



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

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ABSTRACT

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

1. Introduction

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

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

Among other allergens such as enolase and aldolase (Kuehn et al., 2013), β -parvalbumin (PV) is considered to be the major fish allergen responsible for most of the IgE-mediated reactions (Kuehn, Swoboda, Arumugam, Hilger, & Hentges, 2014; Sharp & Lopata, 2014). PV is a small (molecular weight 10–12 kDa), acidic (isoelectric point between pH 4 and 5), calcium-binding muscle protein. Its remarkable stability to thermal, chemical and proteolytic denaturation seems to contribute to its allergenic potency, which is not altered during the gastrointestinal processing (Sánchez et al., 2016). Fish PV is an EF-hand protein, characterized by presenting two functional EF-hand domains (motifs of helix-loop-helix structures) capable of chelating one divalent metal ion each, with high binding affinity for calcium (Arif, 2009). Fish may express multiple PV isoforms differing by their allergenicity, an observation that is not well understood so far (Kuehn, Hutt-Kempf, Hilger, & Hentges, 2011). In teleost (bony) fish, IgE cross-reactivity among PVs

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of different species is highly common, due to the high amino acid and structural identity between the homologs, explaining the high clinical cross-reactions to various fish in allergic patients (Stephen et al., 2017). Monosensitivity to specific fish species or single parvalbumin isoforms has also been reported (Kuehn et al., 2011). To date, the only effective clinical management of fish-allergic patients relies on complete avoidance of any fish-containing food (Kuehn et al., 2014). However, patients might also be exposed accidentally to the offending food. Other common strategies to induce immunological tolerance, like allergen-specific immunotherapies, which relies on the administration of allergenic extracts, are complicated and may induce side reactions or sensitization to new allergens present in the crude extract (Reisacher & Davison, 2017). Hence, the need of new developments to overcome this prevalent allergy is urgent.

Several studies showed a reduction in the PV's IgE-binding capacity upon calcium depletion. This was explained by a global-folding rearrangement of the protein structure, affecting major epitope regions. Studies proved that calcium-binding is essential to maintain the parvalbumin stable structure and its IgE-reactivity (Kobayashi, Ichimura, Kobayashi, & Shiomi, 2016; Permyakov et al., 2008). Swoboda et al. successfully produced hypoallergenic mutant allergens from carp PV (Cyp c 1), with point mutations in the two functional calcium-binding sites and clearly reduced the molecule's IgE-reactivity (Swoboda et al., 2013). Tomura et al. was able to reduce Pacific mackerel PV IgE-reactivity by substituting specific amino acid residues in the calcium-binding sites (Tomura, Ishizaki, Nagashima, & Shiomi, 2008). Moraes et al. and Kumeta et al. performed important studies regarding the 3D-structure, dynamics and stability of cod (Gad m 1) and Pacific mackerel (Sco j 1) parvalbumins, respectively, targeting on the various IgE-binding modes of these allergens (Kumeta, Nakayama, & Ogura, 2017; Moraes et al., 2014).

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

2. Materials and methods

2.1. Fish rearing conditions and sampling procedure

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

Four different diets were tested: control (CTRL), without EDTA supplementation in the commercial feed, EDTA 3% (EDTA3), EDTA 5% (EDTA5) and EDTA 8% (EDTA8). Experimental conditions were settled in triplicate, with randomly sorted tank numbers. A zootechnical characterization performed during fish rearing, showed that fish from dietary treatments EDTA5 and EDTA8 did not grow and were not commercially suitable. Thus, samples from these treatments were not considered for further analyses (see supplementary material 1).

After 98 days of trial, five random fish from each tank were anesthetized with tricaine methanesulfonate (MS-222; Sigma Aldrich, St. Louis, Missouri, USA) for blood collection. Blood samples of approximately 2 ml were collected from the caudal vein with a heparinized syringe and centrifuged at 2000 xg for 20 min. Plasma samples were taken and stored at -80°C until posterior analyses. For proteomic analysis, the same fish were then slaughtered with an overdose of anesthetic and muscle samples were collected, immediately frozen in liquid nitrogen, and stored at -80°C . For biochemical measurements, seven random fish from each tank were slaughtered with an overdose of the same anesthetic and stored in polystyrene boxes with ice during the analyses (72 h). Five random fish from each tank were killed, by immersion in water and ice, for sensory analysis with a human panel (EFSA, 2009). This killing method was exceptionally applied in this case, since fish were used for human consumption, according to the Portuguese legislation n°113/2013 – 2013-08-07 and the Council Regulation (EC) n°1099/2009 of 24th September 2009 on the protection of animals at the time of killing.

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

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

2.2. Cortisol measurement

Plasma cortisol was measured ($n = 10$) using a commercial Cortisol ELISA kit RE52061 (IBL International, Hamburg, Germany), following the manufacturer's instructions. The plate was read at 450 and 620 nm along with a prepared standard curve on a microplate reader Biotek Synergy 4 Hybrid Technology™ (Biotek Instruments Inc., Winooski, USA).

2.3. Biochemical and quality characterization of fish muscle

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

At the same post-mortem periods, rigor mortis index was assessed ($n = 12$) as previously described (Matos, Gonçalves, Nunes, Dinis, & Dias, 2010).

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

cohesiveness, adhesiveness, springiness and chewiness.

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

2.4. High-performance liquid chromatography (HPLC) determination of EDTA

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

2.5. Muscle proteome analysis

2.5.1. Protein extraction

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

2.5.2. Protein labeling

Samples' pH was checked with a pH-indicator paper, Sigma-P4536 (Sigma Aldrich, St. Louis, Missouri, USA) and adjusted to 8.5 using 0.1 M NaOH. For DIGE minimal labeling, 50 µg of protein from each sample was labeled, as described by the manufacturer, using CyDye™ DIGE Fluor minimal labeling kit 5 nmol (GE Healthcare, Little Chalfont,

UK), with 400 pmol fluorescent amine reactive cyanine dyes freshly dissolved in anhydrous dimethylformamide (DMF). Labeling was performed on ice for 30 min, in the dark, and the reaction quenched with 1 mM of lysine for 10 min. Six samples per experimental condition were labeled with Cy3 and six with Cy5 in order to reduce impact of label difference, while an internal standard consisting of a pool of all samples, with equal amounts, was labeled with Cy2. Samples were randomly sorted in order to reduce variable confounding, due to inherent dye-dependent bias.

2.5.3. Protein separation by 2D-E

Labeled proteins from each experimental condition were mixed with the internal standard and rehydration buffer (8 M urea, 2% CHAPS, 50 mM DTT), 0.001% bromophenol blue, 0.5% Bio-lyte 3/10 ampholyte (Bio-Rad Laboratories, Hercules, California, USA) to complete 450 µl. Passive rehydration was conducted for 15 h on 24 cm Immobiline™ Drystrips (GE Healthcare, Little Chalfont, UK) with linear pH 3–7, on an IPG Box (GE Healthcare, Little Chalfont, UK). Rehydration was followed by isoelectric focusing (IEF), performed in 5 steps: 500 V gradient 1 h, 500 V step-n-hold 1 h, 1000 V gradient 1 h, 8000 V gradient 3 h and 8000 V step-n-hold for a total of 60,000 Vhr using Ettan IPGphor at 20 °C (GE Healthcare, Little Chalfont, UK). Prior to second dimension, strips were reduced and alkylated with 6 ml of equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol and 2% SDS) with 1% (w/v) dithiothreitol (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 DALTsix Large Vertical System (GE Healthcare, Little Chalfont, UK) at 10 mA/gel for 1 h followed by 60 mA/gel until the bromophenol blue line reaches the end of the gel, using a standard Tris-Glycine-SDS running buffer. Two rounds were performed, six gels each.

2.5.4. Gel image acquisition and analysis

Gels were scanned on a Typhoon laser scanner 9400 (GE Healthcare, Little Chalfont, UK) using three laser emission filters (520 BP40 for Cy2, 580 BP30 for Cy3 and 670 BP30 for Cy5) at 100 µm resolution, with emission and excitation wavelengths recommended by the manufacturer. Gel images were analyzed using SameSpots software (TotalLab, Newcastle, UK). Analysis includes background subtraction, filtering, spot detection, spot matching, normalization and statistical analysis. Statistical significance was assessed using Student's *t*-test ($p < .05$) and average fold-ratio (ratio > 1.0). Resultant protein spots with statistically different intensities were considered for further manual excision and identification by mass spectrometry (MS).

2.5.5. Protein identification

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

2.6. IgE-reactivity to PV with fish-allergic patients' sera

2.6.1. Patient sera samples

Twenty (12 male, 8 female; mean age 21.5 years) patients with fish allergy were recruited at the Centre Hospitalier de Luxembourg (CHL). The study was approved by the National Committee for Medical Research Ethics in Luxembourg (Ref. 201,307/04) and all participants signed the informed consent. Inclusion criteria for study participation were a clinical history of an IgE-mediated codfish allergy, a positive skin prick test with cod extract and a positive specific IgE-titer to cod extract (mean titer 11.2 kU_A/L) as detected by ImmunoCAP (> 0.35 kU_A/L positive; Phadia-ThermoFisher, Uppsala, Sweden).

2.6.2. Protein extraction

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

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

2.6.3. 1D-PAGE IgE immunodetection

IgE immunoblots using patient sera were performed as described before (Kuehn et al., 2016). Briefly, two pools of muscle samples from CTRL and EDTA3 were prepared and 450 µg of the protein extracts were loaded on 7 cm preparative SDS-PAGE gels (15%), 2 preparative gels for each fish, and separated in two steps: 100 V for 20 min followed by 200 V for 55 min. Resultant gels were blotted onto 0.22 µm PVDF membranes (Millipore, Burlington, Massachusetts, EUA) for 1 h at 14 V, and blocked in buffer containing 3% BSA overnight at 4 °C. Membranes were cut using an Accutran Strip Cutter (Inotech, Nabburg, Germany) and each immunoblot stripe incubated with 700 µl of patient serum (1:3 diluted in blocking buffer containing 3% BSA) overnight, at room temperature and constant agitation. A mix of two in-house antibodies (1:10,000; rabbit pAb against cod/salmon PV; rabbit pAb against herring/mackerel PV) was used as positive control for PV-detection (C). Following, immunoblot stripes were washed 4 times for 5 min each with Tris-buffered saline/Tween-20 (TBS-T) buffer. Each patient stripe was incubated with 1 ml of anti-human IgE Southern Biotech 9160-04-alkaline phosphatase (AP) antibody (1:10,000) for 2 h at room temperature with constant agitation. Control strip was incubated with a mix of anti-mouse IgG A2429-AP (1:10,000) and anti-rabbit IgG Sigma A3812-AP (1:10,000). Alkaline phosphatase buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM MgCl₂) was used for the revelation with addition of AP substrate solution (nitro-blue tetrazolium, NBT: 5-bromo-4-chloro-3'-indolylphosphate, BCIP) (Promega, Madison, WI) to perform a total volume of 1 ml. The colorimetric reaction was stopped by washing with TBS-T buffer.

2.7. Statistical analyses

To assess normality of distributions, a Shapiro-Wilk test was applied, while homoscedasticity assumption was checked by Levene's test. Cortisol data were primarily subjected to Grubb's test for outliers' detection. Statistical significance in plasma cortisol levels was assessed using Student's *t*-test. *Rigor mortis* data were previously transformed by arcsine square root. Differences in pH, *rigor*, and texture, between

conditions, were assessed using Student's *t*-test, if homogeneity of variances was verified. In case any assumption was violated, data was subjected to a non-parametric Mann-Whitney *U* test for statistical significance. Results are presented in mean ± standard deviation (S.D.). Statistical analyses were performed using SPSS Statistics 25 software (IBM Analytics, Armonk, New York, USA) for MacOSX. Data regarding sensory analyses was subjected to the non-parametric Mann-Whitney *U* test using STATISTICA analytics 8.0 (StatSoft) for Windows. A significance level of $\alpha = 0.05$ was used in all tests performed.

3. Results and discussion

3.1. Zootechnical characterization and the EDTA effect on gilthead seabream growth performance

A negative effect of high EDTA concentrations was observed over the fish growth performance and feed efficiency. Growth and daily feed intake tended to be lower the higher the EDTA supplementation. Fish raised in tanks of EDTA8 condition were recorded to have the lowest growth rate and the highest mortalities. A one-way analysis of variances (ANOVA) performed to the final body weight showed significant differences between all experimental conditions ($p < .05$) (See supplementary material 1). Samples from dietary treatments EDTA5 and EDTA8 were not considered for the variables in this study, once growth performance showed that fish from these conditions were not commercially suitable and was not possible to ensure that the results obtained were induced by EDTA supplementation and not a consequence of the observed low feed intake. However, EDTA supplementation did not affect fish growth rates when applied in low concentrations (EDTA3). Values from fish specific growth rate (SGR) belonging to CTRL and EDTA3 experimental conditions correspond to previous nutritional studies with gilthead seabream (unpublished results), presenting similar initial body weights and reared under the same conditions of temperature and salinity (De Francesco et al., 2007; Fountoulaki et al., 2009). Contrarily, fish from EDTA5 and EDTA8 conditions showed low and negative growth rates, respectively, which is supported by the feed conversion ratio (FCR) obtained. FCR is a major indicator of feed efficiency in fish farming and related research. Its values are lower, the higher the weight gain obtained with certain feed. FCR values for CTRL and EDTA3 fish correspond to previously reported for this species (De Francesco et al., 2007; Fountoulaki et al., 2009). Low palatability, digestibility and unpleasant odor might be in the origin of the low feed intake and negative weight gain observed. Further studies and improvements must be performed as the addition of an attractive ingredient or EDTA microencapsulation might favor the diet acceptability by the fish.

3.2. Plasma cortisol quantification and the physiological response to stress

No significant differences ($p = .788$) were found in plasma cortisol between both conditions (5.18 ± 4.85 and 5.78 ± 4.09 ng/ml in CTRL and EDTA3, respectively), suggesting that EDTA level included in the diet had no impact over the plasma cortisol concentration (Fig. 1.A). A high standard deviation was verified due to an enormous biological variability. Minimizing stress conditions and preserving fish welfare is nowadays one of the main concerns in aquaculture, since growth performance and health status are strongly associated with the stress status of the animal. Several stressors have been extensively reported in aquaculture: *pre-* and *post-mortem* handling practices, high stocking densities, presence of diseases, among others (Alves et al., 2010). Results of the current study show that gilthead seabream exposed to EDTA did not experienced stress, as indicated in plasma cortisol levels in comparison with the basal levels of control fish. No literature regarding the effect of EDTA on cortisol release and fish welfare was found. Plasma cortisol concentration levels measured in fish from both treatments are according to the reference levels for this species

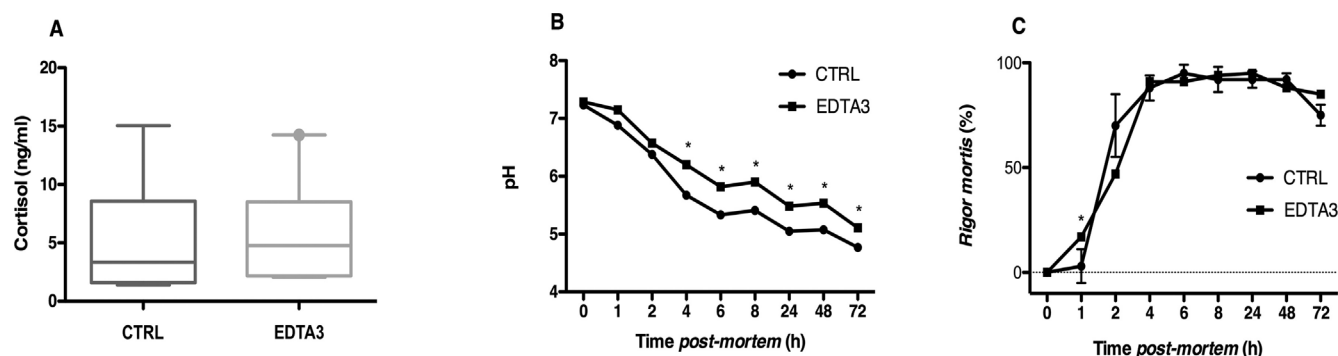


Fig. 1. A - Plasma cortisol levels in blood samples from gilthead seabream (*Sparus aurata*) submitted to control and EDTA supplemented feeds ($n = 10$). The box includes observations from 25th to the 75th percentiles; the horizontal line within the box represents the median value. Lines outside the box represent the 10th and 90th percentiles. No significant differences were found among conditions ($p > .05$). B - Post-mortem changes in muscle pH of gilthead seabream (*Sparus aurata*) stored in ice for 72 h. Data points are the mean of $n = 9$ for each sampling time. $*P < .05$ vs. control at 4, 6, 8, 24, 48 and 72 h post-mortem. C - Development of rigor mortis of gilthead seabream (*Sparus aurata*) stored in ice for 72 h. Data points are the mean of $n = 12$ for each sampling time. $*P < .05$ vs. control at 1 h post-mortem.

(Alves et al., 2010; Yildiz, 2009). However, both groups registered a high individual variability most likely due to individual differences in stress responses.

3.3. Biochemical and muscle quality characterization

Flesh quality of the fish product was determined in this study by several physical-chemical parameters. Several authors have reported the properties of EDTA as a shelf-life extender in fish, added at the storage step, but only a few have studied its effect on other quality attributes and in particular on the organoleptic properties of fish flesh (Ghaly, Dave, Budge, & Brooks, 2010). Muscle pH declined between T0 and T72, ranging from pH 7.2 and 7.3 after slaughtering to 4.8 and 5.1, in fish from CTRL and EDTA3 groups, respectively (Fig. 1.B). Significant differences between both conditions were found at 4, 6, 8, 24, 48 and 72 h post-mortem ($p < .05$). Results from the present study followed the same pH trends as previous studies on the same species. The muscle pH registered immediately after slaughtering are concordant with the values reported by Bagni et al., 2007, who registered an initial muscle pH ranging from 7.0 to 7.3 in gilthead seabream 30 months aged. Nevertheless, pH values registered at 48 h post-mortem, 5.1 and 5.5, for CTRL and EDTA3 respectively, are below the reported by Matos et al., 2013 and Silva et al., 2012, at the same time. The values 4.8 and 5.1, for CTRL and EDTA3, respectively, at the last measuring time, are below the so-called ultimate pH considered around 5.5, suggesting a total depletion of glycolytic energy reserves. Lower pH levels indicate higher levels of glycogen in the muscle immediately after slaughtering (Toldrá, 2010).

The onset of rigor mortis (Fig. 1.C) occurred 1 h post-mortem in both groups, when muscle pH registered 6.9 and 7.1 for CTRL and EDTA3 groups, respectively. Maximum rigor strength was reached at 6 h post-mortem in both groups, with $95 \pm 3\%$, and $96 \pm 4\%$ in CTRL and EDTA3, respectively, which is in accordance with Matos et al., 2013. Significant differences were observed between both conditions at 1 h post-mortem ($p < .05$). The process of rigor mortis occurs when ATP levels in muscle decrease. When reaching low levels, actin and myosin bind together to form the actomyosin complex inducing stiffness of the fish body. Contrarily, resolution of rigor mortis makes fish muscle less rigid, losing its elasticity (Delbarre-Ladrat, Chéret, Taylor, & Verrez-Bagnis, 2006). In this study, the measurement at 72 h was performed since rigor was still on at 48 h post-mortem. The onset of rigor mortis started 1 h after slaughtering, which is consistent with previous studies (Venugopal, 2002). The non-resolution of this state after 72 h is not consistent with the 36 h reported for sea bass stored at 0°C (Delbarre-Ladrat et al., 2006).

Significant differences observed in muscle pH and rigor mortis between both conditions can possibly be explained by a delayed

tenderization of the fish muscle. Tenderization is the process fish muscle undergoes within hours after death, responsible for the softening of the muscle through the degradation of key structures in the muscle sarcomere (Delbarre-Ladrat et al., 2006). This can be due to a low availability of calcium ions caused by the presence of EDTA. The onset of rigor mortis along with the pH drop, leads to an increase in sarcoplasmic calcium ions content, from 0.1–0.8 μM to about 0.2 mM, which will induce the activation of calcium-dependent proteases (e.g. collagenases, calpains and cathepsins). This increased protease activity is responsible for the destruction of Z-disks and the actomyosin complex, causing softening of the flesh and the resolution of rigor mortis (Salem, Kenney, Killefer, & Nath, 2005). Calcium also induces Ca^{2+} -dependent phosphorylase kinase, which is responsible for the regulation of glycolysis, by activating the glycogen phosphorylase. This enzyme catalyzes glycogen molecules consequently lowering the pH (Berg, Tymoczko, & Stryer, 2002). Results observed in this study for the pH and rigor measures might suggest an EDTA effect: a possible low availability of free calcium ions due to the presence of EDTA, leads to a non-activation/delayed activation of the calcium-dependent proteins, consequently slowing the rate of pH drop (Ayala et al., 2010; Delbarre-Ladrat et al., 2006) (see supplementary material 2).

EDTA supplementation affected significantly the instrumental texture of raw gilthead seabream muscle (Table 1). Five textural parameters were evaluated, whereas 3 demonstrated significant differences between both conditions, being these adhesiveness ($p = .029$), springiness ($p = .018$) and chewiness ($p = .017$). Fish muscle texture is one of the most important quality parameters and has a higher impact on consumers' acceptance (Delbarre-Ladrat et al., 2006). Instrumental texture of gilthead seabream muscle suggests a possible EDTA effect, since 3/5 textural parameters revealed significant differences between conditions ($p < .05$). Values obtained for hardness, in both conditions, are practically 2-fold higher than the reported for seabream, with

Table 1

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

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

Values are means $\pm$ standard deviations ($n = 14$). In each row values with different letters are significantly different ($p < .05$).

Table 2
Differently expressed ($p < .05$) muscle proteins between gilthead seabream (*Sparus aurata*) submitted to control and EDTA 3% supplemented diets, identified by MALDI-TOF/TOF MS after separation by 2D-DIGE. Differences in expression are indicated in the “Fold change” column.

Spot n°	Identified protein (species)	NCBI accession number	Score ^a	Mw T/C ^b	pI T/C ^c	Sequence coverage (%) ^d	Fold change (highest volume) ^e	FDR ^f	N° of matched peptides ^g	Best peptide match sequence	Protein function
866	Contractile apparatus PREDICTED: tropomyosin alpha-1 chain isoform X1 [<i>Asyanamexicanus</i>]	gi 597,755,671	80	32,774/ 18255	4.69/4.9	35	−1.52 (EDTA3)	0.0447	6	K.LDKENALDRA	Calcium-dependent regulation of muscle contraction

^a Score – a non-probabilistic protein score obtained from the ions score.

^b Theoretical (T) and calculated (C) molecular weight (Mw).

^c Theoretical (T) and calculated (C) isoelectric point (pI).

^d Sequence coverage (%) -percentage of protein covered by matched peptides.

^e Fold change (Highest volume) - Significant changes in protein abundance across conditions.

^f FDR – False discovery rate.

^g N° of matched peptides –N° of peptides matched to entry with significant E-value < 0.05.

similar body weights, stored in ice (Ayala et al., 2010). The ones registered for springiness are about 3-fold lower, while the registered for cohesiveness and adhesiveness are consistent. The same relation is observed with other studies (Ayala et al., 2011).

Five descriptors were selected during the sensory evaluation. The terms “typical odor” and “typical flavor” were used to describe the attributes odor and flavor, the color “white” was selected for appearance, while “firmness” and “juiciness” were the descriptors employed for texture. The panelists found no differences between the organoleptic properties of fish fed commercial and EDTA supplemented diets (Fig. 2). The typical odor and flavor of the fish were present in only 70% and 90% of the samples belonging to EDTA3 group and two members of the panel indicated that the white color of EDTA3 fish flesh was not the characteristic white but instead, very intense, “almost milky”. Half of the panel did not perceive different odors, flavors or colors on CTRL samples, contrarily to 30% in EDTA3 samples. No significant differences were found between the sensorial attributes of both conditions ($p > .05$).

3.4. EDTA accumulation in fish muscle

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

3.5. IgE immunoblots

Comparative tests of IgE-reactivity performed with crude muscle protein extracts from gilthead seabream showed differences between CTRL (Fig. 3.A) and EDTA3 fish (Fig. 3.B). A total of 20 cod-allergic patients' sera were used, from which 17 showed a band in a narrow molecular weight range (ca. 10–12 kDa), testing positive for IgE-reactivity to gilthead seabream PV in CTRL samples. Regarding EDTA3 samples, only 8 had detectable IgE-binding signals for PV (indicated by a black star in Fig. 3). A positive control using a mix of anti-PV antibodies (C) confirmed the presence and identity of the allergen, in both conditions, at the molecular weight around 10–12 kDa, which is also in concordance with the theoretical Mw of gilthead seabream PV (UniProtKB accession number: D0VB96). Parvalbumin-negative patients (3 out of 20 patients) showed other visible bands (ca. 21, 31 or 45 kDa) (data not shown), suggesting the presence of IgE antibodies to other fish allergens (e.g. enolase from gilthead seabream presents a Mw of 28 kDa). Sequencing of these bands would have to be performed to confirm protein identity. No IgE-reactivity to the EDTA3 samples was found for the last 9 patients. Results suggest that PV epitope regions were affected by the EDTA-supplemented diet, possibly originated by calcium depletion, which is known to result in lower IgE-reactivity (Kubota, Kobayashi, Kobayashi, Shiomi, & Hamada-Sato, 2016; Tomura et al., 2008). Further analysis of the calcium-binding status of PVs from control and EDTA3-fish will be required to confirm this hypothesis. In a follow-up study, an IgE-ELISA with the purified allergen would have to be performed to corroborate the reduction in the IgE-binding capacity upon EDTA presence. Additionally, patient's sera with clinical reactivity to gilthead seabream would improve the results.

3.6. Effect of EDTA over the gilthead seabream muscle proteome

Total protein content of gilthead seabream muscle revealed very clear 2D gels, where a statistical analysis to protein spots' intensity identified 2 spots as significantly different between the experimental

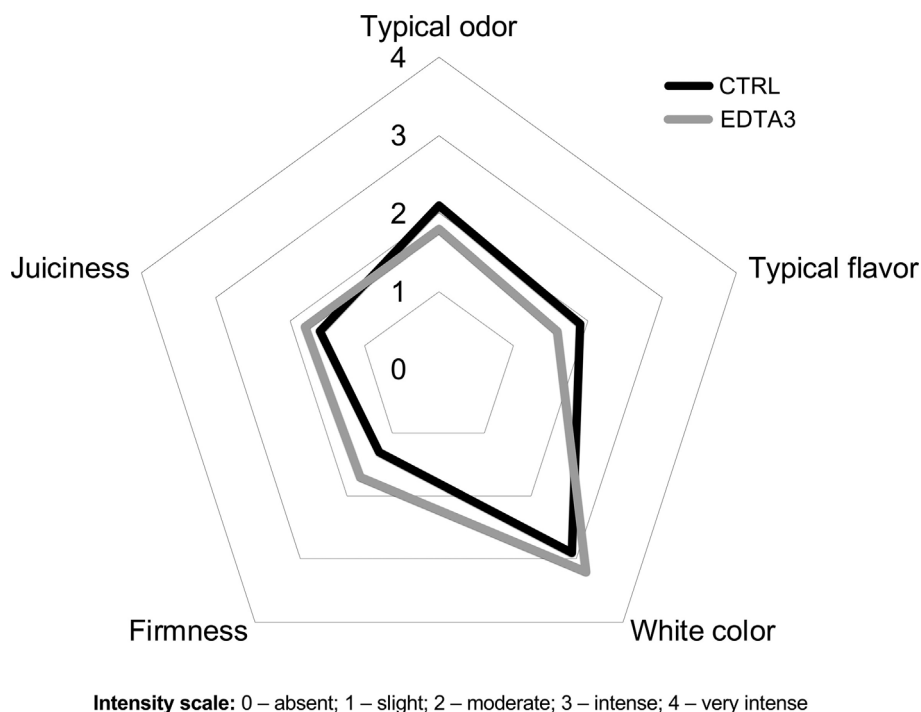


Fig. 2. Sensory profile of gilthead seabream (*Sparus aurata*) muscle from CTRL (black line) and EDTA3 (grey line) experimental conditions. Scores of the attributes are given as absent-very intense. No significant differences were found between treatments ($p > .05$).

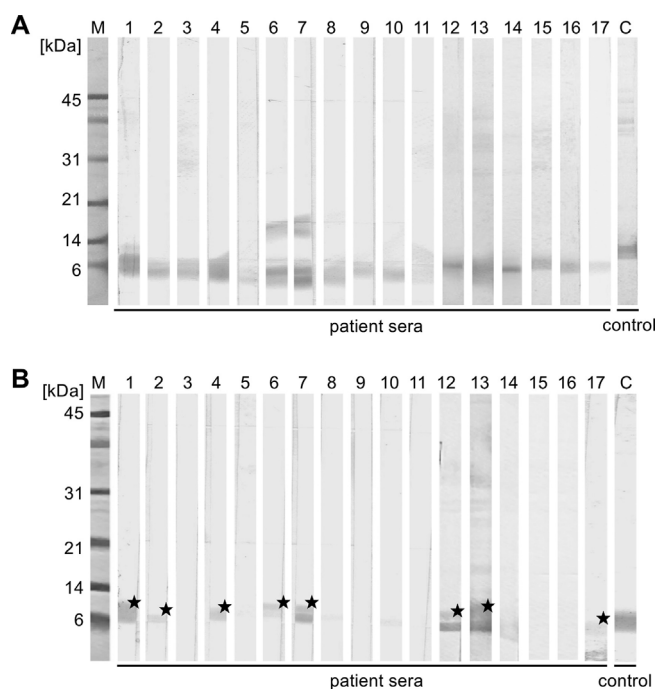


Fig. 3. IgE-reactivity of single patient sera (1–17) to gilthead seabream (*Sparus aurata*) PV from control (A) and EDTA3 (B) experimental conditions. Black stars indicate positive IgE-binding signals. Control antibodies (C) were used to detect PV. M, marker.

conditions ($p < .05$). After spots' excision and MS analysis, identification for 1 protein spot was obtained, which was then classified according to their cell function as belonging to the contractile apparatus. Fig. 4 shows a representative 2D gel of the muscle proteome from gilthead seabream, with the identified spot circled. This spot, identified by PMF in a MALDI-TOF/TOF MS as tropomyosin, registered a theoretical Mw of 18 kDa, a pI of 5, and the higher normalized spot volume

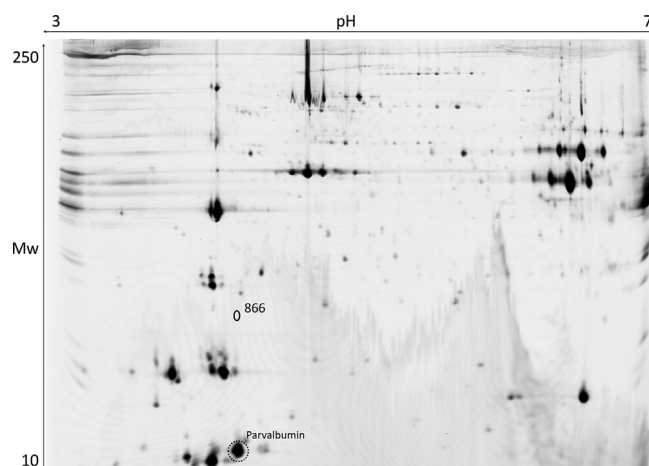


Fig. 4. Representative 2D gel of gilthead seabream (*Sparus aurata*) muscle on a 12.5% polyacrylamide 2D gel. Circled spot, representing a significantly different expressed spot ($p < .05$) between different conditions, were sequenced and identified (Table 2).

in fish fed with EDTA 3% enriched diets. Myosin molecules, composed of two heavy and four light chains subunits, constitute the myosin filaments in muscle cells, whose heads, located on the surface of the filament, interact with actins for muscle contraction. Tropomyosins, found in the fish fast skeletal muscle, form a family of actin-binding proteins, responsible for covering the myosin binding sites on actin filaments. In the presence of calcium ions, tropomyosins uncover the myosin-binding sites, by binding to the troponin complex. The myosin heads attach to the thin filaments of actin, sliding horizontally and originating muscle contraction (Cooper, 2000). The up-regulation of this protein can be explained by the reduced proteolysis of the myofibrillar proteins, during the tenderization process, due to the presence of EDTA, as explained above.

Parvalbumin was identified in spot Ex85 (data not shown) with a calculated pI matching the theoretical one and a calculated Mw of

8403 Da, 3 kDa lower than the theoretical one. PV's expression was not significantly different between conditions ($p > .05$). EDTA was used to induce a rearrangement of the allergen structure, a calcium-depleted hypoallergenic conformation, rather than reducing its expression. Comparative proteomics showed no significant differences in parvalbumin's spot intensity in fish muscles from different EDTA-conditions. Its location in the representative 2D gel of the muscle proteome is circled in dashed line, obtained in previous identifications (data not shown). Mw (~12 kDa) and pI (~5) are consistent with the theoretical values, obtained through the protein accession number.

4. Conclusions

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

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

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2019.125508>.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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SUPPLEMENTARY MATERIAL 1. Growth performance and feed utilization of gilthead seabream (*Sparus aurata*) reared for 98 days under different feeding conditions. Values are mean $\pm$ SD (n = 75).

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

Different letters indicate significant differences among conditions ($p < 0.05$).

SUPPLEMENTARY MATERIAL 3. EDTA accumulation in the muscle of gilthead seabream (*Sparus aurata*), per sample. Values are in ppm (n = 15).

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