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Effect of harvesting stress and storage conditions on protein degradation in fillets of farmed gilthead seabream (*Sparus aurata*): A differential scanning calorimetry study

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ABSTRACT

A trial was undertaken to evaluate Differential Scanning Calorimetry (DSC) as a fast analytical tool to differentiate gilthead seabream subjected to variable conditions of slaughter stress and post-mortem storage. Fish were subjected to different harvesting stress conditions: profound anaesthesia (PA, low stress) and net crowding (NC, high stress). Fish were slaughtered in an ice-salt water slurry, and subsequently stored on ice (7 days). Additional NC fish were frozen (−20 °C) and subjected to a freeze–thaw cycle. Dorsal muscle was assessed for cathepsins activity, liquid loss and DSC analysis. It is demonstrated that DSC analysis is capable of differentiating fresh, frozen and thawed-re-frozen fish, while liquid loss and cathepsin B activity are good markers to distinguish fresh from frozen fish. Harvesting stress had little effect on myosin and actin enthalpy transitions, as observed by DSC at 49 and 74 °C, respectively, but a lower ΔH actin/myosin ratio was found in PA fish, suggesting that intense exercise prior to slaughter promoted partial denaturation of muscle myosin.

1. Introduction

Nowadays, consumer awareness of seafood quality is rising, and given the increasing demand for quality control and assurance in most products, it is inevitable that these concerns extend to aquaculture products. Moreover, the industry itself benefits from the development of systems of control and quality management, especially considering the highly perishable product that is fish.

Texture is considered to be one of the most important quality attributes in fish. During the post-mortem ageing of muscle under chilled conditions or frozen storage, degradation of muscle proteins and connective tissue contributes to the rapid softening of the fish muscle (Caballero et al., 2009). Flesh texture in fish is determined by a complex set of intrinsic traits such as the muscle chemical composition (*e.g.* fat content and fatty acid profile, glycogen stores) and muscle cellularity. It is also strongly influenced by a variety of extrinsic factors, including pre- and post-slaughter handling procedures (Bahuaud et al., 2010). Additionally, storage conditions are known to influence not only texture but also microbiological safety (Mackie, 1993). Since fresh fish has a higher market value than frozen products, it is important to be able to detect fraudulent practices such as retailing fish as fresh when it has been previously frozen (Nott, Evans, & Hall, 1999).

The conventional monitoring of fish freshness by the industry relies on sensory assessment, evaluating characteristics like outer appearance, odour and colour (Olafsdottir et al., 2004). The use of enzymatic and biochemical methods for fish quality assessment is expensive and time consuming and requires specialised personnel. The development of rapid and simple instrumental techniques capable of evaluating flesh quality without relying on trained assessors is therefore of great importance for the industry. Several appropriate methods like quantitative magnetic resonance imaging (Nott et al., 1999), torrymeter measurements (Olafsdottir et al., 2004), and differential scanning calorimetry (DSC) (Radičević, Raičević, & Niketić, 2002) have been successfully used to distinguish between fresh and frozen fish.

Differential scanning calorimetry (DSC) is a widely used thermal analytical technique in food research and its utility in quality assurance in the food chain has been demonstrated (Schubring, 2009). Conformational changes of food proteins affected by various environmental factors, thermal denaturation of tissue proteins and freezing-induced denaturation of food proteins could potentially be monitored by DSC (Schubring, 2009). However, the use of DSC as a fast analytical tool to differentiate pre-harvesting farming practices in aquaculture products is to our knowledge nonexistent. Therefore, a trial was undertaken to evaluate the feasibility of using DSC to distinguish fillets of gilthead seabream (*Sparus aurata*) subjected to two different levels of harvesting stress. Additionally, fish fillets were compared after storage on ice and being subjected to different conditions of frozen storage.

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2. Materials and methods

2.1. Fish and rearing conditions

Eighteen gilthead seabream (body weight: 421 ± 74 g) were stocked in 1000 l circular tanks at CCMAR experimental facilities. Fish were farmed in this system for several months prior to the study, during which they were hand fed a commercial diet (AquaGold, crude protein 45%, crude fat 16%, Sorgal, S.A., Portugal) to apparent satiety in one daily meal. Fish were subjected to natural photoperiod and the rearing system was supplied with flow-through aerated seawater (salinity: 35 ppt; temperature: 18–25 °C; oxygen content: >60% saturation). Prior to harvesting, fish were starved for 48 h.

2.2. Pre-slaughter harvesting stress

Prior to slaughter, fish were subjected to two harvesting conditions: a) profound anaesthesia (PA, low stress condition), in which six fish were anesthetised with a water dispersible isoeugenol-based liquid anaesthetic (50 μl l^{-1}, AQUI-S™, New Zealand Ltd.), approved in several non-EU countries with a zero withdrawal period for human consumption; and b) net crowding (NC, high stress condition), in which 12 fish were confined for 20 min to an extremely high density (approximately 140 kg m^{-3}) inside a cylindrical net, in an attempt to simulate the common industrial practice in the Mediterranean region. Following such harvesting stress conditions, fish from both treatments were slaughtered by immersion in an ice-saltwater slurry in a proportion of 4:1 (common practice in the industry). After 30 min, fish were removed from the slaughter bins and stored with the ventral side upwards in insulated and bottom perforated polystyrene boxes, covered with a plastic film and flaked ice.

2.3. Storage conditions

After slaughter, PA fish ($n = 6$), packed in an insulated polystyrene box and covered with ice, were stored for 7 days in a temperature controlled room (4–5 °C). On day 8, fish were filleted and muscle samples were analysed by differential scanning calorimetry. Muscle samples were also collected for enzyme extraction (cathepsins) and measurement of water holding capacity. All these procedures were done on the sampling day.

NC fish were divided into two groups at the time of slaughter. One group ($n = 6$) was subjected to the same storage conditions as the PA groups, and analysed on day 8 of ice storage. The second group ($n = 6$) was filleted at slaughter time, immediately frozen at −20 °C and stored at this same temperature for 40 days. At this time, the left fillets (single-frozen fillets, NC_{SF}) were processed for DSC analysis, cathepsins extraction and measurement of water holding capacity. The right fillets (double-frozen fillets, NC_{DF}) were allowed to thaw for 8 h (to reach room temperature, 23 °C) and re-frozen at −20 °C for an additional 7 days. Following this period, double frozen fillets were also analysed for the previously described criteria.

2.4. Analytic methods

2.4.1. Differential scanning calorimetry

A heat-flux differential scanning calorimeter (DSC-60, Shimadzu Scientific Instruments Inc., Maryland, USA) was used to perform DSC analysis. Indium was used as a standard for temperature and enthalpy calibration. Muscle samples (approximately 30 mg) were sealed in an aluminium capsule and scanned from 30 to 90 °C at a rate of 5 °C.min^{-1}, against an empty reference capsule. After baseline subtraction in each thermogram, total denaturation enthalpy (ΔH, J g^{-1}), myosin and actin peak denaturation enthalpy (ΔH, J g^{-1}) were estimated by integrating the area under the complete thermogram and under the myosin and actin peaks, respectively. DSC analysis was performed by using the TA-60WS software package (Shimadzu). The myosin and actin peak maximum denaturation temperature (T_{max}, °C) was also registered. Peak identification was done according to Schubring (2009). The actin/myosin enthalpy ratio was calculated from the respective denaturation enthalpies. Three replicates were used for each sample.

2.4.2. Liquid loss

Liquid loss was determined in duplicate according to Hultmann, Røra, Steinsland, Skarad, and Rustad, (2004), with some modifications. Weighed (F1) paper filters (Whatman Cat. No. 1822 047, Whatman International Ltd., Maidstone, England) were placed inside centrifuge tubes, and a sample of muscle of about 1 g (muscle weight: M) was placed on top and centrifuged at 1000 *g* for 15 min, at room temperature. After centrifugation, the paper filters were weighed (F2) and afterwards placed on a drying oven at 100 °C for 48 h. The dry filters were weighed again (F3), and liquid loss, fat loss and water loss were determined according to the following equations:

$$\text{Liquid loss}\,(\%) = \frac{F2 - F1}{M} \times 100$$

$$\text{Fat loss}\,(\%) = \frac{F3 - F1}{M} \times 100$$

$$\text{Water loss}\,(\%) = \frac{F2 - F3}{M} \times 100$$

2.4.3. Cathepsins activity

Cathepsins (L, B, H) extraction and activity measurement were performed according to Cheret, Delbarre-Ladrat, De Lamballerie-Anton, and Verrez-Bagnis (2007). Briefly, 2 g of muscle was homogenised twice for 30 s using an Ultra Turrax T25 Basic (IKA Labortechnik) in 6 ml of extraction buffer (50 mM Tris–HCl (pH 7.5), 10 mM β-mercaptoethanol, 1 mM EDTA). The supernatant was collected after centrifugation at 10,000*g* for 40 min at 4 °C and frozen at −80 °C prior to analysis. Cathepsin activities in the protein extracts were determined at 30 °C in a 298 μl reaction (6 μl 5% CHAPS, 1 μl of 1.40 M β-mercaptoethanol, 16 μl of 5% (w/v) Brij™ 35, 5 μl of synthetic fluorogenic substrate prepared in ultrapure water and 70 μl of 0.4 mM acetate/acid acetic (pH4) buffer containing 10 mM β-mercaptoethanol and 1 mM EDTA). Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride (10 mM), Z-Phe-Arg-7-amido-4-methylcoumarin hydrochloride (20 mM) and L-Arginine-7-amido-4-methylcoumarin hydrochloride (5 mM) were used as the substrates for Cathepsin B, Cathepsin B and L, and Cathepsin H, respectively. The reaction was initiated by adding 200 μl of protein extract. Samples were run in triplicate and a standard curve prepared with 7-amido-4-methylcoumarin (AMC) as well as a control with extraction buffer instead of enzyme extract were run in parallel. One unit of enzyme activity was defined as the amount of enzyme that hydrolyses 1 nmol of substrate per minute, and the results were expressed as U g^{-1} (protein). Protein content in the enzyme extracts was determined according to Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin as standard.

2.5. Statistical analysis

All results are expressed as mean ± standard deviation. A one-way ANOVA was used to test the effects of the treatments on all

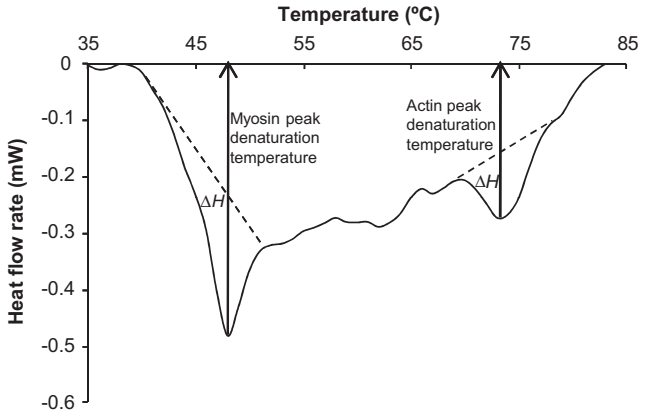

Fig. 1. Typical thermogram for gilthead seabream muscle, showing the different endothermic peaks. The first well defined peak, corresponding to myosin denaturation, is followed by a series of smaller undefined peaks (typically the denaturation of collagen and sarcoplasmic proteins). The second well defined peak corresponds to the denaturation of actin. Denaturation enthalpies (ΔH) were calculated by integrating the area below the dashed lines. Denaturation temperatures were measured as indicated by the arrows in the figure.

parameters. Data were previously checked for normal distribution and homogeneity of variances and when necessary transformed using the multiplicative inverse. Following ANOVA and if appropriate, means were compared by the Student–Newman–Keuls multiple range test. Statistical significance was defined as $P < 0.05$. All tests were run with SPSS ver.17.0 (SPSS Inc., Chicago, USA). For principal component analysis, mean centering and scaling by 1/s (where s is the sample standard deviation) were performed on each variable to normalise the contribution of each variable to the total variance. All PCA calculations and plots were performed using the R environment for statistical computing (v.2.9.2, R Foundation for Statistical Computing).

3. Results

The thermograms of seabream muscle samples showed two well defined endothermic transitions, the first one peaking at a temperature between 47 and 50 °C, corresponding to the denaturation of myosin (Fig. 1). This peak was followed by a series of smaller undefined peaks, which could be attributed to the denaturation of collagen and sarcoplasmic proteins. The second well defined transition peaked on average at a temperature between 73 and 75 °C, corresponding to the denaturation of actin (Fig. 1). Total denaturation enthalpy (ΔH) of the samples (Fig. 2A) ranged from -2.60 to -3.85 J g^{-1} and was significantly different between treatments ($P < 0.001$). There was no distinction between harvest stress levels, but fresh fish (PA and NC treatments) required a higher amount of energy to denaturate all the muscle proteins than both frozen treatments. Moreover, ΔH was also significantly different between single frozen (NC_{SF}, -3.25 J g^{-1}) and double frozen (NC_{DF}, -2.60 J g^{-1}) fillets.

The myosin peak maximum denaturation temperature (T_{max}, Fig. 2B) differed significantly between treatments ($P < 0.001$). Fresh seabream from the profound anaesthesia (PA, 49.44 °C) and net crowding treatments (NC, 49.75 °C) and single frozen seabream fillets (NC_{SF}, 49.11 °C) had a significantly higher T_{max} than double frozen fillets (NC_{DF}, 47.79 °C). T_{max} of the actin denaturation peak ranged from 73.94 to 74.50 °C (Fig. 2C). Fresh seabream (PA and NC) actin T_{max} differed significantly from NC_{DF} fillets ($P = 0.011$),

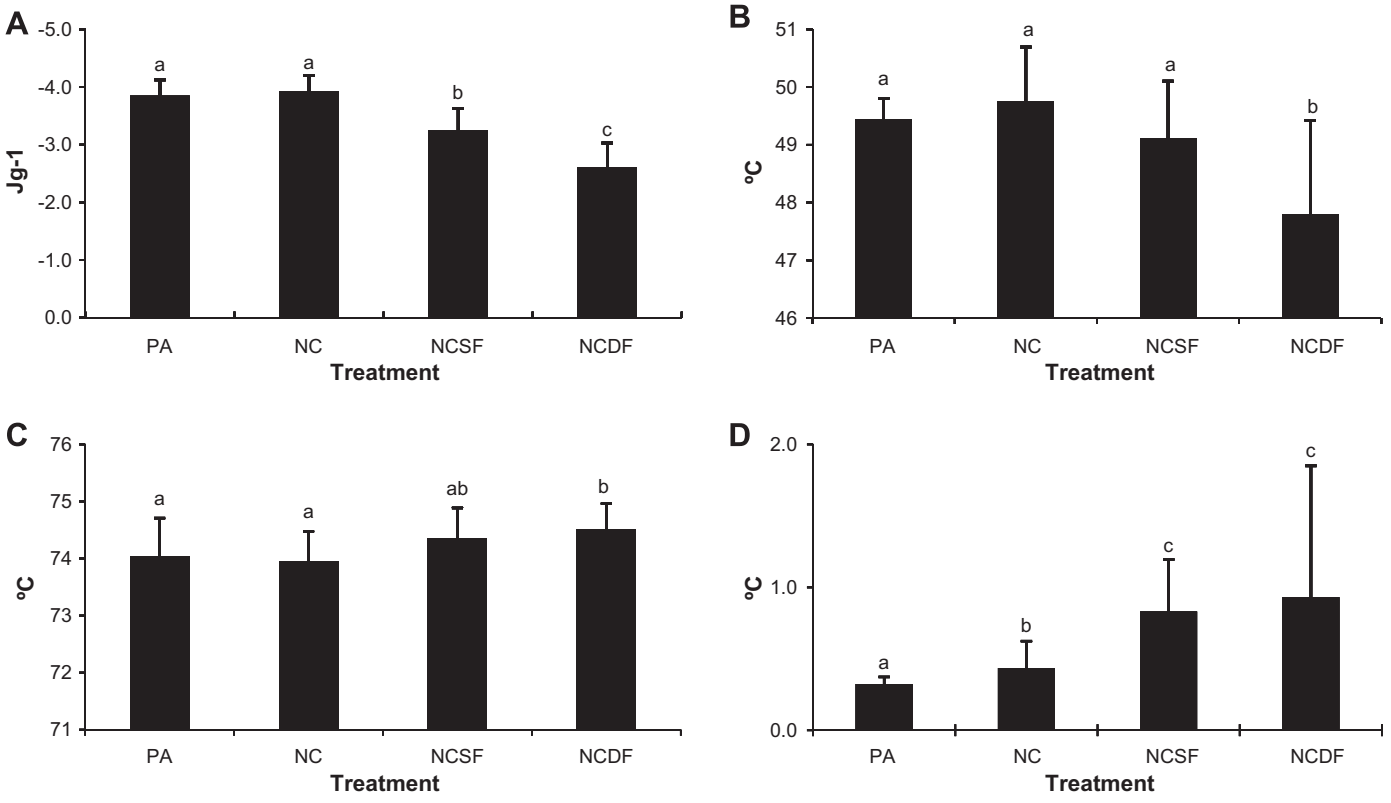

Fig. 2. (A) Total denaturation enthalpy, (B) myosin denaturation peak temperature, (C) actin denaturation peak temperature and (D) actin/myosin denaturation enthalpy ratio of gilthead seabream muscle subjected to different harvesting conditions and subsequently stored under different conditions (PA: profound anaesthesia, stored in ice; NC: net crowding, stored in ice; NCSF: net crowding, single frozen; NCDF: net crowding, double frozen). Values are means ± standard deviation ($n = 6$). Bars with different superscript letters differ significantly ($P < 0.05$). Absence of superscript indicates no significant difference between treatments.

although there were no significant differences between neither fresh fillets and single frozen fillets nor between frozen treatments. The ΔH actin/myosin enthalpy ratio was significantly different between treatments ($P < 0.001$, Fig. 2D). Statistical analysis shows that PA treated fish had the lowest ratio (0.32), followed by NC treated fish (0.43). Both frozen treatments showed the highest ratios (0.83 and 0.93 for NC_{SF} and NC_{DF} respectively) and the highest within-treatment variability.

The results from the DSC analysis were subjected to principal component analysis. A plot of the first two principal components (PC), explaining 81% of the variation within the data set is shown in Fig. 3. PC1 explains 53.7% of the total observed variation and seems to clearly separate the samples in three clusters without overlap; the first group contains the fresh fish samples (PA and NC treatments), the second refers to the NC_{SF} treatment and the third to the NC_{DF} treatment. The variables with highest PC1 loadings are the actin T_{max} and the total denaturation enthalpy of the samples, suggesting that these two variables carry most of the information that can be used to distinguish samples from the three observed clusters.

Liquid loss ranged from 15.71 ± 2.89% to 22.06 ± 1.94% of muscle weight and was not significantly different between harvest stress levels or between frozen storage conditions (Fig. 4). However, frozen fillets had significantly higher liquid loss than fresh fish ($P < 0.001$). Similarly, fat loss and water loss were higher for frozen fillets ($P < 0.001$). Fat loss represented 16 to 18% of total liquid loss.

Cathepsin B activity was significantly higher for fresh seabream ($P = 0.001$), with the frozen seabream having a five-fold lower cathepsin B activity (Fig. 5A). Cathepsin L and H activities (Fig. 5B and C) were not significantly affected by neither harvest stress conditions nor frozen storage ($P = 0.597$ and $P = 0.069$, respectively). Cathepsin L activity was very low, ranging from 1.20 to 4.64 U g^{-1}. Although there were no statistical differences, the lowest activity for cathepsin H was measured in seabream subjected to profound anaesthesia (PA, 45.89 U g^{-1}), while both fresh and frozen seabream subject to net crowding (NC treatments) showed higher cathepsin H activities ranging from 57.96 to 69.98 U g^{-1}.

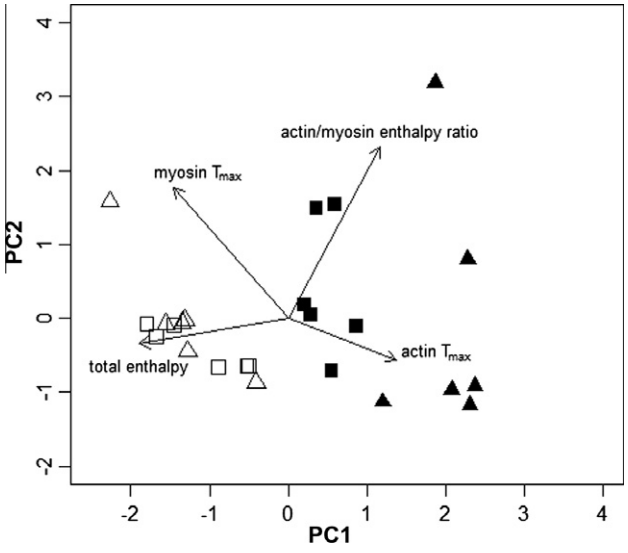

Fig. 3. Principal component analysis biplot (PC1 and PC2) of DSC variables of gilthead seabream muscle subjected to different harvesting conditions and subsequently stored under different conditions (□: profound anaesthesia, stored in ice; △: net crowding, stored in ice; ■: net crowding, single frozen; ▲: net crowding, double frozen). Mean centering and autoscaling were performed prior to the analysis. Each point represents the mean value of three measurements on the same muscle sample ($n = 6$). Arrows indicate the loading of each original variable for the first two components.

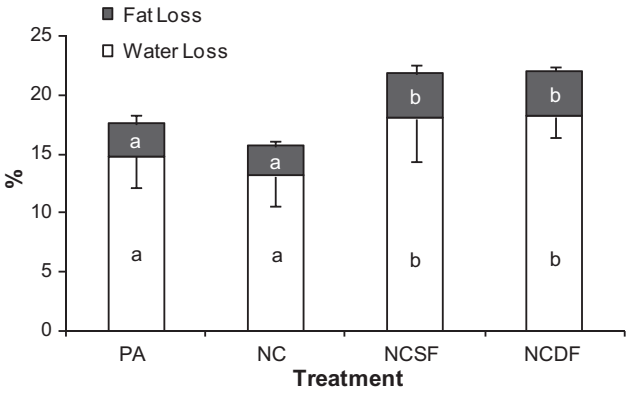

Fig. 4. Liquid loss, represented by the sum of water loss and fat loss, in gilthead seabream muscle subjected to different harvesting conditions and subsequently stored under different conditions (PA: profound anaesthesia, stored in ice; NC: net crowding, stored in ice; NCSF: net crowding, single frozen; NCDF: net crowding, double frozen). Values are means ± standard deviation ($n = 6$). Bars with different superscript letters differ significantly ($P < 0.05$). Absence of superscript indicates no significant difference between treatments.

4. Discussion

The development and implementation of a general scheme of quality assurance in the fish industry has been the object of recent research (*e.g.* Olafsdottir et al., 2004), but there is still a need to develop fast and reliable online methods to evaluate fish quality and authenticity. Differential scanning calorimetry has already proved to be useful in identifying whether fish has suffered some thermal treatment, such as freezing and heating (Radičević et al., 2002; Schubring, 2008), although its utility in indentifying conditions such as freeze–thaw cycles seems to be species specific (Beyrer & Rüsch gen. Klaas, 2007; Hurling & McArthur, 1996; Schubring 1999), and there is no information regarding its ability to identify other factors, such as harvesting or slaughter stress. However, being a simple, fast and practical technique that requires small samples, DSC has the potential to become widely used in fish quality assessment.

4.1. Effect of harvesting stress

The growing awareness on fish welfare issues by the consumer has led to an increase in research pertaining to the evaluation of the effects of traditional aquaculture practices on fish welfare, from an ethical as well as a product quality point of view (Poli, 2009). Alternatives to traditional harvest (crowding) and slaughter methods (ice-saltwater slurry) for gilthead seabream continue to be investigated (Giuffrida et al., 2007). Recent studies show that fish subjected to stress prior to and during slaughter, especially salmonids, display a softer texture and lower flesh quality (*e.g.* Bahuaud et al., 2010; Kiessling, Espe, Ruohonen, & Mørkøre, 2004). The effects of harvest and slaughter stress on gilthead seabream flesh texture have been the object of few studies, most of which found that seabream textural parameters are little affected by slaughter stress (*e.g.* Tejada & Huidobro, 2002). A previous study showed that the use of isoeugenol during harvest improves gilthead seabream muscle pH and delays *rigor mortis*, but no effects were discernible on muscle connective tissue and instrumental texture (Matos, Gonçalves, Nunes, Dinis, & Dias, 2010).

To the best of our knowledge, there is no published information on the effects of harvesting stress on fish protein thermal stability.

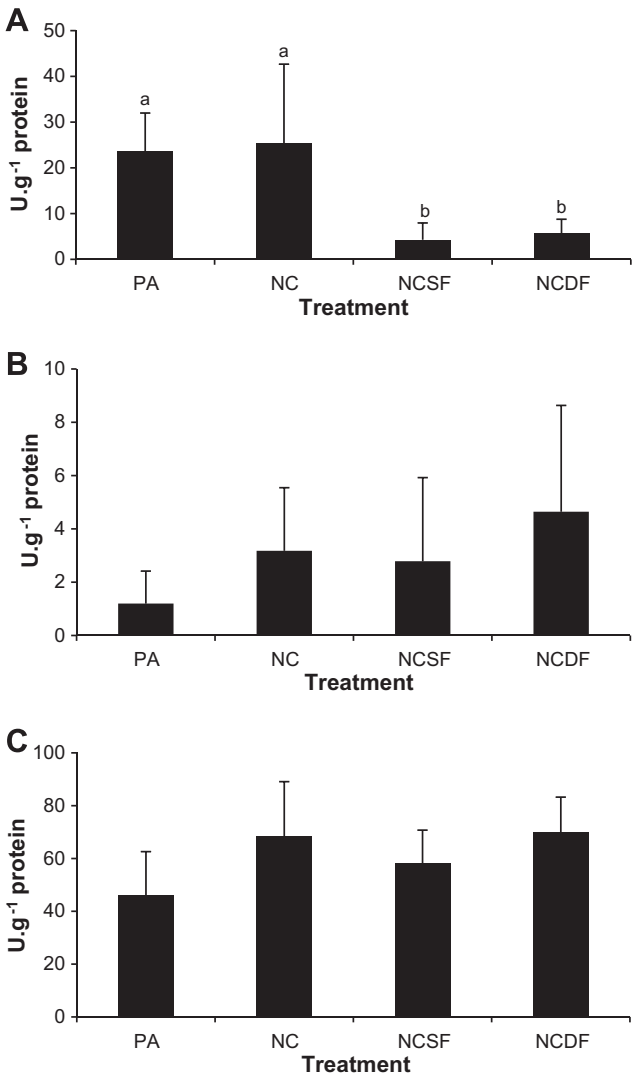

Fig. 5. (A) Cathepsin B, (B) cathepsin L and (C) cathepsin H activities of gilthead seabream muscle subjected to different harvesting conditions and subsequently stored under different conditions (PA: profound anaesthesia, stored in ice; NC: net crowding, stored in ice; NCSF: net crowding, single frozen; NCDF: net crowding, double frozen). Values are means ± standard deviation ($n = 6$). Bars with different superscript letters differ significantly ($P < 0.05$). Absence of superscript indicates no significant difference between treatments.

Although most of the DSC parameters measured showed no significant differences between harvesting stress treatments, the results of the present study clearly show a lower ΔH actin/myosin ratio for fish subjected to profound anaesthesia (PA) prior to slaughter, while no effect was evident on the thermal stability of neither actin nor myosin (T_{max}). This lower ratio results from a higher ΔH value for myosin in PA treatment, since ΔH for actin was not significantly different between harvesting stress treatments (data not shown). The denaturation enthalpy of a protein is a measure of the amount of protein left in its native state. This implies that intense exercise prior to slaughter (net crowding) promoted denaturation of part of the myosin present in the muscle.

Although liquid loss has been reported to be linked to myosin stability (Mackie, 1993; Medina, González, Iglesias, & Hedges, 2009; Ofstad et al., 1996), the results of the present study show no significant differences in water and fat loss between both harvesting stress treatments. Likewise, Tejada and Huidobro (2002) reported no clear influence of different stress levels during slaughter on the water holding capacity of gilthead seabream. A similar study with barramundi (*Lates calcarifer*) also showed no differences on drip loss between conventional harvest methods and the use of isoeugenol as a rested harvest technique (Wilkinson, Paton, & Porter, 2008). However, Kiessling et al. (2004) established that Atlantic salmon subjected to isoeugenol anaesthesia prior to slaughter had significantly lower liquid loss than salmon anaesthetised with CO_2. The differences in ΔH actin/myosin ratio between PA and NC treatments observed in the present study, although significant, are very slight, suggesting that even though some myosin denaturated due to crowding stress, it was not enough to influence the liquid holding capacity of gilthead seabream muscle.

The amount of potentially active cathepsins present in the muscle did not differ significantly between harvesting stress treatments. A study with Atlantic salmon showed a higher cathepsin B activity with pre-slaughter crowding stress (Bahuaud et al., 2010), and a negative correlation with post-mortem muscle pH, suggesting the higher cathepsin B activity was due to enzyme activation by low pH, presumably outside the lysosomal environment. In a previous study (Matos et al., 2010) it was shown that although gilthead seabream muscle pH was significantly affected by pre-slaughter crowding stress at the beginning of the storage period, the muscle pH was similar between treatments after 48 h of storage. However, since the methodology adopted measured the cathepsin B potential activity, it cannot be known whether cathepsin B had been previously released and activated due to lysosome rupture. Cheret, Hernandez-Andres, Delbarre-Ladrat, De Lamballerie, and Verrez-Bagnis (2006) measured cathepsin activity in European seabass (*Dicentrarchus labrax*) muscle, and found that cathepsin B previously released from lysosomes as a result of high-pressure treatment remained active and stable during refrigerated storage up to 8 days post-mortem. In the present study, cathepsin H displayed slightly lower activity in PA treated fish, although this effect was not significant. Cathepsin H in European seabass muscle is reported to be present in small amounts (Cheret, Delbarre-Ladrat, De Lamballerie-Anton, & Verrez-Bagnis, 2005). Aoki, Yamashita, and Ueno (2000) reported that the activity of cathepsins B, L and D on fish muscle differs greatly from species to species. Lysosomal cathepsins are synthesised as proenzymes, which mature by cleavage of the N-terminal propeptide. This can occur due to autolytic cleavage or by action of other enzymes (Turk, Turk, & Turk, 2000). Several studies in mice show an increase in lysosomal enzyme activities due to exercise (*e.g.* Salminen & Vihko, 1984). It is possible that cathepsin H is activated in gilthead seabream during the strenuous exercise caused by crowding prior to slaughter, in order to provide energy from muscle protein turnover. To the best of our knowledge no work has been published regarding gilthead seabream muscle cathepsin activity and further studies are necessary to test this hypothesis.

4.2. Effect of frozen storage

Gilthead seabream is one of the major farmed species in Europe and is traditionally sold fresh. With the increasing growth of the aquaculture sector, there is need to find suitable market alternatives, such as freezing (Huidobro & Tejada, 2004). In the present study, gilthead seabream was subjected not only to freezing, but also to a freeze–thaw cycle, in order to investigate the possible effects of temperature abuse during frozen storage, which can occur during transport and storage.

There are several studies published on the thermal stability of fish subjected to different freeze–thaw cycles. Schubring (1999) studied the effect of double freezing on the thermal properties of saithe (*Pollachius virens*), cod (*Gadus mohrua*) and Alaska pollock (*Theragra chalcogramma*), and concluded that differential scanning calorimetry was not able to differentiate between single and double frozen fillets from these species. Hurling and McArthur (1996)

also found no significant differences between the total denaturation enthalpies of single and double frozen cod fillets. Beyrer and Rüsch gen. Klaas (2007), however, established that double freezing of herring fillets (*Clupea harengus*) led to a significant decrease in specific heat capacity, when compared with fresh and single frozen fillets. In the present work, seabream single frozen fillets had significantly higher total denaturation enthalpy than double frozen fillets. This implies that thawing and refreezing further denaturated muscle proteins. Although the ΔH actin/myosin ratio was not significantly different, this is probably due to the high variation observed within NC_{DF} fillets, since myosin ΔH was significantly lower for this treatment and actin ΔH was slightly but not significantly higher (results not shown). Therefore, the decrease in ΔH observed in NC_{DF} fillets is probably due to further denaturation of myosin, collagen and sarcoplasmic proteins. Additionally, myosin denaturation temperature is significantly lower (ranging from 44.5 to 51.4 °C) in double frozen fillets. This parameter is a measure of the protein thermal stability and a lower T_{max} is a result of conformational changes, with the partial unfolding of the protein's structure (Lörinczy & Belágyi, 1995).

Liquid loss did not differ significantly between single and double frozen fillets. Hurling and McArthur (1996) reported a lower capacity to retain liquids in cod subjected to double freezing when compared to once-frozen fish, although this effect was less apparent at the beginning of the storage period. In the present study, during the thawing process (NC_{DF} treatment), we observed some accumulation of fluids inside the storage bag. Although the moisture content in the muscle was not determined, it is possible that the NC_{DF} fillets had a lower percentage of water at the time of the measurement, in which case liquid loss was underestimated.

Cathepsin B, L and H activities also did not differ significantly between freezing treatments, suggesting these are not good markers to test temperature abuse in gilthead seabream.

4.3. Discrimination between fresh and frozen fish

When frozen storage is performed correctly, it is very difficult to distinguish fresh from frozen-thawed fish through sensory analysis. Fresh fish generally attain a higher market value, and it is therefore important to be able to detect whether previously frozen fish is being retailed as fresh, since this is a fraudulent practice (Nott et al., 1999).

DSC analysis can be particularly useful in distinguishing fresh from frozen fish. The present study shows that fresh fish proteins require a higher amount of energy to fully denaturate (ΔH), implying that the freezing process denaturates some muscle proteins, probably due to the formation of ice crystals (Badii & Howell, 2002). This result is not unexpected, since several studies show that thermal stability of fish proteins, especially myosin, is significantly lowered by frozen storage (*e.g.* Hurling & McArthur, 1996; Radičević et al., 2002). This is supported by the actin/myosin ΔH ratio, which is significantly higher for frozen fish. However, the thermal stability of myosin and actin was not affected in the same way by the freezing treatments. Although the double frozen fillets showed lower myosin and higher actin denaturation temperatures, the single frozen fillets' myosin and actin denaturation temperatures did not differ from those of fresh fish fillets (PA and NC treatments). Gilthead seabream muscle sensory and textural properties are reported to be fairly stable during frozen storage (Huidobro & Tejada, 2004). It is likely that the temperature abuse suffered during the freeze–thaw cycle caused unfolding of some microdomains in the myosin structure. Another possible cause could be an increase in ionic strength in the double frozen muscle due to the observed liquid loss during thawing, which would lower the myosin denaturation temperature (Radičević et al., 2002). The actin T_{max} of double frozen fillets, although higher, shows only an increase of about 0.5 °C when compared to fresh fish. Schubring (2008) reported similar results for rainbow trout (*Oncorhynchus mykiss*). This slight increase in T_{max} could eventually be due to actin filament aggregation or dehydration effects (Badii & Howell, 2002). It is interesting to note, from the PCA analysis, that fish from the frozen treatments show a higher dispersion than the fresh fish. Within-sample variability was also higher for these treatments. Muscle has a heterogeneous composition and this pattern suggests that not only do individual fish "react" differently to frozen storage and temperature abuse, but also that muscle protein degradation is irregular within the same fillet. In this study the samples used for DSC analysis had only about 30 mg of muscle per replicate, and it would be interesting to use larger samples, as it could conceivably lower within-sample variability, enhancing the efficiency of the DSC analysis.

Olsson, Olsen, and Ofstad (2003) suggested that loss of water holding capacity in halibut (*Hippoglossus hippoglossus*) is mostly influenced by the detachment and widening of space between myofibrils, which is consistent with the present results. Medina et al. (2009) reported a loss of water holding capacity in frozen horse mackerel (*Trachurus trachurus*) muscle as storage time increased and Mørkøre, Hansen, Unander, and Einen (2002) reported a higher liquid loss in frozen rainbow trout compared to fresh fish, associated with myosin denaturation. Additionally, Ofstad et al. (1996) suggested that liquid loss is caused by the expulsion of water from the myofibrils during shrinkage, with increasing intermyofibrillar and extracellular spaces, as well as lower stability of myosin. In the present work fresh seabream were stored whole, reducing the shrinkage ability of the muscle as a result of *rigor mortis* (Kristoffersen et al., 2006), while seabream from the NC_{SF} and NC_{DF} treatments were filleted and frozen pre-rigor. It is likely that during the thawing process the fillets experienced some degree of shrinkage and drip loss due to thaw *rigor* (Cappeln & Jessen, 2001), leading to the higher liquid loss observed.

Yamashita and Konagaya (1990) have shown that cathepsin L is responsible for post-mortem tenderization and for the acceleration of muscle degradation during frozen storage in chum salmon (*Oncorhynchus keta*), possibly caused by the release of the enzyme as a consequence of the disruption of the lysosomal membrane, due to the formation of ice-crystals during freezing. In the present study, however, measured activity of cathepsin L was very low and widely variable, and cannot be clearly associated with the experimental conditions, suggesting that this enzyme has only a minor role in post-mortem proteolysis of seabream muscle. In the present study, cathepsin B activity was significantly higher for fresh seabream. When muscle is frozen, the formation of ice crystals leads to the rupture of lysosomes, causing the release of enzymes into the muscle fibres (Karvinen, Bamford, & Granroth, 1982). The method used in this study destroys the lysosome membranes during extraction and uses a synthetic substrate, effectively measuring the proteolytic potential of lysosomal enzymes present. Even if some lysosome rupture occurred during ice-storage, it is likely that cathepsin B was not inactivated due to autolysis during the storage period (Cheret et al., 2006). On the other hand, it is possible that cathepsin B was irreversibly inactivated during frozen storage, due to oxidation of the cysteine residues (Bano, Kunapuli, Bradford, & Colman, 1996). Thiol-dependent proteases such as cathepsin B are prone to inactivation by a wide range of amino acid, peptide and protein hydroperoxides (Headlam, Gracanin, Rodgers, & Davies, 2006). Additionally, studies show that frozen storage of fish muscle can increase lipid and protein oxidation (*e.g.* Medina et al., 2009), further supporting the present results. However, cathepsin H, which maintained its activity potential throughout frozen storage, is also a thiol-dependent protease with cysteine residues, and there is no evidence that it should be less susceptible to inactivation due to oxidation than cathepsin B. One alternative

explanation is that cathepsin B exists in seabream muscle at the time of death only as proenzyme, becoming mature during post-mortem storage, and this process is halted by frozen storage immediately after death, but further studies are necessary to elucidate the mechanisms of cathepsin activation in gilthead seabream muscle.

5. Conclusion

Differential scanning calorimetry is a promising technique in gilthead seabream quality assurance. It is capable of discerning between fresh and frozen seabream, as well as determining whether temperature abuse occurred during *post-mortem* storage. Although the present results indicate limited use to evaluate harvesting stress, it is likely that with a small increase in sample size this drawback could be overcome. Although both liquid loss and cathepsin B activity were equally useful in distinguishing fresh from frozen seabream, both methods require much larger muscle samples. Additionally, enzymatic methods are not easily applicable as online control systems by the industry, as they require highly specialised personnel and are costly. DSC analysis is a fast, simple and low-cost technique that proved reliable in discerning seabream subjected to different storage conditions.

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