypothesis.

Five proteins, differently expressed between fish from control and EDTA8 groups, were identified as belonging to the contractile apparatus (Figure 4.2), whereas four out of these were characterized as myosin chains, light and heavy, and one as a tropomyosin. Myosin filaments in muscle cells, known as thick filaments, are polymers of myosin molecules whose heads, located of the surface of the filament, interact with the actins to generate force for muscle contraction (Huxley 1963). A myosin molecule is composed of two heavy chain and four light chain subunits (Berg *et al.* 2001). Tropomyosins form a family of actin-binding proteins, covering the myosin binding sites on actin filaments, and can be found in the fast skeletal muscle of the fish (Huang & Ochiai 2005). Muscle contraction (Figure 4.2) occurs when calcium ions induce the tropomyosin strands to uncover the myosin-binding sites, present in the actin filaments, by binding to the troponin complex (TnI, TnC and TnT). Once myosin heads attach to the thin filaments of actin, both slide horizontally originating muscle contraction (Squire 1997). In the present study, four of these identified proteins presented higher expression in fish muscle from EDTA8 group. This is consistent with the schematized hypothesis in figure 4.1, since in the presence of EDTA the proteolytic systems of the tenderization process, responsible for the proteolysis

of the myofibrillar proteins (actin, myosin, tropomyosin, tinin, among others), might present a reduced activity or be inactivated. In this case, according to this theory, fish muscle from control group, which possibly contains a higher content of free calcium ions to activate the proteolytic enzymes, would have lower concentration of partial myosin and tropomyosin. This corresponds entirely to the results obtained, where only spot nº 663 present a higher expression of myosin in control group. The one-way ANOVA statistical test identified one single protein (spot nº 866) as differently expressed between control and EDTA3 conditions, equally belonging to the contractile apparatus, named tropomyosin alpha-1 chain isoform X1, with upregulation in EDTA3 condition. This suggests an EDTA effect, as explained above and described in figure 4.1, therefore supporting this theory.

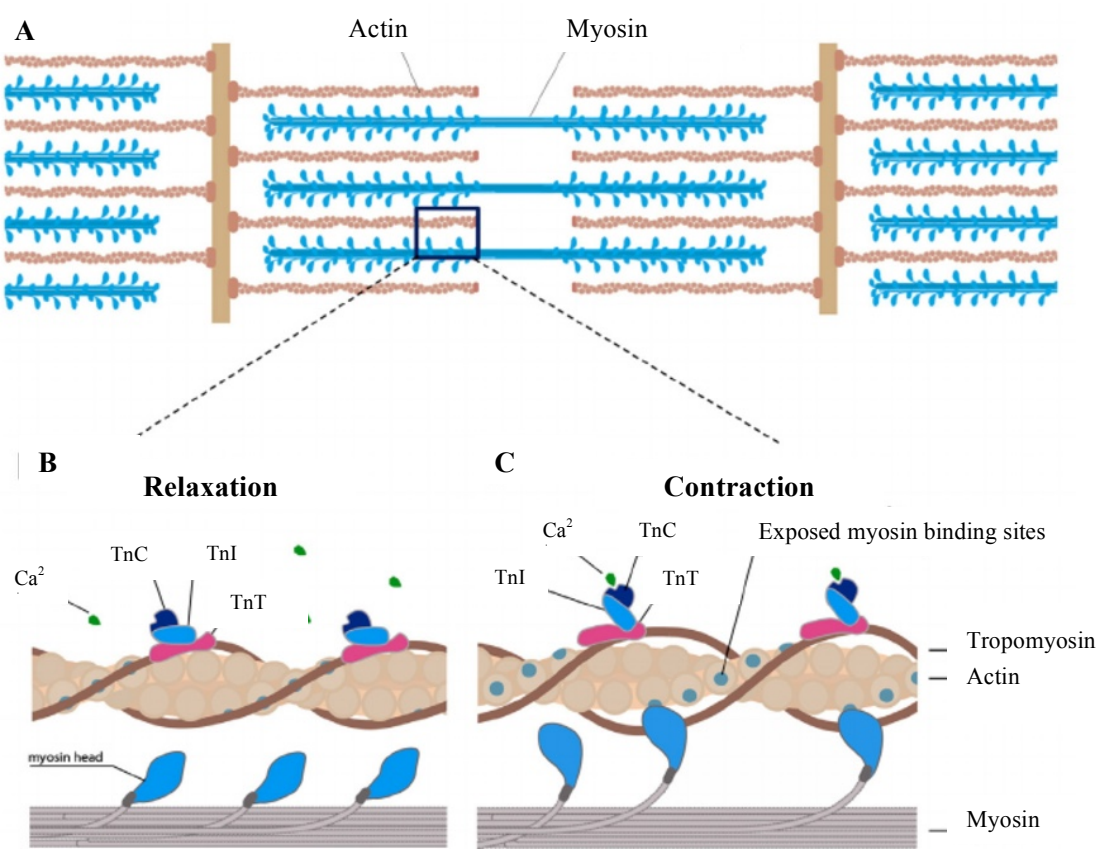


Figure 4.2. Main myofibrillar proteins forming the muscle fibers of the fish muscle implicated on the process of relaxation and contraction in the skeletal muscle. A - Sarcomere; B – event during muscle relaxation; C – events during muscle contraction (Adapted from: Streng *et al*. 2013)

Actins are highly conserved structural proteins. Vertebrates express three main isoforms of actin, the alpha, beta and gama isoforms. Alpha-actin constitutes the thin filaments of the skeletal muscle fibers (Figure 4.2) that, together with myosin, form cylindrical bundles named myofibrils (Cooper 2000; Dominguez & Holmes 2011; Krasnov *et al.* 2003). Results obtained in the present study show three spots differently expressed between fish muscle from control and EDTA8 groups, identified as cell structure proteins, from which two (nº 643 and 773) were characterized as skeletal alpha-actin, both overexpressed in control fish. The remarkable difference observed in the calculated pI of these two spots might be due to the various forms of post-translational modifications that alpha- actin tend to undergo, like methylation of the His73 and acetylations, followed by cleavage of the cysteine residues (Dominguez & Holmes 2011). The upregulation of alpha-actin in control group, considering the levels of cortisol observed, suggest a response to the stress. Krasnov *et al.* (2005) states that cytoskeleton motor proteins could be implicated in the transport of vesicles and the establishment and rearrangement of neuronal networks as a stress response in fish. The same authors reported in previous studies a high activity of skeletal alpha-actin and myosin light chain 2 promoters in the neural tissues of rainbow trout embryos (Krasnov *et al.* 2003). Myosin light chain 2 was also an identified protein in the current study (spot nº 933), although with higher expression in EDTA8 groups. Spot nº743 was identified as signal-induced proliferation-associated 1 like protein 1 (SIPA1L1), one protein responsible for inducing actin cytoskeleton reorganization in rat neurons (Pak *et al*. 2001). Another structural protein was identified, more precisely a skeletal alpha-actin, although differently expressed between EDTA3 and EDTA8 conditions, with more intensity in EDTA8. This fact might also be explained by the reduced activity of the Ca^{2+}-dependent proteolytic enzymes upon the presence of EDTA (Figure 4.1).

Spots nº 611 and 760 were differently expressed between control and EDTA8 fish and identified as transcription and translational regulators, respectively. So far, little or practically no detailed literature is available regarding the processes where these proteins are implicated.

The uncharacterized protein LOC100701294 from *Oreochromis niloticus*, in which the closest identification in gilthead sea bream by BLAST is Allantoicase (http://www.uniprot.org/uniprot/S4W3N7), located in the peroxisomes of marine fish liver and muscle, is composed of two identical subunits and responsible for the degradation of

purines into urate in the hepatic and muscle uricolytic pathway (Hayashi *et al.* 1989; Nogushi *et al.* 1986).

The red circle in figure 3.12 locates the gilthead sea bream parvalbumin in the muscle proteome represented in the 2D gel in a pH range from 3 to 7, according to protein identifications obtained previously to this study (data not shown). Mw (~12 kDa) and pI (~5) are consistent with the theoretical values. Results show the unchanged expression of parvalbumin towards the presence of EDTA, as it was expected.

Results of the preliminary skin-prick tests performed at CHL (Table 3.2) show a slight positive effect of the EDTA over the fish-allergic patients' skin reaction although with minor differences between the two conditions (patients 1 and 2). SPTs are widely used for assessing specific IgE-mediated sensitization and to detect atopic predisposition, however, positivity on a test may precede clinical symptoms of allergy and persist without clinical relevance (Pesonen *et al*. 2015). Several factors may limit SPTs such as sample size, use of different SPTs devices, techniques and non standardized sources of allergens and therefore the sensitivity of a positive test becomes compromised (Ta *et al*. 2011). Giving the high difficulty to accomplish these tests, regarding patients' availability and ethical acceptance, SPTSs are still being performed and a higher number of results are required to check whether there is a significant effect of EDTA in the reduction of the fish allergenicity.

In order to compare the IgE-reactivity patterns to gilthead sea bream subjected to the control and EDTA3 diets in fish-allergic patients, 1D-PAGE immunoblot analysis were performed (Figure 3.10). The new extraction and SDS-PAGE protein separation performed at LIH, using gilthead sea bream muscle samples from control and EDTA3 groups showed a clear and large dynamic distribution of bands similar to those described in the skeletal muscle of other fish species (Figure 3.9) (Grzyb & Skorkowski 2005; McLean *et al*. 2007; Verrez-Bagnis *et al.* 2001). Two narrowly spaced parvalbumin-like bands for control and EDTA3 gilthead sea bream were observed on a Mw range between 7 and 13 kDa, corresponding to the theoretical Mw of PV (~12 kDa) and to results obtained by Brownridge *et al*. (2009) with the common carp (*Cyprinus carpio*). This suggests no effect of EDTA on the gilthead sea bream 1D protein profile. The two differentiated bands might be a result of the slightly distinct parvalbumin isoforms present in this fish species. The high intensity of all protein bands from gilthead sea bream control suggested a high fat

content in the samples, explaining the chloroform-methanol clean-up procedure performed subsequently.

In the present study, fourteen fish-allergic patients' sera were used to compare their IgE-reactivity to proteins from gilthead sea bream belonging to control and EDTA3 conditions by immunoblot assays, revealing, for gilthead sea bream EDTA3 two bands on the Mw corresponding to parvalbumin (~10 kDa) and one single band on the lineblots from gilthead sea bream control (Figure 3.10). It is possible to observe that parvalbumin is clearly the major allergen in fish, since no other bands were detected, with exception to patient 2, which showed other bands corresponding to higher Mws, although with very low intensities. These bands might correspond to oligomeric forms of parvalbumin, which has a monomeric form at ~12 kDa, and a dimeric, trimeric and tetrameric forms at respectively 20, 40 and 50 kDa, approximately (Fæste & Plassen 2008). In control blots (C1-4), an anti-parvalbumin antibody was used to confirm the presence of this allergen, showing thus the parvalbumin band at ~10 kDa but also its potentially oligomeric forms. Besides all fourteen patients were allergic to fish, only 6 showed a reaction to the IgE suggesting potential cases of monosensitivity. Patients 1 and 2 reacted strongly to parvalbumin from both conditions, with no visible effect of EDTA; patient 3 reacted equally strong to parvalbumin from gilthead sea bream control and with less intensity to EDTA3; patients 10 and 11 reacted only in gilthead sea bream control. Therefore, a slightly effect of the EDTA can be observed, since patients 10 and 11 did not react to fish supplemented with the chelator.

Multiplex 2D gel performed at LIH (Figure 3.11) revealed a slight but visible effect of the EDTA. Proteins from control and EDTA3 gilthead sea bream were separated according to their Mw and pI and it is possible to find parvalbumin-like intense spots around Mw 7 and 13 kDa and pI around 6, corresponding to its theoretical Mw and pI. Results show 3 spots more expressed on EDTA3 gilthead sea bream, pointed by purple arrows on figure 3.12. The spot corresponding to parvalbumin in EDTA3 gilthead sea bream presents a big intensity, possible due to the different isoforms with similar Mws also observed on the immunoblots performed (Figure 3.10). The bigger protein pointed with a purple arrow on the gel above parvalbumin might be the potential oligomeric forms of parvalbumin mentioned above and likewise observed on the immunoblots.

5. Conclusions and future perspectives

This preliminary study revealed that specifically designed diets are a valuable tool to try to modulate farmed fish allergenicity while simultaneously, EDTA showed to be a promisor supplement to develop a potentially hypo-allergenic fish product.

Preliminary skin-prick tests and immunoblots of fish-patients sera's IgE-reactivity showed that EDTA might slightly affect gilthead sea bream allergenicity, which in lower concentrations showed to have a positive impact in reducing this allergenicity without influencing negatively fish growth performance, feed utilization, organoleptic properties, and *post-mortem* modifications like pH and WHC. *Rigor mortis* was extended in both control and EDTA3 fish, however, this data might also be explained by the stress levels observed. Contrarily to these results, higher concentrations of EDTA showed a strong negative impact over the growth performance of the fish, which turned to be a non-economically suitable fish for production of a hypo-allergenic product. Considering that skin-reactions do not entirely correspond to patient's clinical reaction, further analysis like basophile histamine release assays and a double-blind placebo-controlled food challenge (DBPCFC) would be needed to optimize and confirm the results. Tests regarding the concentration of EDTA that is retained in the organism of the fish and its toxicity to the animal might explain the poor growth performance of the specimens subjected to the higher supplementations of EDTA.

Concentrations of 8% of EDTA demonstrated to modulate the expression of several proteins with known functions related to muscle contraction, cell structure, DNA processes and energy metabolism. Through these identifications it is also possible to show the importance of proteomic analysis in the food allergy assessment field. Further deeper studies will be required to elucidate the role of these proteins under a situation of low availability of calcium ions. Although the lowest concentration of EDTA did not present any significant effect over the fish muscle proteome it possibly induced a potentially less allergenic apo-form.

As expected, parvalbumin's expression was not affected by the chelator, showing that a potentially reduced allergenicity might be due to the induction of a less allergenic conformation of this allergen, the so-called apo-parvalbumin. Conformational studies on this modified protein along with a structural comparison with the non-modified parvalbumin would be required to understand how the apo-parvalbumin becomes less allergenic. The design of specific antibodies against the gilthead sea bream parvalbumin

epitopes followed by separated immunoblots, using purified apo-parvalbumin, to test each single antibody could elucidate a possible correlation between a reduced allergenicity and a destruction of the epitopes recognized by the IgE. *In vitro* digestibility tests, more specifically, a resistance to pepsin digestion test with the apo-parvalbumin could also be useful in the allergy assessment of this protein.

To our knowledge, this was the first work reported regarding the use of supplemented diets to modulate fish allergenicity, however, this preliminary experiment can be the origin of a wide range of future works. The results obtained can improve the knowledge about the effect of EDTA on the modulation of fish allergenicity and contribute to the assessment and reduction of a worldwide prevalent allergy.

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7. Annexes

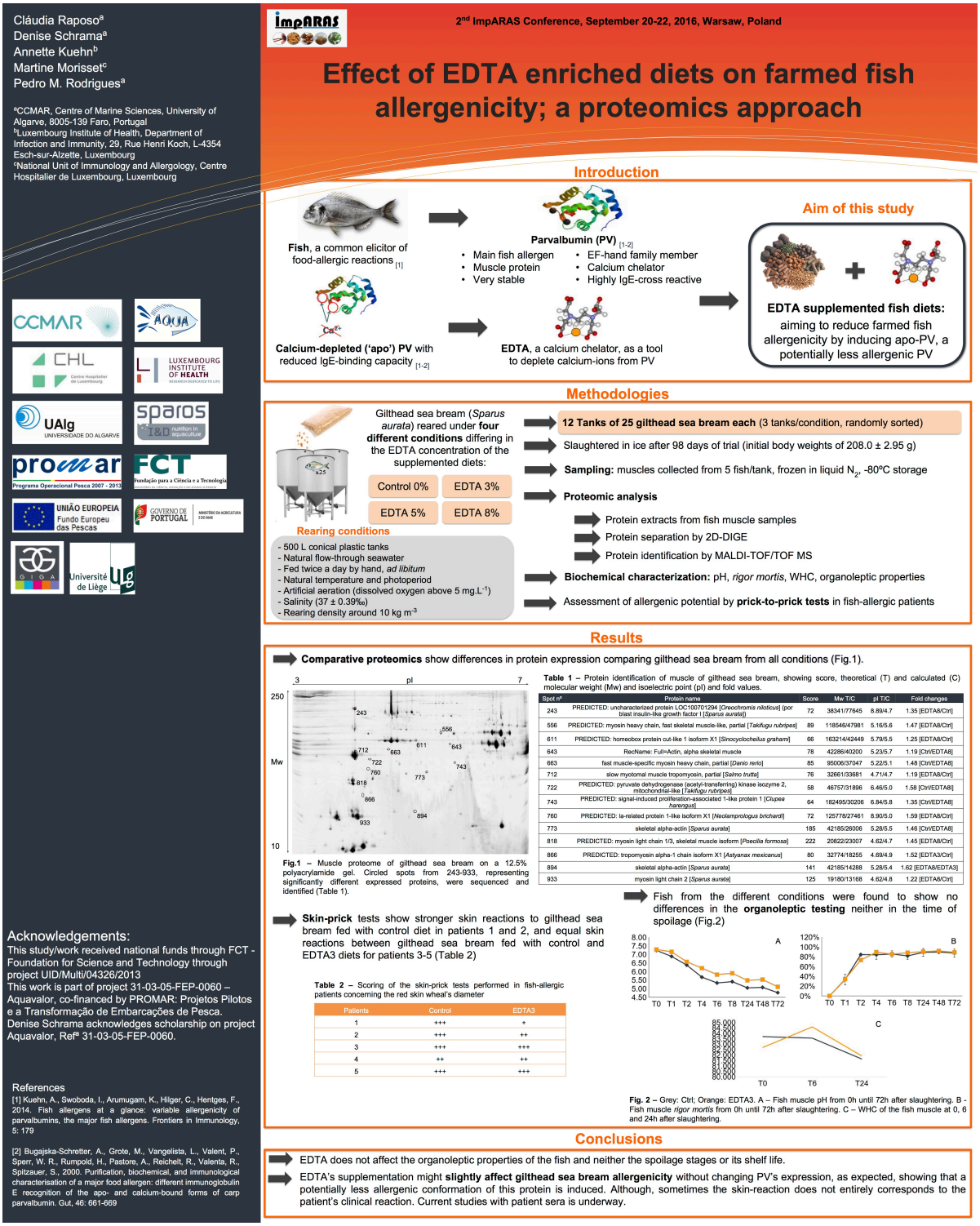

Figure 7.1. Poster presented in COST Action 1402: Improving Allergy Risk Assessment Strategy for New Food Proteins, 2nd ImpARAS Conference, September 20-22, Warsaw, Poland