Universidade de Aveiro Departamento de Química

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Effect of high-pressure pre-treatment on the enzymatic activity related to quality loss in frozen hake

Efeito de pré-tratamento de alta pressão na atividade enzimática relacionada com perda de qualidade de pescada congelada

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, ramo Alimentar, realizada sob a orientação científica do Doutor Jorge Manuel Alexandre Saraiva, Investigador Auxiliar do Departamento de Química da Universidade de Aveiro.

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palavras-chave

Processamento por alta pressão; Conservação de peixe; Atividade enzimática; Armazenamento congelado; *Merluccius merluccius*

resumo

A exigência dos consumidores por produtos alimentares de elevada qualidade sem conservantes e com tempo de prateleira alargado, mantendo a sua qualidade, levou ao desenvolvimento de novas tecnologias para o seu processamento. Uma dessas tecnologias, abordada neste trabalho, é o Processamento por Alta Pressão (HPP), que tem apresentado bons resultados na extensão do tempo de prateleira em peixe, com especial importância para a segurança dos produtos, ao reduzir a carga microbiana (maioritariamente patogénicos) em peixe refrigerado e inibir a atividade de certas enzimas degradativas, principalmente em peixe congelado.

Os produtos da pesca são normalmente armazenados em refrigeração ou congelados, de modo a manter os seus padrões de qualidade e segurança. No entanto, há diversas reações que ainda assim causam a degradação do peixe. Por exemplo, mesmo enquanto congelado, o peixe sofre alterações devido à atividade enzimática. De acordo com a literatura, o HPP mostrou grande potencial na inativação dessas enzimas e, consequentemente, na extensão do tempo de prateleira do peixe.

Este trabalho visou determinar o efeito de um pré-tratamento por alta pressão (150 MPa por 2 minutos) e temperatura de armazenamento (-10, -20 e -30 ºC) na atividade enzimática do músculo de pescada congelada (*Merluccius merluccius*). Verificou–se que o pré-tratamento de HP possibilitou a diminuição de atividade das calpaínas (59 %) após 12 meses a -20 ºC e a manutenção das atividades da fosfatase ácida, catepsina B e calpaínas após 12 meses a -30 ºC. Verificou-se ainda um aumento no conteúdo em grupos tiol (SH) com o pré-tratamento (até 190%) e ao longo do tempo de armazenamento (até 120%), estando esse aumento possivelmente relacionado com a desnaturação proteica do músculo de peixe.

Este trabalho apresenta assim informação importante relativa à aplicação de pré-tratamentos por alta pressão para reduzir a atividade enzimática associada a deterioração da pescada congelada (*Merluccius merluccius*). Este tipo de processamento pode assim ser relevante para uma melhor preservação da qualidade da pescada congelada, bem como possivelmente de outras espécies.

keywords

High Pressure Processing; Fish preservation; *Merluccius merluccius*; Enzymatic activity; Frozen Storage.

abstract

Consumers demand for high quality food stuffs with no preservatives and with extended shelf-life, retaining the quality properties, led to the development of new processing technologies. One of those technologies, studied in this work, is High Pressure Processing (HPP), which has shown good results on fish shelf-life extension, with great importance in fish safety by inactivation of microbial load (mostly pathogenic microorganisms) in refrigerated fish, and inactivation of certain degradative enzymes, mainly in frozen fish.

Fish products are usually stored under refrigeration or frozen to maintain their quality and safety patterns. However, there are still several reactions that cause fish degradation. For instance, even under frozen storage, enzymatic activity still occurs. According to the literature, HPP has shown to be a promising pre-treatment to inhibit these enzymes and extend frozen fish-shelf-life.

This work aimed to determine the effect of HPP (150 MPa for 2 minutes) and storage temperature (-10, -20 and -30 ºC) on the quality of frozen hake muscle (*Merluccius merluccius*). It was verified that HPP resulted in a decrease of calpains activity (59 %) after 12 months of storage at -20 ºC and maintained the activity of acid phosphatase, cathepsin B and calpains after 12 months at -30 ºC Furthermore, the quantification of sulfhydryl (SH) groups indicated that the HP treatment results in higher SH content (increase up to 190%), as well as increased storage time (increase up to 120%), being this increase probably related with protein denaturation of fish muscle.

This work presents relevant information concerning the employment of HP pre-treatments to reduce enzymatic activity associated to quality loss in frozen European hake (*Merluccius merluccius*). This type of processing can so be of interest to enhance the quality preservation of frozen hake, as well as of other species.

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List of Abbreviations

Abbreviation	Designation
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
a_w	Water activity
CFU	Colony forming unit
IMP	Inosine 5'-monophosphate
FDA	Food and Drug Administration
FFA	Free fatty acids
HP	High pressure
HPP	High pressure processing
Mb	Myoglobin
PUFAs	Polyunsaturated fatty acids
RT	Room temperature
SSO	Specific spoilage microorganisms
TMAO	Trimethylamine nitrogen-oxide
TMA-N	Trimethylamine nitrogen

1. Introduction

Fish and some other aquatic species are products of great economic importance for many countries, showing high nutritional value and high digestibility and being highly appreciated by the consumers [1, 2]. They are rich in proteins, essential amino acids (like lysine, methionine and others), lipid-soluble vitamins (namely A and D), minerals (like I, F, Ca, Cu, Zn, Fe) and polyunsaturated fatty acids (PUFAs), with special emphasis on omega-3 [3–5]. These last compounds are now the subject of greater attention, since they have shown a positive role in preventing cardiovascular events such as heart attack and stroke [6]. Some fish species, despite its recognized healthy characteristics, remain underutilized due to its short shelf-life [7]. Being among the most perishable foods, these products demand a rapid and efficient processing and storage after its capture or harvest [3]. Most technologies have already been applied to try to reduce the perishability of fish, and consequently increase its short shelf-life [8].

Although the quality of fresh fish is often regarded as superior to that of the preserved products, adequate freezing immediately after catch and processing can result in products that are as good or even better than the fresh fish [9]. Right after death, the freshness state is modified due to the action of endogenous enzymes, causing undesirable changes in texture, flavour and odour [10, 11]. The freshness and, consequently, the shelf-life of fish products can also be affected by the action of some specific microorganisms [12].

Consumers' health and wellness-oriented lifestyles and consequent demand for convenient, mildly processed and fresh tasting foods with minimal or no preservatives led to the development of several nonthermal technologies for food processing. High Pressure Processing (HPP) has emerged as one of the most valuable approaches to satisfy these requirements and is now the most successfully commercialized nonthermal processing technology [13–15].

The quality of frozen hake, which is addressed in this work, may be improved using this technology, since this method has been shown to extend its shelf-life by inactivating certain enzymes [16], while preserving its original sensory and nutritional properties [17].

1.1. Fish Degradation Mechanisms

Fish products are highly perishable foods, due to biochemical characteristics like high water activity ($a_{w)}$, neutral pH (both factors that contribute to microbial proliferation) and high content in soluble nitrogen compounds (that accelerates autolysis) and unsaturated lipids [18, 19]. The degradation process is caused by the action of three mechanisms: endogenous enzymes, microbial spoilage and lipid hydrolysis/oxidation [20].

The post-mortem softening of fish muscle may be caused by two different types of reaction: biochemically induced reactions (by degradation of myofibrils and collagen) and physical reactions due to the separation of myotomes, a phenomenon called "gaping" (a consequence of the failure of fibres/tissues to connect the muscle blocks along the fillet). Then, for the rest of the post-mortem period, many biochemical mechanisms lead to a reduction in hardness, like the disintegration of the extracellular matrix structure or changes in structural links and bonds caused by enzymes [21].

Loss of sensorial quality can also be a result of enzymatic (activity of lipases) and/or non-enzymatic rancidity (due to the presence of pro-oxidant molecules like transition metals) [22] and it results in a decrease of the product's shelf-life [2, 23, 24].

Degradative microorganisms and endogenous enzymes cause post-mortem autolytic degradation, which leads to a rapid loss of freshness and hence of quality [25]. The activity of these enzymes can be quantified and used as final quality indicators [16].

The degradation of proteins that occurs during the post-mortem degenerative process creates ideal conditions for the development of microorganisms and contamination of the fish muscle [21]. The freshly caught fish is normally sterile, but microorganisms can be found on the gills, skin and intestinal tract, and these bacteria can promote subsequent spoilage [26]. Other contaminations can occur from equipment and humans during handling and processing.

1.1.1. Microbial Spoilage

Microbial spoilage of food products is a matter of global concern, since 25% of all food produced is lost post-harvest due to microbial activity [27]. In fresh, unprocessed products, the multiplication of microorganisms is very fast (especially at non-refrigerated temperatures) and results in loss of quality, potentially causing public health problems [28].

The seafood microbiology is quite complex, being influenced by factors like contaminations of the live animal from the environment and specific intrinsic/extrinsic factors that may create growth conditions for the microorganisms (a_w, pH, microbial interactions, etc) [29].

A part of the total fish microorganisms, the Specific Spoilage Organisms (SSOs), are responsible for the degradation of fish into biochemical components and therefore are associated with loss of freshness [12]. These organisms are normally present in low number and represent a small fraction of the fish microflora [27]. Microorganisms are the major cause of fish spoilage [27], but only the SSOs give rise to off-flavours, since they produce ammonia, organic acids, biogenic amines (like putrescin, cadaverine and histamine), and sulphur compounds from amino acids, acetate from lactate, hypoxanthine from adenosine triphosphate (ATP) degradation products and other compounds like ketones, alcohols or aldehydes [20, 27].

1.1.2. Lipid Hydrolysis and Oxidation

Lipid hydrolysis and consequent free fatty acids (FFA) production causes undesirable effects, as in the case of acceleration of lipid oxidation, development of off-odours and texture degradation [30]. Also, FFA accumulation contributes to an accelerated protein denaturation in frozen fish [31].

In fatty fish species, the most important quality loss factor is lipid oxidation, since rancidity is caused by the abundance of highly unsaturated fatty acids and pro-oxidant molecules (like endogenous enzymes and transition metals) in the muscle [2]. Although lipid oxidation is not commonly regarded as a relevant problem in lean fish species, it has been shown to occur and became an important factor on the approval of these species in the recent past. [32] The demersal fishes, which have low lipid content, also develop off flavours during frozen storage.

Haem proteins like myoglobin (Mb) are considered as the most important endogenous promoters of lipid oxidation in fish muscle [33]. Oxidation typically involves the double bonds in fatty acids, and so polyunsaturated fatty acids of fish are highly susceptible to this alteration [20]. Perturbations in cell membrane (like the ones created by high pressure) can expose phospholipids, which are also key substrates for lipid oxidation [30].

1.1.3. Enzymatic Activity

The presence of lysosomal enzymes (such as cathepsins) is responsible for the degradation of myofibrillar and connective tissue [16]. The activity of proteases results in soft texture and consequent low acceptance of the fish product [34], while lipases cause the development of rancidity, even under frozen conditions [2]. In its turn, phosphatases have an important role in the regulation of several metabolic processes that involve phosphorylation and desphosphorylation [7]. Therefore, the enzymatic activity of fish muscle has been commonly used as an indicator of quality changes.

The degradation of ATP in fish muscle, catalysed by phosphatase, generates adenosine diphosphate (ADP), adenosine monophosphate (AMP) and inosine monophosphate (IMP) [16], resulting in the later formation of inosine and hypoxanthine, and consequently in a lower fish freshness and a higher K-value [7], which is currently a standard index for fish freshness. The K-value is defined as the ratio of inosine (HxR) and hypoxanthine (Hx) to the total ATP and related compounds in fish muscle extract. These related compounds are ADP, AMP, IMP, HxR and Hx [10, 35]. Equation (1) represents the K-value:

$$K - value\ (\%) = \frac{[HxR]+[Hx]}{[ATP]+[ADP]+[AMP]+[IMP]+[HxR]+[Hx]} \times 100 \quad (1)\ [36]$$

Cathepsins B and D are lysosomal proteinases responsible for the tenderization of fish muscle, one of the most negative quality changes [37].

Cathepsin B is a cysteine proteinase, which means it has cysteine and histidine residues as the essential groups in its catalytic sites. This type of enzymes is inhibited by compounds like heavy metal ions and alkylating or oxidizing agents [38]. Cathepsin B has optimum pH activity in the alkaline region [39, 40] and is responsible for the hydrolysis of several myofibrillar proteins of high importance, such as connectin, myosin and nebulin. This causes a sever degradation of the muscle structure, reducing the quality of the product [16].

Cathepsin D is an aspartic protease, and so its activity depends on a pair of aspartic acid residues in the active site (Asp-32 and Asp-215) [38]. This enzyme has optimum pH within the acidic range [39] and it is the most abundant muscle proteinase in fish muscle [41], having great importance in the degradation of fish muscle, since there is no specific inhibitor of its activity [42].

The ability of cathepsins to degrade tissue is well documented and, although cathepsin D is believed to be a major muscle proteinase, cysteine endo-proteinases (like cathepsin B) have the most notorious effect on texture, thanks to their thermo-stability and ability to cleave internal peptide bonds [39].

Calpains are intracellular calcium-dependent proteinases that selectively degrade proteins in the cytosol of eukaryotic and some prokaryotic cells [43]. Similar to cathepsins, these enzymes are responsible for the tenderization of fish muscle and its degradation during post-mortem storage [37, 44], causing the weakening and disintegration of the Z-line of fish myofibrils [45]. Calcium plays a key role in the activity of calpains, since it leads to its dissociation/autoproteolysis, even when another substrate is available [37]. Calpains are heterodimers composed by two subunits of about 80 and 28 kDa. The catalytic role is played by the large subunit while the small subunit has a regulatory function. Calpains are active in its dissociated form (when calcium is present) [44]. Calpastatin is known to be the endogenous specific inhibitor of calpains [37]. Calpains have optimum activity at neutral pH, and so are classified under neutral muscle proteinases [39].

Lipases or acylglycerol hydrolases are enzymes that hydrolyse esters of long-chain aliphatic acids from glycerol. The optimum pH of most lipases is between 7 and 9 [39]. Lipases can also catalyse transesterification, acidolysis, esterification and alcoholysis reactions [46]. Lipase activity is the main cause of hydrolysis and formation of free fatty acids (FFA) in fish, during frozen storage [7]. Lipolysis occurs in great extension in fish muscle post mortem and is responsible for quality loss in the frozen tissue [16]. The FFA formed by enzymatic activity may interact with some types of proteins, leading to alterations in texture [16].

The degradation of trimethylamine nytrogen-oxide (TMAO) to dimethylamine (DMA) and formaldehyde (FA) may by enhanced by the presence of the endogenous enzyme TMAOase, although this series of reactions happens readily after death, even in the absence

of the enzyme [47]. The degradation of TMAO is believed to destabilize proteins and cause their aggregation [47].

1.2. Fish Storage

Refrigeration is one of the main preservation techniques used for fish. This method has the capacity to retard microbial growth (or to eliminate certain microorganisms) and preserve the sensory attributes of the products, by reducing the rates of chemical and biochemical reactions [8]. However, this happens for a very limited time. Freezing can be a good alternative to refrigeration, since it can extend the shelf-life of fish products from 2 weeks [8] to 6 months [20] in average, maintaining the advantages of chilled storage (absence of appreciable changes in quality). Freezing and consequent cold storage constitute an effective way of making seasonal species available all year round [47].

Freezing is the process of reducing the temperature of food below its freezing point (the food never freezes at 0 ºC, because there are lots of constituents, other than water). Frozen storage generally uses temperatures below -10 ºC, being -18 ºC the commercial frozen storage temperature. [48].

Freezing and frozen storage have been largely used to preserve the sensory and nutritional characteristics of fish products, whether for direct consumption or to use as raw material for further processes [2, 49]. Despite being the most common storage method, it comprises several problematic factors including the formation of large ice crystals, dehydration, freezer-burn, protein degradation, activity of endogenous enzymes and consequent changes in chemical constituents cause texture, colour (oxidation of blood pigments [47]) and flavour deterioration, resulting in loss of quality [16, 19]. It is also believed that the decrease on the distance between sarcomeres after prolonged frozen storage contributes to the formation of cross-linkages and consequent stiffening of the fibers. The hydrogen-bonding system that stabilizes the protein structure is also disrupted when the water molecules freeze out [47]. The fact that enzymatic reactions can continue under freezing conditions results in intrinsic physical and chemical changes [20].

Species like hake, which originally have a bland flavour, tend to develop off-flavours during prolonged frozen storage, mainly due to the presence of carbonyl compounds and acids that result from lipid oxidation [47].

The problems of drying, dehydrating and lipid oxidation (and consequent rancidity) may be prevented by glazing the products. This process is applied during the freezing process and consists on the application of a layer of ice to the surface of the product by spraying water or immersing it in a water bath. This layer will exclude air from the products' surface and consequently reduce the rate of oxidation [50] and the freezer-burn [51].

Other protective treatments may be used to prevent lipid oxidation and its negative effects on sensory acceptance: vacuum packaging, modified atmosphere packaging and even the application of natural antioxidants [52].

Matsumoto (1979) [53] concluded that frozen fish exhibits many types of quality deterioration processes. A great part of those changes is due to the denaturation of proteins or their subunits during frozen storage, which results in aggregation or conformational changes. Generally, a rapid freezing process and lower storage temperatures result in a lower rate of protein denaturation.

The limiting factor of frozen storage in lean fish (which is the case of hake, studied in this work) is the aggregation of proteins, that results in firmer fillets with low water-holding capacity, while in fat fish the major problem is lipid oxidation. The freezing and thawing processes may also cause the disintegration of membrane structures and consequent release of enzymes that are normally retained in intracellular organelles. These enzymes are regarded as markers for membrane damage and their activity may be used to differentiate frozen from fresh fish [54]. The lysosomal enzymes released will be responsible for the decomposition of protein, carbohydrate, fat and nucleic acids. There are reported results that confirm the higher lysosomal enzyme activity in frozen and thawed fish, when compared to fresh fish. Thanks to these factors, and although the deterioration processes are slowed down, frozen fish can gradually develops off-flavours and off-odours [55].

One of the major concerns related to the freezing of fish products is that changes in the texture or flavour of the final cooked product may occur. These changes can be enhanced by the shortening of diffusion distances between reactants (like enzymes and substrates) that is caused by the freezing process [56]. In terms of texture, the reported problem is a tendency to express liquid on initial compression in the mouth, while the remaining material is fibrous, dry and hard [56].

The deterioration of frozen fish products is also dependent on some extrinsic factors that may be monitored, such as the speed of freezing, storage temperature and contact of the product with oxygen. The thawing method is also an important variant [51].

1.3. High Pressure Processing

1.3.1. History

The first applications of HPP on food date to the late nineteenth century and aimed to diminish micro-organisms and extend the milk shelf-life. Hite et al. (1914) reported that the shelf-life of several fruits could be increased by high pressure (400-820 MPa), since it results in the inhibition of yeasts and moulds (the microorganisms that can grow in acidic products) [57]. Later, in 1924, high pressure was found to be able to successfully preserve fruit juices, which would become one of its major applications [58].

The first commercial installation for HPP appeared in 1990 in Japan and HP-treated fruit and fruit jams were introduced to the market [30, 59]. Lately, a wider range of food products like smoothies, guacamole and some fish and meat products also started to be commercialised [30].

HPP has been officially approved as a non-thermal pasteurization technology by the FDA (Food and Drug Administration) and, since then, its wide range of applications has increased the development and market demand for high pressure equipment. Among the other novel alternatives to food processing, HPP was adopted with the highest rate. This is proved by the increase in the number of HPP installations around the world, as it is shown in **Figure 1** [60]. The annual output value of the HPP market has nearly reached 10 billion USD as this technology is employed on the cold pasteurization of ready-to-eat meals, fruit juices, fishery and meat products worldwide [13, 61].

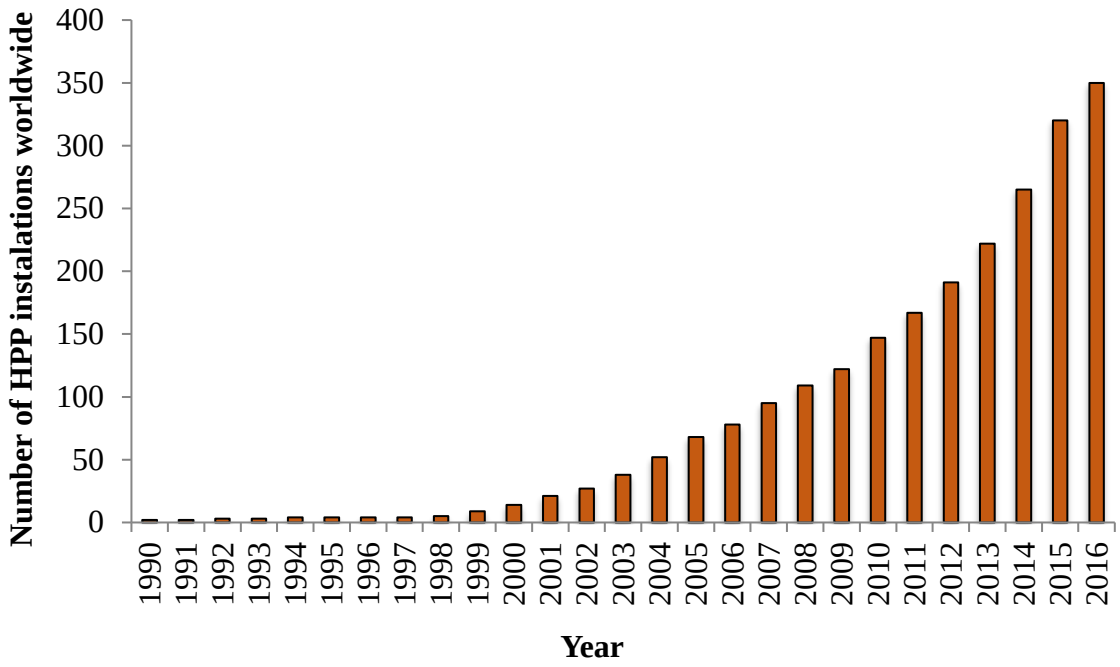

Figure 1 - Total number of high pressure processing equipment currently operating worldwide. Source: NC Hyperbaric, Burgos, Spain (www.nchyperbaric.com)

1.3.2. Basic Principles of HPP

HPP is a non-thermal technique, applied commercially in the range of 100 to 700 MPa, although at laboratory scale pressures up to 1,000 MPa may be applied [7, 30]. For industrial application, HP treatment conditions vary greatly depending on processing purposes [30].

HPP has been proving to inactivate microbial activity, leading to safety assurance and shelf-life extension while maintaining or causing minimal effect on the sensory and nutritional properties of foods, since the thermal exposure is reduced [15, 17]. Pressure can be used to process both liquids and solid foods, as long as the latter have high-moisture-content [59]. Some commercial applications in the food industry are the pasteurization of fruits, sauces, yoghurts, meat or vegetables and the decontamination of heat sensitive ingredients like vitamins or flavourings [62]. Because of its unique features, HPP is especially used for the treatment of fresh/ready-to-eat foodstuffs [1].

The basic governing principles that determine the food behaviour under pressure are:

1. Le Chatelier's principle: this principle states that any phenomenon (reaction, conformational change, phase transition) accompanied by a decrease in volume is favoured by pressure.

2. Isostatic Principle: this principle presumes that the food products are compressed by uniform pressure that acts equally in every direction, as it is represented in **Figure 2**. The effects are instantaneously and homogenously distributed within the product, regardless of its size and geometry. If the product contains sufficient moisture, pressure will not damage its macroscopic structure and the original shape will be recovered once the treatment is over.

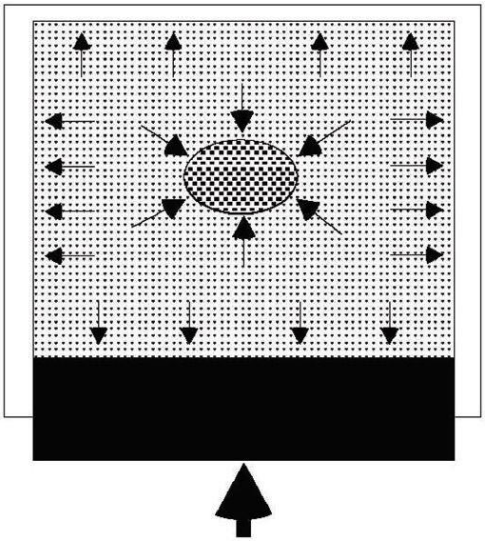

Figure 2 – The principle of isostatic processing [59]

3. Hydrostatic pressure changes interatomic distances, affecting the interactions for which bonding energy depends on distance (electrostatic, hydrogen and van der Waals bonding). Covalent bonds are unlikely to be affected because their bonding distance is minimally compressed by pressure. Altering the interatomic distances can result in changes in equilibrium processes, rates of processes and physical properties. The inactivation of microorganisms results from combinations of these phenomena (changes in membrane lipids, changes in chemical equilibrium that modify the internal pH and changes in the rate of some physiological functions) [15].

For applying pressure to food, the surrounding fluid is compressed. Once the desired pressure is reached, the pump stops, the valves close and pressure level can be maintained without further energy expenditure. After the chosen holding time, the vessel is decompressed by releasing the pressure-transmitting fluid [59].

The physical compression of the product results in an increase of temperature. Due to adiabatic heating, the temperature of water increases about 3 ºC per 100 MPa of increased pressure at room temperature (fats and oils have higher heat compression values) [59, 62].

The pressure and temperature profiles of a typical HPP treatment are represented in **Figure 3**.

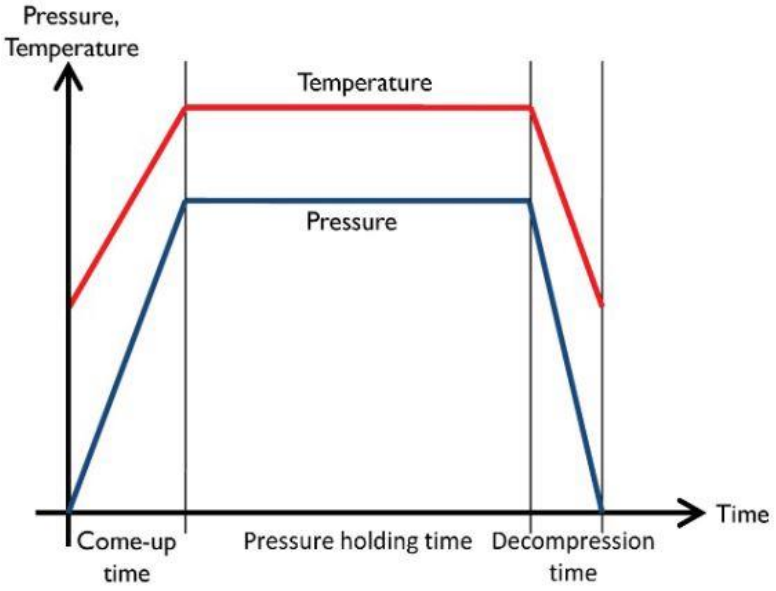

Figure 3 – Pressure and temperature profiles of a typical HPP treatment. Source: High pressure processing: insights on technology and regulatory requirements, Covance white paper series volume 10, July 2013.

1.3.3. Equipment

The typical components of batch HPP equipment are the following:

1. Pressure vessel;
2. Two end closures to cover the cylindrical vessel;
3. Yoke (structure that restrains the end closures);
4. High pressure pump and intensifier;
5. Process control and instrumentation;
6. Handling System for loading and removing the product.

The food product is typically packed in vacuum and placed inside a loading basket that is then loaded into the vessel containing the pressure-transmitting fluid [15]. This fluid may be water (the most common), glycol solutions, silicone oil, ethanol solutions, inert gases, sodium benzoate solutions or castor oil [59]. The selection of the fluid depends on several characteristics like its corrosion prevention properties, viscosity, changes under pressure, ability to seal under pressure and heat of compression [15].

The packaging material must be specific for HPP. At least one interface of the package should be flexible enough to transmit pressure. The headspace air should be minimized because the presence of oxygen can have adverse effects on the product quality at high pressure-temperature conditions [15].

With further advances of this technology and its commercialization, it is expected that the cost of the equipment will become lower in the near future and therefore the safe and nutritious high-pressure processed products will soon be available to all consumers at an affordable cost [14].

1.3.4. Main Effects of HPP

One of the major goals for the application of HPP is microbial inactivation, being this effect dependent on factors like number and type of microorganisms [59]. The resistance of microorganisms to high pressure varies relevantly, in the following increasing order: vegetative bacteria < yeasts and moulds < viruses < bacterial spores [58]. Some researchers have reported that HPP is able to eliminate *Listeria monocytogenes* and can be also effective in the inactivation of other hazardous microorganisms like *Escherichia coli*, *Vibrio* and *Salmonella*, as well as other bacteria, moulds or yeasts responsible for food spoilage [59]. The extension of microbial reduction is dependent of the treatment conditions: pressure level and holding time. Typically, increases in pressure and holding time result in higher magnitude of microbial reduction. It is possible that the Gram property and optimum growth temperature of bacteria are determinant factors for their resistance to HPP [30].

As for thermal processing, there is a threshold value for HPP, below which no inactivation occurs. This value is specific for each microorganism. Above the threshold, the lethal effect tends generally to increase with the use of higher pressure [58].

An additional advantage of HPP is the possibility of inactivating hydrolytic (lipases, phospholipases) and oxidative (peroxidases, lipoxygenases) enzymes. This results in positive effects when HPP is employed before frozen or refrigerated storage of food products [2]. This effect has a special relevance in seafood products, since the autolysis of fish (together with microbial spoilage) is one of the events that causes accelerated putrefaction and softening of fish muscle [30]. The inactivation of enzymes occurs due to the disruption of the bonds that determine their secondary, tertiary and quaternary conformations, since covalent bonds are not affected [16]. The breaking of covalent bonds is associated to an

increase in volume and so, according to the Le Chatelier Principle, it is not enhanced by HPP. The limited effect of HPP on covalent bonds is an unique feature that allows it to preserve food chemistry [59]. Low-molecular weight food components like vitamins and other compounds like anthocyanins, lycopene and conjugated linoleic acid are therefore not affected by pressure [15, 23]. When proteins are exposed to HPP, the electrostatic and hydrophobic interactions are the most vulnerable, while hydrogen bonds are not affected (the α-helical and ß-pleated sheets remain stable) [59, 63]. HPP can induce changes on the functional properties of food and, so, it can be used by the food industry to create new textures [24].

Moreover, HPP can be considered an environment-friendly technology. It produces a small carbon footprint and negligible effluent since it uses normal potable water as pressure medium and consumes low power [30].

Nonetheless, the application of high pressure in the food industry has some limitations: the systems are semicontinuous or operated in batch, which limits the throughput, and the incapacity of pressure alone to inactivate bacterial spores [15].

Bacterial spores are highly resistant to pressure, requiring values over 1200 MPa for their inactivation [14]. For effective elimination of bacterial endospores in low-acid food products, a combination of heat with HPP is needed [64]. Moderate pressures (200-400 MPa) along with 20-50ºC can trigger spore germination. However, it is not easy to ensure 100% germination and therefore it is difficult to warrant microbiological safety of the product [15].

1.3.5. Effect of HPP in Fish

The application of high pressure at ambient or refrigerated temperatures may be useful for the pasteurization of several liquid and solid foods, including fish products. The refrigeration (or freezing) of the HP-treated products is necessary, as the bacterial spores remain active after the treatment.

The inactivation at high pressure of proteolytic enzymes can improve the tenderness of fish. [65]. The behaviour of enzymes under high pressure depends on the enzyme structure. For example, cathepsin D is less resistant to HPP than cathepsins B and B+L [30]. This susceptibility suggests that HPP can be an efficient tool for controlling unwanted phenomena that may occur during storage, such as enzymatic softening [66]. The enzymatic activity in

fish muscle under high pressure is influence by the acceleration/inhibition effect of HPP, by the protective effect of the matrix of the muscle and by the release of enzymes after cell rupture [30].

HPP has also effects on the colour of fish: the denaturation of myofibrillar and sarcoplasmic proteins results on a loss of translucency of the flesh, that consequently acquires a cooked appearance [65].

Among bacterial cells, there is a huge variation in the resistance to pressure of different strains. Concerning vegetative bacteria, Gram-positive bacteria and cells in the stationary growth phase tend to have higher pressure resistance than Gram-negative or cells in the exponential phase [15]. In fish muscle, H_2S-producing bacteria (including *Shewanella putrefaciens*) and psychotrophic Gram negative *Pseudomonas* are possibly the spoilage bacteria that are the most sensitive to high pressure. HPP has been shown to be an effective treatment to delay the spoilage of fish muscles during storage, by reducing the population of spoilage microorganisms [30].

1.3.6. Refrigerated HP-treated Fish

During refrigerated storage, there are several degradation processes like hydrolysis, breakdown and aggregation of certain chemical components. These processes happen due to endogenous muscle enzymes in an initial stage and later due to the action of microbial enzymes [5].

In what concerns to enzymes, the activities of cathepsin B, cathepsin B+L and calpains were reduced at all pressures up to 300 MPa in cold-smoked salmon [45]. At 300 MPa, the calpains were almost completely inactivated. However, it was verified an increase in cathepsin B+L and calpains activity after 12 days of refrigerated storage. In another study, made with sea bass (*Dicentrarchus labrax*), the HPP resulted in a decrease of calpains activity, without affecting its inhibitor (calpastatin) [67]. The evolution of the activity during storage depended on the level of pressure. The activity of cathepsins was also modified by HPP and the storage time: the initial increase of activity is explained by the disruption of lysosomes, while the decrease in the last days of storage may be caused by enzyme denaturation.

Regarding other biochemical parameters, a combination of 330 MPa/5 min/3 ºC resulted in an increase of 3 days (from 12 to 15 days) on the shelf-life of Red Mullet (*Mullus surmetulus*), when compared to the untreated fish, by analysis of colour, trimethylamine-nitrogen (TMA-N) and lipid oxidation (by thiobarbituric acid index, TBA-i) [18]. In coho salmon, a treatment of 170 and 200 MPa for 30s resulted in a decrease of the sarcoplasmic protein content, namely phosphoglycerate mutase, showing similar K-value after treatments at 135, 170 and 200 MPa. [5] Comparing to untreated fish, the FFA content increased initially, but decreased at the end of storage due to the inhibition of microbial activity (and consequent inhibition of microbial enzymes). In a study using sea bass fillets, there was an increase on lipid oxidation in HP-treated samples (250 and 400 MPa, 5 min), especially in the sample treated at 400 MPa [24]. A treatment of 550 MPa for 4 min resulted in a reduction of TMA-N production in refrigerated Chilean jack mackerel (*Trachurus murphyi*), allowing to obtain a shelf-life extension [68].

In terms of texture and sensory properties, a HP treatment of sea bass (*D. labrax*) up to 500 MPa for 5 minutes had slight (and probably imperceptible) effects on the colour of cooked fish [21]. During 0, 7 or 14 days of refrigerated storage, the pre-treatment reduced the exudation and water-holding capacity, while increasing the fish hardness (proving the ability to improve textural quality and to maintain total aerobic counts equivalent to untreated fish). For the same species, both 250 and 400 MPa for 5 minutes increased the microbiological shelf-life of refrigerated fillets and resulted in firmer consistency, increase of whiteness and loss of translucency [24]. The appearance of trout fillets was nearly unaffected by a treatment of 600 MPa for 5 minutes, while catfish fillets appeared to be paler and like cooked products [1].

Microbial growth was delayed by HPP in vacuum-packed herring (*Clupea harengus*) and haddock (*Melanogrammys aeglefinus*), resulting in the extension of microbiological shelf-life (stored in ice at 2 ºC) [69]. The microflora of both species was not significantly altered during storage. A treatment of 200 MPa for 3 minutes increased the shelf-life of these species from 4 to ~13 days on ice. In a different study, HPP resulted in a reduction of *L. monocytogenes* and *E. coli* by >6 $\log_{10}$ CFU/g in mild smoked rainbow trout fillets (*Oncorhynchus mykiss*) and fresh European catfish fillets (*Silurus glanis*) [1]. However, it was detected subsequent growth of *L. monocytogenes* during storage. The initial microbial counts of Chilean jack mackerel (*Trachurus murphyi*) were reduced by a treatment of 550

MPa for 4 min, with the additional benefit of slowing down the microbial growth during chilled storage [68]. These effects translate in a shelf-life extension of 34 days (from 6 to 40 days) compared to control samples. This extension refers only to microbial shelf-life, since sensorial analysis was not performed.

1.3.7. Frozen HP-treated Fish

Despite being widely employed to retain the desired fish properties, frozen storage eventually causes quality losses. Several factors, including the activity of endogenous enzymes may be responsible for texture, flavour and colour deterioration [16]. Nevertheless, the number of articles concerning the effect of HP pre-treatment in frozen fish is still scarce. The effect of HPP on enzymatic activities during frozen storage was recently studied by several authors using Atlantic mackerel (*Scomber scombrus*) [7, 23], horse mackerel (*Trachurus trachurus*) [16], sardine (*Sardina pilchardus*) [17], barramundi (*Lates calcarifer*) [70] and hake (*Merluccius merluccius* – work not yet published). Some of these assays were made under accelerated storage conditions (-10 ºC), so that the effect of storage time at typical storage temperatures can be rapidly predicted, while others, more recently, were made under the conditions that are used commercially (-18 ºC). The effects of HPP on the enzymes activity in different fish species are summarized in **Table 1.** Different effects on enzymes activity were verified for different fish species.

An HPP treatment of up to 450 MPa, with 0 to 5 min holding time, before frozen storage (-10 ºC, 3 months) of Atlantic horse mackerel (*T. trachurus*) decreased the activity of both cathepsin B and acid phosphatase (particularly at 450 MPa), while the activity of cathepsin D increased with lower pressure treatments and only decreased with 450 MPa [16]. The activity of lipase tended to increase during the storage time, although there is no acceptable predictive model. It is noteworthy that the effect of holding time was lower than the effect of pressure level or frozen storage time.

Table 1 - Effects of HPP on enzymes activity in different fish species during frozen storage.

Species	Enzymes	Storage Conditions	Pressure Conditions	Outcome	Reference
Trachurus trachurus	Cathepsin B, Acid Phosphatase	-10 ºC, 3 months	150, 300, 450 MPa (0.0, 2.5, 5.0 min)	Decrease of activity	Fidalgo et al. (2015) [16]
	Cathepsin D			Increase of activity at lower pressures and decrease at 450 MPa	
Scomber scombrus	Acid Phosphatase	-10 ºC, 3 months	150, 300, 450 MPa (0.0, 2.5, 5.0 min)	No effect with low pressure and slight decrease of activity at 450 MPa	Fidalgo et al. (2014) [7]
	Cathepsin B, Lipases			Decrease of activity with increase of pressure and higher holding time at 150 MPa	
	Lipases			Decrease of activity with the increase of holding time at 300 MPa	
	Cathepsin D			Increase of activity at 300 MPa and decrease at 450 MPa	
Sardina pilchardus	Cathepsin B, Cathepsin D, Acid Phosphatase	-18 ºC, 9 months	125, 150, 175, 200 MPa (0 min)	No significant effect on the activity	Méndez et al. (2017) [17]

The same HPP conditions (up to 450 MPa, with 0 to 5 min holding time) were applied in frozen Atlantic mackerel (*S. scombrus*) [7]. There was nearly no effect on acid phosphatase activity. Contrariwise, increasing pressure reduced the activity of both cathepsin B and lipase. Cathepsin D activity decreased only with the treatment of 450 MPa, while lower pressure values resulted in a higher activity value. A pre-treatment of 125-200 MPa was tested in commercially frozen (-18 ºC, 9 months) sardine (*S. pilchardus*) [17]. There were no substantial changes on the activities of acid phosphatase and cathepsins B and D, as well as on the electrophoretic patterns of sarcoplasmatic and myofibrillar protein fractions. Similarly, a treatment of up to 200 MPa (0 min) did not affect the enzymes activity in Atlantic mackerel (*S. scombrus*) frozen at commercial conditions (9 months, -18 ºC) [23]. However, this treatment had effect on the sarcoplasmic protein fracture, causing the disappearance of the band that corresponded to phosphoglycerate mutase on the SDS-PAGE. These results show that pressure level must be superior to 200 MPa for HPP to have the desired effect on the enzymes activity, while lower pressures may affect other parameters. Cathepsin D activity seems to only decrease with higher pressure values [7, 16].

In frozen (-10 ºC, 5 months) hake (*Merluccius merluccius*), a recent study showed that HPP (150-450 MPa for 2 min) inhibits the formation of dimethylamine, FFA, formaldehyde, trimethylamine, total volatile amine and fluorescent compounds (tertiary lipid oxidation compounds) [71]. All these effects can be explained on the basis of the enzyme damage caused by the pre-treatment.

The texture and sensory properties can also vary according to fish species and parameters used on the pre-treatment. For commercially frozen (-18 ºC, 18 weeks) barramundi (*L. calcarifer*), a treatment higher than 200 MPa for 3 minutes resulted in a loss of visual freshness and increased drip loss of the muscle [70]. However, HPP at 200 MPa increased hardness and maintained drip loss similar to control samples (without altering significantly the appearance of cooked fish). Other authors verified that HPP causes changes on texture parameters before and after cooking of frozen (-10 ºC, 5 months) European hake (*M. merluccius*) [25]. After cooking, the cohesiveness and chewiness values were similar to cooked fresh hake, showing that HPP improves the quality of frozen hake. Pressure values of up to 169.27 MPa did not cause alterations on the colour parameters of raw muscle. The adhesiveness values were similar to the ones observed in non-treated fresh muscle. Positive results were also obtained for horse mackerel (*T. trachurus*) [72]: a treatment of 150 MPa

resulted in a similar texture to fresh fish muscle, both for raw and cooked samples. A consequent high degree of acceptability of the products was verified, despite the decrease of quality during frozen storage (that also occurs in untreated fish).

HPP with pressure levels up to 200 MPa was shown to keep the characteristic sensory properties of fresh fish (that are appreciated by the consumers) in different fish species and even improve some parameters, while higher values led to quality losses. Considering that low pressures have the best outcome in terms of sensory properties, but higher pressures are needed to the inactivation of enzymes, it is necessary to establish a compromise. Keeping in mind that the effects of these treatments depend on the target species, it is possible that lower pressures can also inactivate enzymes, allowing the preservation of the organoleptic quality.

The effects of HPP on lipids in different fish species are summarized in **Table 2.** In Atlantic mackerel (*S. scombrus*), the FFA formation was clearly inhibited during frozen storage (-18 ºC, 3 months), after a treatment at 175 MPa [23]. There was also a partial inhibition of tertiary lipid oxidation in samples treated at 175/200 MPa. On the other hand, HPP did not affect significantly the primary and secondary lipid oxidation and PUFA levels. In another study, using frozen Atlantic mackerel (-10 ºC, 3 months), increasing pressure level (from 150 to 450 MPa) or holding time (from 0 to 5 minutes) resulted in a marked inhibition of the formation of FFA and tertiary lipid oxidation, while primary and secondary oxidation compounds content barely changed [73]. In frozen (-10 ºC, 3 months) Atlantic horse mackerel (*T. trachurus*), similar conclusions were withdrawn: inhibition of lipid hydrolysis with an increase of both pressure level and holding time. In what concerns to lipid oxidation, increasing pressure value resulted in partial inhibition during frozen storage, while the holding time did not lead to a definitive trend [2]. In barramundi fillets, HPP (at 200 MPa) delayed lipid oxidation [70] and in sardine, increasing pressure level resulted in inhibition of lipid hydrolysis, without increasing lipid oxidation [17].

Higher pressure levels and holding times have invariably positive effects in what concerns to the lipid content of fish products, both at accelerated and commercial frozen storage.

Table 2 - Effects of HPP on lipids in different fish species during frozen storage.

Species	Storage Conditions	Pressure Conditions	Outcome	Reference
Scomber scombrus	-18 ºC, 9 months	125, 150, 175, 200 MPa (0 min)	Partial inhibition of tertiary oxidation at 175 and 200 MPa; Inhibition of FFA formation at 175 MPa	Pázos et al. (2015) [23]
	-10 ºC, 3 months	150, 300, 450 MPa (0.0, 2.5, 5.0 min)	Inhibition of FFA and tertiary lipid oxidation compounds formation with increasing pressure	Vázquez et al. (2013) [73]
Sardina pilchardus	-18 ºC, 9 months	125, 150, 175, 200 MPa (0 min)	Inhibition of lipid hydrolysis with increasing pressure with no increase in lipid oxidation	Méndez et al. (2017) [17]
Trachurus trachurus	-10 ºC, 3 months	150, 300, 450 MPa (0.0, 2.5, 5.0 min)	Inhibition of lipid hydrolysis and partial inhibition of lipid oxidation with increasing pressure	Torres et al. (2013) [2]
Lates calcarifer	-18 ºC, 9 months	150, 200, 250, 300 MPa (3 min)	Delaying of lipid oxidation at 200 MPa	Truong et al. (2015) [19]

Another recent work [74] studied the effect of HPP on the physical properties and colour of frozen hake (*M. merluccius)* stored at -21 ºC for 12 months. The results revealed that HPP is beneficial to maintain the level of expressible water until 6 months of frozen storage. Although raw HP-treated samples showed increases in luminosity, hardness, adhesiveness and springiness, cooked hake showed good results (particularly at 300 MPa and 6 months of frozen storage), proving that HPP improves the quality of frozen hake.

2. Framework and Main Objectives

This master's thesis is part of a research project in collaboration with a Spanish institution, with the main objective of increasing the quality of frozen hake (*M. merluccius*) using HP technology. The effect of different pressure levels (150-450 MPa) for 2 minutes was already studied, using accelerated storage at -10 ° C, and the chemical changes (DMA, FA, FFA, trimethylamine, volatile amine total), lipid oxidation, enzymatic activities, colour and textural properties were evaluated.

Results regarding chemical changes are already published [71], reporting the inhibition of DMA, FA, FFA, trimethylamine, total volatile amine and tertiary lipid oxidation compounds formation in HP-treated fish muscle, being verified that this effect increased with the pressure level applied. On the contrary, no significant effect was observed on K-value, polyene index, formation of peroxides and thiobarbituric acid reactive substances. All these results may be related with the damage caused by HP to different types of enzymes that decreases their activity during storage [71]. Furthermore, Bottom-Up proteomics to study the effects of HP pre-treatment (150-450 MPa for 2 min) on frozen hake (-10 ºC for 5 months) were also studied [75]. Pressures of 150-170 MPa did not affect the abundance of the selected protein biomarkers for quality change, while higher pressure levels ($\geq$ 430 MPa) resulted in a significant degradation of some of those proteins. HP also increased the intensity of the electrophoretic profiles of biomarkers assigned to tropomyosin and glyceraldehyde-3-phosphate dehydrogenase [75]. Colour parameters (L*, a* and b*), expressible water and texture parameters were also evaluated on raw and cooked muscles, showing that a low-pressure level (150 MPa) allowed adequate expressible water for raw muscle up to 2.5 months of frozen storage time, and values of 150 or 169.27 MPa did not cause significant changes on L* values of raw muscle. Pressures of 150–300 MPa for 5 months of frozen storage resulted in cohesiveness and chewiness values for cooked muscle like cooked fresh hake, showing that the HP improves the quality of frozen hake [25]. Furthermore, there is also another work not yet published, which concluded that HP pre-treatments may in fact reduce deleterious enzymatic activity during frozen storage of hake.

This previous work allowed selecting of the optimized HP conditions, using different frozen storage temperatures, and being also studied according chemical changes, colour and textural properties and enzymatic activities. Thus, the objective of this work was to evaluate

the effect of a HP pre-treatment when it comes to the enzymatic activities and protein damage in hake (*Merluccius merluccius*) muscle, during 12-month frozen storage. The pressure level (150 MPa) and holding time (2 min) were selected considering the results obtained in previous studies [25, 71, 75]. The effect of different storage temperatures (-10 ºC, -20 ºC or -30 ºC) and storage times (0, 3, 6, 9 or 12 months) on the activity of enzymes such as cathepsins B, acid phosphatase, and calpains and on sulfhydryl groups content was studied. Fresh fish (before freezing and HP) was also analysed, for comparison to be possible. This work intended so to deepen the studies for the possible potential of the application of a HP pre-treatment to improve the quality of frozen hake.

3. Materials and Methods

3.1. Chemicals

Sodium dodecyl-sulphate (SDS), ethylenediaminetetraacetic acid (EDTA), *p*-nitrophenol, dithiothreitol (DTT), 2-bis-(2-hydroxyethyl)-amino-2-(hydroxymethyl)-1,3-propanediol (Bis-Tris), urea, NaCl, and 5,5'-dithio(2-nitrobenzoic acid) (DTNB) were obtained from Sigma-Aldrich (Steinheim, Germany).

Potassium phosphate monobasic (KH_2PO_4) was obtained from Scharlab S.L., Sentmenat, Barcelona, Spain. Calcium chloride dihydrate and acetic acid were acquired from Chem-Lab (Zedelgem, Belgium).

Other chemicals, such as potassium hydroxide (KOH), sodium acetate tri-hydrate and potassium phosphate dibasic (K_2HPO_4) were acquired from Panreac Quimica S.L.U. (Barcelona, Spain).

Substrates used in the determination of enzymatic activity, e.g. *p*-nitrophenyl phosphate disodium salt hexahydrate (*p*-NPP, #N22002), Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride and L-methionine-7-amido-4-methylcoumarin trifluoroacetic salt were also obtained from Sigma-Aldrich.

3.2. Raw material, processing and storage conditions

European hake (*Merluccius merluccius*) was obtained at the Vigo harbour (Galicia, northwest Spain), being caught close to the Galician coast and immediately transported to

the “Plataforma Tecnológica Multidiscipinar Alta Pressão” (University of Aveiro) in a refrigerated truck. Samples were packed in polyethylene bags and vacuum sealed (400 mbar).

HPP was carried out in a high-pressure equipment (55-L 6000-55; Hiperbaric, Spain). The HPP level used was 150 MPa and it was achieved at 250 MPa/min. The holding time was 2 min and the decompression time was approximately 1 s.

After the treatment, the fish was stored at different temperatures: the fresh fish was immediately filleted and stored at -80 ºC in polyethylene bags (so that the diverse parameters were maintained), the month 0 sample was stored at -20 ºC for only 24 hours and the remaining samples were stored at -10, -20 or -30 ºC for 3, 6, 9 and 12 months. After the pre-determined storage time at different temperatures, the hake was thawed (12 hours at 4 ºC) and the white muscle was removed. The muscle was then grinded and stored at -80 ºC in polyethylene bags, until the analysis were performed.

3.3. Enzymatic Activity

3.3.1. Preparation of Enzymatic Extract

The preparation of enzymatic extracts was performed according to the procedure described by Lakshmanan et al. (2005) [45]. Twelve grams of fish samples were homogenised with 20 mL of cold-distilled water for 2 min (8000 rpm, Miccra D9 45187, Miccra GmbH, Heitersheim, Germany). The homogenate was then centrifuged at 14600 g and 4 ºC (Heraeus Biofuge Stratos Centrifuge, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The supernatant was filtered (MN 640 w) and stored at -80 ºC, prior to enzymatic activity quantification. The data referent to the enzymatic extracts is displayed in **Table 3**, in the **Appendix A** of this document.

3.3.2. Acid Phosphatase Activity

Acid phosphatase activity was assayed with *p*-NPP as substrate, following the methodology described by Ohmori et al. (1992) [76] with only minor modifications. Enzymatic extract (250 µL) was mixed with 4 mM *p*-NPP (225 µL) in 0.1 mM sodium acetate buffer and 1 mM EDTA (pH 5.5). After incubation at 37 ºC for 15 min, the reaction

was stopped by adding 1000 μL of 100 mM KOH. The *p*-NP released was measured at 400 nm (Microplate Spectrophotometer Multiskan Go, Thermo Scientific, USA). Acid phosphatase activity was expressed as ABS/min/g of fish muscle. Three replicates were performed for each condition. The data referent to the determination of Acid Phosphatase activity is displayed in **Table 4**, in the **Appendix B** of this document.

3.3.3. Cathepsin B Activity

The activity of cathepsin B was assayed by the methodology described by Lakshmanan et al. (2005) [45]. Enzyme extract (100 μL) and substrate solution (100 μL) containing 0.0625 mM Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride in 100 mM Bis-Tris buffer, 20 mM EDTA and 4 mM DTT (pH 6.5) were incubated at 37 ºC for 5 min. The reaction was stopped by adding 1000 μL 3% SDS (w/v) in 50 mM Bis-Tris (pH 7.0). The free 7-amino-4-methylcoumarin (AMC) liberated was determined by fluorescence (excitation 360 nm, emission 460 nm; F-2000 Fluorescence Spectrophotometer, Hitachi, Tokyo, Japan). Cathepsin B activity was expressed as fluorescence units (FU)/min/g of fish muscle. Three replicates were performed for each condition. The data referent to the determination of Cathepsin B activity is displayed in **Table 5**, in the **Appendix C** of this document.

3.3.4. Calpains Activity

The activity of calpains was assayed by the methodology described by Sasaki et al. (1984). [77] Enzyme extract (50 μL) was added to substrate solution (50 μL) containing 0.125 mM L-methionine-7-amido-4-methylcoumarin trifluoroacetic salt in 100 mM Bis-Tris at pH 6.5, including 5 mM calcium chloride, previously incubated at 37ºC. The reaction was stopped by adding 1,5 mL of 9 mM sodium acetate buffer at pH 6.5, including 30 mM monochloroacetic acid and 21 mM acetic acid. The 7-amino-4-methylcoumarin liberated was determined by fluorescence (excitation 360 nm, emission 460 nm; F-2000 Fluorescence Spectrophotometer, Hitachi, Tokyo, Japan). Calpains activity was expressed as fluorescence units (FU)/min/g of fish muscle. Three replicates were performed for each condition. The

data referent to the determination of calpains activity is displayed in **Table 6**, in the **Appendix D** of this document.

3.4. Sulfhydryl Groups (SH content)

The total SH content was determined using 5,5–dithiobis(2-nitrobenzoic acid) (DTNB), according to the Ellman's method (1959) with some modifications. 0.5 g of sample were homogenised in 10 mL of 0.05 M phosphate buffer pH 7.2 for 30 s (8000 rpm, Miccra D9 45187, Miccra GmbH, Heitersheim, Germany). 1 mL of the homogenate was mixed with 9 mL of 0.05 M phosphate buffer pH 7.2 containing 0.6 M NaCl, 6 mM EDTA and 8 M urea. This mixture was centrifuged for 15 minutes at 14000 g and 5 ºC (Heraeus Biofuge Stratos Centrifuge, Thermo Fisher Scientific, Waltham, Massachusetts, USA). 3 mL of the supernatant was mixed with 0.04 mL of 0.01 M DTNB solution in 0.05 M sodium acetate and incubated at 40 ºC for 15 minutes. Finally, the absorbance was measured at 412 nm (Lambda 35 UV/Vis spectrometer, PerkinElmer Instruments Inc., MA, USA). A blank was prepared by replacing the first homogenate with 0.05 M phosphate buffer pH 7.2 containing 0.6 M NaCl, 6 mM EDTA and 8 M urea. SH content was calculated using a molar extinction coefficient of 13600 $M^{-1}cm^{-1}$ and results expressed in micromomoles of SH per gram of fish. The data referent to the determination of SH group content is displayed in **Table 7**, in the **Appendix E** of this document.

3.5. Statistical Analysis

All analyses were performed in triplicate of samples and are expressed as mean ± standard deviation. The effect of HP pre-treatment and frozen storage time up to 12 months was tested with a two-way Analysis of Variance (ANOVA) followed by a multiple comparisons test (Tukey's honestly significant difference, HSD), to identify differences between the conditions. The effect of storage temperature at each storage time and pressure/control condition and the differences between the fresh fish and all the other samples were tested with one-way ANOVA, followed by Tukey's HSD. The level of significance was established at $p < 0.05$.

4. Results and Discussion

4.1. Acid Phosphatase Activity

According to Ohmori et al. [76], 40-60% of this enzyme is bounded to lysosomes membranes, having these authors verified that a mild HP pre-treatment increases its activity on the cytosolic fraction, as a result of lysosomes membranes disruption. Acid phosphatase is related to the freshness of fish muscle, as it degrades molecules like ATP, ADP and IMP [78].

The data for acid phosphatase activity evolution along storage are presented in **Figure 4**. Samples from the month zero correspond to those stored only 24h at -20 ºC, while the remaining samples were stored at different temperatures: -10, -20 and -30 ºC, during 3, 6, 9 and 12 months.

Comparing fresh to frozen hake (month 0), it is possible to verify a significant increase ($p < 0.05$) of activity (85%), possibly related with the freezing process and the consequent rupture of lysosomes and liberation of the enzyme [54]. It was also observed an increase ($p < 0.05$) of the activity for the control samples stored at -10 ºC for 9 months, at -20 º C for 3 and 9 months and at -30 ºC for 3 months and for the HP-treated sample stored at -20 ºC for 12 months. The only sample that had a significant decrease ($p < 0.05$) was the HP-treated one stored at -10 ºC for 12 months.

During storage at -10 ºC, there was a significant decrease ($p < 0.05$) in the activity values after 12 months of storage, for both control and HP-treated samples (*ca.* 65 and 64%, respectively). However, control samples showed an increase of activity on the 9th month of sampling, which could be explained by the enzyme release from the lysosomes membranes [54], while the denaturing effect of the low temperature used in frozen storage explains the further denaturation for longer storage times [53]. On HP-treated samples, the only significant effect verified was the denaturation of the enzyme after longer storage times, possibly caused by the combined effect of pressure and frozen storage [53]. Comparing control and HP-treated samples, there are only significant differences ($p < 0.05$) at month 0 and month 9, where the pressure treatment resulted in a decrease of the acid phosphatase activity. This means that this enzyme may be partially inactivated by pressure, as it is referred in some previous works [36, 76]. It is noteworthy to refer that, when samples stored at -10 ºC were collected at month 12, it was found that the freezer had suffered a power

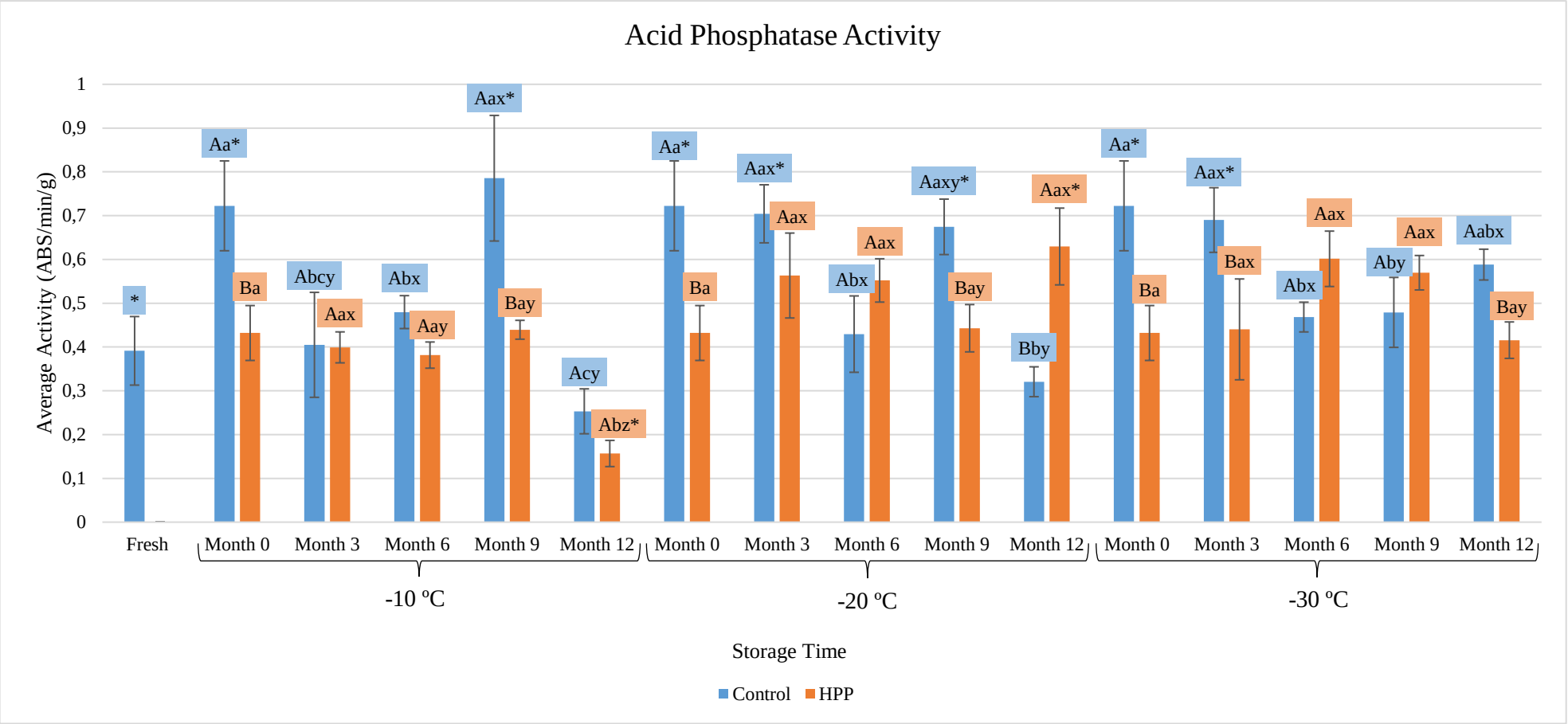

Figure 4 – Activity of acid phosphatase. Different letters represent significant differences ($p < 0.05$): **capital letters** between control and HP-treated samples (for each combination of storage time and temperature); **noncapital a, b and c** through storage time (for each combination of pressure treatment and storage temperature); **noncapital x, y and z** between storage temperatures (for each combination of pressure treatment and storage time); **asterisks** mark the values that are significantly different from the value of fresh fish.

failure for an undetermined period of time and the fish was partially thawed. This might have affected the results and be the reason for activity decrease found for these samples at month 12.

At -20 ºC, in control samples, activity was maintained ($p > 0.05$) until the 3rd month of storage, having then a decrease ($p < 0.05$) of 56% at month 6. Similar to storage at -10 ºC, this activity increased ($p < 0.05$) at month 9 and decreased again at month 12, reaching values similar to the ones verified after 6 months. On the other hand, for HP-treated samples, the effect of storage time was not significant ($p > 0.05$). Comparing to the controls, HP-treated samples were significantly different ($p < 0.05$) at months 0 and 9 (where a decrease is verified) and for month 12 (where there is an increase of activity). This higher value for the HP-treated sample at month 12 may be explained by the disruption of lysosomes due to the prolongated frozen storage, which possibly resulted in activity recovery [54].

At the lowest temperature tested (-30 ºC), control samples showed a decrease ($p < 0.05$) on the acid phosphatase activity of about 35% after 6 months of storage, maintaining ($p > 0.05$) the same value for the remaining storage time. On the other hand, HP-treated samples did not change ($p > .05$) along frozen storage, showing values between 0.416 and 0.601 ABS/min/g.

4.2. Cathepsin B Activity

This enzyme is a cysteine protease and influences the quality of frozen fish by promoting the hydrolysis of myofibrillar proteins [40]. Being contained in the lysosomes, it is expected that this enzyme is released to the cytoplasm and intercellular spaces by the HP pre-treatment, resulting in an increase of its activity [42].

The data for cathepsin B activity evolution along storage are presented in **Figure 5.** Comparing fresh to frozen hake (month 0), no significant ($p > 0.05$) changes in the activity of cathepsin B were observed. There was an increase ($p < 0.05$) of the activity for the HP-treated samples stored at -10 ºC for 9 months, at -20 º C for 3 and 12 months and also for the control samples stored at -30 ºC for 9 and 12 months.

At -10 ºC, the activity values for the control and HP-treated samples showed a significant ($p < 0.05$) increase of about 102 and 189%, respectively, which could be explained by the disruption of lysosomes at frozen temperature and consequent release of the enzyme [54], increasing its activity. At month 12, there was a decrease of activity for both control and HP-treated samples (93 and 98%, respectively). As for acid phosphatase, the considerable activity decrease at month 12 might be due to the power failure of the freezer and the partial thawing of the samples.

The control samples stored at -20 ºC showed an increase ($p < 0.05$) of activity at month 6, followed by a decrease at months 9 and 12. The breakage of lysosomes [54] and posterior denaturation of the enzymes liberated [53], both due to the low temperature, may explain this activity evolution. As to the HP-treated samples, there was an increase of activity at month 3 and month 12, which can be explained by the rupture of lysosomes and consequent release of these enzymes [36], while the decrease in month 6 is probably due to the denaturation of the enzyme due to frozen storage [53]. However, it was also observed a possible recovery of enzymatic activity along the storage.

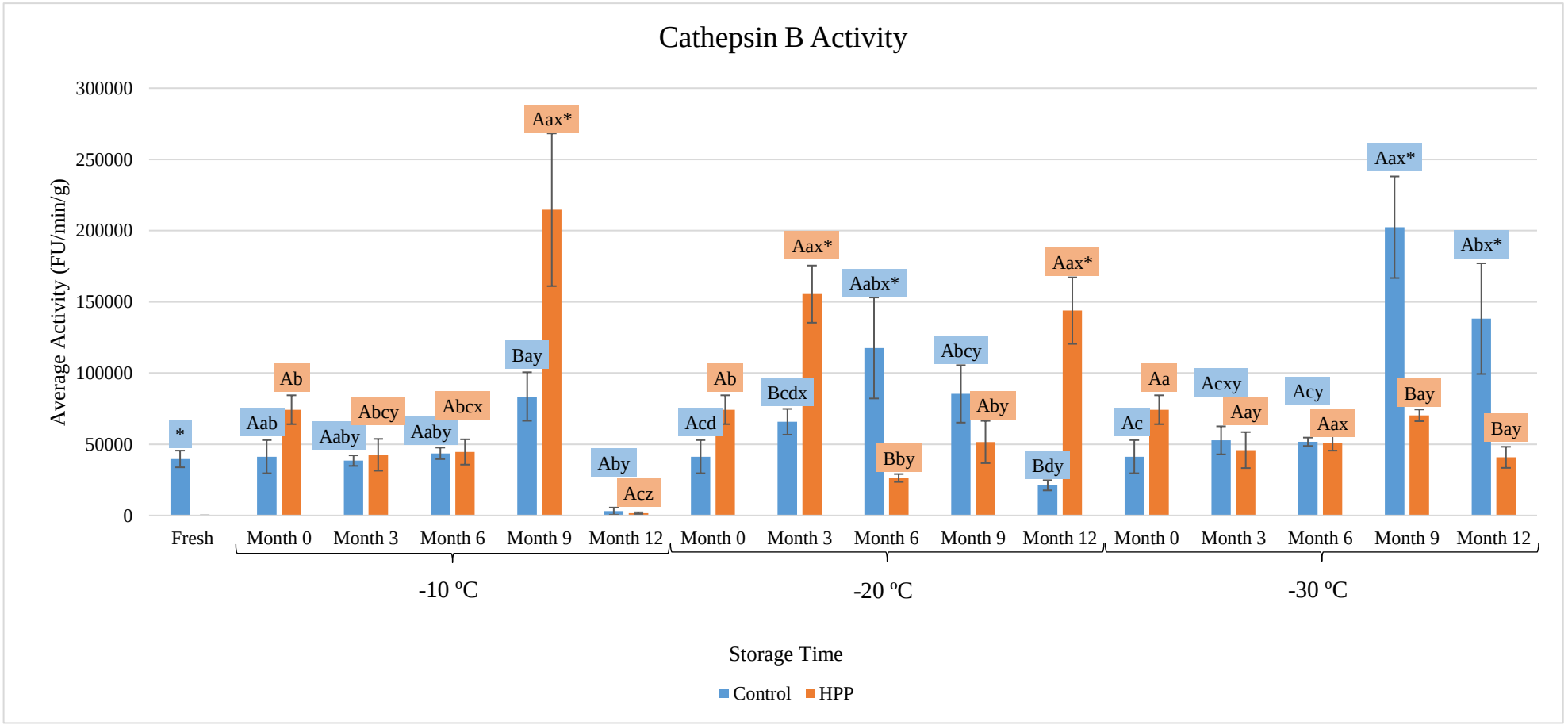

Figure 5 – Activity of cathepsin B. Different letters represent significant differences ($p < 0.05$): **capital letters** between control and HP-treated samples (for each combination of storage time and temperature); **noncapital a, b and c** through storage time (for each combination of pressure treatment and storage temperature); **noncapital x, y and z** between storage temperatures (for each combination of pressure treatment and storage time); **asterisks** mark the values that are significantly different from the value of fresh fish

At -30 ºC, the control samples showed a significant ($p < 0.05$) increase at months 9 and 12, of about 390 and 235%, respectively. This increase might be due to the heterogeneity of the fish samples. The HP-treated samples do not show significant changes ($p > 0.05$) in cathepsin B activity with storage time.

The effect of HPP is significant at months 9 and 12, in which a great decrease of activity (about 65 and 70%, respectively) is verified due to the denaturation of the enzyme with pressure [7]. This might result in a better frozen fish quality, since the tenderization of fish muscle might be reduced.

4.3. Calpains Activity

Calpains are another proteinase group that hydrolyses myofibrillar proteins, affecting the texture of post-mortem fish muscle, being sarcoplasmic enzymes.

The data for calpains activity evolution along storage are presented in **Figure 6.** Comparing fresh to frozen hake (month 0), no significant ($p > 0.05$) change in the activity of calpains was verified. There was a decrease ($p < 0.05$) for both control and HP-treated samples stored at -10 ºC for 12 months and an increase ($p < 0.05$) for the control sample stored at -30 ºC for 3 months.

At -10 ºC, the control samples only showed a significant decrease ($p < 0.05$) of calpains activity after 12 months, while there are no statistical differences between the other storage times tested ($p > 0.05$). In the HP-treated samples, there is also a significant decrease ($p < 0.05$) of activity from month 0 to months 3, 6 and 9 (47% less activity at month 9) and then a further decrease to month 12. In both samples, it was observed a strong effect of frozen storage time, since calpains activity significantly decreased after 12 months, which could be explained by the protein denaturation caused by the constant exposition to frozen temperature. The effect of HPP was not significant, as no major differences in the activity values were observed ($p > 0.05$). Similar to acid phosphatase and cathepsin B, also for calpains a considerable decrease of activity was found at month 12, what might be related to the power failure of the freezer and the partial thawing of the samples.

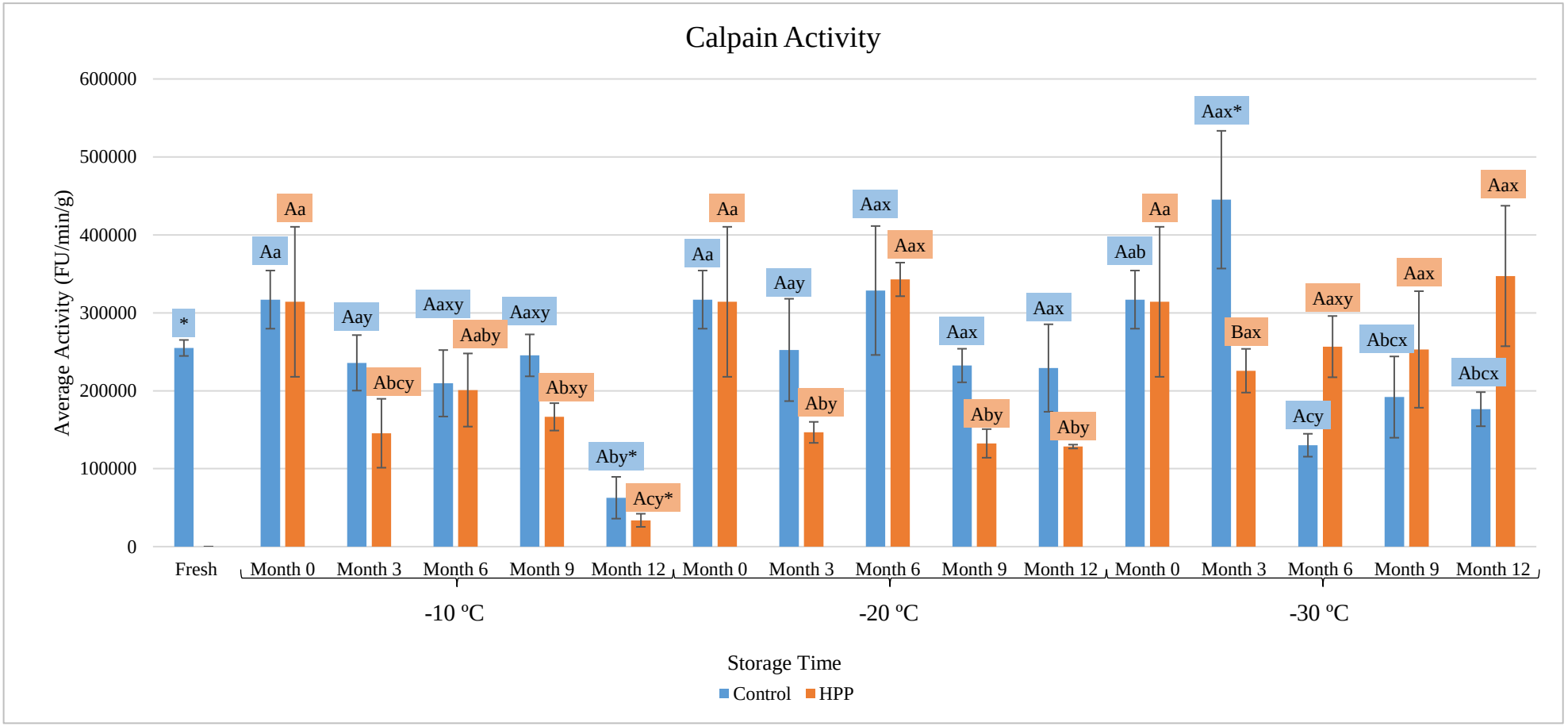

Figure 6 – Activity of calpains. Different letters represent significant differences ($p < 0.05$): **capital letters** between control and HP-treated samples (for each combination of storage time and temperature); **noncapital a, b and c** through storage time (for each combination of pressure treatment and storage temperature); **noncapital x, y and z** between storage temperatures (for each combination of pressure treatment and storage time)); **asterisks** mark the values that are significantly different from the value of fresh fish.

For the control samples stored at -20º C, there were not significant changes ($p > 0.05$) in the activity values throughout the storage time, while for HP-treated samples there was a significant decrease ($p < 0.05$) after 9 and 12 months. Pressure was effective to decrease activity during storage, as it was previously reported in other works [36, 42], probably due to the denaturation and consequent dissociation of the subunits of the enzyme, when it is subjected to the HP level.

The control samples stored at -30 ºC showed a significant ($p < 0.05$) decrease of calpains activity after month 6 (59% less activity, comparing to month 0), maintaining similar values from then until month 12 ($p > 0.05$). Contrarily, the HP-treated samples showed no significant changes ($p > 0.05$) throughout storage time.

4.4. Sulfhydryl (SH) Groups Content

The content in SH groups is a parameter that gives indications about the level of protein oxidation in the samples, since it relates with the formation or disruption of disulphide bonds in its structure [79].

The data for SH group content evolution along storage are presented in **Figure 8**. Comparing the values of fresh and frozen fish, there are significant ($p < 0.05$) differences only for the HP-treated samples stored for 9 months at -10 ºC and -20 ºC.

At -10 ºC, control samples showed no significant changes ($p > 0.05$) in their content in sulfhydryl groups during frozen storage, it was only verified a very considerable significant difference between 9 and 12 months (a decrease of about 76%). HP-treated samples showed a constant increase ($p < 0.05$) with storage time up to month 9, with an increase of about 122%, comparing to month 0. This effect may result of the denaturation of muscle proteins, due to both frozen temperature and enzymatic activity, which results in higher exposition of SH groups. This increasing effect was more pronounced in HP-treated samples, probably due to changes on protein conformation which promote greater exposure of the previously “hidden” SH groups [80, 81], and possible breakage of S-S bonds. Noteworthy to highlight is the considerable decrease of SH groups content at month 12, what, as for the enzymes activity, might related to the power failure of the freezer and the partial thawing of the samples.

For the samples stored at -20 ºC, there were not significant differences ($p > 0.05$) in both control and HP-treated samples, being verified an average increase in SH groups content after 12 months.

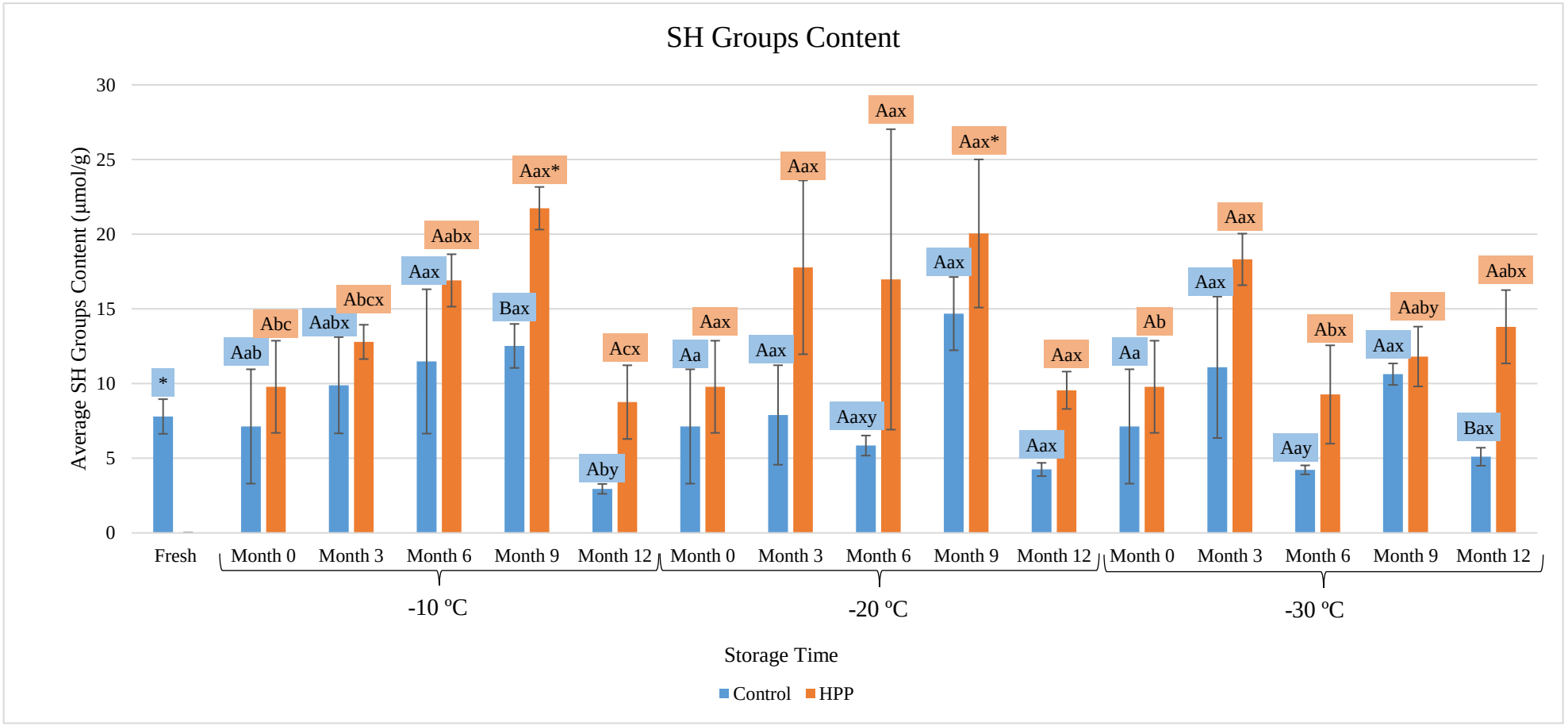

Figure 7 – Sulfhydryl group content. Different letters represent significant differences ($p < 0.05$): **capital letters** between control and HP-treated samples (for each combination of storage time and temperature); **noncapital a, b and c** through storage time (for each combination of pressure treatment and storage temperature); **noncapital x, y and z** between storage temperatures (for each combination of pressure treatment and storage time); **asterisks** mark the values that are significantly different from the value of fresh fish.

At -30 ºC, control samples did not show significant differences ($p > 0.05$). On the other hand, there was a significant increase of SH groups content on HP-treated samples at month 3, which may be related to the denaturation of muscle proteins, and consequent exposure of those groups. However, this value decreased for further storage times, probably due to the oxidation of fish muscle proteins during storage [82].

Compared to the control samples, the effect of HP was statistically significant ($p < 0.05$) for the samples stored for 12 months at -30 ºC and 9 months at -10 ºC, as an increase of SH content is verified. This reinforces the idea that HPP reveals some SH groups that were previously "hidden" in the protein structure, allowing its measurement [80, 81].

5. Conclusions and Future Work

This work aimed determining the effect of HP (150 MPa for 2 minutes) and storage temperature (-10, -20 and -30 ºC) on the quality of frozen hake muscle (*Merluccius merluccius*). The enzymatic activities (acid phosphatase, cathepsin B and calpains) and SH groups were studied. HP induced significant changes in the activities of the studied enzymes during frozen storage time of European hake.

Generally, at -10 ºC, a considerable frozen storage effect was observed on enzymatic activity, verifying a decrease of acid phosphatase, cathepsin B and calpains after 12 months in both control and HP-treated samples, but this might be related with the power failure of the freezer and partial thawing of the samples.

For storage at -20 ºC, acid phosphatase activity decreased after 12 months in control samples, maintaining in similar values for HP-treated samples during all storage time. Cathepsin B activity in control samples increased after 6 months and decrease at month 12 to similar values obtained initially at month 0. Otherwise, on HP-treated samples, cathepsin B activity increased after 12 months. Calpains activities did not change during frozen storage, contrarily to HP-treated samples, in which a decrease of activity was observed after 9 and 12 months of frozen storage.

Using a lower frozen storage (-30 ºC) in control samples increased cathepsin B activity after 9/12 months and decreased acid phosphatase and calpains activity after 6 months. HP-treated samples showed no significant effect on acid phosphatase, cathepsin and calpains activities.

Furthermore, SH groups content on control samples did not change at -20 and -30 ºC, verifying variations only at -10 ºC, increasing after 9 months and decreasing at month 12. The same behaviour was observed for HP-treated samples, except for the ones stored at -30 ºC, where an increase of SH groups content was obtained after 3 months, revealing that HP has an increasing effect on SH groups, partially due to the disruption of disulphide bonds. The differences between control and HP-treated samples, when significant, confirmed the increasing effect of HP on the SH groups content.

Although, the findings here presented can lead to improvements of frozen fish quality, additional research is needed to understand the effect of HP pre-treatment in the quality attributes of European hake during frozen storage. The quantification of carbonyl groups is an interesting analysis to be performed in the future, since it complements the information about protein oxidation obtained with the sulfhydryl groups quantification. Protein carbonylation is a type of oxidation that may be promoted by reactive oxygen species, converting side chains of amino acids like lysine, threonine, proline or arginine in reactive ketones or aldehydes (that can be quantified with 2,4–dinitrophenylhydrazine). Also, although the activity quantification of the enzymes studied reveals that they might be active during frozen storage, this might not be the case, because of the low temperature and the need of contact between the enzymes and its substrate. This way, to see the possible effect of these enzymes during storage, their products of reaction should be quantified. As this is not an easy task, the utilization of fish model systems could be a good alternative.

For further conclusions to be withdrawn, it is fundamental to combine the results here presented with the ones obtained in the other studies which are part of this collaboration project with colleagues of a Spanish institution.

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7. Appendices

7.1. Appendix A – Data referent to the enzymatic extracts

Table 3 - Data referent to the enzymatic extracts.

Condition	Replica	Fish Mass (g)	Extract Volume (mL)	Assay Fish Mass - Calpains (g)	Assay Fish Mass - Cathepsin B (g)	Assay Fish Mass - Acid Phosphatase (g)
Fresh	1	12,004	18,0	0,006669	0,003334	0,041681
	2	12,015	19,0	0,006324	0,003162	0,039523
	3	12,007	16,0	0,007504	0,003752	0,046902
Month 0, Control	1	12,012	16,0	0,007508	0,003754	0,046922
	2	12,069	19,0	0,006352	0,003176	0,039701
	3	12,018	19,0	0,006325	0,003163	0,039533
Month 0, HPP	1	12,018	19,0	0,006325	0,003163	0,039533
	2	12,021	20,5	0,005864	0,002932	0,036649
	3	12,059	20,0	0,006030	0,003015	0,037684
Month 3, Control, -10 ºC	1	12,016	17,0	0,007068	0,003534	0,044176
	2	12,035	19,0	0,006334	0,003167	0,039589
	3	12,011	16,0	0,007507	0,003753	0,046918
Month 3, HPP, -10 ºC	1	12,036	20,0	0,006018	0,003009	0,037613
	2	12,027	21,0	0,005727	0,002864	0,035795
	3	12,074	20,0	0,006037	0,003019	0,037731
Month 3, Control, -20 ºC	1	12,008	18,0	0,006671	0,003336	0,041694
	2	12,017	20,0	0,006009	0,003004	0,037553
	3	12,060	17,5	0,006891	0,003446	0,043071
Month 3, HPP, -20 ºC	1	12,016	21,0	0,005722	0,002861	0,035762
	2	12,008	21,0	0,005718	0,002859	0,035738
	3	12,055	19,5	0,006182	0,003091	0,038638
Month 3, Control, -30 ºC	1	12,038	18,0	0,006688	0,003344	0,041799
	2	12,044	21,0	0,005735	0,002868	0,035845

	3	12,030	20,0	0,006015	0,003008	0,037594
Month 3, HPP, -30 ºC	1	12,056	18,5	0,006517	0,003258	0,040730
	2	12,022	19,5	0,006165	0,003083	0,038532
	3	12,037	19,5	0,006173	0,003086	0,038580
Month 6, Control, -10 ºC	1	12,064	18,0	0,006702	0,003351	0,041889
	2	12,040	18,0	0,006689	0,003344	0,041806
	3	12,015	17,0	0,007068	0,003534	0,044173
Month 6, HPP, -10 ºC	1	12,060	19,0	0,006347	0,003174	0,039671
	2	12,018	19,0	0,006325	0,003163	0,039533
	3	12,003	20,5	0,005855	0,002928	0,036595
Month 6, Control, -20 ºC	1	12,036	18,0	0,006687	0,003343	0,041792
	2	12,038	18,0	0,006688	0,003344	0,041799
	3	12,052	17,0	0,007089	0,003545	0,044309
Month 6, HPP, -20 ºC	1	12,007	18,0	0,006671	0,003335	0,041691
	2	12,008	18,5	0,006491	0,003245	0,040568
	3	12,036	19,0	0,006335	0,003167	0,039592
Month 6, Control, -30 ºC	1	12,039	19,0	0,006336	0,003168	0,039602
	2	12,011	19,0	0,006322	0,003161	0,039510
	3	12,017	16,0	0,007511	0,003755	0,046941
Month 6, HPP, -30 ºC	1	12,052	20,0	0,006026	0,003013	0,037663
	2	12,019	21,0	0,005723	0,002862	0,035771
	3	12,014	20,0	0,006007	0,003004	0,037544
Month 9, Control, -10 ºC	1	12,027	21,5	0,005594	0,002797	0,034962
	2	12,033	22,0	0,005470	0,002735	0,034185
	3	12,074	21,5	0,005616	0,002808	0,035099
Month 9, HPP, -10 ºC	1	12,057	21,0	0,005741	0,002871	0,035884
	2	12,037	22,0	0,005471	0,002736	0,034196

	3	12,076	22,0	0,005489	0,002745	0,034307
Month 9, Control, -20 ºC	1	12,071	19,5	0,006190	0,003095	0,038689
	2	12,061	19,0	0,006348	0,003174	0,039674
	3	12,031	20,0	0,006016	0,003008	0,037597
Month 9, HPP, -20 ºC	1	12,072	20,0	0,006036	0,003018	0,037725
	2	12,036	20,0	0,006018	0,003009	0,037613
	3	12,017	20,5	0,005862	0,002931	0,036637
Month 9, Control, -30 ºC	1	12,080	19,0	0,006358	0,003179	0,039737
	2	12,044	20,0	0,006022	0,003011	0,037638
	3	12,058	20,0	0,006029	0,003015	0,037681
Month 9, HPP, -30 ºC	1	12,060	20,0	0,006030	0,003015	0,037688
	2	12,031	20,5	0,005869	0,002934	0,036680
	3	12,018	20,5	0,005862	0,002931	0,036640
Month 12, Control, -10 ºC	1	12,015	19,0	0,005059	0,003162	0,047428
	2	12,050	20,0	0,004820	0,003013	0,045188
	3	12,033	17,5	0,005501	0,003438	0,051570
Month 12, HPP, -10 ºC	1	12,180	14,0	0,008700	0,004350	0,054375
	2	12,020	14,0	0,008586	0,004293	0,053661
	3	12,065	13,0	0,009281	0,004640	0,058005
Month 12, Control, -20 ºC	1	12,014	18,5	0,006494	0,003247	0,040588
	2	12,007	18,0	0,006671	0,003335	0,041691
	3	12,037	18,5	0,006506	0,003253	0,040666
Month 12, HPP, -20 ºC	1	12,032	20,0	0,004813	0,003008	0,045120
	2	12,032	21,0	0,004584	0,002865	0,042971
	3	12,044	20,0	0,004818	0,003011	0,045165
Month 12, Control, -30 ºC	1	12,012	18,5	0,006493	0,003246	0,040581
	2	12,031	19,0	0,006332	0,003166	0,039576

	3	12,033	19,0	0,006333	0,003167	0,039582
Month 12, HPP, -30 ºC	1	12,083	19,0	0,006359	0,003180	0,039747
	2	12,029	20,0	0,006015	0,003007	0,037591
	3	12,028	20,0	0,006014	0,003007	0,037588

7.2. Appendix B – Data referent to the acid phosphatase activity

Table 4 - Data referent to the determination of acid phosphatase activity.

Condition	Replica	Time	Abs (400 nm)	Time (min)	Abs	Activity (ABS/min)	Fish Mass (g)	Activity (ABS/min/g)	Average Activity (ABS/min/g)	Standard Deviation
Fresh	1	T1	0,0423	0	0,0000	0,01585	0,04168	0,38019	0,39126	0,07828
		T2	0,1632	7,5	0,1209					
		T3	0,2759	15	0,2336					
	2	T1	0,0229	0	0,0000	0,01875	0,03952	0,47449		
		T2	0,1662	7,5	0,1433					
		T3	0,2989	15	0,2760					
	3	T1	0,0404	0	0,0000	0,01497	0,04690	0,31910		
		T2	0,1668	7,5	0,1264					
		T3	0,2366	15	0,1962					
Month 0, Control	1	T1	0,0382	0	0,0000	0,02838	0,04692	0,60484	0,72245	0,10259
		T2	0,2561	7,5	0,2179					
		T3	0,4538	15	0,4156					
	2	T1	0,0442	0	0,0000	0,03150	0,03970	0,79352		
		T2	0,2951	7,5	0,2509					
		T3	0,4875	15	0,4433					
	3	T1	0,0462	0	0,0000	0,03040	0,03953	0,76898		
		T2	0,2864	7,5	0,2402					

		T3	0,4778	15	0,4316					
Month 0, HPP	1	T1	0,0330	0	0,0000	0,01422	0,03953	0,35978	0,43199	0,06268
		T2	0,1400	7,5	0,1070					
		T3	0,2457	15	0,2127					
	2	T1	0,0272	0	0,0000	0,01732	0,03665	0,47250		
		T2	0,1397	7,5	0,1125					
		T3	0,3217	15	0,2945					
	3	T1	0,0352	0	0,0000	0,01747	0,03768	0,46368		
		T2	0,1572	7,5	0,1220					
		T3	0,3154	15	0,2802					
Month 3, Control, -10 ºC	1	T1	0,0333	0	0,0000	0,02158	0,04418	0,48857	0,40494	0,11986
		T2	0,1932	7,5	0,1599					
		T3	0,3610	15	0,3277					
	2	T1	0,0396	0	0,0000	0,01816	0,03959	0,45863		
		T2	0,1663	7,5	0,1267					
		T3	0,3309	15	0,2913					
	3	T1	0,0537	0	0,0000	0,01256	0,04692	0,26763		
		T2	0,1587	7,5	0,1050					
		T3	0,2204	15	0,1667					
Month 3, HPP, -10 ºC	1	T1	0,0136	0	0,0000	0,01540	0,03761	0,40944	0,39918	0,03536
		T2	0,1258	7,5	0,1122					
		T3	0,2512	15	0,2376					
	2	T1	0,0430	0	0,0000	0,01533	0,03579	0,42828		
		T2	0,1564	7,5	0,1134					
		T3	0,2761	15	0,2331					
	3	T1	0,0276	0	0,0000	0,01358	0,03773	0,35983		
		T2	0,1261	7,5	0,0985					

		T3	0,2379	15	0,2103					
Month 3, Control, -20 ºC	1	T1	0,0374	0	0,0000	0,03072	0,04169	0,73671	0,70422	0,06630
		T2	0,2753	7,5	0,2379					
		T3	0,4831	15	0,4457					
	2	T1	0,0372	0	0,0000	0,02809	0,03755	0,74801		
		T2	0,2460	7,5	0,2088					
		T3	0,4623	15	0,4251					
	3	T1	0,0360	0	0,0000	0,02705	0,04307	0,62795		
		T2	0,2372	7,5	0,2012					
		T3	0,4450	15	0,4090					
Month 3, HPP, -20 ºC	1	T1	0,0370	0	0,0000	0,02114	0,03576	0,59123	0,56325	0,09700
		T2	0,1827	7,5	0,1457					
		T3	0,3799	15	0,3429					
	2	T1	0,0677	0	0,0000	0,02299	0,03574	0,64320		
		T2	0,2524	7,5	0,1847					
		T3	0,3879	15	0,3202					
	3	T1	0,0364	0	0,0000	0,01759	0,03864	0,45534		
		T2	0,1528	7,5	0,1164					
		T3	0,3314	15	0,2950					
Month 3, Control, -30 ºC	1	T1	0,0403	0	0,0000	0,02546	0,04180	0,60911	0,69006	0,07382
		T2	0,2328	7,5	0,1925					
		T3	0,4191	15	0,3788					
	2	T1	0,0414	0	0,0000	0,02536	0,03585	0,70739		
		T2	0,2335	7,5	0,1921					
		T3	0,4179	15	0,3765					
	3	T1	0,0428	0	0,0000	0,02833	0,03759	0,75367		
		T2	0,2755	7,5	0,2327					

		T3	0,4274	15	0,3846					
Month 3, HPP, -30 ºC	1	T1	0,0558	0	0,0000	0,02296	0,04073	0,56380	0,44025	0,11515
		T2	0,2417	7,5	0,1859					
		T3	0,3729	15	0,3171					
	2	T1	0,0983	0	0,0000	0,01294	0,03853	0,33591		
		T2	0,1852	7,5	0,0869					
		T3	0,3128	15	0,2145					
	3	T1	0,0325	0	0,0000	0,01624	0,03858	0,42103		
		T2	0,1689	7,5	0,1364					
		T3	0,2470	15	0,2145					
Month 6, Control, -10 ºC	1	T1	0,0281	0	0,0000	0,01956	0,04189	0,46695	0,47970	0,03760
		T2	0,1731	7,5	0,1450					
		T3	0,3249	15	0,2968					
	2	T1	0,0398	0	0,0000	0,02182	0,04181	0,52202		
		T2	0,1970	7,5	0,1572					
		T3	0,3801	15	0,3403					
	3	T1	0,0306	0	0,0000	0,01988	0,04417	0,45013		
		T2	0,1933	7,5	0,1627					
		T3	0,3017	15	0,2711					
Month 6, HPP, -10 ºC	1	T1	0,0262	0	0,0000	0,01537	0,03967	0,38752	0,38153	0,02991
		T2	0,1370	7,5	0,1108					
		T3	0,2658	15	0,2396					
	2	T1	0,0207	0	0,0000	0,01380	0,03953	0,34908		
		T2	0,1288	7,5	0,1081					
		T3	0,2185	15	0,1978					
	3	T1	0,0182	0	0,0000	0,01493	0,03659	0,40798		
		T2	0,1284	7,5	0,1102					

		T3	0,2457	15	0,2275					
Month 6, Control, -20 ºC	1	T1	0,0304	0	0,0000	0,01703	0,04179	0,40758	0,42945	0,08723
		T2	0,1822	7,5	0,1518					
		T3	0,2378	15	0,2074					
	2	T1	0,0321	0	0,0000	0,02197	0,04180	0,52554		
		T2	0,2002	7,5	0,1681					
		T3	0,3549	15	0,3228					
	3	T1	0,0356	0	0,0000	0,01574	0,04431	0,35523		
		T2	0,1738	7,5	0,1382					
		T3	0,2314	15	0,1958					
Month 6, HPP, -20 ºC	1	T1	0,0357	0	0,0000	0,02064	0,04169	0,49507	0,55206	0,04947
		T2	0,1858	7,5	0,1501					
		T3	0,3547	15	0,3190					
	2	T1	0,0488	0	0,0000	0,02369	0,04057	0,58405		
		T2	0,2194	7,5	0,1706					
		T3	0,4184	15	0,3696					
	3	T1	0,0512	0	0,0000	0,02285	0,03959	0,57705		
		T2	0,2259	7,5	0,1747					
		T3	0,3872	15	0,3360					
Month 6, Control, -30 ºC	1	T1	0,0336	0	0,0000	0,01814	0,03960	0,45797	0,46842	0,03396
		T2	0,1663	7,5	0,1327					
		T3	0,3123	15	0,2787					
	2	T1	0,0372	0	0,0000	0,01742	0,03951	0,44090		
		T2	0,1594	7,5	0,1222					
		T3	0,3154	15	0,2782					
	3	T1	0,0370	0	0,0000	0,02377	0,04694	0,50638		
		T2	0,2183	7,5	0,1813					

		T3	0,3875	15	0,3505					
Month 6, HPP, -30 ºC	1	T1	0,0856	0	0,0000	0,02433	0,03766	0,64609	0,60145	0,06325
		T2	0,2988	7,5	0,2132					
		T3	0,3892	15	0,3036					
	2	T1	0,0385	0	0,0000	0,02251	0,03577	0,62919		
		T2	0,2267	7,5	0,1882					
		T3	0,3373	15	0,2988					
	3	T1	0,0359	0	0,0000	0,01986	0,03754	0,52907		
		T2	0,1969	7,5	0,1610					
		T3	0,3098	15	0,2739					
Month 9, Control, -10 ºC	1	T1	0,0400	0	0,0000	0,03007	0,03496	0,85998	0,78552	0,14344
		T2	0,2637	7,5	0,2237					
		T3	0,4946	15	0,4546					
	2	T1	0,0294	0	0,0000	0,02996	0,03418	0,87642		
		T2	0,2497	7,5	0,2203					
		T3	0,4876	15	0,4582					
	3	T1	0,0292	0	0,0000	0,02177	0,03510	0,62015		
		T2	0,1916	7,5	0,1624					
		T3	0,3574	15	0,3282					
Month 9, HPP, -10 ºC	1	T1	0,0238	0	0,0000	0,01506	0,03588	0,41978	0,43936	0,02154
		T2	0,1370	7,5	0,1132					
		T3	0,2493	15	0,2255					
	2	T1	0,0199	0	0,0000	0,01581	0,03420	0,46243		
		T2	0,1373	7,5	0,1174					
		T3	0,2595	15	0,2396					
	3	T1	0,0174	0	0,0000	0,01495	0,03431	0,43587		
		T2	0,1238	7,5	0,1064					

		T3	0,2532	15	0,2358					
Month 9, Control, -20 ºC	1	T1	0,0362	0	0,0000	0,02804	0,03869	0,72467	0,67445	0,06331
		T2	0,2527	7,5	0,2165					
		T3	0,4443	15	0,4081					
	2	T1	0,0262	0	0,0000	0,02394	0,03967	0,60333		
		T2	0,2066	7,5	0,1804					
		T3	0,3835	15	0,3573					
	3	T1	0,0316	0	0,0000	0,02614	0,03760	0,69536		
		T2	0,2497	7,5	0,2181					
		T3	0,3797	15	0,3481					
Month 9, HPP, -20 ºC	1	T1	0,0326	0	0,0000	0,01855	0,03773	0,49172	0,44291	0,05403
		T2	0,1672	7,5	0,1346					
		T3	0,3199	15	0,2873					
	2	T1	0,0480	0	0,0000	0,01701	0,03761	0,45215		
		T2	0,1673	7,5	0,1193					
		T3	0,3196	15	0,2716					
	3	T1	0,0375	0	0,0000	0,01410	0,03664	0,38485		
		T2	0,1338	7,5	0,0963					
		T3	0,2679	15	0,2304					
Month 9, Control, -30 ºC	1	T1	0,0731	0	0,0000	0,01616	0,03974	0,40668	0,47904	0,07989
		T2	0,1835	7,5	0,1104					
		T3	0,3371	15	0,2640					
	2	T1	0,0448	0	0,0000	0,02126	0,03764	0,56477		
		T2	0,1866	7,5	0,1418					
		T3	0,3989	15	0,3541					
	3	T1	0,0228	0	0,0000	0,01755	0,03768	0,46566		
		T2	0,1327	7,5	0,1099					

		T3	0,3294	15	0,3066					
Month 9, HPP, -30 ºC	1	T1	0,0421	0	0,0000	0,02165	0,03769	0,57437	0,56966	0,03934
		T2	0,2006	7,5	0,1585					
		T3	0,3745	15	0,3324					
	2	T1	0,0287	0	0,0000	0,01937	0,03668	0,52817		
		T2	0,1762	7,5	0,1475					
		T3	0,3149	15	0,2862					
	3	T1	0,0461	0	0,0000	0,02222	0,03664	0,60644		
		T2	0,2151	7,5	0,1690					
		T3	0,3747	15	0,3286					
Month 12, Control, -10 ºC	1	T1	0,0345	0	0,0000	0,01144	0,04743	0,24114	0,25312	0,05133
		T2	0,1048	7,5	0,0703					
		T3	0,2370	15	0,2025					
	2	T1	0,0666	0	0,0000	0,01398	0,04519	0,30938		
		T2	0,1769	7,5	0,1103					
		T3	0,2654	15	0,1988					
	3	T1	0,0682	0	0,0000	0,01077	0,05157	0,20884		
		T2	0,1479	7,5	0,0797					
		T3	0,2319	15	0,1637					
Month 12, HPP, -10 ºC	1	T1	0,0113	0	0,0000	0,00939	0,05438	0,17269	0,15676	0,02988
		T2	0,0814	7,5	0,0701					
		T3	0,1528	15	0,1415					
	2	T1	0,0251	0	0,0000	0,00941	0,05366	0,17530		
		T2	0,0939	7,5	0,0688					
		T3	0,1697	15	0,1446					
	3	T1	0,0213	0	0,0000	0,00709	0,05800	0,12229		
		T2	0,0725	7,5	0,0512					

		T3	0,1317	15	0,1104					
Month 12, Control, -20 ºC	1	T1	0,0248	0	0,0000	0,01432	0,04059	0,35282	0,32063	0,03411
		T2	0,1303	7,5	0,1055					
		T3	0,2434	15	0,2186					
	2	T1	0,0369	0	0,0000	0,01188	0,04169	0,28487		
		T2	0,1306	7,5	0,0937					
		T3	0,2058	15	0,1689					
	3	T1	0,0415	0	0,0000	0,01318	0,04067	0,32419		
		T2	0,1323	7,5	0,0908					
		T3	0,2554	15	0,2139					
Month 12, HPP, -20 ºC	1	T1	0,0573	0	0,0000	0,03264	0,04512	0,72333	0,62957	0,08768
		T2	0,3152	7,5	0,2579					
		T3	0,5206	15	0,4633					
	2	T1	0,0732	0	0,0000	0,02646	0,04297	0,61576		
		T2	0,2764	7,5	0,2032					
		T3	0,4606	15	0,3874					
	3	T1	0,0819	0	0,0000	0,02482	0,04517	0,54961		
		T2	0,2769	7,5	0,1950					
		T3	0,4366	15	0,3547					
Month 12, Control, -30 ºC	1	T1	0,0551	0	0,0000	0,02343	0,04058	0,57736	0,58823	0,03519
		T2	0,2487	7,5	0,1936					
		T3	0,3708	15	0,3157					
	2	T1	0,0635	0	0,0000	0,02484	0,03958	0,62757		
		T2	0,2366	7,5	0,1731					
		T3	0,4624	15	0,3989					
	3	T1	0,0636	0	0,0000	0,02216	0,03958	0,55976		
		T2	0,2319	7,5	0,1683					

		T3	0,3917	15	0,3281					
Month 12, HPP, -30 ºC	1	T1	0,0156	0	0,0000	0,01724	0,03975	0,43383	0,41564	0,04161
		T2	0,1402	7,5	0,1246					
		T3	0,2837	15	0,2681					
	2	T1	0,0157	0	0,0000	0,01673	0,03759	0,44506		
		T2	0,1454	7,5	0,1297					
		T3	0,2582	15	0,2425					
	3	T1	0,0147	0	0,0000	0,01383	0,03759	0,36803		
		T2	0,1314	7,5	0,1167					
		T3	0,1963	15	0,1816					

7.3. Appendix C – Data referent to the cathepsin B activity

Table 5 - Data referent to the determination of cathepsin B activity.

Condition	Replica	Time	Fluorescence (360/460 nm)	Time (min)	FU	Activity (FU/min)	Fish Mass (g)	Activity (FU/min/g)	Average Activity (FU/min/g)	Standard Deviation
Fresh	1	T1	74,51	0	0,00	150,247	0,00333	45059,08	39681,00	5858,08
		T2	421,10	2,5	346,59					
		T3	883,80	5	809,29					
	2	T1	70,51	0	0,00	128,197	0,00316	40545,04		
		T2	368,60	2,5	298,09					
		T3	756,30	5	685,79					
	3	T1	72,07	0	0,00	125,469	0,00375	33438,89		
		T2	370,70	2,5	298,63					
		T3	729,50	5	657,43					

Month 0, Control	1	T1	66,99	0	0,00	139,353	0,00375	37123,68	41305,73	11650,91
		T2	381,60	2,5	314,61					
		T3	831,30	5	764,31					
	2	T1	69,43	0	0,00	173,001	0,00318	54470,44		
		T2	494,70	2,5	425,27					
		T3	948,90	5	879,47					
	3	T1	68,18	0	0,00	102,226	0,00316	32323,08		
		T2	298,30	2,5	230,12					
		T3	630,20	5	562,02					
Month 0, HPP	1	T1	64,26	0	0,00	268,202	0,00316	84803,43	74258,45	10139,89
		T2	600,90	2,5	536,64					
		T3	1673,00	5	1608,74					
	2	T1	80,32	0	0,00	215,184	0,00293	73392,76		
		T2	455,90	2,5	375,58					
		T3	1481,00	5	1400,68					
	3	T1	120,50	0	0,00	194,690	0,00302	64579,15		
		T2	575,70	2,5	455,20					
		T3	1157,00	5	1036,50					
Month 3, Control, -10 ºC	1	T1	108,40	0	0,00	135,450	0,00353	38326,40	38519,10	3704,55
		T2	385,00	2,5	276,60					
		T3	909,70	5	801,30					
	2	T1	100,10	0	0,00	134,020	0,00317	42316,24		
		T2	405,10	2,5	305,00					
		T3	830,30	5	730,20					
	3	T1	100,00	0	0,00	131,050	0,00375	34914,66		
		T2	394,70	2,5	294,70					
		T3	821,10	5	721,10					

Month 3, HPP, -10 ºC	1	T1	118,20	0	0,00	108,360	0,00301	36011,96	42597,77	11171,40
		T2	328,10	2,5	209,90					
		T3	782,00	5	663,80					
	2	T1	95,74	0	0,00	158,918	0,00286	55496,43		
		T2	452,10	2,5	356,36					
		T3	972,20	5	876,46					
	3	T1	93,98	0	0,00	109,526	0,00302	36284,91		
		T2	351,90	2,5	257,92					
		T3	673,40	5	579,42					
Month 3, Control, -20 ºC	1	T1	99,31	0	0,00	189,147	0,00334	56706,30	65790,45	9030,38
		T2	551,70	2,5	452,39					
		T3	1086,00	5	986,69					
	2	T1	99,88	0	0,00	224,616	0,00300	74766,08		
		T2	623,40	2,5	523,52					
		T3	1299,00	5	1199,12					
	3	T1	97,97	0	0,00	227,069	0,00345	65898,96		
		T2	629,30	2,5	531,33					
		T3	1306,00	5	1208,03					
Month 3, HPP, -20 ºC	1	T1	137,50	0	0,00	380,050	0,00286	132840,40	155425,40	20060,00
		T2	1047,00	2,5	909,50					
		T3	2119,00	5	1981,50					
	2	T1	147,70	0	0,00	489,390	0,00286	171172,40		
		T2	1312,00	2,5	1164,30					
		T3	2713,00	5	2565,30					
	3	T1	147,80	0	0,00	501,560	0,00309	162263,30		
		T2	1348,00	2,5	1200,20					
		T3	2763,00	5	2615,20					

Month 3, Control, -30 ºC	1	T1	70,89	0	0,00	172,963	0,00334	51725,10	52762,02	9843,56
		T2	485,60	2,5	414,71					
		T3	971,10	5	900,21					
	2	T1	88,94	0	0,00	180,898	0,00287	63083,00		
		T2	558,60	2,5	469,66					
		T3	958,60	5	869,66					
	3	T1	87,30	0	0,00	130,760	0,00301	43477,97		
		T2	386,30	2,5	299,00					
		T3	796,90	5	709,60					
Month 3, HPP, -30 ºC	1	T1	58,06	0	0,00	119,162	0,00326	36570,95	45918,05	12633,50
		T2	351,60	2,5	293,54					
		T3	662,60	5	604,54					
	2	T1	56,93	0	0,00	185,851	0,00308	60291,04		
		T2	515,40	2,5	458,47					
		T3	998,50	5	941,57					
	3	T1	82,30	0	0,00	126,210	0,00309	40892,17		
		T2	386,90	2,5	304,60					
		T3	735,20	5	652,90					
Month 6, Control, -10 ºC	1	T1	103,40	0	0,00	143,260	0,00335	42750,00	43611,80	4032,84
		T2	428,80	2,5	325,40					
		T3	885,20	5	781,80					
	2	T1	97,89	0	0,00	160,553	0,00334	48005,88		
		T2	500,50	2,5	402,61					
		T3	898,20	5	800,31					
	3	T1	96,12	0	0,00	141,634	0,00353	40079,53		
		T2	417,80	2,5	321,68					
		T3	869,10	5	772,98					

Month 6, HPP, -10 ºC	1	T1	73,64	0	0,00	130,328	0,00317	41065,21	44595,54	8890,10
		T2	338,90	2,5	265,26					
		T3	846,40	5	772,76					
	2	T1	68,39	0	0,00	173,023	0,00316	54708,55		
		T2	446,20	2,5	377,81					
		T3	1043,00	5	974,61					
	3	T1	53,15	0	0,00	111,285	0,00293	38012,87		
		T2	321,30	2,5	268,15					
		T3	629,70	5	576,55					
Month 6, Control, -20 ºC	1	T1	111,70	0	0,00	495,690	0,00334	148262,20	117598,30	35441,11
		T2	1336,00	2,5	1224,30					
		T3	2620,00	5	2508,30					
	2	T1	96,50	0	0,00	420,450	0,00334	125736,80		
		T2	1090,00	2,5	993,50					
		T3	2314,00	5	2217,50					
	3	T1	95,24	0	0,00	279,308	0,00355	78795,82		
		T2	745,40	2,5	650,16					
		T3	1588,00	5	1492,76					
Month 6, HPP, -20 ºC	1	T1	70,14	0	0,00	80,438	0,00334	24117,33	26324,37	2776,40
		T2	273,30	2,5	203,16					
		T3	468,20	5	398,06					
	2	T1	101,90	0	0,00	95,550	0,00325	29441,62		
		T2	310,10	2,5	208,20					
		T3	641,00	5	539,10					
	3	T1	62,98	0	0,00	80,496	0,00317	25414,16		
		T2	246,80	2,5	183,82					
		T3	500,30	5	437,32					

Month 6, Control, -30 ºC	1	T1	122,90	0	0,00	169,210	0,00317	53409,59	51750,52	2934,51
		T2	488,40	2,5	365,50					
		T3	1084,00	5	961,10					
	2	T1	84,69	0	0,00	152,863	0,00316	48362,28		
		T2	461,30	2,5	376,61					
		T3	860,10	5	775,41					
	3	T1	74,09	0	0,00	200,833	0,00376	53479,70		
		T2	551,80	2,5	477,71					
		T3	1127,00	5	1052,91					
Month 6, HPP, -30 ºC	1	T1	69,09	0	0,00	148,993	0,00301	49450,05	50694,82	5175,85
		T2	409,20	2,5	340,11					
		T3	878,80	5	809,71					
	2	T1	65,98	0	0,00	132,366	0,00286	46254,86		
		T2	374,30	2,5	308,32					
		T3	773,00	5	707,02					
	3	T1	67,53	0	0,00	169,336	0,00300	56379,56		
		T2	491,40	2,5	423,87					
		T3	913,15	5	845,62					
Month 9, Control, -10 ºC	1	T1	147,90	0	0,00	246,490	0,00280	88127,30	83519,34	17029,28
		T2	685,30	2,5	537,40					
		T3	1538,00	5	1390,10					
	2	T1	132,40	0	0,00	267,380	0,00274	97770,46		
		T2	803,00	2,5	670,60					
		T3	1465,00	5	1332,60					
	3	T1	118,80	0	0,00	181,560	0,00281	64660,26		
		T2	540,50	2,5	421,70					
		T3	1091,00	5	972,20					

Month 9, HPP, -10 ºC	1	T1	106,20	0	0,00	716,640	0,00287	249638,20	214632,10	53615,92
		T2	2023,00	2,5	1916,80					
		T3	3439,00	5	3332,80					
	2	T1	130,80	0	0,00	660,260	0,00274	241351,20		
		T2	1656,00	2,5	1525,20					
		T3	3683,00	5	3552,20					
	3	T1	140,80	0	0,00	419,660	0,00275	152906,90		
		T2	1150,00	2,5	1009,20					
		T3	2319,00	5	2178,20					
Month 9, Control, -20 ºC	1	T1	96,92	0	0,00	266,144	0,00310	85988,04	85363,19	20164,71
		T2	733,60	2,5	636,68					
		T3	1485,00	5	1388,08					
	2	T1	94,24	0	0,00	205,968	0,00317	64893,33		
		T2	558,70	2,5	464,46					
		T3	1225,00	5	1130,76					
	3	T1	135,00	0	0,00	316,440	0,00301	105208,20		
		T2	918,20	2,5	783,20					
		T3	1733,00	5	1598,00					
Month 9, HPP, -20 ºC	1	T1	103,10	0	0,00	207,070	0,00302	68611,66	51589,64	14839,66
		T2	623,50	2,5	520,40					
		T3	1133,00	5	1029,90					
	2	T1	79,53	0	0,00	134,751	0,00301	44782,65		
		T2	426,40	2,5	346,87					
		T3	733,30	5	653,77					
	3	T1	77,74	0	0,00	121,268	0,00293	41374,62		
		T2	362,20	2,5	284,46					
		T3	721,50	5	643,76					

Month 9, Control, -30 ºC	1	T1	121,90	0	0,00	512,430	0,00318	161194,90	202364,30	35655,05
		T2	1320,00	2,5	1198,10					
		T3	2850,00	5	2728,10					
	2	T1	137,40	0	0,00	670,380	0,00301	222643,60		
		T2	1724,00	2,5	1586,60					
		T3	3668,00	5	3530,60					
	3	T1	150,00	0	0,00	673,000	0,00302	223254,30		
		T2	1775,00	2,5	1625,00					
		T3	3630,00	5	3480,00					
Month 9, HPP, -30 ºC	1	T1	136,00	0	0,00	226,120	0,00302	74998,34	70321,49	4103,81
		T2	658,60	2,5	522,60					
		T3	1352,00	5	1216,00					
	2	T1	113,70	0	0,00	197,550	0,00293	67322,33		
		T2	578,80	2,5	465,10					
		T3	1159,00	5	1045,30					
	3	T1	109,10	0	0,00	201,210	0,00293	68643,78		
		T2	591,70	2,5	482,60					
		T3	1156,00	5	1046,90					
Month 12, Control, -10 ºC	1	T1	55,26	0	0,00	1,914	0,00316	605,34	3048,78	2522,43
		T2	63,45	2,5	8,19					
		T3	58,02	5	2,76					
	2	T1	53,33	0	0,00	8,729	0,00301	2897,59		
		T2	70,94	2,5	17,61					
		T3	105,40	5	52,07					
	3	T1	56,58	0	0,00	19,402	0,00344	5643,40		
		T2	97,53	2,5	40,95					
		T3	168,70	5	112,12					

Month 12, HPP, -10 ºC	1	T1	70,88	0	0,00	9,046	0,00435	2079,54	1646,82	596,77
		T2	100,80	2,5	29,92					
		T3	101,50	5	30,62					
	2	T1	69,75	0	0,00	4,147	0,00429	966,02		
		T2	74,51	2,5	4,76					
		T3	101,70	5	31,95					
	3	T1	69,02	0	0,00	8,793	0,00464	1894,89		
		T2	97,30	2,5	28,28					
		T3	100,39	5	31,37					
Month 12, Control, -20 ºC	1	T1	68,60	0	0,00	77,330	0,00325	23815,63	21182,03	3591,22
		T2	261,40	2,5	192,80					
		T3	456,30	5	387,70					
	2	T1	76,84	0	0,00	75,508	0,00334	22639,19		
		T2	262,60	2,5	185,76					
		T3	460,40	5	383,56					
	3	T1	70,66	0	0,00	55,602	0,00325	17091,25		
		T2	202,60	2,5	131,94					
		T3	362,80	5	292,14					
Month 12, HPP, -20 ºC	1	T1	118,30	0	0,00	353,730	0,00301	117596,40	143835,90	23363,30
		T2	898,10	2,5	779,80					
		T3	2096,00	5	1977,70					
	2	T1	178,70	0	0,00	465,190	0,00287	162383,50		
		T2	1882,00	2,5	1703,30					
		T3	1424,00	5	1245,30					
	3	T1	126,50	0	0,00	456,250	0,00301	151527,70		
		T2	1895,00	2,5	1768,50					
		T3	1152,00	5	1025,50					

Month 12, Control, -30 ºC	1	T1	87,13	0	0,00	563,461	0,00325	173560,20	138199,60	38853,63
		T2	1205,00	2,5	1117,87					
		T3	3486,00	5	3398,87					
	2	T1	114,40	0	0,00	457,280	0,00317	144432,20		
		T2	1096,00	2,5	981,60					
		T3	2724,00	5	2609,60					
	3	T1	93,56	0	0,00	305,912	0,00317	96606,47		
		T2	894,90	2,5	801,34					
		T3	1550,00	5	1456,44					
Month 12, HPP, -30 ºC	1	T1	70,55	0	0,00	123,535	0,00318	38850,70	40818,73	7383,60
		T2	396,20	2,5	325,65					
		T3	654,60	5	584,05					
	2	T1	79,58	0	0,00	147,316	0,00301	48986,95		
		T2	424,50	2,5	344,92					
		T3	862,90	5	783,32					
	3	T1	78,24	0	0,00	104,098	0,00301	34618,56		
		T2	309,30	2,5	231,06					
		T3	657,10	5	578,86					

7.4. Appendix D – Data referent to the calpains activity

Table 6 - Data referent to the determination of calpains activity.

Condition	Replica	Time	Abs (400 nn)	Time (min)	Abs	Activity (ABS/min)	Fish Mass (g)	Activity (ABS/min/g)	Average Activity (ABS/min/g)	Standard Deviation
Fresh	1	T1	141,8	0	0,0	1777,65	0,00667	266558,6	254916,1	10253,3
		T2	1845,0	1	1703,2					
		T3	3846,0	2	3704,2					
	2	T1	150,7	0	0,0	1586,98	0,00632	250957,4		
		T2	1576,0	1	1425,3					
		T3	3648,0	2	3497,3					
	3	T1	162,9	0	0,0	1855,33	0,00750	247232,4		
		T2	1830,0	1	1667,1					
		T3	4250,0	2	4087,1					
Month 0, Control	1	T1	158,8	0	0,0	2063,90	0,00751	274911,8	316947,8	37294,3
		T2	2246,0	1	2087,2					
		T3	4240,0	2	4081,2					
	2	T1	201,2	0	0,0	2095,35	0,00635	329867,0		
		T2	2552,0	1	2350,8					
		T3	3881,0	2	3679,8					
	3	T1	160,4	0	0,0	2188,95	0,00633	346064,7		
		T2	2189,0	1	2028,6					
		T3	4859,0	2	4698,6					
Month 0, HPP	1	T1	177,7	0	0,0	1817,23	0,00633	287296,3	314122,5	96225,0
		T2	1978,0	1	1800,3					
		T3	3846,0	2	3668,3					
	2	T1	128,9	0	0,0	1373,08	0,00586	234157,2		
		T2	1366,0	1	1237,1					

		T3	3147,0	2	3018,1					
	3	T1	183,8	0	0,0	2537,90	0,00603	420913,8		
		T2	2612,0	1	2428,2					
		T3	5479,0	2	5295,2					
Month 3, Control, -10 ºC	1	T1	138,9	0	0,0	1401,33	0,00707	198256,7	235797,7	35605,9
		T2	1317,0	1	1178,1					
		T3	3388,0	2	3249,1					
	2	T1	131,3	0	0,0	1520,53	0,00633	240049,6		
		T2	1523,0	1	1391,7					
		T3	3430,0	2	3298,7					
	3	T1	147,0	0	0,0	2020,00	0,00751	269086,7		
		T2	2273,0	1	2126,0					
		T3	3975,0	2	3828,0					
Month 3, HPP, -10 ºC	1	T1	69,3	0	0,0	714,95	0,00602	118802,3	145458,8	44199,1
		T2	764,4	1	695,1					
		T3	1539,0	2	1469,7					
	2	T1	59,9	0	0,0	693,53	0,00573	121095,7		
		T2	665,9	1	606,0					
		T3	1622,0	2	1562,1					
	3	T1	93,5	0	0,0	1186,14	0,00604	196478,4		
		T2	1222,0	1	1128,5					
		T3	2581,0	2	2487,5					
Month 3, Control, -20 ºC	1	T1	99,9	0	0,0	1234,11	0,00667	184993,5	252369,7	65655,0
		T2	1284,0	1	1184,2					
		T3	2668,0	2	2568,2					
	2	T1	138,5	0	0,0	1899,63	0,00601	316156,3		
		T2	1887,0	1	1748,5					

		T3	4240,0	2	4101,5					
	3	T1	140,1	0	0,0	1763,93	0,00689	255959,3		
		T2	1682,0	1	1541,9					
		T3	4112,0	2	3971,9					
Month 3, HPP, -20 ºC	1	T1	90,2	0	0,0	805,22	0,00572	140726,3	146599,3	13488,5
		T2	792,7	1	702,5					
		T3	1906,0	2	1815,8					
	2	T1	62,9	0	0,0	926,50	0,00572	162028,6		
		T2	917,9	1	855,0					
		T3	2059,0	2	1996,1					
	3	T1	78,2	0	0,0	847,21	0,00618	137043,1		
		T2	826,2	1	748,0					
		T3	1971,0	2	1892,8					
Month 3, Control, -30 ºC	1	T1	313,8	0	0,0	3436,40	0,00669	513832,9	445151,4	88340,5
		T2	3582,0	1	3268,2					
		T3	7523,0	2	7209,2					
	2	T1	202,4	0	0,0	2730,70	0,00574	476126,7		
		T2	2859,0	1	2656,6					
		T3	5812,0	2	5609,6					
	3	T1	162,8	0	0,0	2078,15	0,00602	345494,6		
		T2	2207,0	1	2044,2					
		T3	4387,0	2	4224,2					
Month 3, HPP, -30 ºC	1	T1	112,6	0	0,0	1673,30	0,00652	256768,8	225695,6	28032,8
		T2	1744,0	1	1631,4					
		T3	3543,0	2	3430,4					
	2	T1	145,9	0	0,0	1344,08	0,00617	218012,5		
		T2	1331,0	1	1185,1					

		T3	3152,0	2	3006,1					
	3	T1	88,9	0	0,0	1248,80	0,00617	202305,4		
		T2	1269,0	1	1180,1					
		T3	2724,0	2	2635,1					
Month 6, Control, -10 ºC	1	T1	87,0	0	0,0	1632,49	0,00670	243573,7	209570,7	42650,8
		T2	1402,0	1	1315,0					
		T3	3987,0	2	3900,0					
	2	T1	115,4	0	0,0	1494,45	0,00669	223422,8		
		T2	1444,0	1	1328,6					
		T3	3436,0	2	3320,6					
	3	T1	121,4	0	0,0	1142,95	0,00707	161715,8		
		T2	1206,0	1	1084,6					
		T3	2524,0	2	2402,6					
Month 6, HPP, -10 ºC	1	T1	69,9	0	0,0	953,26	0,00635	150181,1	200937,4	46944,2
		T2	928,8	1	858,9					
		T3	2165,0	2	2095,1					
	2	T1	97,7	0	0,0	1327,26	0,00633	209835,1		
		T2	1275,0	1	1177,4					
		T3	3052,0	2	2954,4					
	3	T1	126,2	0	0,0	1421,60	0,00586	242796,0		
		T2	1410,0	1	1283,8					
		T3	3245,0	2	3118,8					
Month 6, Control, -20 ºC	1	T1	128,6	0	0,0	1783,05	0,00669	266657,5	328642,0	82713,0
		T2	1773,0	1	1644,4					
		T3	3972,0	2	3843,4					
	2	T1	229,0	0	0,0	2826,00	0,00669	422561,9		
		T2	2886,0	1	2657,0					

		T3	6219,0	2	5990,0					
	3	T1	159,7	0	0,0	2103,48	0,00709	296706,6		
		T2	2114,0	1	1954,3					
		T3	4665,0	2	4505,3					
Month 6, HPP, -20 ºC	1	T1	163,5	0	0,0	2198,13	0,00667	329526,5	342863,4	21539,0
		T2	2189,0	1	2025,5					
		T3	4905,0	2	4741,5					
	2	T1	186,0	0	0,0	2386,75	0,00649	367712,2		
		T2	2443,0	1	2257,0					
		T3	5219,0	2	5033,0					
	3	T1	169,3	0	0,0	2099,03	0,00634	331351,6		
		T2	2229,0	1	2059,7					
		T3	4446,0	2	4276,7					
Month 6, Control, -30 ºC	1	T1	69,0	0	0,0	772,27	0,00634	121879,2	130118,2	14677,1
		T2	755,5	1	686,5					
		T3	1785,0	2	1716,0					
	2	T1	102,7	0	0,0	929,68	0,00632	147063,7		
		T2	949,9	1	847,2					
		T3	2127,0	2	2024,3					
	3	T1	71,2	0	0,0	911,88	0,00751	121411,7		
		T2	936,6	1	865,4					
		T3	1988,0	2	1916,8					
Month 6, HPP, -30 ºC	1	T1	99,3	0	0,0	1799,00	0,00603	298538,8	256624,7	39289,6
		T2	1921,0	1	1821,7					
		T3	3652,0	2	3552,7					
	2	T1	100,0	0	0,0	1262,75	0,00572	220631,9		
		T2	1246,0	1	1146,0					

		T3	2859,0	2	2759,0					
	3	T1	111,7	0	0,0	1505,98	0,00601	250703,3		
		T2	1444,0	1	1332,3					
		T3	3471,0	2	3359,3					
Month 9, Control, -10 ºC	1	T1	77,1	0	0,0	1493,93	0,00559	267062,0	245399,0	26875,8
		T2	1548,0	1	1470,9					
		T3	3111,0	2	3033,9					
	2	T1	75,0	0	0,0	1177,72	0,00547	215323,2		
		T2	1230,0	1	1155,0					
		T3	2476,0	2	2401,0					
	3	T1	76,5	0	0,0	1425,36	0,00562	253811,8		
		T2	1371,0	1	1294,5					
		T3	3189,0	2	3112,5					
Month 9, HPP, -10 ºC	1	T1	64,3	0	0,0	852,25	0,00574	148439,1	166526,6	17567,1
		T2	856,0	1	791,7					
		T3	1890,0	2	1825,7					
	2	T1	59,5	0	0,0	1004,12	0,00547	183522,3		
		T2	1031,0	1	971,5					
		T3	2133,0	2	2073,5					
	3	T1	75,6	0	0,0	920,07	0,00549	167618,4		
		T2	1038,0	1	962,4					
		T3	1831,0	2	1755,4					
Month 9, Control, -20 ºC	1	T1	113,4	0	0,0	1492,70	0,00619	241137,0	232361,3	21533,6
		T2	1491,0	1	1377,6					
		T3	3329,0	2	3215,6					
	2	T1	85,3	0	0,0	1575,05	0,00635	248121,2		
		T2	1696,0	1	1610,7					

		T3	3164,0	2	3078,7					
	3	T1	101,1	0	0,0	1250,18	0,00602	207825,6		
		T2	1103,0	1	1001,9					
		T3	3098,0	2	2996,9					
Month 9, HPP, -20 ºC	1	T1	82,7	0	0,0	795,43	0,00604	131780,2	132440,3	18344,3
		T2	870,9	1	788,2					
		T3	1688,0	2	1605,3					
	2	T1	66,9	0	0,0	909,36	0,00602	151105,8		
		T2	878,5	1	811,6					
		T3	2081,0	2	2014,1					
	3	T1	55,9	0	0,0	670,81	0,00586	114435,0		
		T2	777,4	1	721,6					
		T3	1296,0	2	1240,2					
Month 9, Control, -30 ºC	1	T1	98,2	0	0,0	1165,36	0,00636	183293,0	191872,2	52139,4
		T2	1200,0	1	1101,8					
		T3	2556,0	2	2457,8					
	2	T1	80,8	0	0,0	870,51	0,00602	144554,5		
		T2	901,7	1	820,9					
		T3	1921,0	2	1840,2					
	3	T1	115,6	0	0,0	1493,80	0,00603	247769,1		
		T2	1494,0	1	1378,4					
		T3	3334,0	2	3218,4					
Month 9, HPP, -30 ºC	1	T1	105,8	0	0,0	2021,65	0,00603	335265,3	252994,3	74755,8
		T2	1956,0	1	1850,2					
		T3	4492,0	2	4386,2					
	2	T1	93,9	0	0,0	1110,55	0,00587	189230,5		
		T2	1066,0	1	972,1					

		T3	2592,0	2	2498,1					
	3	T1	92,8	0	0,0	1374,67	0,00586	234486,9		
		T2	1367,0	1	1274,2					
		T3	3043,0	2	2950,2					
Month 12, Control, -10 ºC	1	T1	36,5	0	0,0	325,22	0,00506	64286,1	62745,4	26802,5
		T2	312,2	1	275,7					
		T3	786,1	2	749,6					
	2	T1	44,5	0	0,0	427,75	0,00482	88744,3		
		T2	493,1	1	448,6					
		T3	858,2	2	813,7					
	3	T1	26,5	0	0,0	193,66	0,00550	35205,8		
		T2	198,3	1	171,8					
		T3	457,6	2	431,1					
Month 12, HPP, -10 ºC	1	T1	55,4	0	0,0	287,97	0,00870	33100,0	33786,6	8429,2
		T2	329,3	1	273,9					
		T3	659,6	2	604,2					
	2	T1	41,5	0	0,0	365,22	0,00859	42538,1		
		T2	386,0	1	344,5					
		T3	813,5	2	772,0					
	3	T1	29,5	0	0,0	238,72	0,00928	25721,7		
		T2	251,7	1	222,2					
		T3	540,0	2	510,5					
Month 12, Control, -20 ºC	1	T1	84,3	0	0,0	1335,51	0,00649	205651,2	229163,0	56098,3
		T2	1402,0	1	1317,7					
		T3	2791,0	2	2706,7					
	2	T1	166,0	0	0,0	1955,75	0,00667	293191,5		
		T2	1917,0	1	1751,0					

		T3	4487,0	2	4321,0					
	3	T1	105,1	0	0,0	1227,43	0,00651	188646,4		
		T2	1202,0	1	1096,9					
		T3	2821,0	2	2715,9					
Month 12, HPP, -20 ºC	1	T1	48,3	0	0,0	607,68	0,00481	126262,3	128419,5	2467,0
		T2	590,8	1	542,5					
		T3	1394,0	2	1345,7					
	2	T1	55,3	0	0,0	600,96	0,00458	131109,3		
		T2	650,3	1	595,0					
		T3	1269,0	2	1213,7					
	3	T1	54,8	0	0,0	616,11	0,00482	127886,8		
		T2	707,9	1	653,1					
		T3	1213,0	2	1158,2					
Month 12, Control, -30 ºC	1	T1	14,0	0	0,0	1306,01	0,00649	201141,7	176380,1	21881,9
		T2	1209,0	1	1195,0					
		T3	2848,0	2	2834,0					
	2	T1	96,0	0	0,0	1066,04	0,00633	168354,4		
		T2	1100,0	1	1004,1					
		T3	2352,0	2	2256,1					
	3	T1	95,9	0	0,0	1011,05	0,00633	159644,3		
		T2	1112,0	1	1016,1					
		T3	2108,0	2	2012,1					
Month 12, HPP, -30 ºC	1	T1	139,4	0	0,0	1828,20	0,00636	287476,6	347251,4	90125,6
		T2	1763,0	1	1623,6					
		T3	4205,0	2	4065,6					
	2	T1	172,3	0	0,0	2712,03	0,00602	450914,5		
		T2	2635,0	1	2462,7					

		T3	6095,0	2	5922,7					
	3	T1	143,1	0	0,0	1824,43	0,00601	303363,0		
		T2	1694,0	1	1550,9					
		T3	4339,0	2	4195,9					

7.5. Appendix E – Data referent to the SH group content

Table 7 - Data referent to the determination of SH groups content.

Condition	Replica	Fish Mass (g)	Sobrenatant Volume (mL)	Fish Mass - Assay (g)	Abs (412 nm)	SH content (M)	SH content (mmol/g)	SH Average Content (µmol/g)	Standard Deviation
Fresh	1	0,513	5	0,01539	0,6273	4,61E-05	0,00911	7,80	1,16
	2	0,511	5	0,01533	0,5044	3,71E-05	0,00736		
	3	0,511	5	0,01533	0,4745	3,49E-05	0,00692		
Month 0, Control	1	0,506	5	0,01518	0,3591	2,64E-05	0,00529	7,13	3,83
	2	0,507	5	0,01521	0,311	2,29E-05	0,00457		
	3	0,504	5	0,01512	0,7798	5,73E-05	0,01153		
Month 0, HPP	1	0,502	5	0,01506	0,4942	3,63E-05	0,00734	9,79	3,09
	2	0,516	5	0,01548	0,9176	6,75E-05	0,01325		
	3	0,516	5	0,01548	0,6073	4,47E-05	0,00877		
Month 3, Control, -10 ºC	1	0,51	5	0,0153	0,452	3,32E-05	0,0066	9,89	3,23
	2	0,512	5	0,01536	0,6879	5,06E-05	0,01001		

	3	0,515	5	0,01545	0,902	6,63E-05	0,01305		
Month 3, HPP, -10 ºC	1	0,504	5	0,01512	0,8179	6,01E-05	0,01209	12,79	1,15
	2	0,507	5	0,01521	0,9605	7,06E-05	0,01412		
	3	0,506	5	0,01518	0,8259	6,07E-05	0,01216		
Month 3, Control, -20 ºC	1	0,507	5	0,01521	0,7944	5,84E-05	0,01168	7,90	3,33
	2	0,502	5	0,01506	0,3631	2,67E-05	0,00539		
	3	0,502	5	0,01506	0,4464	3,28E-05	0,00663		
Month 3, HPP, -20 ºC	1	0,51	5	0,0153	1,0907	8,02E-05	0,01594	17,78	5,82
	2	0,506	5	0,01518	1,6503	0,000121	0,0243		
	3	0,505	5	0,01515	0,8888	6,54E-05	0,01311		
Month 3, Control, -30 ºC	1	0,504	5	0,01512	0,7721	5,68E-05	0,01141	11,09	4,73
	2	0,509	5	0,01527	1,0692	7,86E-05	0,01565		
	3	0,508	5	0,01524	0,4227	3,11E-05	0,0062		
Month 3, HPP, -30 ºC	1	0,512	5	0,01536	1,2005	8,83E-05	0,01747	18,31	1,73
	2	0,515	5	0,01545	1,4032	0,000103	0,0203		
	3	0,501	5	0,01503	1,1543	8,49E-05	0,01717		
Month 6, Control, -10 ºC	1	0,506	5	0,01518	0,5858	4,31E-05	0,00863	11,48	4,83
	2	0,502	5	0,01506	1,1494	8,45E-05	0,01706		
	3	0,503	5	0,01509	0,5913	4,35E-05	0,00876		
Month 6, HPP, -10 ºC	1	0,511	5	0,01533	1,029	7,57E-05	0,015	16,91	1,76
	2	0,514	5	0,01542	1,2737	9,37E-05	0,01846		
	3	0,51	5	0,0153	1,1805	8,68E-05	0,01725		
Month 6, Control, -20 ºC	1	0,503	5	0,01509	0,4188	3,08E-05	0,0062	5,85	0,67
	2	0,504	5	0,01512	0,424	3,12E-05	0,00627		
	3	0,503	5	0,01509	0,3425	2,52E-05	0,00507		
	1	0,504	5	0,01512	1,632	0,00012	0,02413	16,97	10,06

Month 6, HPP, -20 ºC	2	0,503	5	0,01509	1,4394	0,000106	0,02132		
	3	0,507	5	0,01521	0,3725	2,74E-05	0,00547		
Month 6, Control, -30 ºC	1	0,514	5	0,01542	0,3144	2,31E-05	0,00456	4,22	0,30
	2	0,512	5	0,01536	0,2842	2,09E-05	0,00414		
	3	0,509	5	0,01527	0,2714	2,00E-05	0,00397		
Month 6, HPP, -30 ºC	1	0,511	5	0,01533	0,8278	6,09E-05	0,01207	9,27	3,29
	2	0,505	5	0,01515	0,684	5,03E-05	0,01009		
	3	0,512	5	0,01536	0,3877	2,85E-05	0,00564		
Month 9, Control, -10 ºC	1	0,502	5	0,01506	0,932	6,85E-05	0,01383	12,52	1,47
	2	0,511	5	0,01533	0,878	6,46E-05	0,0128		
	3	0,515	5	0,01545	0,7552	5,55E-05	0,01093		
Month 9, HPP, -10 ºC	1	0,505	5	0,01515	1,3827	0,000102	0,0204	21,74	1,43
	2	0,511	5	0,01533	1,5939	0,000117	0,02324		
	3	0,504	5	0,01512	1,4591	0,000107	0,02157		
Month 9, Control, -20 ºC	1	0,505	5	0,01515	1,0222	7,52E-05	0,01508	14,68	2,45
	2	0,512	5	0,01536	1,162	8,54E-05	0,01691		
	3	0,501	5	0,01503	0,8105	5,96E-05	0,01205		
Month 9, HPP, -20 ºC	1	0,503	5	0,01509	0,9757	7,17E-05	0,01445	20,05	4,96
	2	0,514	5	0,01542	1,6488	0,000121	0,0239		
	3	0,501	5	0,01503	1,4652	0,000108	0,02179		
Month 9, Control, -30 ºC	1	0,507	5	0,01521	0,7133	5,24E-05	0,01048	10,63	0,72
	2	0,506	5	0,01518	0,6786	4,99E-05	0,00999		
	3	0,515	5	0,01545	0,7886	5,80E-05	0,01141		
Month 9, HPP, -30 ºC	1	0,512	5	0,01536	0,8118	5,97E-05	0,01181	11,81	2,00
	2	0,516	5	0,01548	0,9565	7,03E-05	0,01381		
	3	0,513	5	0,01539	0,6753	4,97E-05	0,00981		

Month 12, Control, -10 ºC	1	0,503	5	0,01509	0,2222	1,63E-05	0,00329	2,95	0,33
	2	0,503	5	0,01509	0,1774	1,30E-05	0,00263		
	3	0,515	5	0,01545	0,2022	1,49E-05	0,00293		
Month 12, HPP, -10 ºC	1	0,507	5	0,01521	0,757	5,57E-05	0,01113	8,76	2,47
	2	0,515	5	0,01545	0,4285	3,15E-05	0,0062		
	3	0,517	5	0,01551	0,6208	4,56E-05	0,00895		
Month 12, Control, -20 ºC	1	0,518	5	0,01554	0,2793	2,05E-05	0,00402	4,25	0,44
	2	0,503	5	0,01509	0,2678	1,97E-05	0,00397		
	3	0,517	5	0,01551	0,3303	2,43E-05	0,00476		
Month 12, HPP, -20 ºC	1	0,516	5	0,01548	0,6879	5,06E-05	0,00993	9,55	1,25
	2	0,512	5	0,01536	0,7261	5,34E-05	0,01057		
	3	0,508	5	0,01524	0,5559	4,09E-05	0,00815		
Month 12, Control, -30 ºC	1	0,52	5	0,0156	0,3688	2,71E-05	0,00528	5,10	0,60
	2	0,521	5	0,01563	0,3911	2,88E-05	0,00559		
	3	0,506	5	0,01518	0,3008	2,21E-05	0,00443		
Month 12, HPP, -30 ºC	1	0,51	5	0,0153	0,7942	5,84E-05	0,0116	13,80	2,46
	2	0,522	5	0,01566	1,1526	8,48E-05	0,01645		
	3	0,522	5	0,01566	0,9357	6,88E-05	0,01336		