Searching for molecular markers of quality in fish reared in aquaculture

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Abstract

One of the most unique characteristics of fish as food is that it is highly perishable. Consequently, freshness is fundamental to the quality of fish, and the time that has passed after catching it and the temperature ‘‘history” of fish are very often the key factors in determining the ultimate quality characteristics of such products. Therefore, the development of reliable methods to assess the freshness of fish, as well as the evaluation of quality, has been the goal of fish research for many years.

During *post mortem* storage, fish muscle degrades and the flesh quality decreases rapidly, depending on the fish species. Loss of freshness, followed by spoilage, is the result of complex biochemical and microbiological processes that begin with a metabolic shift from an aerobic to an anaerobic state. This is followed by transformation of glycogen into lactic acid and the consequent reduction in pH and activation of different proteolytic reactions catalyzed by endogenous enzymes, which produce nutrients that promote subsequent bacterial proliferation. *Post mortem* tenderization is one of the most unfavourable quality changes in fish muscle and contrasts with mammalian and avian meats in which *post mortem* degradation of myofibrillar and cytoskeletal proteins is often desired to obtain a tender product.

Previous studies on fish have investigated *post mortem* phenomena using chemical, physical, histological, and microbiological methods by focusing on indicators such as pH, lactic acid, adenine nucleotides and their degradation products, texture, firmness, and elasticity. Only few studies in the current literature have included a combination of molecular and proteomic investigation. Therefore the objective of the present Thesis was to investigate by molecular biology and proteomic techniques on how different methods of slaughtering and *post mortem* storage conditions influence the freshness quality of fish filet. The target species was cultured European sea bass (*Dicentrarchus labrax*) which is a marine teleost of great interest for Mediterranean aquaculture, representing an excellent fishfood product of high commercial value.

Firstly, microfluidic capillary electrophoresis and real-time PCR were successfully applied to investigate the *post mortem* alterations in RNA extracted from sea bass muscular tissue in relation to three parameters: slaughtering method, and *post mortem* time and storage temperature (Chapter 3). In the course of this study,

we first identified the sea bass cDNA sequences coding for endogenous proteases such as calpains and cathepsins which are assumed to play a major role in *post mortem* degradation of fish muscle. Then we determined the total RNA and μ-calpain- and cathepsin-L-specific mRNAs integrity over 5 days of storage at two different temperatures (1°C and 18°C) in sea bass slaughtered by three different methods (asphyxia in air, hypothermia/asphyxia in ice, and spinal cord transection). The results of this study showed that, RNA degradation is a slow process under the conditions investigated and, although *post mortem* storage temperature negatively affects the integrity of total RNA (higher degradation at 18°C), the transcripts of μ-calpain and cathepsin L are present for up to 5 days *post mortem* in the muscle of sea bass stored at either 1°C or 18°C without showing any significant slaughtering method-related degradation rates.

In the light of these results, and in order to shed light on the influence of temperature on the status of sea bass muscle proteins during *post mortem* storage, a 2-D DIGE (2 Dimensional Difference Gel Electoforesis) and mass spectrometry study was performed on fish (Chapter 4). As expected, the greatest alterations in sea bass filet protein composition were observed upon *post mortem* storage at 18°C, with distinct changes appearing in the 2-D protein profile after 5 days of storage at this temperature. In particular, degradation of the myofibrillar protein myosin heavy chain and of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, among the most abundant muscle proteins, could be clearly observed upon storage at higher temperatures. Although to a lesser extent, however, several proteins were observed to vary in abundance also upon storage for 5 days at 1°C. In particular, one of the most interesting observations was the rapid and significant decrease in the abundance of nucleoside diphosphate kinase B and phosphoglycerate mutase 2, which was observed also at low storage temperatures and appeared to be temperature-independent. The results of this study offer new knowledge on changes occurring in sea bass muscle proteins during *post mortem* storage at different temperatures and provide indications on protein degradation trends that might be useful for monitoring freshness of fish and quality of storage conditions.

Thirdly, 2D DIGE and mass spectrometry were applied to investigate the impact of slaughtering on the *post mortem* integrity of muscle tissue proteins in European sea bass (Chapter 5). Three different slaughtering techniques were evaluated: asphyxia in air (AA), asphyxia in ice (AI), and spinal cord severance (SCS). Principal

components analysis (PCA) revealed a significant divergence of SCS samples, whereas AA and AI samples, although grouped separately, were less divergent and could be included in a single asphyxia cluster. In terms of single proteins, the most significant impact was seen on nucleoside diphosphate kinase B, which was consistently less affected when fish were slaughtered by SCS as compared to asphyxia. Integrity of the sarcomeric proteins myosin heavy chain and myosin binding protein C and of the cytosolic proteins fructose biphosphate aldolase, glyceraldehyde 3-phosphate dehydrogenase, and enolase 1 was also better preserved upon SCS slaughtering. Most interestingly, the influence on muscle protein integrity could be detected since the early *post mortem* phase. The results of this study demonstrated that slaughtering by SCS preserves protein integrity better than death by asphyxia, either in ice or in air. Both asphyxia conditions are comparably more adverse than SCS to muscle protein integrity, although a general trend favoring AI over AA is observed.

The last objective of this Thesis was to realize a proteome map of the sea bass muscle (Chapter 6) for use in traceability and species authenticity studies, and as a reference for identification of possible freshness and quality markers.

In the last study of this Thesis, the proteome profile of European sea bass (*D.labrax*) muscle was analyzed using 2-DE and tandem mass spectrometry (MS/MS) with the aim of providing a more detailed characterization of its specific protein expression profile. A highly populated and well resolved 2-DE map of the sea bass muscle tissue was generated, and the corresponding protein identity was provided for a total of 54 abundant protein spots. Upon Ingenuity Pathway Analysis, the proteins mapped in the sea bass muscle profile were mostly related to glycolysis and to the muscle myofibril structure, together with other biological activities crucial to fish muscle metabolism and contraction, and therefore to fish locomotor performance. The data obtained in this study provide important and novel information on the sea bass muscle tissue-specific protein expression, which can be useful for future studies aimed to improve seafood traceability, food safety/risk management and authentication analysis.

In conclusion, the results of this Thesis indicate that, storage conditions are important for *post mortem* deterioration of fish muscle, and temperature is one of the factors with the strongest impact on this process.

Pre-slaughter and slaughter stressful practices can have an important effect on the fish flesh quality. Therefore, the reduction of stress at slaughter might be a satisfactory strategy for both animal welfare and product quality.

The molecular and proteomic methods used in this Thesis helped to identify nucleoside diphosphate kinase B and phosphoglycerate mutase 2 as candidate biomarkers for the evaluation of sea bass fillet freshness quality.

List of Abbreviation

2D- DIGE	2D-Fluorescence Difference Gel Electrophoresis
2-DE	Two-Dimensional Gel Electrophoresis
AA	Asphyxia in Air
AD	Death by Asphyxiation
ADP	Adenosine Disphosphate
AEC	Adenylate Energy Charge
AI	Asphyxia in Ice
AMP	Adenosine Monophospshate
ANOVA	One-way Analysis of Variance
APC	Aerobic Plate Counts
ATP	Adenosine 5-Triphosphate
BVA	Biological Variation Analysis
CBB	Coomassie Brilliant Blu
cDNA	Complementary Desoxiribonucleic Acid
CHAPS	3-((3-cholamidopropyl)dimethylammonio)-1-PropaneSulfonate
Cy	Cyanine dyes
DIA	Differential In-Gel Analysis
DMA	Dimethylamina
dNTPs	DeoxyNucleotide TriPphosphate
DTT	Dithiothreitol
dTTP	DeoxyThimidine TriPhosphate

dUTP	DeoxyUridine TriPhosphate
EEG	Electrocardiogram
ESI	ElectroSpray Ionization
ESI-MS	Electrospray Ionization/Mass Spectrometry
ESI-QTOF	Electrospray Ionization – Quadrupole Time Of Flight Hybrid
FBA	Fructose-Bisphosphate Aldolase
FDR	False Discovery Rate
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
HAS	Humane Slaughter Award
HD	Death from Hypothermia
HPLC	High-Performance Liquid Chromatography
HPI	Hypothalamic-Pituitary-Interrenal axis
Hx	Hypoxanthine
IEF	Isoelectrofocalisation
IMP	Inosine Monosphospate
Ino	Inosine
IPA	Ingenuity Systems Pathway Analysis
IPG	Immobilized pH Gradients
LC-MS/MS	Liquid chromatography–mass spectrometry
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization Time of Flight
MAPC	Mesophilic Aerobic Plate Count
MbpC	Myosin Binding Protein C
MHC	Myosin Heavy Chain
MLC 2	Myosin light chain 2

M-MLVRT	Moloney muvine leukemia virus
Mr	Molecular Weight
MS	Mass Spectrometry
NDP kinase B	Nucleoside DiPhosphate kinase B
NHS	N-Hydroxysuccinimidyl
PCA	Principal Components Analysis
PCR	Polymerase Chain Reaction
PFF	Peptide Fragment Fingerprinting
Pi	Isoelectric Point
PMF	Peptide Mass Fingerprint
PMT	PhotoMultiplier Tube
PVN	Hypothalamic Paraventricular Nucleus
QIM	Quality Index Method
Real-time RT-PCR	Real time Reverse Transcription PCR
RNA	Ribonucleic Acid
SCD	Death by Severing the Spinal Cord
SCS	Spinal Cord Severance
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis
SPS	Sulphite-Polymyxin-Sulfadiazine
SSMPC	Specific Spoilage Microorganism Plate Count
T0	Time of Death
TMA	Trimethylamine

TMAO	Trimethylamine-Oxide
TVB-N	Total Volatile Basic Nitrogen
TVC	Total Viable Counts
UNG	Uracil-N-Glucosylate

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Chapter 1

General Introduction

1.1 Aquaculture production

By year 2050, the world will have an additional 2 billion people to feed. Since 1990, the global increases in fish production up to present-day levels have been achieved entirely through aquaculture production. Maintaining this growth in future will depend upon development and dissemination of specialized species and varieties adapted for low cost pond culture and low proteins diets (Pickering, 2011; Gemmell, 2001).

A major challenge in the future will not only be to adequately feed the burgeoning population of the world but also to improve the quality of life for those people living in poverty. To meet that challenge requires improvements in food security and in the economic status of many developing countries (Diana, 2009). Aquaculture is often seen as potentially having an effect on the biodiversity through introduction of exotic species, escapes of selectively bred species or the impact on the wider environment through the release of wastes. Conversely, carefully managed aquaculture may enable an increase in the biodiversity of a particular area or ecosystem (Lane et al., 2005).

The fish farming industry has grown at a rate of approximately 8% per year since the mid-1980s (FAO, 2006). Globally, the average annual per-capita fish and fishery products consumption was 16.4 kg from 2003 to 2005 (FAO, 2008) and could increase to as much as 22.5 kg by 2030 (FAO, 2002). As consumption has outpaced the growth of the world’s human population since the 1960s (World Health Organization, 2007), the world’s fisheries are unlikely to satisfy the marketplace. As such, “the seafood industry is beginning to shift from wild harvest to aquaculture, the production of aquatic plants and animals under grower-controlled conditions”(Harvey, 2004).

However, an intensive production of seafood today has determined a more detailed information on the quality of the raw materials and products (Martinsdòttir et al., 2003).

Fish quality is a complex concept involving a whole range of factors which for the consumer include for example: safety, nutritional quality, availability, convenience and integrity, freshness, eating quality and the obvious physical attributes of the species, size and product type (Bisogni et al., 1987,; Botta, 1995; Oehlenschlager et al., 1998; Bremner, 2000). Information about handling, processing and storage techniques, including time/temperature histories that can affect the freshness and quality of the products is very important for the partners in the chain (Abbas et al., 2008). Additionally, seasonal condition, that affect the fishing grounds and capture methods and the occurrence of various quality defects influence the overall quality (Abbas et al., 2008).

One of the most unique characteristics of fish as food is that it is a highly perishable commodity. Consequently time passed after catch and the temperature “history” of fish is very often the key factor determining the final quality characteristics of fish products (Olafsdottir et al., 2004).

1.2 Fish Quality

Quality is one of the most abused words in food science and especially fisheries and aquaculture research (Bremner, 2000). Nowadays, fish fresh quality is an indispensable factor for marketing. It is interesting to note that quality and welfare issues are intrinsically linked as there is evidence that inadequate fish husbandry produces lower meat quality (Huss, 1988; Ribas et al., 2007). It will refer to attributes of the flesh (muscle) which are considered to increase the attraction of the product to the consumer.

Flesh quality in fish is determined by various factors, including husbandry practices, diet, handling at slaughter, the choice of slaughtering method, and *post mortem* handling (Terova et al.,2011; Ruff et al., 2002).

Sensory assessment has always played a key role in quality and freshness evaluation in the fish industry (Abbas et al., 2008). The various sensory characteristics, such as outer appearance, odor and colour are still very important in the quality systems in the fish processing industry (Abbas et al., 2008).

Freshness makes a major contribution to the quality of fish or fishery products (Figure 1.1).

The state of freshness can be described by a variety of definite properties of the fish which can be assessed by various indicators (Raatikainen et al., 2005). These properties, and thus the freshness and quality of the end product, are dependent on different biological and processing factors that influence the degree of various physical, chemical, biochemical and microbiological changes occurring *post mortem* in fish (Huss, 1995; Botta, 1995; Olafsdottir et al., 2004).

Figure 1.1 The relationship between quality and freshness

1.3 Factors that influence the freshness

Under farming conditions, fish quality is known to be influenced by extrinsic factors such as feeding strategies, and diet composition (Izquierdo et al., 2005; Morris et al., 2005; Mourente et al., 2006), as well as pre- and post slaughter handling procedures (Ozogul et al., 2007; Robb et al., 2000; Skjervold et al., 1999). All these factors have an impact on the composition, structure and metabolic characteristics of the muscle tissue (Grigorakis et al., 2005; Johnston, 1999; Mourente et al., 2006). Although the biochemical and physiological processes that occur *post mortem* are fairly well known in fish (Poli et al., 2005; Verres Bagnis et al., 2002), several studies emphasize the need to correlate changes in the pre-and post-slaughter procedures to the consequent biochemical and physiological changes of the organoleptic characteristic of fish, namely texture, flavor and aroma (Ayala et al., 2005; Grigorakis et al., 2005; Periago et al., 2005).

Therefore, investigation on the freshness quality during handling, distribution, and storage of fish in ice are of considerable interest (Scherer et al., 2004).

1.3.1. Pre-slaughter management practices

Stress during capture may influence the animal's physiological reactions and consequently the *post mortem* biochemical processes related to the conversion of muscle to meat, affecting in this way the quality and conservation of the final product (Parisi et al., 2001; Thomas et al., 1999; Sigholt et al. 1997; Marx et al., 1997).

Increased stocking density, handling, disturbance prior to stunning, and slaughter suffering are unavoidably associated with a degree of stress, particularly if it provokes scaling, external abrasion, and protracted agony in the fish (Pankhurst et al., 1992; Tobiassen et al., 1999). In this regard, a higher oxygen level during the crowding condition prior to slaughter, reduce stress (Poli et al., 2005).

Indeed, high density and repeated catching produced different stressful effects and differences in *rigor mortis* phases such as a decrease in *pre-rigor*, a faster decrease in contraction force and a decrease in freshness (Poli et al., 2005).

1.3.2 *Rigor mortis*

Rigor mortis is one of the first *post mortem* changes and it has a major impact on the flesh quality of a fish. When fish is died, muscles are wholly relaxed, but as ATP (adenosine 5-

triphosphate) content decreases below a critical level, actin and myosin make an irreversible bond (acto-myosin) and muscle enters *rigor mortis* (Iwamoto et al., 1987). After some time, fish muscle enters into a process of tenderization, possibly related to the degradation of connective tissue (Ando et al., 1993) and breakage of Z-discs of myofibrils (Seki et al., 1991) and acto-myosin junctions (Yamanoue et al., 1988).

The *rigor mortis* is influenced by the handling procedures before slaughter and the method of slaughtering used (Sikorski et al., 1990). Therefore, an animal that struggles at slaughter goes into rigor very rapidly (Ruff et al., 2002).

Minimal stress for animal results in a slower onset of *rigor mortis* and in a less intense rigor, which helps to prevent undesirable fillet texture problems such as “gaping” (Robb, 2001). Therefore, delayed onset of *rigor* indicates that fish has been handled and treated correctly, thereby promoting high flesh quality (Jerrett et al., 1998) and resulting in higher premiums being demanded for greater quality (Ruff et al., 2002). A delayed time period before *rigor* as well as a long-lasting *rigor* ensures that the fish remains fresh for a long time, as degradative processes only set in with the resolution of *rigor* (Sikorski et al., 1990). Stress and exercise are linked to a reduction of fish quality and this is mainly associated with pre-slaughter stress (Pottinger, 2001). Therefore, choosing the most suitable slaughter method is an important step for assuring a good quality of fish products (Scherer et al., 2005).

1.3.3 Killing methods

Killing methods should result in a rapid and irreversible loss of consciousness (Bagni et al., 2007). When fish are killed rapidly, stress can be reduced, thus improving both welfare and quality (Ottera et al., 2001). Low muscle activity at slaughter has been reported to be obtained by applying killing methods which leave fish immediately insensitive, such as the spiking of the brain (Boyd et al., 1984), a blow to the head (Kestin et al., 1995; Ottera et al., 2001), and the destruction of the spinal cord (Mochizuki et al., 1994).

Indeed, various methods are used to stun and slaughter fish with differing impacts on the animal's welfare. The most common techniques include the followings.

1.3.3.1 Asphyxia in Air

Asphyxia, traditionally used for captured fish, consists in leaving fish to die out of the water. This is the oldest fish slaughtering method and it is extremely aversive. Fish often show violent escape behaviors accompanied by maximum stress response (Robb et al., 2002). When

fish are taken out of water, their gills collapse, preventing oxygen exchange with the environment (Robb et al., 2002).

Fish killed by asphyxia had higher lactate and lower pH, ATP and adenylate energy charge (AEC) in tissues, and an earlier *rigor* onset in comparison with those killed by spiking and stunned by percussion (Poli et al., 2004):

1.3.3.2 Asphyxia in Ice and Live Chilling

Fish may also be asphyxiated by transferring them from water into:

i) ice flakes (solid ice);

ii) liquid ice (also known as super cold water at temperature ranging from -2.3 to -2.8°C);

ii) ice-water slurry (ice flakes and water mixed in a ration ranging from 1:2 to 3:1);

These methods are commonly used in different farmed species such as rainbow trout, gilthead sea bream, sea bass, barramundi, and channel catfish. Intensive rapid cooling causes muscle paralysis. Behavioral indices used to measure aversion cannot be used; however, rapid cooling has been clearly shown to initiate a stress response (Skjervold et al., 2001).

Similar to the method of asphyxiation in ice, live chilling involves immerging fish in chilled water with the intentions of causing fish either to become torpid or stunned before slaughter (Roth et al., 2006, Erikson et al., 2006). This method of cooling muscle immobilizes the animals so that they can be more easily handled (Roth et al., 2006). Live chilling is considered by the aquaculture industry to offer benefits to carcass quality since reducing muscle temperature close to 0°C it eliminates significant thermal energy that would otherwise influence the muscle degradation process that begins soon after death. This method also increases both the time for onset of *rigor mortis* and resolution of *rigor* (Skjervold et al., 2001). The efficiency of hypothermia for killing fish may depend on the temperature of the rearing water and be higher in species acclimated to relatively warm water, such as Mediterranean fish (Acerete at al., 2004)). When the differenze between the ambient temperature of fish and the ice slurry is relatively great, thermal shock may shorten the time of brain function loss (Robb et al., 2002). Loss of brain function due to cooling can be reversed if the fish are removed from the cold water too soon.

1.3.3.3 Carbon-Dioxide Stunning

Commonly used as a stunning method, water saturation with carbon dioxide creates an acidic and hypoxic environment that eventually leads to narcosis. In response to this treatment,

fish have been reported to show aversive behavior and fight reactions (Robb and Kestin, 2002; Southgate and Wall, 2001; Roth et al., 2002, Poli et al., 2005). It was observed that fish were not rendered unconscious immediately and the animals moved vigorously during the application (Robb et al., 2000). In addition to these behavioral responses, some fish such as rainbow trout, carp, and eels also increase mucus production, a possible sign of stress (Conte, 2004; Shephard, 1994) during carbon-dioxide narcosis (Erikson et al., 2006). The fish are customarily removed once movement stops, typically after 2-3 minutes (European Food Safety Authority, 2004).

Although carbon-dioxide stunning is considered an unacceptable method of slaughtering by the HSA, well-run systems leave fish in carbon dioxide-saturated water for a minimum of ten minutes in order to induce unconsciousness (HAS, 2005).

1.3.3.4 Live Chilling with Carbon-Dioxide Stunning

Live chilling at temperatures customarily used by the aquaculture industry may immobilize fish, yet may not induce unconsciousness (Roth et al., 2006). Thus, live-chilled fish may be fully conscious when their gills are cut (Has, 2005)

As carbon dioxide-saturated water causes extreme aversive behavior, chilling first reduces negative reactions by the animals when they are exposed to the gas (Yue, 2010). As such, this dual method may be considered by some to be a more humane way to induce the desired state of hypercapnìa in fish before gill-bleeding (Robb and Roth, 2003).

1.3.3.5 Bleeding without Prior Stunning

This method typically entails removing fully conscious fish from water, manually restraining them, inserting a sharp knife under their opercula, and severing all four arches on one side of their head (Robb et al., 2000). Alternatively, the heart may be pierced, isthmus cut with a knife or the blood vessels in the tail severed (European Food Safety Authority, 2004).

The Scientific Panel of Animal Health and Welfare of the European Food Safety Authority and other have stated that exsanguinations without stunning are inhumane and should not be used for slaughter (EFSA, 2004; Van de Vis et al., 2003). Fish behavior and brain function measures have indeed shown that it is a slow method of slaughtering as fish are not rendered immediately insensible (Kestin et al., 2002).

1.3.3.6 Percussive Stunning

With percussive stunning, fish are rapidly struck on the head, resulting in violent movement of the brain within the skull, causing concussion and cerebral dysfunction (Van de Vis et al., 2003). This method renders fish unconscious immediately and irreversibly if sufficient force is applied to the correct part of the head (HAS, 2005). In the occasion that a fish regains consciousness due to an improper stun or there is any uncertainly whether the stun was effective, the fish should be re-stunning immediately (HAS, 2005).

Innovations are in development that remove the human handling aspect and involve methods that do not require removing fish from water at all. New designs encourage fish to swim along to apparatus (EFSA, 2004), which would improve animal welfare by reducing if not eliminating stress caused by handling. The researches posited that EEG electrodes recording brain activity may have hindered correct application of the blow, preventing immediate loss of brain function (Van de Vis et al., 2003). Lambooij et al., (2003) concluded through EEG readings that captive bolt stunning almost certainly eliminates pain perception and is therefore an effective and humane method of slaughter.

1.3.3.7 Electrical Stunning and Killing

Stunning by use of electricity is known as electronarcosis, whereas killing by using electricity is known as electrocution. Depending on the electrical parameters, such as voltage, frequency and duration, either outcome can be induced. Electric stunning is reversible as normal brain function is disrupted for a short period only; hence electronarcosis must be immediately followed by bleeding before the animal can recover from the stun and regain consciousness. Electrocution, on the other hand, completely destroys brain function and therefore renders the animal unconscious while stopping the breathing reflex from functioning (HAS, 2005). Similarly, if the stun duration is too short, fish can quickly regain consciousness (Lines et al., 2003). Therefore, along with water factors, such as conductivity and temperature (Has, 2005), each of these three electrical parameters must be appropriately managed in order to ensure a proper stun (Robb et al., 2002).

When fish are subjected to a poor stun and rendered analyzed instead of insensible, they cannot express pain or show avoidance behavior and risk being bled while conscious (Yue, 2010).

The electric method has been shown to induce irreversible unconsciousness with a 1 second application of correctly selected electrical parameters (Kestin et al., 1995; Lines et al., 2003); thus

its instantaneous nature has appeal from a humane slaughter perspective. On the other hand, as quick as the application may be, fish have been reported to show violent behavioral reactions, muscle blood spots and fractured vertebrae when subjected to electricity (Poli et al., 2005).

Another advantage of a well-designed and operated electrical system is that it can eliminate or significantly reduce both stressful pre-slaughter handling and the need for removal from water (EFSA 2004). With the electrical system, large numbers of fish can be slaughtered and processed with minimal handling (Robb and Roth, 2003; EFSA, 2004).

1.3.3.8 Pre-slaughter Sedation with Anesthetic

The sedation calms the pre-slaughter stress response in fish, but does not stun or kill the animals (Yue, 2010). After the fish are sedated, they must than be properly stunned and/or killed. The sedative effect does not appear to be stressful to most fish, and sedated fish show significantly less distress when they are removed from eater fro stunning (EFSA, 2004).

1.4 Evaluation of freshness and quality

Due to the growing demand of seafood, the development of protocols for assurance and control of seafood safety is currently a major challenge in fisheries and aquaculture (FAO, 2009). Different methods can be applied at different steps of the fish processing depending on purpose, definition and suitability of different methods.

Sensory methods are widely used in the food industry to judge different quality attributes of raw materials, ingredients and final products (Luten et al., 2003). Sensory analysis has the advantages of being fast, measure several important attributes the same time, relatively accurate in the hands of trained operators, and often it is non-destructive (Luten et al., 2003).

The methods for evaluation of fresh fish quality may be conveniently divided into sensory, microbiological, chemical, biochemical, and other instrumental methods.

1.4.1 Sensory methods

Sensory evaluation is defined as the scientific discipline used to evoke, measure, analyze and interpret reactions to characteristics of food as perceived through the senses of sight, smell, taste, touch and hearing.

In sensory analysis appearance, odour, flavour and texture for whole fish are evaluated using the human senses.

A new method, the Quality Index Method (QIM) originally developed by the Tasmanian Food Research unit, is now used for fresh and frozen cod, herring, redfish, sardines and flounder.

QIM is based on the significant sensory parameters for raw fish when using many parameters and a score system from 0 to 4 demerit points. QIM is using a practical rating system, in which the fish is inspected and the fitting demerit point is recorded. The scores for all the characteristics are then summed to give an overall sensory score, the so-called quality index. QIM gives scores of zero for very fresh fish while increasingly larger totals result as fish deteriorate (Table 1.1). After the literal description, the scores are ranked for each description for all the parameters, giving scores 0-1, 0-2, 0-3 or 0-4. Parameters with less importance are given lower scores. The individual scores never exceed 4, so no parameter can excessively unbalance the score.

Quality parameter		**Description**	**Score**
Appearance	Skin (*Both dark and white side*)	Fresh, bright, metallic, no discolouration	0
		Bright, but without shine	1
	Mucus	Mat, rather dull, slight green/blue or purple discolouration	2
		Dull, green/blue, purple discolouration	3
		Clear, not clotted	0
		Slightly clotted and milky	1
		Clotted and slightly yellow	2
		Yellow and clotted	3
Eyes	Form	Convex	0
		Convex but slightly sunken	1
		Flat or swollen (like a ballon)	2
		Flat, sunken in the middle	3
	Brightness	Clear, black shining pupil	0
		Rather mat, black pupil	1
		Mat, opaque pupil	2
		Milky, grey pupil	3
Gills	Odour	Fresh oil, seeweedy, metallic, peppery	0
		Neutral, oily, grassy, slightly musty	1
		Musty, bread, beer, malt, slightly rancid	2
		Rancid, sour, rotten, sulphurous	3
	Colour	Bright, light red	0
		Slightly discoloured, especially at the end of gill filaments	1
		Discoloured	2
		Yellowish, brown, grey	3
	Mucus	No mucus	0
		Clear	1
		Yellowish, slightly clotted	2
		Yellow, brown, clotted	3
Flesh, fillets	Colour	Fresh, translucent, bluish	0
		Waxy, milky	1
		Dull, slightly discoloured, yellowish	2
		Opaque, discoloured, yellow, brown	3

Quality Index	0-24

Table 1.1. Quality Index Method (QIM) scheme for plaice

QIM is primarily used in the evaluation of whole and gutted fish. The method is difficult to use with fish fillets and schemes for lightly preserved seafoods are not yet available. QIM is a promising sensory method and the practical usefulness of this tool would be further increased if new schemes applicable for groups of fish species or products could be developed. It also needs to be noted that different schemes provide different score-values corresponding to the end of product shelf life (Table 1.1) (Martinsdòttir et al., 2003). QIM can be used to estimate shelf life and remaining shelf life. Shelf life is defined as the time period a food product is fit for human consumption. With whole, gutted fish it represents the time from catch until it becomes unfit for human consumption. Shelf life of fillets or other fish products can also been defined as shelf life from processing or packaging. Predicted storage time in ice is defined as the number of days that the fish has been stored in ice. An estimate can be calculated for the remaining shelf life. It should be emphasized that remaining shelf life should be used with some precaution due to the uncertainty in its estimation. Various factors can affect the remaining shelf life such as fish handling rapid cooling after the catch and uninterrupted cold storage, different fishing gear, bleeding and gutting methods, season, and catching ground.

1.4.2 Microbiological methods

Bacteria naturally exist on the skin, gills, and in the gut but they are not able to cause spoilage because of the natural defensive mechanism of healthy living fish. After slaughtering of fish, bacteria find access into the fish flesh not only due to the lost sterility, but also because of the various mechanical and autolytic changes that rupture gut walls and soften fish flesh, making it easy for bacteria to access fish tissues. Indeed, microbial activity is responsible for the spoilage of most fresh and of several lightly preserved seafoods. Possibility for this reason, the total number of microorganisms, named total viable counts (TVC) or aerobic plate counts (APC), have been used in mandatory seafood standards in some European Countries in Japan and in the USA.

Fish bacterial flora is composed mainly of psychrotrophic bacteria, micro-organism that are capable of surviving or even thriving in a cold environment. Although a wide variety of spoilage microorganisms can contaminate seafood, a limited number of analysis techniques may serve to control its microbiological quality. Mainly psychrotrophic aerobes, lactic acid and related bacteria

analyses might serve to control seafood spoiling, coliforms and streptococci analysis might serve to verify eventual faecal exposure. The most found foodborne pathogen microorganisms are Vibro spp., *Clostridium botulinum* and *Listeria monocytogenes*, whereas all other pathogenic microorganisms such as *E. coli* and Salmonella spp, have their origin in the human activity, being the mostly found between them (Amarita, 2007).

Parameters like **mesophilic aerobic plate counts (MAPC)** have been used broadly as an indicator of food quality. MAPC is related to all micro-organisms capable of growing at 30°C, being spoilage or non-spoilage micro-organisms.

Specific spoilage microorganism plate count (SSMPC) could be a better indicator of food quality, and thus, depending on the nature of the attended bacterial flora these microorganisms might be evaluated by growth at 20°C up to four days or at 30°C up to two days.

***E. coli* and/or coliforms bacteria analysis** may be used to verify counts of this specific group.

Faecal streptococci analysis may be employed as a good indicator for hygiene and appropriate handling of frozen food.

Salmonella spp. and Shigella spp. can be analyzed by spotting food samples in the selective **Salmonella-Shigella agar plates**, after several preparative steps in broth for its concentration. Presumptive Salmonella and Shigella will grow at 37°C as colorless colonies.

Clostridia may be analyzed by anaerobic growth in **sulphite-polymyxin-sulfadiazine agar (SPS)**, they will form black colonies in 24 h at 37°C.

Listeria monocytogenes may be analyzed by one of the several methods offered by culture media producers, as **RAPID'L.**

Vibrio spp. may become a serious problem in seafood derived from warm waters. This genus can be detected by using **TCBS agar**.

1.4.3 Chemical, biochemical and other instrumental methods

During *post mortem* storage microbiological spoilage causes the formation of volatile bases, which can be determined to measure indirectly the freshness quality of such seafood. There are a few substances that are usually determined in order to evaluate fish raw material freshness.

TVB-N, TMA and other volatile amines: total volatile basic nitrogen (TVB-N) primarily includes trimethylamine (TMA), ammonia, and dimethylamina (DMA).

The TVB-N remains constant for the first days of storage or increases slowly but it rises fast later in the spoilage process. Therefore TVB-N is a very good indicator of spoilage in fish (Oehlenschlager, 1992). The TVB-N determination is not as good to detect the early stages of deterioration of freshness quality as the TMA measurement, but it can be used for measuring later stages of deterioration (Botta, 1995).

Critical limits of 25, 30 and 35 mg-TVB-N/100g were established for different groups of fish species.

TMA is a microbial metabolite and it can only be used as an index of spoilage and not as an index of freshness. Development of TMA in seafood depends primarily on the content of the trimethylamine-oxide (TMAO) in the fish raw material.

In seafood with high contents of free amino acids, e.g. squid, crustaceans and some dark fleshed fish like herring, substantial amounts of ammonia can be formed during chill storage. This is a slow process but DMA can be a useful index of spoilage.

Many seafood spoilage bacteria produce one or more of the biogenic amined agmatine, cadaverine, histamine, putrescine, spermidine, spermine, and tyramine. Biogenic amines are heat stable, and are therefore appropriate for the evaluation of freshness of the raw material used in canned products.

Production of biogenic amines in seafood depends on the concentration of the free amino acid substrates and is, therefore, strongly species-dependent. A quality index was suggested and validated for grading of determined species e.g. tuna, rockfish, salmon.

$$\text{Quality index} = \frac{\text{mg/kg histamine} + \text{mg/kg putrescine} + \text{mg/kg cadaverine}}{1 + \text{mg/kg spermidine} + \text{mg/kg spermine}}$$

The practical importance of quality indices relying on biogenic amines will increase substantially if such biosensors could replace the more cumbersome chromatographic techniques previously used for the detection of these amines.

Adenosine triphosphate (ATP) is degraded into adenosine disphosphate (ADP), adenosine monophospshate (AMP), inosine monosphospate (IMP), inosine (Ino) and hypoxanthine (Hx) during processing and storage of fresh and lightly preserved seafood. IMP is formed by autolytic enzymes whereas spoilage bacteria contribute to Ino and Hx formation. Hx has a bitter taste which may be part of the off-flavour in stale fish.

The K-value was suggested by Japanese research in 1959 as an objective index of fish freshness. In most fish, K-values increase linearly during the first days of chilled storage and it is often and excellent index of freshness. However, the K-value cannot be used in general as an index of spoilage because maximum values, for many fish species, are reached long before sensory rejection.

$$\text{K-value} = \frac{[Ino] + [Hx]}{[ATP] + [ADP] + [AMP] + [IMP] + [Ino] + [Hx]} \times 100$$

Ratios of catabolites like the K-value have been indicated to be less prone to fish-to-fish or species-to-species variability than single compound quality indices.

$$K_i\text{-value} = \frac{([Ino] + [Hx]) \times 100}{[IMP] + [Ino] + [Hx]}$$

The K_i-value is quantitatively similar to the K-value, but it reduces this problem.

Whereas, Hx-index alone has also been suggested to be an useful quality index in specific seafoods.

$$\text{Hx-index} = \log [(Hx) + 5]$$

Concentration of the adenine nucleotides required for the calculation of the K- and K_i-value can be determined in seafood by chromatographic methods.

On the basis of numerous volatile compounds, determined by gas chromatography, it has been possible to grade fresh fish products in agreement with sensory analyses. Recent results further suggest that multivariate statistical methods allow identification of a limited number of the most important compounds. These volatile compounds can then be determined by visual spoilage

indicators or electronic noses and in this way be used as indicators of seafood spoilage. The measurements require little sample preparation and the time of analysis is relatively short, approximately 10-90 min. Different gas sensors including electrochemical, metal oxide and organic polymers are available. Responses of electrochemical and conducting polymer sensors have been shown to correlate with other objective measures of quality in fresh seafood. However, the stability of correlation between sensory data and electronic nose response still represents a problem for practical application of gas sensors in seafood shelf life evaluation.

1.4.4 Other instrumental methods

Measurements of dielectric properties have been tested and used for almost 40 years for quality grading and remaining shelf life determination of various fish. The Intellectron Fishtester, the Torrymeter and RT-Freshtester represent instruments with increasing degrees of sophistication. Readings from all instruments reflect di-electical properties of fish which decrease with storage time, almost following a straight line.

Electrical properties of fish are not directly responsible for spoilage and it is, therefore, to be expected that numerous factors influence the relationship between such measurements and seafood spoilage. Indeed, these instruments need calibration depending on the season and fish handling procedures. They are unsuitable for grading frozen/thawed fish, partially frozen fish, i.e. superchilled fish, fish chilled in refrigerated seawater, and for fish fillets. These and the high cost of the instruments limit their practical use in the seafood sector for freshness evaluation. However, electrical measurements can also be used to test if fish was previously frozen.

The use of alternative rapid methods for fish grading in fish production does not only improve scientific knowledge or technical performance but can add value for both producers and consumers in the fish processing chain (Amerongen et al., 2007).

1.4.5 Proteomics technologies

More recently, modern proteomic technologies for understanding striated muscle biology have been successfully used for to give valuable insight into the composition of the raw material; quality involution within the product before, during, and after processing or storage; and the interactions of the proteins with one another, with other food components, or with the human

immune system after consumption (Vilhelmsson et al., 2005) and the discovery of biomarkers for freshness and quality (Fornè et al., 2010).

The changes may be brought about by growth, differentiation, senescence, change in the environment, genetic manipulation, or other events (Pineiro et al., 2003).

In cultured teleosts, such as carp or sea bass, proteomics studies were aimed at determining the effect of confinement conditions or additives on the processing and quality muscle (Martinez et al., 2004; McLean et al., 2007; Monti et al., 2005) or to characterize *post mortem* changes in the muscle of different species under different storage condition (Martinez et al., 2004; Kjærsgard et al., 2006).

1.4.5.1 A state of the art

The concept of the proteome is based on a relatively old technique of protein separation (Han et al., 2008). The conventional 2D-GE (Vercauteren et al., 2004), in combination with advanced mass spectrometric techniques, has facilitated the rapid characterization of thousands of proteins in a single polyacrylamide gel (Kislinger et al., 2006).

1.4.5.2 *Sample preparation*

The first step is usually protein extraction, using an aqueous extraction buffer, which contains detergents, chaotropes, reducing agents and proteases inhibitors. The purpose of this step is to attempt thorough solubilization of all the proteins present in a given bodily fluid, organ, tissue or cell extract. To improve protein separation and identification, they are divided according to their isoelectric point (pI) in a step called isoelectrofocalisation (IEF). This is done with Immobilized pH Gradients (IPG) strips which allow very accurate separation of proteins with high reproducibility.

1.4.5.3 Protein separation and quantification

The two most important analytic techniques in proteomics are two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS). Currently, 2-DE is still, the most common strategy for proteins separation and quantification, enabling the separation, detection and quantification of hundreds of different proteins from a single extract, taking advantage of the fact that the pI of a protein is mostly uncorrelated to its molecular weight.

Classically, detection and quantification methods for 2-DE are usually based on Coomassie Brilliant Blu (CBB) or silver staining, which enable estimation of protein quantity by scanning 2-DE gels in the visible range. Consequently, proteolysis is typically applied to the protein samples and much information that is important to distinguish protein isoforms can be lost in not captured before proteolysis (Timms et al., 2008). Therefore, labeling techniques that can provide a read-out at the protein level with a high dynamic range are good alternatives to Ms-based quantitative methods (Timms et al., 2008).

In 1997, Unlü et al., combined and applied 2-DE and fluorescent tag for quantitative protein analysis and called this new approach 2D- Fluorescence Difference Gel Electrophoresis (DIGE). The fluorescent tags are synthetic N-hydroxysuccinimidyl (NHS) ester derivatives of the cyanine dyes Cy3 and Cy5 (Von Eggeling et al., 2001). These reagents react with primary amine groups (N-terminal α-amino and lysine Ɛ-amino groups) in the target protein sample through a nucleophilic substitution reaction. The samples are typically labeled in denaturing 2-DE lysis buffer at an optimal pH of 8.0-8.5 in the absence of primary amines and commonly used reducing agents, which show reactivity towards the labeling reagents. Equal protein amounts of the differentially labeled samples are then mixed and subjected to 2-DE separation followed by fluorescent imaging/scanning to generate two super-imposable images. Computer-aided image analysis is performed where the signals from labeled protein spots are determined and the normalized intensities or spots are determined and the normalized intensities or spot volume for each spot from Cy3 and Cy5 channels are compared to define differentially expressed protein isoforms between the samples (Timms et al., 2008). Differentially expressed protein spots of interest are then picked from the gel for identification by MS (Figure 1.2).

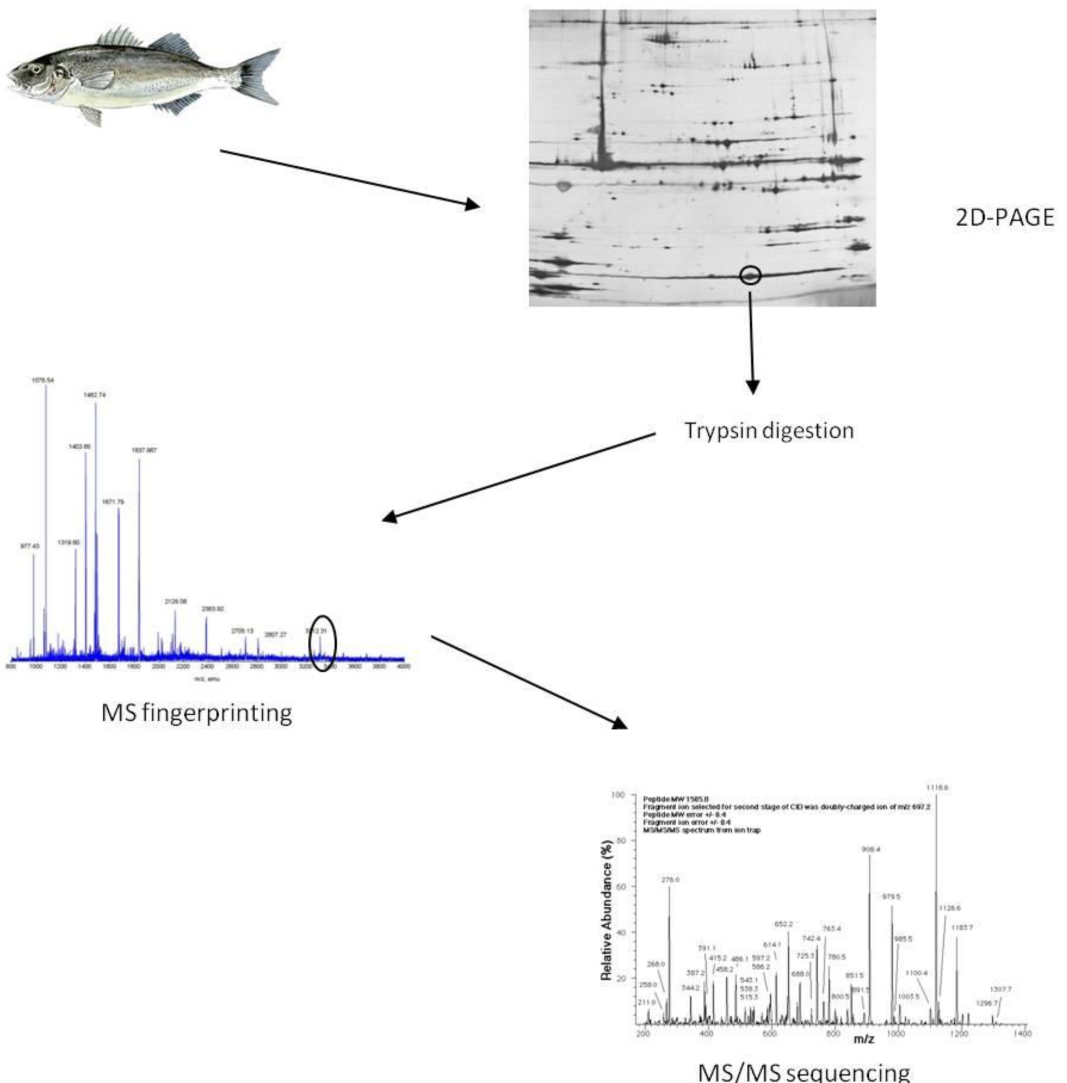
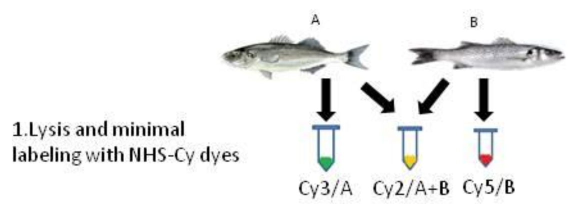


Figure 1.2 - An overview over the "classical approach" in proteomics. First, a protein extract (crude or fractionated) from the tissue of choice is subjected to two-dimensional polyacrylamide gel electrophoresis. Once a protein of interest has been identified, it is excised from the gel and subjected to degradation by trypsin (or other suitable protease). The resulting peptides are analyzed by mass spectrometry, yielding a peptide mass fingerprint. In many cases this is sufficient for identification purposes, but if needed, peptides can be dissociated into smaller fragments, and small partial sequences can be obtained by tandem mass spectrometry.

Importantly, the two fluorophores Cy3 and Cy5 are spectrally distinct (with emission maxima at 590 and 680 nm, respectively), allowing detection of the signal from one sample without appreciable contribution from the other (Timms et al., 2008). A third fluorophores Cy2 used to be simultaneously analyse on a single 2D gel, thus reducing the number of gels that need to be run in an experiment. Introduction of a third dye has also allowed the introduction of an internal standard sample, which can be run on all gels against pairs of test samples (Alban et al., 2003; Gharbi et al., 2002) (Figure 1.3). Use of the internal standard not only improves the accuracy of relative quantitation of protein spots across gels by acting as a loading control, but also aids in spot matching across gels, thus alleviating somewhat the inherent gel-to-gel variation encountered in a multi-gel 2D-DIGE experiment.

This recent improvement of the 2-DE method has been increasing in popularity in aquaculture species proteome studies with some successful results in several species (Unlü et al., 1997; Tang et al., 2008; Stasyk et al., 2007; Lull et al., 2008; Warren eta l., 2008).



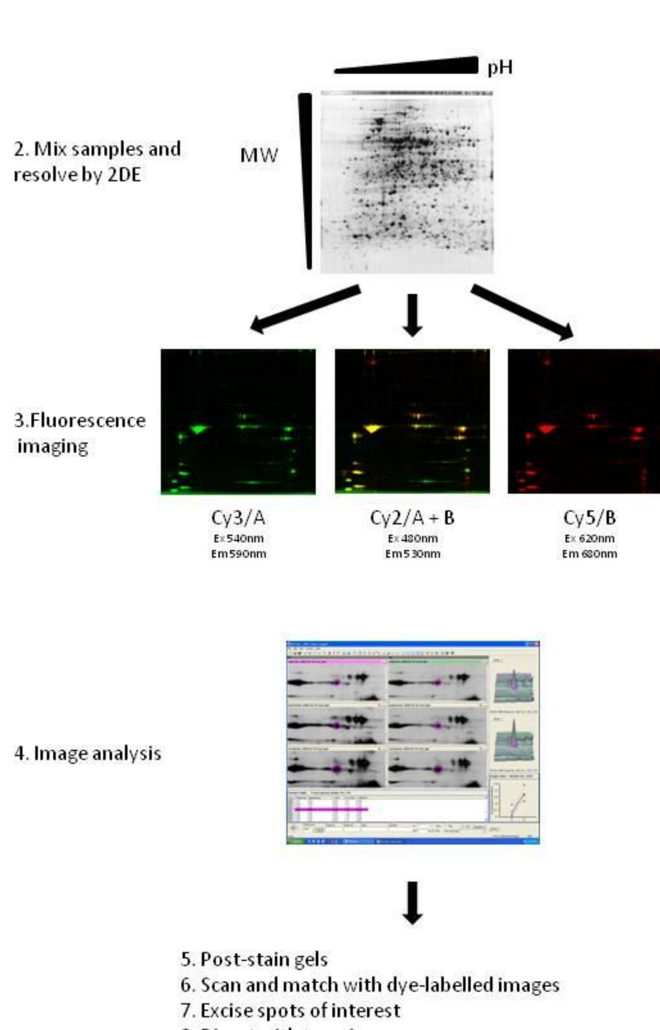


Figure 1.3. Schematic representation of the 2D-DIGE protocol for minimal lysine labeling using an internal standard for normalization and cross gel spot matching.

1.4.5.4 Protein identification and characterization

After computer image analysis, spots (or proteins) of interest can be excised from 2DE-gels and further processed (including trypsin digestion) before their final identification (Rémignon et al., 2006) and characterization by mass spectrometry (MS). Instruments currently employed for this purpose include ESI-MS (Electrospray Ionization/Mass Spectrometry), MALDI-TOF (Matrix-

Assisted Laser Desorption Ionization Time-of-Flight) and ESI-QTOF (CERCARE ACRONIMO) mass spectrometers to a lesser extent (Rodrigues et al., 2012). Identification of proteins can be assessed either directly through its peptide mass fingerprint (PMF), for the case of organisms with fully sequenced genome, or by analysis of the fragmentation spectra of such peptides (PFF, peptide Fragment fingerprinting or even *de novo* sequencing) obtained through tandem MS (Rodrigues et al., 2012).

1.5 Dicentrarchus labrax Linnaeus, 1758 (Moronidae)

From: FAO 2005-2010. Cultured Aquatic Species Information Programme.

The European Sea bass, Dicentrarchus labrax, is of great interest for Mediterranean aquaculture as it is an excellent food fish, with high commercial value; it is often marketed as Mediterranean seabass, branzino, in Northern Italy, or spigola in other parts of Italy.

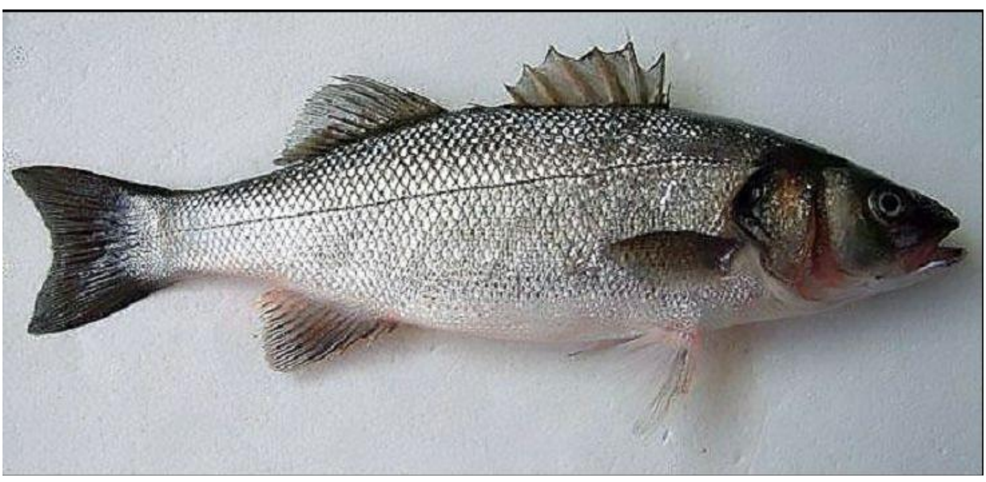


Figure 1.4. *Dicentrarchus labrax*

1.5.1 Features, habitat and biology

Its body is rather elongate; the opercle has two flat spines and the preopercle has large, forward-directed spines on its lower margine. The mouth is terminal and moderately protractile with vomerine teeth in a crescentic band, without a backward extension on midline of roof of mouth. Sea bass has two separate dorsal fins; the first with 8 to 10 spines; the second with 1 spine and 12 or 13 soft rays. The anal fin has 3 spines and 10 to 12 soft rays. The scales are small; the lateral line is complete, but not extending onto caudal fin, that is moderately forked. The colour is silvery

grey to bluish on the back, silvery on the sides, sometimes tinged with yellow on the belly. Young specimens have some dark spots on upper part of body.

The European sea bass are eurythermic (5-28 °C) and euryhaline (3‰ to full strength sea water); thus they are able to frequent coastal inshore waters, and occur in estuaries and brackish water lagoons. Sometimes they venture upstream into freshwater. There is only one breeding season per year, which takes place in winter in the Mediterranean population (December to March), and up to June in Atlantic populations. Sea bass spawn small (1.02-1.39 mm) pelagic eggs in water with salinities lower than 35‰, near to river mouths and estuaries or in littoral areas where the salinity is high (≥30‰). Being not particularly sensitive to low temperature some fish may over-winter in coastal lagoons instead of returning to the open sea. Sea bass are predators and their feeding range includes small fish, prawns, crabs and cuttlefish.

1.5.2 Historical background

Sea bass were historically cultured in coastal lagoons and tidal reservoirs before the race to develop the mass-production of juveniles started in the late 1960s. During that time, France and Italy competed to develop reliable mass-production techniques for juvenile sea bass and, by the late 1970s, these techniques were well enough developed in most Mediterranean countries to provide hundreds of thousands of larvae. The European sea bass was the first marine non-salmonid species to be commercially cultured in Europe and at present is the most important commercial fish widely cultured in Mediterranean areas. Greece, Turkey, Italy, Spain, Croatia and Egypt are the biggest producers.

1.5.3 Production cycle in intensive system

The bulk of sea bass aquaculture production comes from sea cage farming. To secure a reliable and sufficient supply of good quality fish eggs, most hatcheries have established their own broodstock units, where breeders of different age groups are maintained longterm. Parents may come either from a farm or from the wild. The management of captive broodstock in the breeding stations includes natural maturation, the induction of ovulation by photoperiod manipulation or hormonal treatments, fertilisation in spawning tanks and incubation in an open-water circulation system. At the onset of the spawning season it is necessary to move selected batches of breeders from their long term holding facilities to the spawning tanks, where they can be better treated and their performance can be easily monitored. When fertilised eggs are required outside the natural spawning period, out-of-season sexual maturation is obtained by promoting gametogenesis by manipulating the photoperiod and temperature. The hatchery management

decides on the periods of egg production according to its marketing and/or farm needs. Hormonal with human chorionic gonadotropin treatment is used to trigger the last phase of egg maturation.

In intensive production, on growing units are supplied with fry from hatcheries and controlled diet is provided. Juveniles are sold to farmers as on growing stock at a size of 1.5-2.5 g. The on growing juveniles reach 400-450 g in 18-24 months. Cages can be of different kinds but the principle is the same; all types are based on a natural exchange of water through pens. The quality of sites is therefore highly variable, according to local conditions such as tide and current. Tanks are usually supplied with seawater (38‰) maintained in a continuous flow-through system under ambient temperature. High stocking densities are applied (20-35 kg/m^3); this means that accurate control of water quality and careful observations of fish health are essential. A recirculation system, to control water temperature (between 13-18 °C) is used during autumn/winter, frequently full-time in hatchery and the pre-fattening phase of the production cycle; this system is also used for fattening in high technology farms.

This practice improves growth but can be highly expensive due to the required technology for water quality control (filtering, air stripping, UV treatment, catabolite removal). Killing methods should result in rapid and irreversible loss of consciousness. Methods that kill fish rapidly result in a reduction of stress, thus an improvement in welfare and in quality. Prolonged crowding before harvesting is avoided, to ensure high product quality and fish welfare. Greater muscle activity at slaughter leads to a rapid decrease in energy reserves (i.e. adenosine triphosphate, ATP), and to the build up of lactic acid and consequently a drop in post-mortem pH. An animal that struggles at slaughter goes into rigor very rapidly, adversely affecting the quality of fish fillets by softening the muscle texture.

Although a sturdy species, sea bass are subject to a wide range of diseases under rearing conditions. These outbreaks have important effects on commercial production and could prevent the expansion of the industry in some countries. Stress is considered an important factor co-responsible for disease outbreaks; thus improved husbandry is generally suggested to reduce stress. Another problem is the lack of authorized effective therapeutants, particularly for parasites, in most European countries.

1.5.4 Market and trade

One of the largest success stories in European aquaculture has been the Mediterranean sea bass industry, which in less than 15 years grew from a few thousand tonnes to 57 000 tonnes today, having peaked at nearly 71 000 tonnes in 2000. When farmed bass started getting to market in

the late 1980s and early 1990s, the farmed quality was seen to complement the wild species and prices were very high. Prices of the wild product mayhave suffered initially, as the volumes from aquaculture continued growing, but today there is clear distinction in the market between wild and farmed product, with the prices for wild bass several times higher than those of the farmed fish.

Objectives

The objectives of this Thesis were:

1**. To investigate the integrity of total RNA extracted from muscle of sea bass (*Dicentrarchus labrax*) slaughtered with three different techniques and stored for 5 days *post mortem* at two different temperatures of 1°C and 18°C.**

2. To identify potential biological markers enabling the evaluation of fish filet freshness quality under different storage temperatures.

3. To investigate on the effects of different slaughtering methods*,* namely, *asphyxia in air, hypothermia/asphyxia in ice,* and *spinal cord transection*, on *post mortem* proteome changes of the fish fillet and, hence, on its quality.

4. Provide a reference proteome map of the sea bass (*D. labrax*) muscle to be used in traceability and species authenticity studies, and as a reference for identification of possible freshness and quality markers.

Chapter 2

General Material & Methods

2.1. Animals and experimental protocol

The experiment was carried out at the Department of Biotechnology and Molecular Sciences of the University of Insubria (Varese, Italy). One month before slaughter, sea bass (D. labrax) were randomly distributed into three tanks of 1 m3 each, 40 fish per tank, and fed Hendrix-Skretting® Power Excel feed for marine fish. The tanks were connected to a sea water recirculation system under the following water conditions: temperature 20 ± 2 °C, pH 7, total ammonia <0.2 mg/L, and dissolved oxygen over 99% of the saturation.
At the start of the experiment and after 4 days of starvation, 30 fish (average body weight 710 ± 157.87 g) were removed from water and sacrificed in three different ways: 10 (immersion in ice cold water at a fish: ice ratio 2:1) (Figure 2.1A and B), another 10 by asphyxiation (Figure 2.1 C), and the last ones by severing the spinal cord. Fish from the three groups (HD, death from hypothermia; AD, death by asphyxiation; and SCD, death by severing the spinal cord) were then divided into two subgroups of five fish each, which were stored ungutted for up to 5 days p*ost mortem* either in a refrigerator at 1°C or in a thermostat at 18°C.

Figure 2.1: A and B: Fish sacrificed in ice; C: Fish sacrificed in air

The first sampling was performed at the time of death (T0) by taking a fragment of white muscle tissue (2x2x2cm) from the lateral-dorsal muscle quadrant (epiaxial) of each fish. The next six samplings were performed similarly after 2, 4, 24, and 48 hand then after 3 and 5 days of *post mortem* storage at the respective temperatures for a total of 210 muscle samples (Figure2.2A). The muscle fragments were taken alternately from both sides (right and left) of the epiaxial muscular quadrant, leaving a thickness of about 1 cm of tissue between one fragment and the other to avoid contamination (Figure 2.2B).

Furthermore, throughout the trial, fish were all kept flat on the belly to prevent contact with the dorsal walls of the container or with other fish in the group. All 210 muscle fragments taken in this way were immediately frozen at -80 °C and stored at this temperature until the molecular biology analysis.

All procedures were approved by the Animal Care Committee of the University of Insubria and conducted according to the guidelines of the Italian Committee on Animal Care.

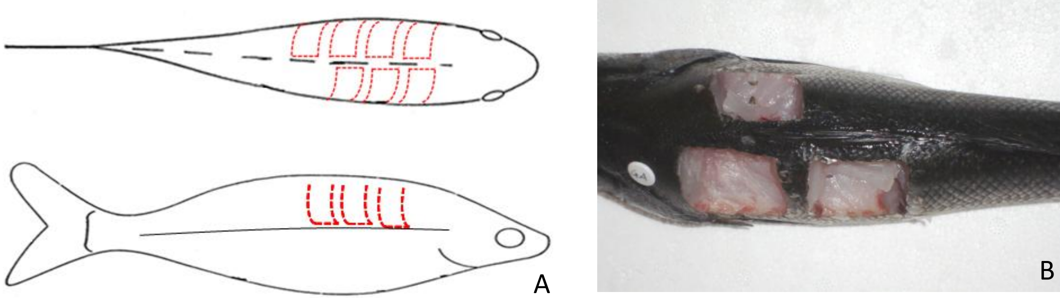


Figure 2.2: A: Schematic representation of the sampling of the muscle; B: Lateral-dorsal muscle section of a fish.

2.2. Quantitative real-time RT-PCR

2.2.1. RNA purification and first strand cDNA synthesis

Molecular biology analysis was carried out at the University of Insubria (Varese, Italy). Total RNA was extracted from sea bass muscle, using PureYield RNA Midiprep System (Promega, Italy). Briefly, 2 ml of ice-cold Lysis Solution containing b-mercaptoethanol were transferred to a 10 ml tube. Tissues of interest were excised, placed in the tube and then homogenized until no visible tissue fragments remained. 2ml of the lysate prepared above were transferred to a 15 ml centrifuge tube, and 4 ml of RNA Dilution Buffer were added. The tube was sealed, mixed thoroughly by inverting it 3-4 times, and vortex. 1ml of thoroughly mixed Clearing Agent was added to the diluted lysate mixture which was then mixed inverting 2-3 times, and vortex until homogeneous. Samples were incubated at 70°C for 5 minutes to denature Tubes were then removed, and cool at room temperature for at least 5 minutes. One blue PureYield™ Clearing Column for each sample was placed in a 50 ml collection tube. Each sample was mixed by vortexing or vigorously shaking until homogeneous and the mixture was immediately poured into the assembled PureYield™ Clearing Column/collection tube. The PureYield™ Clearing Column assembly was then centrifuged in a swinging bucket rotor at 2,000 × g at 22-25°C for 10 minutes to clear the lysate. The blue Clearing Column was discarded, whereas the cleared lysate was saved in the collection tube. For additional information, please see the PureYield™ RNA Midiprep System Technical Manual #TM279, available online at: http://www.promega.com/tbs.

The quantity and purity of RNA was assessed by spectrophotometer. Visualization on 1% agarose gel stained with ethidium bromide showed that RNA was not degraded. After DNAse treatment (Invitrogen, Milan, Italy), 3 µg of total RNA was reverse transcribed into complementary DNA (cDNA) in a volume of 12 µl, including 1 µl of oligo dT16 primer (50 pmol) and 1 µl of 10 mM deoxynucleotide triphosphates (dNTPs). This mix was heated at 65ºC for 5 minutes, chilled on ice and then 4 µl of 5X reverse transcription buffer, 2 µl 0.1M DTT, 1 µl RNAse out and 1 µl of Moloney murine leukemia virus (M-MLVRT) was added. After incubation at 37ºC for 50 minutes, the reaction was stopped by heating at 75ºC for 15 minutes.

2.2.2 Generation of in vitro-transcribed mRNAs for standard curves

The number of each target gene transcript copies could be absolutely quantified by comparing them with a standard graph constructed using the known copy number of mRNAs of each gene. For this, a forward and a reverse primer were designed based on the mRNA sequences of *Dicentrarchus labrax*. These primer pairs were used to create templates for the *in vitro* transcription of mRNAs for each gene. The forward primers were engineered to contain a T7 or a T3 phage polymerase promoter gene sequence to their 5' end and used together with the reverse specific primer in a conventional RT-PCR of total sea bass larvae RNA. RT-PCR products were then checked on a 2.5% agarose gel stained with ethidium bromide, cloned using pGEM®-T Easy cloning vector system (Promega, Milan, Italy) and subsequently sequenced in the SP6 direction.
In vitro transcription was performed using T7 or T3 RNA polymerase and other reagents supplied in the Promega RiboProbe *In Vitro* Transcription System kit according to the manufacturer's protocol.
The molecular weight (MW) of the *in vitro*-transcribed RNAs were calculated according to the following formula:
MW = [(*nº of A bases*) x 329.2) + (*nº of U bases*) x 306.2) + (*nº of C bases*) x 305.2) + (*nº of G bases*) x 345.2)] + 159.
Spectrophotometry at 260 nm gave a concentration of each mRNA. Therefore, the concentration of the final working solutions were calculated and expressed as n° of molecules μl^{-1}.

2.2.3 Generation of standard curves

The mRNAs produced by in vitro transcription were used as quantitative standards in the analysis of experimental samples. Defined amounts of mRNAs at 10-fold dilutions were subjected in triplicates to real-time PCR using one-step TaqMan EZ RT-PCR Core Reagents (Applied Biosystems, Italy), including 1x Taqman buffer, 3 mM MnOAc, 0.3 mM deoxynucleotide triphosphates (dNTP) except deoxythymidine triphosphate (dTTP), 0.6 mM deoxyuridine triphosphate (dUTP), 0.3 µM forward primer, 0.3 µM reverse primer, 0.2 µM FAM-6 (6-carboxyfluorescein-labeled probe), 5 units rTH DNA polymerase, and 0.5 units AmpErase UNG enzyme in a 25 µl reaction. AmpErase® uracil-N-glycosylase (UNG) is a 26-kDa recombinant enzyme encoded by the *Escherichia coli* uracil-N-glycosylase gene. UNG acts on single-and double-stranded dUcontaining DNA. It acts by hydrolyzing uracil-glycosidic bonds at dU-containing DNA sites. The enzyme causes the release of uracil, thereby creating an alkali-sensitive apyrimidic site in the DNA. The enzyme has no activity on RNA or dT-containing DNA.

For Taqman® assays, AmpErase® UNG treatment can prevent the reamplification of carry over PCR products from previous PCR reactions. When dUTP replaces dTTP in PCR amplification, AmpErase® UNG treatment can remove up to 200,000 copies of amplicon per 50 µl reaction. RT-PCR conditions were: 2 min at 50°C, 30 min at 60°C, and 5 min at 95°C, followed by 40 cycles consisting of 20 s at 92°C, 1 min at 62°C. The Ct (cycle threshold) values obtained by amplification were used to create standard curves for target genes (Figure 2.3).

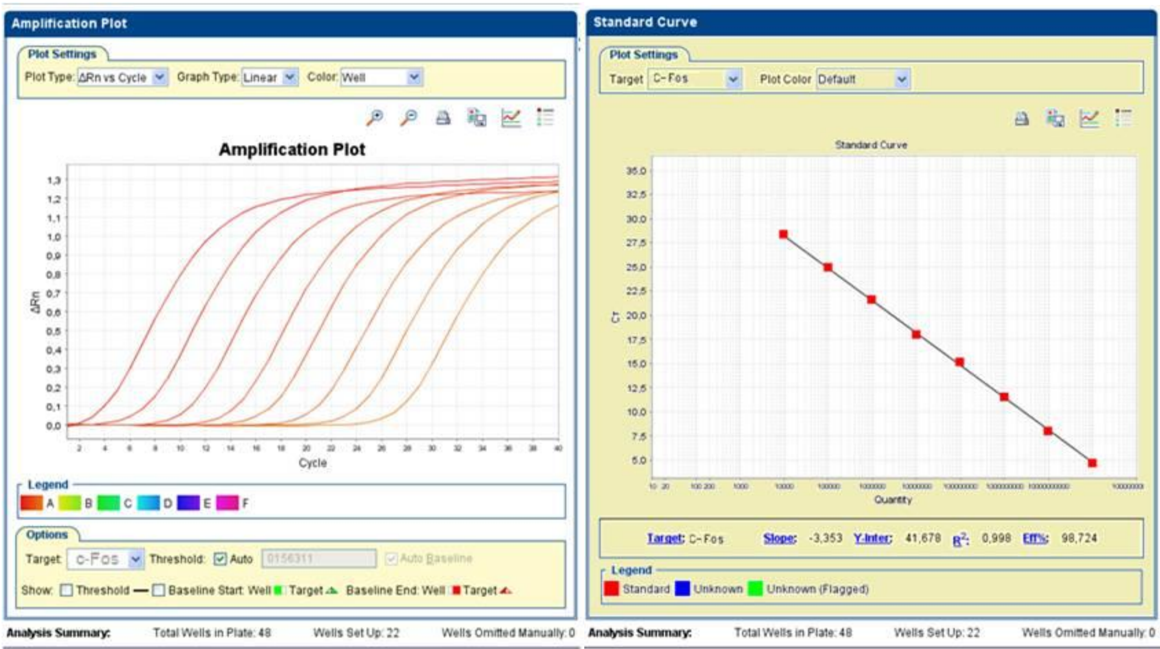


Figure 2.3 Standard curve and amplification plot of myosin heavy chain gene

2.2.4 Quantitation of transcripts by one-step RT-PCR TaqMan system

A hundred nanograms of total RNA extracted from the experimental samples were subjected, in parallel to triplicates of 10-fold-diluted, defined amounts of standard mRNAs, to real-time PCR under the same experimental conditions as for the establishment of the standard curves. Real-time Assays-by-Design™ PCR primers and gene-specific fluorogenic probes were designed by Applied Biosystems (ABI). Taqman® probes of the obtained target genes are showed at Table 2.1.

Gene	Primer	Sequence 5'–3'	Melting temperature (°C)
Cathepsin L	Catep_fw1	GCTGTGTTGCAGTGTGC	58.2
	Catep_fw2	GGTGTGGGAGAAGAACCTGA	59.4
	Catep_rv1	TAAGGACCAGGGTCAGTGTG	59.4
	Catep_fw3	TGAGTGAGCAGAACCTGGTG	59.4

	Catep_rv2	ATGGACCAGGCCTTCCAGTA	59.4
	Catep_rv3	GAAAGAACCACTGTGGAATTGC	58.4
µ-Calpain	Capn1_fw1	ACCACAACGCAGTCAAGTTC	57.3
	Capn1_rv1	GAGAAAGCCTACGCCAAGCT	57.3
	Capn1_rv2	TCATAGTGGATGGAGCCACT	59.4
	Capn1_fw2	GGCATCTTCCACTTCCAGTT	57.3
	Capn1_fw3	GACATGGAGGCTGTGACCTT	59.4
	Capn1_rv2a	AGGACGGAGAGTTCTGGAT	59.4
	Capn1_fw4	AAGTGGAGCTCAGCCGTCTA	59.4
	Capn1_fw5	CGCTCTGAGCTCTTCATCAAC	59.8
	Capn1_rv3	TCAGATTGCAGCTTCCTGGT	57.3
	Capn1_rv4	ATCATCGTCCCTTCCACCTT	59.4
	Capn1_fw6	GACATGGAGATCAGTGCCACA	59.8
	Capn1_rv5	TGCTGTGGGAGAAGATTAAACG	58.4
	Capn1_rv6	CTTCCAGTGGATCACCCTGA	59.4

Table 2.1 TaqMan® probes of genes employed in the present Thesis.

Data from TaqMan® PCR runs were collected using ABI´s Sequence Detector Program. Cycle threshold (Ct) values corresponded to the number of cycles at which the fluorescence emission monitored in real time exceeded the threshold limit. The Ct values were used to create standard curves to serve as a basis for calculating the absolute amounts of mRNA in total RNA. To reduce pipetting errors, master mixes were prepared to set up duplicate reactions (2 x 30 µl) for each sample.

2.2.5 Statistical analysis

The data were statistically compared using one-way analysis of variance (ANOVA). The level of statistical significance was set at $p < 0.05$.

2.3 Microfluidic capillary electrophoresis to assess RNA integrity

Total RNA was extracted from sea bass white muscle using PureYield RNA Midiprep System (Promega, Italy), following the manufacturer's protocol. Seven hundred milligrams of muscle were lysed and homogenized in cold lysis solution using sterile tubes. Dilution buffer and clearing agents were added to lysates and the reaction tube was then heated at 70°C in a water bath for 5 min. The heated samples were cooled at room temperature and then poured into a PureYield™ Clearing Column and centrifuged.

Total RNA was precipitated by adding isopropanol to the cleared lysate and then purified by Vacuum On PureYield™ Binding Column. After two washing steps with ethanol, total RNA was eluted with nuclease-free water. The integrity of total RNA extracted was evaluated by using a microfluidic capillary electrophoresis technique with the Experion™ Automated Electrophoresis System (Bio-Rad) (Figure 2.4). Provided with the Experion™ expert software is a tool, the RNA Quality Indicator (RQI), designed to automatically assign an integrity number to an eukaryotic total RNA sample. With this tool, sample integrity is no longer determined by the ratio of the 28S/18S ribosomal bands, but rather by the entire electrophoregram of the RNA sample, including the presence of degradation products (Figure 2.3). The RQI is independent of sample concentration, instrument, and analyst and therefore represents a de facto standard for RNA integrity. Total RNAs with an RQI value of 7.0–9.7 or greater represent high-quality, intact RNA, whereas values from 1.0 to 4.0 correspond to highly degraded RNA (Figure 2.4).

The Experion system calculates quantity and also performs a qualitative visual assessment of the RNA sample in a single step. The results are displayed in an electrophoregram and simulated gel view (Figure 2.4), which indicates whether the sample has been degraded. Thus, the Experion system obviates the need to run a gel for visual assessment or to take spectrophotometer readings to determine concentration.

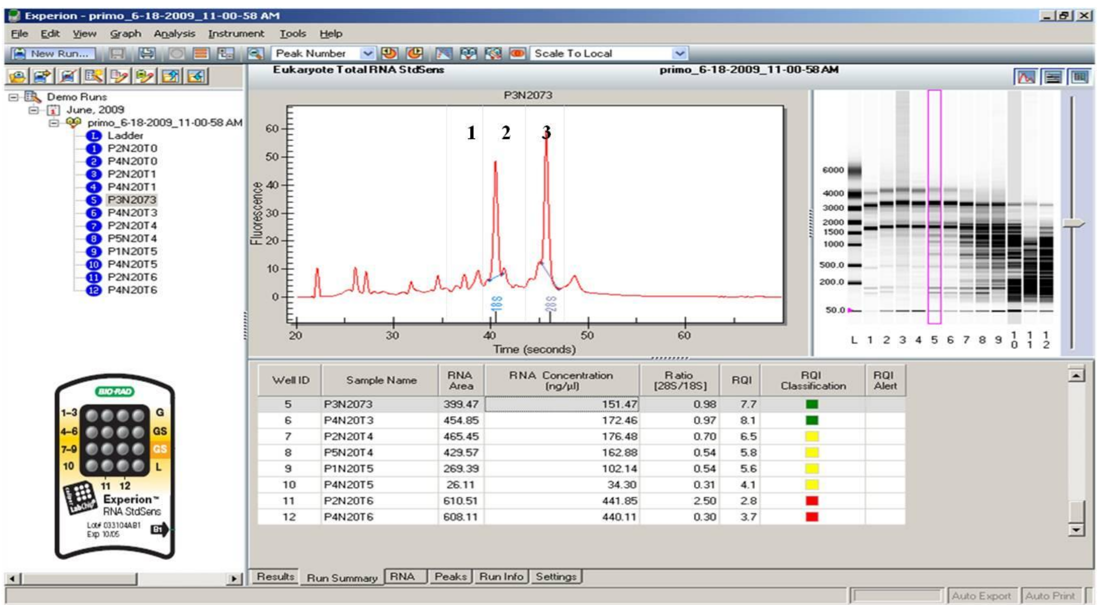


Figure 2.4 - RNA setting in the Experion software interface. Left panel: three electrophenogram regions used for RQI determination are indicated on a electropherogram of partially degraded RNA. 1, pre-18S peak area; 2, 18S peak area; 28S peak area. Panel below: screen capture of Run Summary page that displays the RQI value for each RNA sample along with a color-coded RNA classification. The default setting are: , 1 ≤ RQI ≤ 4; , 4 < RQI ≤ 7; , 7< RQI ≤ 10. right panel, virtual gel image of 12 RNA degradation samples.

2.4. Proteomic analysis

2.4.1 Protein extraction

Proteomic experiments were carried out at the Proteomics Laboratory of Porto Conte Ricerche (Tramariglio, Alghero). Proteins were extracted from frozen muscle tissues using a TissueLyser mechanical homogenizer (Qiagen, Hilden, Germany) as reported previously (Addis et al., 2009) Addis, M. F., Tanca, A., Pagnozzi, D., Crobu, S. et al., Generation of high-quality protein extracts from formalinfixed, paraffin-embedded tissues. *Proteomics* 2009, *9*,3815–3823.). A small portion (50 mg) of frozen fish muscle was minced with a sterile scalpel, placed in a 2-mL Eppendorf safe-lock tube (Eppendorf, Hamburg, Germany), and then immersed in lysis buffer (8M urea, 2% CHAPS, and 0.5% IPG buffer; GE Healthcare, Little Chalfont, UK) at a 5% w/v ratio. Three cycles of 1.5 min at 30 cycles/s in the TissueLyser mechanical homogenizer were employed for each sample. All extracts were then clarified for 15 min at 12 000xg at 4°C, quantified by the Bradford method (Sigma-Aldrich, St. Louis, MO, USA), tested for the quality and quantity by SDS-PAGE as described previously (Addis et al., 2009), and then stored at -80°C until analysis.

2.4.2 Sample labelling with DIGE fluorophores

Sixty micrograms of proteins extracted from six muscle fragments sampled at the time of death (T0) and after 5 days of *post mortem* storage at either 1°C (n=3) or 18°C (n=3) were labelled with

400 pmol N-hydroxysuccinimidyl-ester of cyanine dye Cy-3 and Cy5 (GE Healthcare), respectively. A pooled sample comprising equal amounts of each of the specimens in the study (T0+$T5_1$ and T0+$T5_{18}$), which served as internal standards, was labelled with Cy-2. Samples were maintained under dark conditions and on ice during 30 min for labelling. After quenching with 10mM lysine solution (Sigma-Aldrich), the labelled proteins of T0, $T5_1$, $T5_1$8, and those of the internal standard were mixed in suitable combinations and adjusted to a concentration of 1%. IPG buffer (GE Healthcare) and Destreak Rehydration Solution (GE Healthcare) were added to a final volume of 450 mL (Table 5.1- Chapter 5).

A second analysis has been conducted with sixty micrograms of protein extracted from each of the nine muscle specimens sampled at the time of death (T0), and from the other nine sampled after 5 days of *post mortem* storage at 1°C (T5) were labelled with 400 pmol N-hydroxysuccinimidyl-ester of cyanine dye Cy3 and Cy5 (GE Healthcare), respectively (Table 6.1 – Chapter 6). A pooled sample, which served as the internal standard, was labeled with Cy2, as described previously (Terova et al., 2011). After quenching with lysine, the labeled proteins were mixed in suitable combinations as summarized in Table 6.1 (Chapter 6). IPG buffer (GEHealthcare) and Destreak Rehydration Solution (GE Healthcare) were added to a final volume of 200 µl for each mix.

2.4.3 2-D gel electrophoresis

First-dimension isoelectric focusing (IEF) was performed using 24 cm precast IPG strips (pH 3–11 NL; GE Healthcare). Mixtures of samples (450 mL) were applied onto the strips by passive rehydration overnight at room temperature. Six strips were run together on an IPGphor equipped with the EttanTM IPGphorTM3 loading manifold (GE Healthcare). The strips were focused at 20°C for a total of about 90 000V h.

After IEF, the strips were sequentially incubated in a freshly prepared solution of 1% DTT and 2% iodoacetamide in 50mM Tris-HCl (pH 8.8), 6M urea, 20% glycerol, and 2% SDS for 10 min.

The second-dimension SDS-PAGE was conducted on six 8–16.5% polyacrylamide gradient gels, using an EttanTM DALTtwelve electrophoresis system (GE Healthcare), for 30 min at 5 W/gel and then for 5 h at 17 W, at 25°C.

2.4.4 Image acquisition and analysis

After 2-D electrophoresis, gels were scanned on a Typhoon Trio1 image scanner (GE Healthcare) at 100 mm of resolution and with the photomultiplier tube (PMT) voltage was set between 500 and 700 V. The scanned gel images were then transferred to the ImageQuant V5.2 software

package (GE Healthcare), cropped, and exported to the DeCyder Batch Processor and differential in-gel analysis (DIA) modules (GE Healthcare) for statistical analysis. To compare protein spots across gels, a match set was created from the images of all gels. The statistical analysis of protein level changes was performed by the DeCyder-BVA (Biological Variation Analysis, v.6.5) module. Preparative cyandye- labeled gels were scanned and matched with the master gel in order to assign the right correspondence for spot picking. Calibration of the electrophoretic gels for protein molecular weight (Mr) and isoelectric point (pI) was performed by using markers for 2-DE electrophoresis (Sigma-Aldrich) and Precision Plus All Blue Protein Standards (Bio-Rad Laboratories, Hercules, CA, USA). Specifically, seven 7-cm IPG strips (pH 3–11, NL) were loaded as follows: one strip was loaded only with 2-DE markers, three strips were loaded with 100 mg of proteins from samples T0, $T5_1$, and $T5_{18}$, and three strips were loaded with a combination of each sample with 2-DE markers. All strips were then subjected to IEF as above for a total of about 30 000 V h and to SDS-PAGE in 15% polyacrylamide gels. All samples were analyzed in the same experiment in order to ensure the uniformity ofrunning conditions. After staining with the Simply Blue Safe Stain (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions; gels images were digitalized with Image Scanner II (GE Healthcare). Image analysis, including spot detection, matching, and calibration, was performed using the Image Master 2-D Platinum v6.0.1software (GE Healthcare). The Mr and pI values for the respective proteome patterns were determined by interpolation with the Image Master 2-D Platinum v6.0.1 calibration tools using Mr/pI markers associated with each gel. Isoelectric points of experimental samples were then cross-checked by using the method described for Immobiline DryStrip gels in the GE Healthcare Data File no. 18-1177-60 AB.

2.4.5 Spot picking and *in situ* tryptic digestion

For protein identification, preparative 2-D PAGE gels were set up by overnight rehydration loading of 300 mg of protein extract into 3–11 NL 24-cm IPG strips. Strips were then focused and subjected to 2-DE as described above. All blue molecular weight markers (Bio-Rad Laboratories) were also loaded on an electrode wick and run together with the isoelectrofocused strips for calibrating molecular weight across the gel. After electrophoresis, the gel slab was subjected to mass-compatible silver staining as described previously (Addis et al., 2009). Visible protein spots of interest weremanually excised from the gels, destained with 15mM $K_3Fe(CN)_6$ in 50mM $Na_2S_2O_3$, washed with water, and then stored in ACN. The spots were then subjected to an O/N tryptic digestion at 37°C in 50mM $(NH_4)HCO_3$, pH 8.0, by using trypsin in the range from 40 to 100 ng, depending on spot intensity. Peptide mixtures were then collected by squeezing with ACN and

centrifugation. Peptides were subsequently acidified with 20% TFA, dried, resuspended in formic acid 0.2%, and then stored at -20°C.

2.4.6 MS/MS analysis

LC-MS/MS analysis was performed on an XCT Ultra 6340 ion trap equipped with a 1200 HPLC system and a chip cube (Agilent Technologies, Palo Alto, CA, USA). After loading, samples were concentrated and desalted at 4 mL/min on a 40-nL enrichment column (75 µm x 43 mm, Agilent Technologies), with 0.2% formic acid. Peptides were then fractionated on a C18 reverse-phase capillary column at a flow rate of 300 nL/min, with a linear gradient of eluent B (0.2% formic acid in 95% ACN) in A (0.2% formic acid in 2% ACN) from 3–60% in 20 min. ESI parameters were as follows: capillary voltage 1730 V; dry gas (N2), 5.00 L/min; dry temperature, 325°C; trap drive, 100; skimmer 30 V; lens 1, -5.00 V; octopole RF amplitude, 200 Vpp; capillary exit, 90 V. The ion-trap mass spectrometer was operated in a positive-ion mode. Trap ICC smart target was 30 0000 units and maximal accumulation time was 100 ms. MS/MS was operated at a fragmentation amplitude of 1.3 V, and threshold ABS was 6000 units. Scan speed was 8100 UMA/s in MS and 26 000 UMA/s in MS/MS scans. Peptide analysis was performed scanning from m/z 250 to m/z 2200 in AutoMS (n) precursor selection mode of the three most intense ions (fragmentation mass range from 100 to 2200m/z). Dynamic exclusion was used to acquire a more complete survey of the peptides by automatic recognition and temporary exclusion (0.15 min) of ions from which definitive mass spectral data had previously acquired. Data analysis software, provided by the manufacturers, was used to analyze MS/MS spectra and to generate a peak list that was introduced in the in-house MASCOT MS/MS ion search software (Version 2.3, Matrix Science, Boston, MA, USA) for protein identification in NCBI database using the chordata (vertebrates and relatives) taxonomy. Search parameters were as follows: peptide tolerance 300 ppm, MS/MS tolerance 0.6 Da, charges state +2 and +3, +4, enzyme trypsin, allowing 2 missed cleavage.

2.4.7 Statistical analysis

The results related to T0 and 5 days of *post mortem* storage at either 1°C ($T5_1$) or 18°C ($T5_{18}$) were compared and statistically evaluated using univariate analysis one-way ANOVA. One-way ANOVA was performed using the DeCyder-BVA module and applying the false discovery rate (FDR) to minimize the number of false positives. A q-value of 0.05 was specified corresponding to the lowest FDR at which the protein is called significant. Data were normalized for computing the fold

changes. Protein spots with a statistically significant variation ($p<0.05$), showing an over twofold difference in volume, were selected as differentially expressed and analyzed by MS/MS.

2.5 Data and network pathway analysis

The list of protein identification was imported in the online software package IPA (Ingenuity Systems, Redwood City, CA), and network analyses were performed by substituting UniProt IDs with the UniProt ID (Uniprot Consortium, 2008) for the closest human protein equivalent in order to enable the best exploitation of the knowledge-based IPA software, version 12718793, updated May 2012.

Chapter 3

Applying transcriptomics to better understand the molecular mechanisms underlying fish filet quality

This work was published in Terova *et al.* (2011) *Food Chemistry* 124, 1268–1276

Keywords:

Post mortem aging
μ-Calpain mRNA
Cathepsin L mRNA
Marine fish
Sea bass
Microfluidic capillary electrophoresis
Real-time PCR

ABSTRACT

Microfluidic capillary electrophoresis and real-time PCR were successfully applied to investigate the *post mortem* alterations in RNA extracted from fish muscular tissue in relation to three parameters: slaughtering method, time, and storage temperature. *Post mortem* proteolytic degradation of fish filets, which leads to textural changes such as muscle softening and gaping, is a major problem for fish freshness. Endogenous proteases such as calpains and cathepsins are assumed to play the major role in this process, although the exact mechanisms underlying fish meat tenderization have yet to be determined. In the course of the present study we first identified the cDNA sequences coding for μ-calpain and cathepsin L in sea bass (*Dicentrarchus labrax*). Then we determined the total RNA and μ-calpain and cathepsin-L-specific mRNA integrity over 5 days of storageat two different temperatures (1°C and 18°C) in sea bass slaughtered by three different methods (asphyxia in air, hypothermia, and immediately severing the spinal cord). The results of this study show that, RNA degradation is a slow process under the conditions investigated and, although *post mortem* storage temperature negatively affects the integrity of total RNA (higher degradation at 18°C), the transcripts of μ-calpain and cathepsin L are present for up to 5 days *post mortem* in the muscle of sea bass stored at either 1°C or 18°C.

3.1 Introduction

One of the most unique characteristics of fish as food is that it is highly perishable. Consequently, freshness is fundamental to the quality of fish, and the time that has passed after catching it and the temperature ‘‘history’’ of fish are very often the key factors in determining the ultimate

quality characteristics of such products. During *post mortem* storage, fish muscle degrades and the flesh quality decreases rapidly, depending on the fish species. Loss of freshness, followed by spoilage, is the result of complex biochemical and microbiological processes that begin with a metabolic shift from an aerobic to an anaerobic state. This is followed by transformation of glycogen into lactic acid and the consequent reduction in pH and activation of different proteolytic reactions catalyzed by endogenous enzymes, which produce nutrients that promote subsequent bacterial proliferation (Delbarre-Ladrat et al., 2006). *Post mortem* tenderization is one of the most unfavourable quality changes in fish muscle and contrasts with mammalian and avian meats in which *post mortem* degradation of myofibrillar and cytoskeletal proteins is often desired to obtain a tender product (Geesink, Morton, Kent, & Bickerstaffe, 2000). The results of a number of recent studies indicate that the main groups of enzymes involved in fish muscle protein degradation during *post mortem* storage are calpains and cathepsins (Chéret et al., 2007; Delbarre-Ladrat et al., 2006). Calpains are calcium-dependent cytosolic proteases existing in two main ubiquitous forms, according to the concentration of Ca^{2+} required for their activation: μ-calpain and m-calpain. Cathepsins are proteinases which play an importantrole in the lysosome. Cathepsin L is believed to contribute more than cathepsin D to the autolytic degradation of fish muscle since it is far more active at acidic pH and has been shown to digest both myofibrillar proteins (actomyosin) and connective tissue (Ogata et al., 1998). In mammals, the calpains have received a great deal of attention due to their roles in: (1) muscle protein turnover and growth and (2) in *post mortem* proteolysis and the muscle food texture. However, in fish, little is known about their role in *post mortem* protein degradation, and the μ-calpain gene has only been cloned and sequenced from two aquaculture fish species, such as rainbow trout (*Oncorhynchus mykiss*) (Salem et al., 2005), and Atlantic salmon (Salmo salar) (accession No. BT059271).

Mammalian cathepsins L have also been isolated and cloned from a wide range of sources, whereas piscine cathepsin L gene has only been cloned and sequenced from a few species, such as gilthead sea bream (*Sparus auratus*) (Carnevali et al., 2008), rainbow trout (*Oncorhynchus mykiss*) (Kwon et al., 2001), common carp (*Cyprinus carpio*) (Tsunemoto et al., 2004), and channel catfish (*Ictalurus punctatus*) (Yeh et al., 2009). Previous studies on fish have investigated *post mortem* phenomena using chemical, physical, histological, and microbiological methods by focusing on indicators such as pH, lactic acid, adenine nucleotides and their degradation products, texture, firmness, and elasticity. Only one study in the current literature has included a molecular investigation. In fact Seear and Sweeney (2008) were the first researchers to analyze the integrity of skeletal muscle RNA extracted at different *post mortem* times in Atlantic salmon. One parameter that was not taken into account in this study, though, was the physiological status of fish before it was killed. Several studies have shown that preslaughter handling stress, exhausting

muscular activity, and asphyxia increased muscle ischemia in the *premortem* period, thus deteriorating the ultimate quality of the filet (Poli et al., 2005). Direct mechanical stress of the muscle fibrils or connective tissue, causing the release of proteases, could also contribute to the acceleration of muscle structure degradation (Roth et al., 2006).

A wide variety of slaughter techniques are used among and within farmed fish species. Current slaughter methods for fish farmed in the Mediterranean consist of live chilling in ice or in a mixture of ice and seawater and by asphyxia in air (EFSA, 2009). Methods that kill fish rapidly, such as those involving an overdose of anaesthetics or immediate excising of the spinal cord, are also used in order to reduce stress levels, thus improving animal welfare and food quality.

Still, nothing is known about the impact of such slaughtering methods on total RNA quality and mRNA integrity extracted from fish muscle at different *post mortem* intervals. Consequently, we were interested in performing a RNA stability time course study on the marine teleost sea bass (*Dicentrarchus labrax*), economically an important species for Mediterranean aquaculture. The objective of our experiment was to show that RNA degradation in *post mortem* samples can be measured quantitatively and might be used as an indicator of the *post mortem* interval. We therefore first investigated the integrity of total RNA extracted from dorsal white muscle of sea bass slaughtered with three different techniques and stored for 5 days *post mortem* at two different temperatures (1°C and 18°C). Subsequently, the presence and integrity of two specific mRNA transcripts encoded by the sea bass μ-calpain and cathepsin L genes were determined by real-time RT-PCR analyses after isolating the respective cDNA sequences by molecular cloning and sequencing techniques.

3.2. Materials and methods

3.2.1. Animals and experimental protocol

The experiment was carried out at the Department of Biotechnology and Molecular Sciences of the University of Insubria (Varese, Italy). One month before slaughter, sea bass (*D. labrax*) were randomly distributed into three tanks of 1 m3 each, 40 fish per tank, and fed Hendrix-Skretting®Power Excel feed for marine fish.

The tanks were connected to a sea water recirculation system under the following water conditions: temperature 20 ± 2°C, pH 7, total ammonia <0.2 mg/L, and dissolved oxygen over 99% of the saturation.

At the start of the experiment and after 4 days of starvation, 30 fish (average body weight 710 ± 157.87 g) were removed from water and sacrificed in three different ways: 10 specimens by hypothermia (immersion in icecold water at a fish:ice ratio 2:1), another 10 by asphyxiation, and

the last ones by severing the spinal cord. Fish from the three groups (HD, death from hypothermia; AD, death by asphyxiation; and SCD, death by severing the spinal cord) were then divided into two subgroups of five fish each, which were stored ungutted for up to 5 days *post mortem* either in a refrigerator at 1°C or in a thermostat at 18°C.

The first sampling was performed at the time of death (T0) by taking a fragment of white muscle tissue (2 x 2 x 2 cm) from the lateral-dorsal muscle quadrant (epiaxial) of each fish. The next six samplings were performed similarly after 2, 4, 24, and 48 h and then after 3 and 5 days of *post mortem* storage at the respective temperatures for a total of 210 muscle samples. The muscle fragments were taken alternately from both sides (right and left) of the epiaxial muscular quadrant, leaving a thickness of about 1 cm of tissue between one fragment and the other to avoid contamination.

Furthermore, throughout the trial, fish were all kept flat on the belly to prevent contact with the dorsal walls of the container or with other fish in the group.

All 210 muscle fragments taken in this way were immediately frozen at -80°C and stored at this temperature until the molecular biology analysis.

All procedures were approved by the Animal Care Committee of the University of Insubria and conducted according to the guidelines of the Italian Committee on Animal Care.

3.2.2. Microfluidic capillary electrophoresis to assess RNA integrity

Total RNA was extracted from sea bass white muscle using PureYield RNA Midiprep System (Promega, Italy), following the manufacturer's protocol. Seven hundred milligrams of muscle were lysed and homogenized in cold lysis solution using sterile tubes. Dilution buffer and clearing agents were added to lysates and the reaction tube was then heated at 70°C in a water bath for 5 min. The heated samples were cooled at room temperature and then poured into a PureYield™ Clearing Column and centrifuged. Total RNA was precipitated by adding isopropanol to the cleared lysate and then purified by Vacuum On PureYield™ Binding Column. After two washing steps with ethanol, total RNA was eluted with nuclease-free water.

The integrity of total RNA extracted was evaluated by using a microfluidic capillary electrophoresis technique with the Experion ™ Automated Electrophoresis System (Bio-Rad). Provided with the Experion™ expert software is a tool, the RNA Quality Indicator (RQI), designed to automatically assign an integrity number to an eukaryotic total RNA sample. With this tool, sample integrity is no longer determined by the ratio of the 28S/18S ribosomal bands, but rather by the entire electrophoregram of the RNA sample, including the presence of degradation products. The RQI is independent of sample concentration, instrument, and analyst and therefore

represents a de facto standard for RNA integrity. Total RNAs with an RQI value of 7.0–9.7 or greater represent high-quality, intact RNA, whereas values from 1.0 to 4.0 correspond to highly degraded RNA.

The Experion system calculates quantity and also performs a qualitative visual assessment of the RNA sample in a single step.

The results are displayed in an electrophoregram and simulated gel view, which indicates whether the sample has been degraded.

Thus, the Experion system obviates the need to run a gel for visual assessment or to take spectrophotometer readings to determine concentration.

3.2.3. Sea bass µ-calpain and cathepsin L cDNA sequence isolation

3.2.3.1. Sea bass µ-calpain and cathepsin L cDNA sequences

At the beginning of this research, the µ-calpain and cathepsin L sequences were not available in public databases for D. labrax; in fact, despite the high commercial interest in this animal, information on its genome, transcriptome, or proteome is minimal. Considering this lack of information, we aimed to obtain the open reading frame for both genes.

A BlastN search was performed on the Genbank nucleotide database for orthologues of µ-calpain and cathepsin L, in other fish species. A multiple sequence nucleotide alignment was then carried out on coding sequences for each target gene and a strategy based on regions of strong nucleotide conservation was used to design the primers (Table 3.1).

In the case of µ-calpain primer design was based on the alignment of two teleost µ-calpain coding sequences available on the NCBI Genbank database: *Danio rerio* (accession no. AF282675) and *Oncorhynchus mykiss* (accession no. AY573919), whereas cathepsin L primer design was based on the alignment of *Lates calcarifer* (accession no. AY795481) and *Ictalurus punctatutus* (accession no. EU915299) cathepsins L. In both cases the aligned sequences presented several conserved regions within the sequence where µ-calpain and cathepsin L primers could be reasonably designed.

3.2.3.2. cDNA synthesis

After extraction, 3 lg of total RNA were reverse transcribed into cDNA with MMLV reverse transcriptase (Invitrogen), following manufacturer’s instructions. After extraction, 3 < mu > g of

total RNA were reverse transcribed into cDNA with MMLV reverse transcriptase (Invitrogen), following manufacturer's instructions.

To perform PCR, an aliquot of 4 µl of the resulting cDNA was amplified with 1µl GoTaq Polymerase (Promega) in 50 µl of final volume containing 5 µl buffer, dNTPs 10 mM, and 50 pmol of each of the designed µ-calpain and cathepsin L RT-PCR primer sets (Table 3.1).

The PCR products from each target gene primer amplifications were then cloned using the pGEM®-T Easy cloning vector system (Promega) and subsequently sequenced in both directions (T7 and SP6).

Gene	Primer	Sequence 5'–3'	Melting temperature (°C)
Cathepsin L	Catep_fw1	GCTGTGTTGCAGTGTGC	58.2
	Catep_fw2	GGTGTGGGAGAAGAACCTGA	59.4
	Catep_rv1	TAAGGACCAGGGTCAGTGTG	59.4
	Catep_fw3	TGAGTGAGCAGAACCTGGTG	59.4
	Catep_rv2	ATGGACCAGGCCTTCCAGTA	59.4
	Catep_rv3	GAAAGAACCACTGTGGAATTGC	58.4
µ-Calpain	Capn1_fw1	ACCACAACGCAGTCAAGTTC	57.3
	Capn1_rv1	GAGAAAGCCTACGCCAAGCT	57.3
	Capn1_rv2	TCATAGTGGATGGAGCCACT	59.4
	Capn1_fw2	GGCATCTTCCACTTCCAGTT	57.3
	Capn1_fw3	GACATGGAGGCTGTGACCTT	59.4
	Capn1_rv2a	AGGACGGAGAGTTCTGGAT	59.4
	Capn1_fw4	AAGTGGAGCTCAGCCGTCTA	59.4
	Capn1_fw5	CGCTCTGAGCTCTTCATCAAC	59.8
	Capn1_rv3	TCAGATTGCAGCTTCCTGGT	57.3
	Capn1_rv4	ATCATCGTCCCTTCCACCTT	59.4
	Capn1_fw6	GACATGGAGATCAGTGCCACA	59.8
	Capn1_rv5	TGCTGTGGGAGAAGATTAAACG	58.4
	Capn1_rv6	CTTCCAGTGGATCACCCTGA	59.4

Table 3.1 Primers used for the molecular cloning and sequencing of the target genes.

3.2.4. Quantitative real-time RT-PCR

3.2.4.1. Generation of in vitro-transcribed µ-calpain and cathepsin L mRNAs for standard curves

The transcript copy number of each target gene could be absolutely quantified by comparing them with a standard curve constructed using a known mRNA transcript copy number of this gene. For this, forward and reverse primers were designed based on the mRNA sequences of D. labrax cathepsin L and µ-calpain identified in this study (accession nos. FJ807676, FJ821591, respectively). These primer pairs were used to create templates for the in vitro transcription of mRNAs for each target gene. The forward primer was engineered to contain a T3 phage polymerase promoter gene sequence to its 50 end and used together with the reverse primer in a

conventional RT-PCR of total sea bass muscle RNA. RT-PCR products were then checked on a 2.5% agarose gel stained with ethidium bromide, cloned using pGEM®-T Easy cloning vector system (Promega) and subsequently sequenced in the SP6 direction.

In vitro transcription was performed using T3 RNA polymerase and other reagents supplied in the Promega RiboProbe in vitro Transcription System kit according to the manufacturer's protocol. The molecular weight (MW) of the in vitro-transcribed RNAs for each target gene was calculated according to the following formula as described in Molecular Cloning, a Lab Manual (Sambrook & Russell, 2001): Gene MW= (129 (no. of A bases) x 329.2) + 69 (no. of U bases) x 306.2) + 66 (no. of C bases) x 305.2) + 98 (no. of G bases) x 345.2)) + 159.

The result was 191785.2 for µ-calpain, and 144,192 for cathepsin L. Spectrophotometry at 260 nm gave a concentration of 171 ng/µl for µ-calpain, and 125 ng/µl for cathepsin L. Therefore, the concentrations of the final working solutions were 5.37 x 1011 molecules/µl for µ-calpain, and 5.22 x 1011 molecules/µl for cathepsin L.

3.2.4.2. Generation of standard curves for µ-calpain and cathepsin L

The mRNAs produced by in vitro transcription were used as quantitative standards in the analysis of experimental samples. Defined amounts of mRNAs at 10-fold dilutions were subjected in triplicates to real-time PCR using one-step TaqMan EZ RT-PCR Core Reagents (Applied Biosystems), including 1x Taqman buffer, 3 mM MnOAc, 0.3 mM dNTP except dTTP, 0.6 mM dUTP, 0.3 µM forward primer, 0.3 µM reverse primer, 0.2 M FAM-6 (6-carboxyfluorescein-labelled probe), 5 units rTH DNA polymerase, and 0.5 units AmpErase UNG enzyme in a 25 µl reaction. RT-PCR conditions were: 2 min at 50 °C, 30 min at 60 °C, and 5 min at 95°C, followed by 40 cycles consisting of 20 s at 92°C, 1 min at 62°C.

Data from Taqman®PCR runs were collected with ABI's Sequence Detector Program. CT (cycle threshold) values corresponded to the number of cycles at which the fluorescence emission monitored in real time exceeded the threshold limit. The CT values were used to create standard curves to serve as a basis for calculating the absolute amounts of target mRNAs in total RNA.

3.2.4.3. Quantitation of µ-calpain and cathepsin L transcripts by onestep RT-PCR TaqMan system

A hundred nanograms of total RNA extracted from the experimental samples was subjected, in parallel to triplicates of 10- fold-diluted, defined amounts of standard mRNA, to real-time RT-PCR

under the same experimental conditions as for the establishment of the standard curves. Real-time Assays-by-DesignSM PCR primers and gene-specific fluorogenic probes were designed by Applied Biosystems. TaqMan® PCR was performed on a StepOne™ Real-Time PCR System (Applied Biosystems).

3.2.5. Calculation and statistical analysis

The data were statistically compared using Student T-test (Sigma Plot 8.0). The level of statistical significance was set at p<0.05.

3.3 Results

3.3.1 RNA integrity assessment by microfluidic capillary electrophoresis

The integrity of total RNA extracted from 210 samples of muscle tissue was assessed using the Experion™ Automated Electrophoresis System (Bio-Rad), which both calculates quantity and performs a qualitative visual assessment of the RNA sample in a single step. Figure 3.1A shows the virtual electrophoretic gel and Figure 3.1B shows the pattern of RQI values of the total RNA extracted from the muscle.

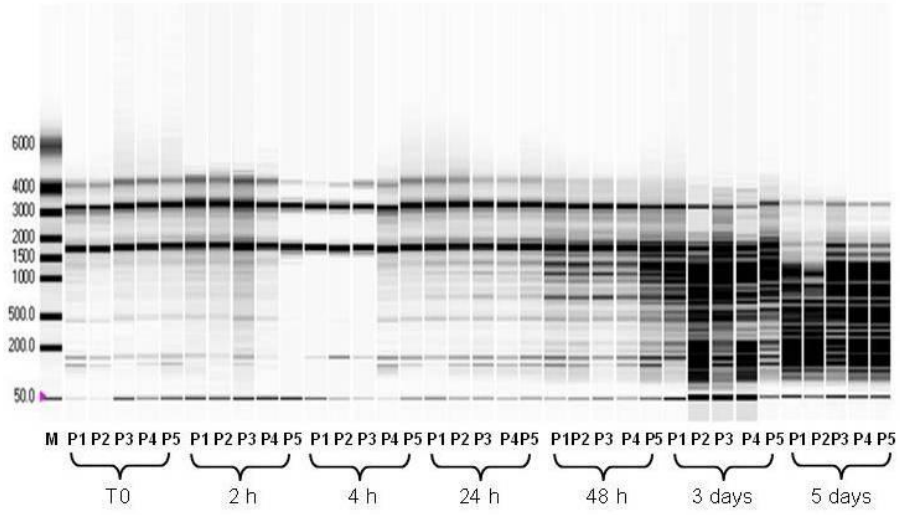


Figure 3.1A - An example of the virtual electrophoresis gel that it was assessed using the Experion™ Automated Electrophoretic System (Bio-Rad).

As shown from the chart, the RQI value of the total RNA extracted from muscle samples stored at 1 °C is stable up to 24 h *post mortem*, then decreases slightly until the fifth day *post mortem*. The

RQI values of fish of the same group, but stored at 18°C, remain constant until 24 h *post mortem*, like those stored at 1°C, but thereafter they decrease sharply, reaching a value of 3 at 5 days *post mortem*, indicating a highly degraded RNA.

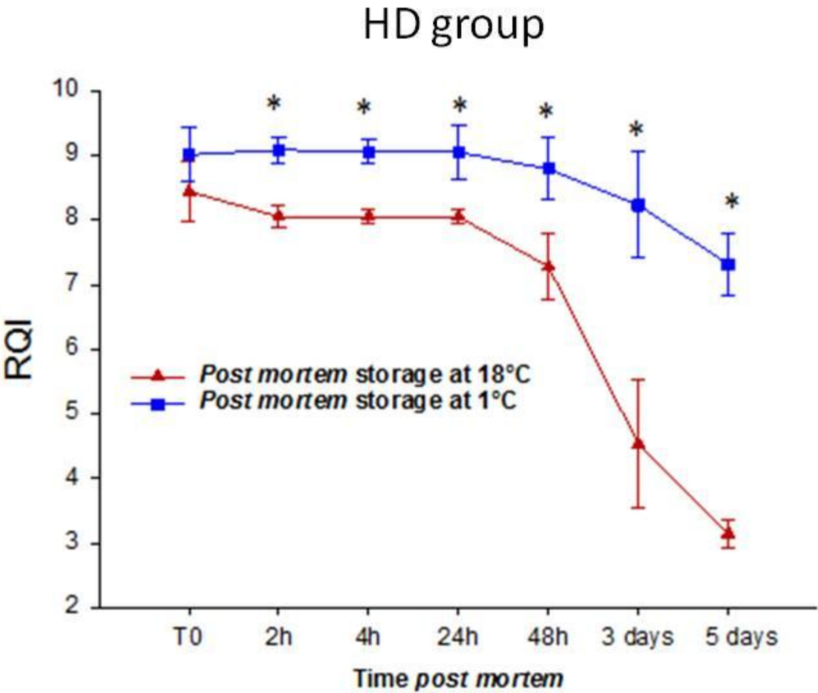


Figure 3.1B - The pattern of the RQI value in muscle of the sea bass group that died of hypothermia and were stored for 5 days *post mortem* at the temperature of 1°C or 18°C. (*) Indicates significant differences between the two groups ($p<0.05$).

The pattern of the RQI value in muscle of the sea bass group that died of asphyxiation and was stored for 5 days *post mortem* at a temperature of 1°C or 18°C is shown in Figure 3.2. The RQI value for the group stored at 1°C shows a gradual and slight decrease up to 24 h *post mortem*, drops to a value of 7 at 48 h, and then remains constant until the fifth day *post mortem*. The pattern of RQI for sea bass kept at 18°C, in contrast, is initially very similar to that for those kept at 1°C for up to 48 h *post mortem*, after which the RQI values decline rapidly. These values are also significantly higher in the group stored at 1°C than at 18°C for the time points zero, 3, and 5 days *post mortem*.

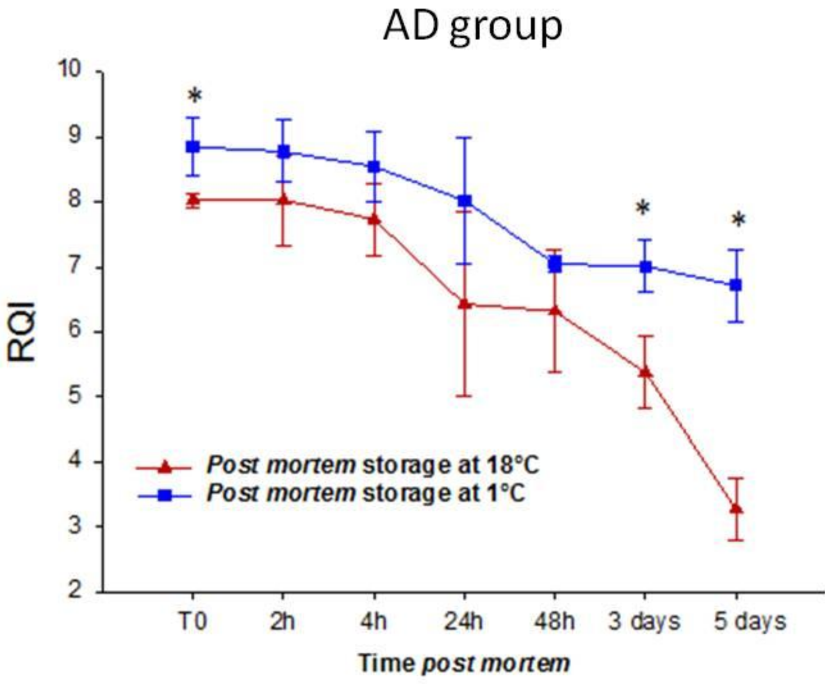


Figure 3.2 - The pattern of the RQI value in muscle of the sea bass group that died of asphyxiation and were stored for 5 days *post mortem* at the temperature of 1°C or 18°C. (*) Indicates significant differences between the two groups ($p<0.05$).

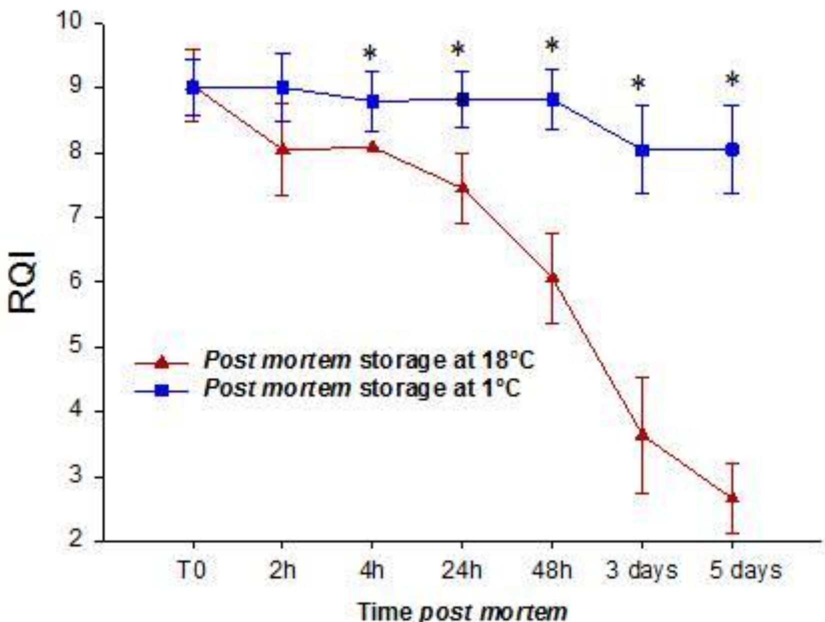


Figure 3.3 - The pattern of the RQI value in muscle of the sea bass group that died of severing the spinal cord and were stored for 5 days *post mortem* at the temperature of 1°C or 18°C. (*) Indicates significant differences between the two groups ($p<0.05$).

The graph presented in Figure 3.3 shows the pattern of the RQI in the experimental group of sea bass that died of severing the spinal cord and were stored for 5 days *post mortem* at a temperature of 1°C or 18°C. As shown, total RNA extracted from the muscle of samples stored at 1°C shows a slight decrease in the RQI value starting from 48 h *post mortem*, a value which then remains stable up to 5 days *post mortem*. The RQI value for RNA extracted from the muscle of the group stored at 18°C continuously decreases starting from 4 h *post mortem*. Furthermore, comparing the values of RQI at 1°C with those at 18°C, the differences are statistically significant ($p<0.05$) from 4 h to 5 days *post mortem*.

3.3.2. Sea bass μ-calpain and cathepsin L cDNA sequences

Two cDNA fragments were obtained using the primers designed for μ-calpain: the first one using Capn1_fw1 + Capn1_rv1, and the second using Capn1_fw2 + Capn1_rv2a (Table 3.1). Then, by connecting the sequences of the partially overlapping clones, a partial coding sequence (543 bp) for sea bass μ-calpain was determined and deposited in GenBank under the accession no. FJ821591.
Following the same strategy we could isolate a partial coding sequence for sea bass cathepsin L. Three cDNA fragments were obtained using these primers: the first one using Catep_fw1 + rv1, the second using Catep_fw2 + rv2 and the last using Catep_fw3 + rv3 (Table 3.1). Then, by connecting the sequences of the partially overlapping clones, a partial coding sequence of 971 bp which corresponded to sea bass cathepsin L was determined and deposited in GenBank under the accession no. FJ807676.

3.3.3. Cathepsin L mRNA copy number in sea bass dorsal white muscle

The levels of transcripts coding for cathepsin L in the muscle of sea bass that died of asphyxiation (AD), by severing the spinal cord (SCD), or of hypothermia (HD) and kept for 5 days *post mortem* at a temperature of 1°C or 18°C are shown in Figure 3.4. In each graph the two *post mortem* storage temperatures of transcripts (at 1°C and 18°C) are compared for a given mode of slaughter. As shown in the figure, the levels of messenger encoding cathepsin L in the three groups were significantly higher in samples stored at 1°C than in those kept at 18°C from 4 h to 5 days *post mortem*.

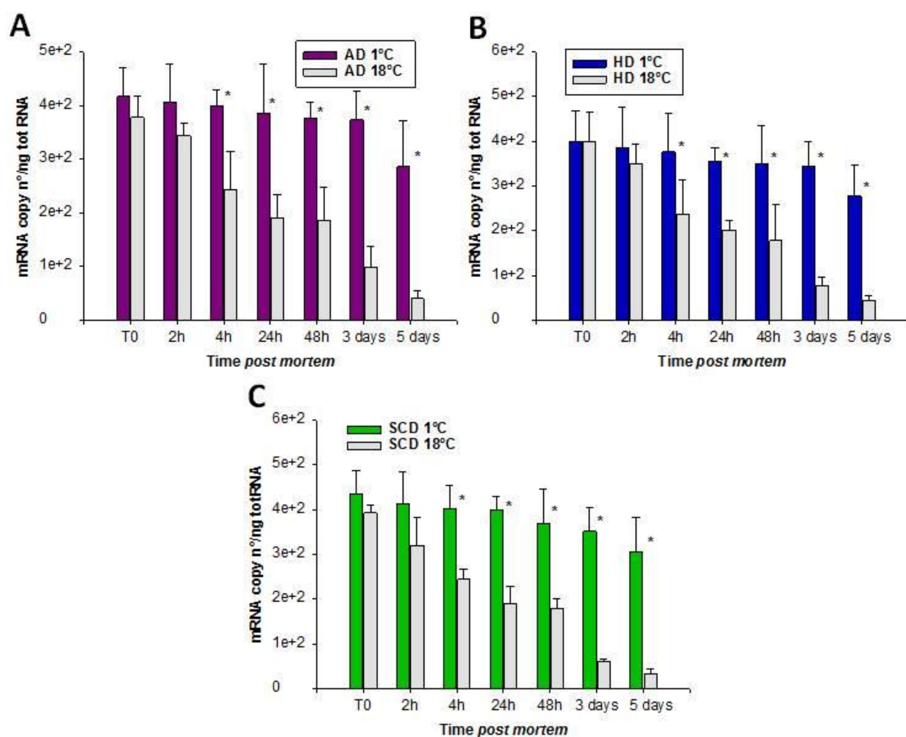


Figure 3.4 - The levels of transcripts coding for cathepsin L in the muscle of sea bass that died of asphyxiation (A), of hypothermia (B) or by severing the spinal cord (C) and were kept for 5 days *post mortem* at the temperature of 1°C or 18°C. in each graph the two *post mortem* storage temperatures of transcripts (at 1°C and 18°C) are compared for a given mode of slaughter. The mean of five animals for each sampling time point is shown. The bars indicate the standard deviation from the mean. The differences were determined using the student T-test (Sigma Plot 8.0). (*) Indicates significant differences between the subgroups stored at different temperatures ($p<0.05$).

The cathepsin L mRNA copy number does not appear to be influenced by the type of slaughter of animals. Indeed, the pattern of transcripts of this gene is very similar among the groups AD, HD, and SCD for a given *post mortem* storage temperature: at 1°C the number of copies of mRNA which is 4.169×10^2 at time zero for the AD group, 3.987×10^2 for the HD, and 4.346×10^2 for the SCD, decreases gradually to 5 days *post mortem*, whereas at 18°C there is a rapid decrease starting from 4 h *post mortem* until the end of the retention period (5 days). Furthermore, the number of transcripts of cathepsin L in fish stored at 1°C is significantly lower at 5 days *post mortem* than that at T0, for either the AD, HD, or the SCD group, whereas in the samples stored at 18°C the number of copies of mRNA is significantly lower than that at T0 starting from 4 h until 5 days *post mortem* ($p < 0.05$).

3.3.4. µ-Calpain mRNA copy number in sea bass dorsal white muscle

The absolute mRNA levels of µ-calpain in the muscle of sea bass in response to three different types of slaughter and to 5-day *post mortem* storage at a temperature of 1°C or 18 °C are presented in Figure 3.5. As one can see, the transcripts of this gene are less stable than those of

the other target gene considered in this study, in which levels gradually decreased in samples kept at 1°C, while transcripts of µ-calpain show a high degradation at the same temperature starting from 2 h *post mortem*.

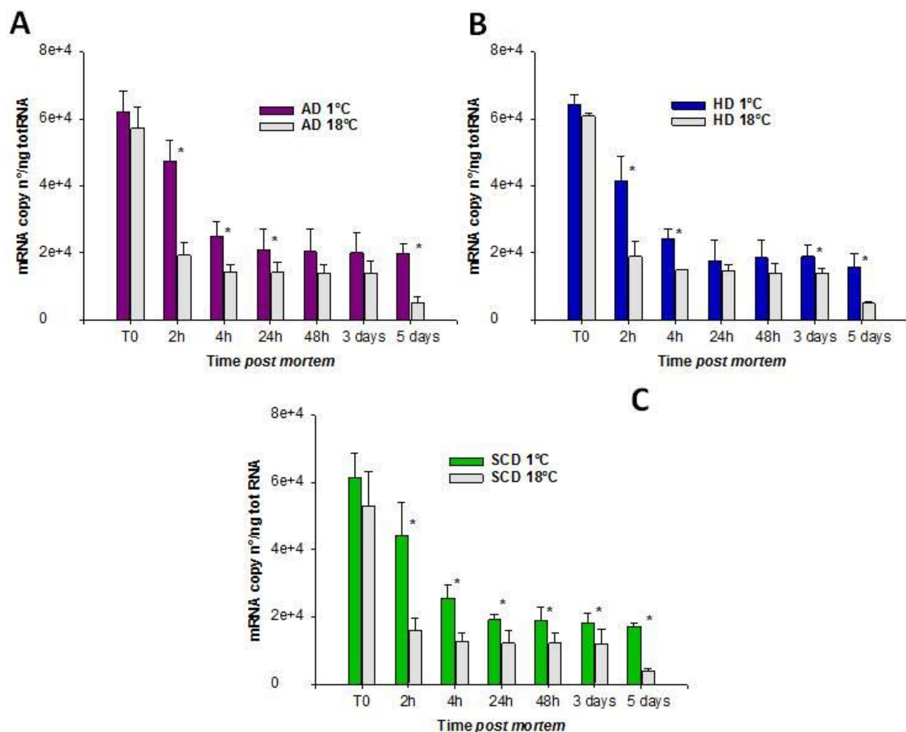


Figure 3.5 - The levels of transcripts coding for µ-calpain in the muscle of sea bass that died of asphyxiation (A), of hypothermia (B) or by severing the spinal cord (C) and were kept for 5 days *post mortem* at the temperature of 1°C or 18°C. in each graph the two *post mortem* storage temperatures of transcripts (at 1°C and 18°C) are compared for a given mode of slaughter. The mean of five animals for each sampling time point is shown. The bars indicate the standard deviation from the mean. The differences were determined using the student T-test (Sigma Plot 8.0). (*) Indicates significant differences between the subgroups stored at different temperatures ($p<0.05$).

The levels of messenger RNA encoding µ-calpain in the group that died of asphyxia (Figure 3.5A) are significantly higher in samples stored at 1°C than in those kept at 18°C from 2 h to 24 h and at 5 days *post mortem*, whereas in the group that died of hypothermia (Figure 3.5B), the mRNA copy number of samples stored at 1°C is significantly higher than in those stored at 18°C from 2 up to 4 h and from 3 to 5 days *post mortem*. Finally, the mRNA levels of µ- calpain in samples from the group that died by severing the spinal cord and subsequently stored at 18°C (Figure 3.5C), are significantly lower ($p<0.05$) than those stored at 1°C from 2 h *post mortem* to the end of the experiment.

Similar to what was seen for cathepsin L transcripts; there are no significant differences due to different methods of slaughter, but only to the storage temperature. The pattern of µ-calpain transcripts of samples stored at 1°C initially shows a decrease for up to 4 h *post mortem* and then remained constant at this value for all subsequent times *post mortem*, while in the samples stored

at 18°C the number of transcripts decreased rapidly up to 2 h *post mortem*, remained constant at this value for up to 3 days *post mortem*, and then decreased further up to 5 days *post mortem*. Statistical analysis of these results show significant differences from the value at T0 in the level of transcripts of samples from the three groups stored at 1°C from 4 h up to 5 days *post mortem*, whereas in those stored at 18°C, the differences are significant ($p<0.05$) from 2 h after death onwards.

3.4. Discussion

Changes that occur after death in fish muscle have an important impact on the aquaculture industry due to the significant effect this has on the quality of the filet and subsequent acceptance by consumers. Contrary to what occurs in mammals during *post mortem* storage, the muscle of fish undergoes a rapid process of tenderization due to the action of proteolytic enzymes which contributes to the progressive deterioration of filet quality. Two characterized proteolytic systems, acting in synergy, are currently known to hydrolyze myofibrillar proteins during *post mortem* storage of fish muscle: calpains and cathepsins (Delbarre-Ladrat et al., 2006).

However, despite the increasing number of studies on the *post mortem* modifications and degradation of these proteases at the protein level (Delbarre-Ladrat et al., 2006), no studies have been performed so far on the fate and dynamics of their specific mRNAs in fish muscle tissues.

In the course of the present study we identified the cDNA sequences coding for μ-calpain and cathepsin L for the first time in sea bass (*D. labrax*).

Calpain isoforms are believed to have evolved from a single common ancestral gene and have been markedly conserved during evolution (Jekely & Friedrich, 1999). Their highly conserved structure suggests that they play a fundamental and common role in cell biochemistry, as demonstrated by the fact that the calpain regulatory subunit knockout mouse embryos did not undergo normal embryogenesis (Arthur, Elce, Hegadorn, Williams, & Green, 2000). The sequence analysis of sea bass μ-calpain revealed considerable amino acid similarity with homologous sequences in other teleosts.

Sea bass μ-calpain showed 90%, 89%, and 90% amino acid sequence identity with rainbow trout, zebrafish, and Atlantic salmon μ-calpains, respectively. The lower level of homology (70–76%) between the sea bass and the mammalian μ-calpains, compared to 90% among the mammalian μ-calpains, suggests similar but distinct structural, functional, and regulatory mechanisms for fish μ-calpain. The same low level of homology was also reported between the rainbow trout and the mammalian μ-calpains by Salem et al., (2005), who were the first to hypothesize that such a distinction may explain the low Ca^{2+} sensitivity of fish calpains found in several species.

When the predicted sea bass cathepsin L amino acid sequence was compared with that from other species deposited in the Gen-Bank database, we found that the degree of conservation among species ranged from 65% (vs. human) to 83–87% (vs. other teleosts). Based on mammalian and fish (Yeh et al., 2009) cathepsin L studies, the sea bass cathepsin L amino acid sequence could be structurally divided into three domains: (1) a signal peptide at the amino terminus, (2) a propeptide domain, and (3) a mature peptide at the carboxyl terminus. Several functionally important amino acid residues, described in all known vertebrate cathepsin L mature peptides, are also found in that of sea bass. They include: (1) the catalytic triad at Cys135, His275, and Asn299 residues, (2) potential substrate binding sites at Gln129, Gly177–178, Trp301, and Trp305 residues, and (3) five potential cysteine disulphide linkage sites at 132, 166, 175, 209, and 268 (Tingaud-Sequeira et al., 2007).

After identifying the µ -calpain and cathepsin L proteases, we were interested in investigating the stability and integrity of total RNA and the presence of µ-calpain and cathepsin L mRNAs in muscle fragments of sea bass in relation to three parameters: slaughtering method, *post mortem* time, and *post mortem* storage temperature.

It is commonly assumed that RNA is highly unstable and degrades rapidly after death. However, studies that have investigated RNA stability in a number of *post mortem* tissues from cow (Fitzpatrick et al., 2002), pig (Fontanesi et al., 2008; Malik et al., 2003), rabbit (Marchuk et al., 1998), and rat (Inoue et al., 2002; Wetzel et al., 1994) have found that RNA is commonly stable for between 24 and 48 h *post mortem* at room temperature (depending on the type of tissue), whereas in beef cattle stored at refrigeration temperature, despite evident total RNA degradation after 8 days *post mortem*, specific mRNAs could be detected for up to 22 days after death (Bahar et al., 2007). Extensive RNA stability for extended *post mortem* intervals has also been demonstrated in several human tissues, including the brain, liver, lung, heart, and kidney (Barton, Pearson, Najlerahim, & Harrison, 1993).

These studies have concentrated on mammals so far, however, and only few studies have dealt with RNA integrity of skeletal muscle *post mortem* in fish (Bahuaud et al., 2010; Seear et al., 2008).

The integrity of total RNA was analyzed by microfluidic capillary electrophoresis in 210 fragments of muscle tissue, collected from 30 sea bass slaughtered by using three different sacrifice techniques, and subsequently stored for 5 days *post mortem* at two different temperatures: 1°C and 18°C. Provided with the Experion™ Microfluidic Capillary Electrophoresis software is a tool, the RNA Quality Indicator (RQI), designed to automatically assign an integrity number to an eukaryote total RNA sample. Comparing the obtained RQI values, total RNA extracted from samples stored at 18°C had, as expected, degraded more than RNA extracted from samples stored

at 1°C, without showing any significant slaughtering method-dependent degradation rates. A possible explanation for the slow RNA degradation at 1°C is the minor activity of ribonucleases, enzymes responsible for degrading total RNA at temperatures around zero.

In samples stored at 1°C, the group of fish that died from asphyxia in air showed a significant reduction in the RQI value with respect to the other two groups sacrificed by hypothermia and by severing the spinal cord from 24 h up to 5 days *post mortem*. The exhausting muscular activity of fish slaughtered by asphyxia in air certainly leads to a rapid decline in ATP reserves, with lactic acid formation and a consequent decrease in pH, which contributes to the rapid RNA degradation. It may also be assumed that asphyxia promotes the formation of free radicals during the *premortem* phase, which contributes to the increasing rate of degradation of total RNA.

The presence and integrity of specific mRNAs encoded by the sea bass μ-calpain and cathepsin L genes were determined by real-time RT-PCR in all the 210 fragments of postmortem muscle tissue.

Messenger RNA, which represents only 1–2% of total cellular RNA, is physiologically degraded in the cell to control protein synthesis. Some mRNA molecules such as transcription factor mRNA have very short half-lives of only 5 or 10 min (Guhaniyogiet al., 2001); others, such as globins, exhibit slower turnover rates of up to 24 h or more due to different mechanisms, including varying activities of intracellular ribonucleases. After cell death, these enzymes continue to digest RNA unless the cellular microenvironment interferes with enzyme activity (e.g., due to an increasing pH value). In addition, exogenous ribonucleases originating from bacteria or other sources may contribute to overall RNA degradation.

The cathepsin L mRNA copy number in our study was present for a longer *post mortem* interval in samples stored at 1°C than in those stored at 18°C, and no differences were observed in the mRNA copy number of this gene when correlated to the type of death, only to the *post mortem* storage temperature.

The transcript copy number of μ -calpain was 100 times higher than that of cathepsin L for all the time points tested. Unlike cathepsin L, the μ-calpain mRNA copy number in D. labrax stored at 1°C and 18°C for 5 days *post mortem* showed a rapid clearance starting from 2 h *post mortem* in samples stored at 18°C, and from 24 h in those stored at 1°C for all three different means of slaughter.

To explain this rapid decrease in both storage temperatures, we hypothesize that μ-calpain transcripts are more susceptible to the action of RNase, as compared to cathepsin L ones. However, future studies will help to clarify this hypothesis, ours being the first study to have examined the *post mortem* pattern of such transcripts in the muscle of sea bass over a long interval after death. The data presented here are consistent with previous publications on *post*

mortem stability of mRNAs (Barton et al., 1993). Johnson, Morgan, and Finch (1986) examined rat mRNAs up to 48 h *post mortem* and human mRNAs up to 36 h after death, and they found no evidence that mRNAs changed with *post mortem* interval. Leonard et al. (1993) found some mRNA deterioration in brains stored at -70°C for 5 years, but the tissue was viable for PCR evaluation of gene expression. They found no correlation in degradation of selected mRNAs at *post mortem* intervals of between 7 and 42 h although some tailing of bands by Northern blot analysis was noted. Ross, Knowler, and McCulloch (1992) reported variable degradation of G-protein mRNAs from human brain but were unable to relate this to the time interval between death and freezing of tissue. Wetzel, Bohn, and Hamill, (1994) found rat mRNAs to be remarkably stable *post mortem* when stored up to 72 h at 4°C.

Walker and McNicol (1992) reported stability of rat mRNAs up to 24 h *post mortem* as assessed by in situ hybridization.

In conclusion, the results of this study revealed that microfluidic capillary electrophoresis and real-time RT-PCR are suitable methods to quantify the degree of total RNA fragmentation and the number of copies of specific mRNAs, respectively. Overall, RNA degradation is a slow process under the conditions investigated here and, although *post mortem* storage temperature negatively affects the integrity of total RNA (higher degradation at 18°C), the transcripts of μ-calpain and cathepsin L are present for up to 5 days *post mortem* in the muscle of sea bass stored at either 1°C or 18°C.

Chapter 4

Effects of *post mortem* storage temperature on sea bass (*Dicentrarchus labrax*) muscle protein degradation: Analysis by 2-D DIGE and MS

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ABSTRACT

Storage conditions are known to be important for *post mortem* deterioration of fish muscle, and temperature is one of the factors with the strongest impact on this process. In order to shed light on the influence of temperature on the status of sea bass (*Dicentrarchus labrax*) muscle proteins during *post mortem* storage, a 2-D DIGE and mass spectrometry study was performed on fish kept at either 1 or 18°C for 5 days. As expected, the greatest alterations in sea bass filet protein composition were observed upon *post mortem* storage at 18°C, with distinct changes appearing in the 2-D protein profile after 5 days of storage at this temperature. In particular, degradation of the myofibrillar protein myosin heavy chain and of the glycolytic enzyme glyceraldehyde-3- phosphate dehydrogenase, among the most abundant muscle proteins, could be clearly observed upon storage at higher temperatures. Although to a lesser extent, however, several proteins were observed to vary in abundance also upon storage for 5 days at 1°C. In particular, one of the most interesting observations was the rapid and significant decrease in the abundance of nucleoside diphosphate kinase B and phosphoglycerate mutase 2, which was observed also at low storage temperatures and appeared to be temperature-independent. The results of this study offer new knowledge on changes occurring in sea bass muscle proteins during *post mortem* storage at different temperatures and provide indications on protein degradation trends that might be useful for monitoring freshness of fish and quality of storage conditions.

4.1 Introduction

Freshness is considered to be the most important quality of fish products, and the development of reliable methods for its assessment is of outstanding interest in the field of food science. One of the most unfavorable changes related to fish freshness is the *post mortem* tenderization of muscle, which promotes the progressive loss in quality. Therefore, the biochemical processes that are involved here have been extensively studied in order to identify the potential quality indicators or to control *post mortem* degradation.

Post mortem aging of fish normally follows four stages: *rigor mortis*, resolution of rigor, autolysis (loss of freshness), and bacterial spoilage. These stages can vary, depending upon a range of factors that include species, physiological condition of fish, stress prior to death, and temperature of *post mortem* storage, the last factor being the one with the strongest impact (Ocaño-Higuera et al., 2009). The autolytic processes catalyzed by endogenous enzymes represent the initial steps of fish muscle deterioration. Different proteolytic systems within the muscular fibers seem to be involved in the disorganization of the myofibrillar structure, including the lysosomal cathepsins, cytosolic calpains, cytoplasmatic aminopeptidases, multicatalytic proteasome, as well as some connective tissue hydrolytic enzymes (Delbarre-Ladrat et al., 2005). Several fish muscle proteins were shown to be essentially affected by *post mortem* proteolysis, such as titin and nebulin (Astier et al., 1991), which anchor myofilaments to Z-disks; a-actinin, which cross-links elastic filaments to thin filaments (Papa et al., 1996); troponin T in thin filaments; desmin, a myofibrillar packaging protein (Taylor et al., 1995; Campus et al., 2010); dystrophin, an essential component of the costameric structure, which represents more than 5% of sarcolemma cytoskeletal proteins (Papa et al., 1997); tropomyosin, a myofibrillar component (Astier et al., 1991, Ogata et al., 1998); and myosin heavy chain (MHC), the large protein component of striated muscle myofibrils (Wang et al., 2009). Aquaculture is currently contributing nearly half of the fish consumed by the human population worldwide. This sector has grown extensively in the last 50 years and is still growing more rapidly than other animal food-producing sectors (FAO, 2010). The growth of this market is leading to the steady increase in the amount of fish that is produced, stored, and transported, and new problems concerning quality issues are being generated. A higher quality is needed to maintain an elevated economical value of the aquacultured product, and the identification of markers enabling to monitor this parameter is a growing concern. The increasing importance of aquaculture is also reflected by the increasing number of proteomic studies that have been performed on commercial fish species in the recent years (Delbarre-Ladrat et al., 2006; Papa et al., 1996; Taylor et al., 1995; Alves et al., 2010; Forné

et al., 2010; Parrington et al., 2002; Goll et al 1991; Seki et al., 1984; Verrez-Bagni et al., 1999; Addis et al., 2010).

The recent developments in cutting-edge technologies such as proteomics can provide better insight into the biochemical processes occurring *post mortem* in fish, thus facilitating the identification of markers of fish quality. One of the most popular methods of differential proteome analysis combines protein separation by high-resolution 2-DE with MS identification of selected protein spots (Chevalier, 2010). Indeed, these technologies probably represent the best strategy for poorly characterized animal species: they offer a comprehensive approach for studying biochemical systems by expanding the level of investigation from a single protein to a wide range of proteins present in a cell or a tissue at once, without 'a priori' knowledge (FAO, 2010; Alves et al., 2010). Another advantage offered by the combined 2-D PAGE/MS identification approach relies. In the fact that 2-DE is very well suited for studies on degradation patterns, as this is the only proteomics setup that reads proteins (and not just peptides) with a high precision. In addition to protein identification data, PAGE/MS enables to obtain information also on molecular weight and isoelectric point of the spots of interest, and to compare these data with the theoretical parameters of the intact, unmodified protein. Multiplexing methods such as fluorescent 2-D DIGE (Fornè et al., 2010; Chevalier, 2010; Marouga et al., 2005) represent a further improvement of this analytical strategy, since they substantially reduce the variability by displaying two or more complex protein mixtures labelled with different fluorescent dyes on the same 2-D gel (Ünlü et al., 1997). In the specific field of our investigation, the implementation of the multiplexing approach enables a better comparison of protein profiles generated at different times of storage. Moreover, by loading the samples to be compared into the same gel together with an internal standard, and by subjecting them to exactly the same focusing and PAGE conditions, the 2-D DIGE approach enables robust and reliable sample-to-sample and gel-to-gel comparisons by means of dedicated software tools. In fish, proteomic technologies have been applied primarily to model organisms, such as zebrafish (*Danio rerio*). However, many teleosts of interest in biological research and with potential application in aquaculture have unique physiological characteristics (farmed fish are species that reach a large adult size with important muscle hyperplasia) that cannot be directly investigated from the study of small laboratory fish models (Forné et al., 2010). As a consequence, large scale proteomic studies are being increasingly applied to farmed and wildfish species of economical relevance, such as farmed sea breams (*Sparus aurata*) (Addis et al., 2010) and wild European hake (*Merluccius merluccius*) (Gonzalez et al., 2010). Here, 2-D DIGE and MS/MS were used to trace changes in the protein profiles of aquacultured sea bass (*Dicentrarchus labrax*) muscle proteins over 5 days of *post*

mortem storage at different temperatures, with the aim of identifying potential biochemical markers enabling monitoring and evaluation of fish filet quality.

4.2 Materials and methods

4.2.1 Animals and experimental protocol

The experiment was carried out at the Department of Biotechnology and Molecular Sciences of the University of Insubria (Varese, Italy) based on our previously described protocol (Terova et al., 2011).

One month before slaughter, sea bass (D. labrax) were randomly distributed into three tanks of 1m3 each with 40 fish per tank, and fed Hendrix-Skretting® Power Excel feed for marine fish. The tanks were connected to a sea water recirculation system with the following water conditions: temperature 20±2°C, pH 7, total ammonia o0.2mg/L, and dissolved oxygen level over 99% of the saturation. At the start of the experiment and after 4 days of starvation, six fish (average body weight 710±157.87 g) were removed from water and sacrificed by asphyxia/hypothermia (immersion in ice-cold water at a fish: ice ratio: 2:1). Fish were then divided into two subgroups of three fish each, which were stored ungutted for up to 5 days *post mortem* either in a refrigerator at 1°C or in a thermostat at 18°C. The first sampling was performed immediately after death (T0) by taking a fragment of white muscle tissue (2x2x2 cm) from the lateral–dorsal (epiaxial) muscle quadrant of each fish. The next sampling was performed similarly after 5 days of *post mortem* storage at the respective temperatures. In this way, the first three fish were sampled two times: at T0 and after 5 days of storage at 1°C ($T5_1$) and the other three ones were sampled at T0 and after 5 days of storage at 18°C ($T5_{18}$). When taking the muscle fragments, about 1 cm of tissue was left between one fragment and another to avoid contamination. Furthermore, throughout the trial, fish were all kept flat on the belly to prevent contact with the dorsal walls of the container or with other fish in the group. All muscle fragments taken in this way were immediately frozen at -80°1C and stored at this temperature until the proteomic analysis. All procedures were approved by the Animal Care Committee of the University of Insubria and conducted according to the guidelines of the Italian Committee on Animal Care.

4.2.2 Protein extraction

Proteomic experiments were carried out at the Proteomics Laboratory of Porto Conte Ricerche (Tramariglio, Alghero). Proteins were extracted from frozen muscle tissues using a TissueLyser

mechanical homogenizer (Qiagen, Hilden, Germany) as reported previously (Addis et al., 2009). A small portion (50 mg) of frozen fish muscle was minced with a sterile scalpel, placed in a 2-mL Eppendorf safe-lock tube (Eppendorf, Hamburg, Germany), and then immersed in lysis buffer (8M urea, 2% CHAPS, and 0.5% IPG buffer; GE Healthcare, Little Chalfont, UK) at a 5% w/v ratio. Three cycles of 1.5 min at 30 cycles/s in the TissueLyser mechanical homogenizer were employed for each sample. All extracts were then clarified for 15 min at 12 000xg at 4°C, quantified by the Bradford method (Sigma-Aldrich, St. Louis, MO, USA), tested for the quality and quantity by SDS-PAGE as described previously (Addis et al., 2009), and then stored at –80°C until analysis.

4.2.3 Sample labeling with DIGE fluorophores

Sixty micrograms of proteins extracted from six muscle fragments sampled at the time of death (T0) and after 5 days of *post mortem* storage at either 1°C (n=3) or 18°C (n=3) were labeled with 400 pmol N-hydroxysuccinimidyl-ester of cyanine dye Cy-3 and Cy5 (GE Healthcare), respectively. A pooled sample comprising equal amounts of each of the specimens in the study (T0+$T5_1$ and T0+$T5_{18}$), which served as internal standards, was labeled with Cy-2. Samples were maintained under dark conditions and on ice during 30 min for labeling. After quenching with 10mM lysine solution (Sigma-Aldrich), the labeled proteins of T0, $T5_1$, $T5_{18}$, and those of the internal standard were mixed in suitable combinations and adjusted to a concentration of 1%. IPG buffer (GE Healthcare) and Destreak Rehydration Solution (GE Healthcare) were added to a final volume of 450 mL (Table 4.1).

Gel Number	Cy2	Cy3	Cy5
1	Pooled sample T0+$T5_1$	T0 sample 1	$T5_1$ sample 1
2	Pooled sample T0+$T5_1$	T0 sample 2	$T5_1$ sample 2
3	Pooled sample T0+$T5_1$	T0 sample 3	$T5_1$ sample 3
4	Pooled sample T0+$T5_{18}$	T0 sample 4	$T5_{18}$ sample 1
5	Pooled sample T0+$T5_{18}$	T0 sample 5	$T5_{18}$ sample 2
6	Pooled sample T0+$T5_{18}$	T0 sample 6	$T5_{18}$ sample 3

Table 4.1. Sample preparation for quantitative analysis by 2-D DIGE

4.2.4 2-D gel electrophoresis

First-dimension isoelectric focusing (IEF) was performed using 24 cm precast IPG strips (pH 3–11 NL; GE Healthcare).

Mixtures of samples (450 mL) were applied onto the strips by passive rehydration overnight at room temperature. Six strips were run together on an IPGphor equipped with the EttanTM IPGphorTM3 loading manifold (GE Healthcare).

The strips were focused at 201C for a total of about 90 000V h.

After IEF, the strips were sequentially incubated in a freshly prepared solution of 1% DTT and 2% iodoacetamide in 50mM Tris-HCl (pH 8.8), 6M urea, 20% glycerol, and 2% SDS for 10 min.

The second-dimension SDS-PAGE was conducted on six 8–16.5% polyacrylamide gradient gels, using an EttanTM DALTtwelve electrophoresis system (GE Healthcare), for 30 min at 5 W/gel and then for 5 h at 17 W, at 25°C.

4.2.5 Image acquisition and analysis

After 2-D electrophoresis, gels were scanned on a Typhoon Trio1 image scanner (GE Healthcare) at 100 mm of resolution and with the photomultiplier tube (PMT) voltage was set between 500 and 700 V. The scanned gel images were then transferred to the ImageQuant V5.2 software package (GE Healthcare), cropped, and exported to the DeCyder Batch Processor and differential in-gel analysis (DIA) modules (GE Healthcare) for statistical analysis. To compare protein spots across gels, a match set was created from the images of all gels. The statistical analysis of protein level changes was performed by the DeCyder-BVA (Biological Variation Analysis, v.6.5) module. Preparative cyandye- labeled gels were scanned and matched with the master gel in order to assign the right correspondence for spot picking.

Calibration of the electrophoretic gels for protein molecular weight (Mr) and isoelectric point (pI) was performed by using markers for 2-DE electrophoresis (Sigma-Aldrich) and Precision Plus All Blue Protein Standards (Bio-Rad Laboratories, Hercules, CA, USA). Specifically, seven 7-cm IPG strips (pH 3–11, NL) were loaded as follows: one strip was loaded only with 2-DE markers, three strips were loaded with 100 mg of proteins from samples T0, $T5_1$, and $T5_{18}$, and three strips were loaded with a combination of each sample with 2-DE markers. All strips were then subjected to IEF as above for a total of about 30 000 V h and to SDS-PAGE in 15% polyacrylamide gels. All samples were analyzed in the same experiment in order to ensure the uniformity of running conditions. After staining with the Simply Blue Safe Stain (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions; gels images were digitalized with Image Scanner II (GE Healthcare). Image analysis, including spot detection, matching, and calibration, was performed using the Image Master 2-D Platinum v6.0.1 software (GE Healthcare). The Mr and pI values for the respective proteome patterns were determined by interpolation with the Image Master 2-D Platinum v6.0.1 calibration tools using Mr/pI markers associated with each gel. Isoelectric points

of experimental samples were then cross-checked by using the method described for Immobiline DryStrip gels in the GE Healthcare Data File no. 18-1177-60 AB.

4.2.6 Spot picking and *in situ* tryptic digestion

For protein identification, preparative 2-D PAGE gels were set up by overnight rehydration loading of 300 mg of protein extract into 3–11 NL 24-cm IPG strips. Strips were then focused and subjected to 2-DE as described above. All blue molecular weight markers (Bio-Rad Laboratories) were also loaded on an electrode wick and run together with the isoelectrofocused strips for calibrating molecular weight across the gel. After electrophoresis, the gel slab was subjected to mass-compatible silver staining as described previously (Addie et al., 2009). Visible protein spots of interest were manually excised from the gels, destained with 15mM $K_3Fe(CN)6$ in 50mM $Na_2S_2O_3$, washed with water, and then stored in ACN. The spots were then subjected to an O/N tryptic digestion at 37°C in 50mM (NH4)HCO3, pH 8.0, by using trypsin in the range from 40 to 100 ng, depending on spot intensity. Peptide mixtures were then collected by squeezing with ACN and centrifugation. Peptides were subsequently acidified with 20% TFA, dried, resuspended in formic acid 0.2%, and then stored at -20°C.

4.2.7 MS/MS analysis

LC-MS/MS analysis was performed on an XCT Ultra 6340 ion trap equipped with a 1200 HPLC system and a chip cube (Agilent Technologies, Palo Alto, CA, USA). After loading, samples were concentrated and desalted at 4 mL/min on a 40-nL enrichment column (75 µm x 43 mm, Agilent Technologies), with 0.2% formic acid. Peptides were then fractionated on a C18 reverse-phase capillary column at a flow rate of 300 nL/min, with a linear gradient of eluent B (0.2% formic acid in 95% ACN) in A (0.2% formic acid in 2% ACN) from 3–60% in 20 min.

ESI parameters were as follows: capillary voltage 1730 V; dry gas (N2), 5.00 L/min; dry temperature, 325°C; trap drive, 100; skimmer 30 V; lens 1, -5.00 V; octopole RF amplitude, 200 Vpp; capillary exit, 90 V. The ion-trap mass spectrometer was operated in a positive-ion mode.

Trap ICC smart target was 30 0000 units and maximal accumulation time was 100 ms. MS/MS was operated at a fragmentation amplitude of 1.3 V, and threshold ABS was 6000 units. Scan speed was 8100 UMA/s in MS and 26 000 UMA/s in MS/MS scans. Peptide analysis was performed scanning from m/z 250 to m/z 2200 in AutoMS (n) precursor selection mode of the three most intense ions (fragmentation mass range from 100 to 2200m/z). Dynamic exclusion was used to acquire a more complete survey of the peptides by automatic recognition and temporary exclusion (0.15 min) of ions from which definitive mass spectral data had previously acquired.

Data analysis software, provided by the manufacturers, was used to analyze MS/MS spectra and to generate a peak list that was introduced in the in-house MASCOT MS/MS ion search software (Version 2.3, Matrix Science, Boston, MA, USA) for protein identification in NCBI database using the chordata (vertebrates and relatives) taxonomy. Search parameters were as follows: peptide tolerance 300 ppm, MS/MS tolerance 0.6 Da, charge state 12 and 13, enzyme trypsin, allowing one missed cleavage.

4.2.8 Statistical analysis

The results related to T0 and 5 days of *post mortem* storage at either 1°C ($T5_1$) or 18°C ($T5_{18}$) were compared and statistically evaluated using univariate analysis one-way ANOVA. One-way ANOVA was performed using the DeCyder-BVA module and applying the false discovery rate (FDR) to minimize the number of false positives. A q-value of 0.05 was specified corresponding to the lowest FDR at which the protein is called significant. Data were normalized for computing the fold changes. Protein spots with a statistically significant variation ($p<0.05$), showing an over twofold difference in volume, were selected as differentially expressed and analyzed by MS/MS.

4.3 Results and discussion

The ultimate freshness of fish muscle depends on the extent of proteolysis of key target proteins within muscle fibers, which takes place during the *post mortem* period and promotes disintegration of the muscular structure (Delbarre-Ladrat et al., 2006; Papa et al., 1996, Papa et al., 1997).

This process is known to proceed much faster in fish than in other muscle-based foods, such as mammalian and avian meats. Furthermore, while in pork, beef, and poultry weakening of the muscular structure is desirable as it improves tenderness, this is not the case in fish, in which 'filet tenderization' stands for 'lack of freshness', and 'low quality product'.

Given the recognized importance of storage conditions for *post mortem* deterioration of fish muscle, this study has focused on the storage temperature, which is considered the factor with the strongest impact in this process.

Differences in the abundance of muscle proteins, due to biological *post mortem* processes, were studied by means of 2-D DIGE and MS/MS analysis in sea bass (*D. labrax*).

4.3.1 Changes in the protein profile of fish muscle after 5 days of *post mortem* storage at 1°C

In order to assess the modifications introduced in sea bass muscle proteins by *post mortem* storage, a 2-D DIGE analysis of the total muscle protein profiles at death (T0) and after 5 days of *post mortem* storage at 1°C ($T5_1$) was performed. The overlay signal of a representative map is reported in Figure 4.1.

In general, the 2-D profile appeared well preserved, and the extent of degradation was minimal after 5 days of storage at 1°C. Upon DeCyder analysis, spots 1–7 showed a significant quantitative variation (higher than twofold) during storage and were subjected to LC-MS/MS identification (Table 4.2). Spots 1, 2, 3, and 4 (Figure 4.1), identified as fructose-bisphosphate aldolase A, glycogen phosphorylase, and phosphoglucomutase-1, showed a significant increase in $T5_1$ with respect to T0 ($p<0.05$), whereas spots 5, 6, and 7, identified as nucleoside diphosphate kinase B (NDP kinase B), fatty acid-binding protein, and parvalbumin, respectively, decreased at the same *post mortem* time (Table 4.2). As seen from the data presented in Table 4.2, NDP kinase B showed the highest abundance decrease during storage at 11C, with an average ratio of -20.13 in $T5_1$ with respect to T0 ($p<0.04$), whereas the spots identified as fructose-bisphosphate aldolase A (1 and 2) showed the highest abundance increase at the same conditions ($p<0.03$).
The observation of an increasing spot upon storage might appear surprising. It should be taken into account, however, that the apparent increase in the abundance of a spot in the 2-D map can depend on protein modifications occurring as a result of *post mortem* biochemical processes, such as dephosphorylation, oxidation, or loss of charged amino acid side chains, leading to a shift in the isoelectric point. This might be the case of proteins for which an increase in only one spot of the isoelectric series was detected. As an example, for fructose bisphosphate aldolase A (spots 1 and 2), glycogen phosphorylase (spot 3), and phosphoglucomutase-I (spot 4) the appearance of 'laterally shifted' spots is evident when examining the DIGE overlay patterns. Most interestingly, these 'shifted' forms were detected at the same molecular weight of the 'parent' spots (i.e. spots 1 and 2 with spot 35, having experimental pIus of 7.66 and 7.76 versus 8.10, respectively). Conversely, most interesting was the dramatic disappearance of NDP kinase B at 5 days of storage (-20.13 in fold change), despite the relative stability of the muscle protein pattern in general.

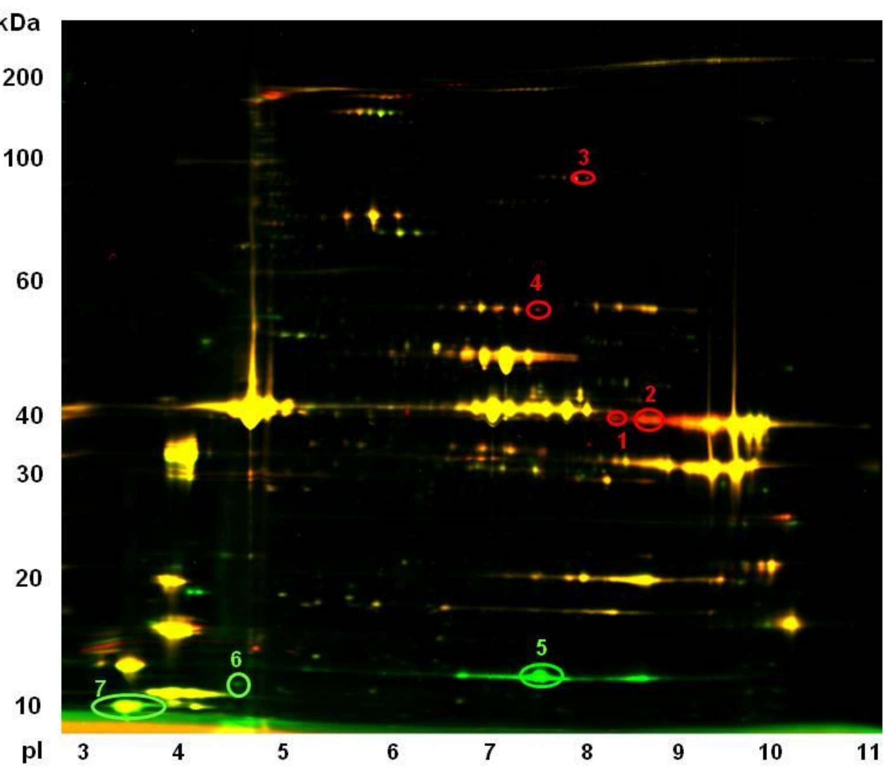

Figure 4.1 - Overlay 2-D DIGE profile of protein patterns corresponding to sea bass muscle at death (T0) and after 5 days of *post mortem* storage at 1°C ($T5_1$). Equal amounts (60 mg) of two pools of proteins extracted from T0 (*n*=3) and ($T5_1$) (*n*=3) samples (green and red signals, respectively), together with a standard mix of equimolar amounts of the six aforementioned biological replicates, were loaded on the same gel. Proteins undergoing a significant variation in abundance (42-fold change) are numbered, circled, and indicated in red (increase) or green (decrease). The resulting overlapping colours (from red to green, through shades of yellow) reflect the expression of each protein along the time scale of investigation.

4.3.2 Changes in the protein profile of fish muscle after 5 days of *post mortem* storage at 18°C

In order to investigate the trend in protein degradation at higher storage temperatures, and to compare it with the degradation trends observed upon storage at 1°C, a 2-D DIGE analysis of the protein profiles of sea bass muscle at death (T0) and after 5 days of *post mortem* storage at 18°C ($T5_{18}$) was also performed. Results are presented in Figure 4.2. As expected, a higher number of spots showed a significant quantitative variation (higher than twofold, $p<0.05$) compared with the T0 samples (Table 4.3). When subjected to MS identification, many of these spots did not produce statistically significant results (spots 9, 10, 13, 14, and 16).

Spot no.	Protein name	Average ratio $T5_1/T0$	1-ANOVA
1	Fructose-biphosphate aldolase A	3.06	0.03
2	Fructose-biphosphate aldolase A	2.9	0.03
3	Glycogen phosphorylase	2.12	0.05
4	Phosphoglucomutase-1	2.15	0.03
5	Nucleoside diohosophate kinase B	-20.13	0.04
6	Fatty acid binding protein	-3.63	0.04
7	Parvalbumin	-3.16	0.044

Table 4.2 - List of protein spots undergoing significant variation in abundance (<2-fold change) after 5 days of *post mortem* storage at 1°C ($T5_1$) with respect to the time of death (T0). Difference were determined by one-way analysis of variance (1-ANOVA) ($p<0.05$).

This might be an indication that this constellation of spots might be represented by a mixture of proteolytic fragments derived from the most abundant muscle proteins, being each present at amounts too low to enable MS identification with the instrumentation used in this work. Other differentially abundant spots produced MS identifications corresponding to proteins having higher molecular weights than those calculated based on spot location, suggesting that they were fragments of larger proteins (spots 8, 17, 19, 20, 22, 27, and 29, Table 4.4). Among these, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and MHC were identified in two strongly increased spots in the basic, low–molecular-weight region, in agreement with proteolytic events occurring during storage (GAPDH in spot 8, at 18 versus 36.2 kDa and pI 8.23 versus 8.7, and MHC in spot 19, at 13 versus 222 kDa and pI 8.24 versus 5.57) (Table 4.4). Similarly, spots 20 and 22 (45 kDa, pI 6.27 and 6.30, respectively) were identified as enolase, with the 'parent' spot located at higher molecular weight and isoelectric point (spot 37, 54 kDa, pI 6.98).

The major changes in the 2-D DIGE analysis of $T5_{18}$ versus T0 samples concerned GAPDH and myosin, which are among the most abundant protein components of striated fish muscle. Myosin is a protein of approximately 500 kDa composed of six subunits: two MHCs and four myosin light chains (MLCs). It is assembled into thick filaments that form the functional myosin in muscle. Some studies have indicated *post mortem* proteolysis of fish MHCs (Busconi et al., 1989), particularly when stored at room temperature (Astier et al., 1991).

Others have found that MHCs are fairly stable when fish muscle is stored in ice for a prolonged period (Verrez-Bagnis et al., 2001; Jasra et al., 2001; Tsuchiya et al., 1991).

In our study, the sequence coverage of protein spots 19 and 27, both identified as MHC, was very low (Table 4.4).

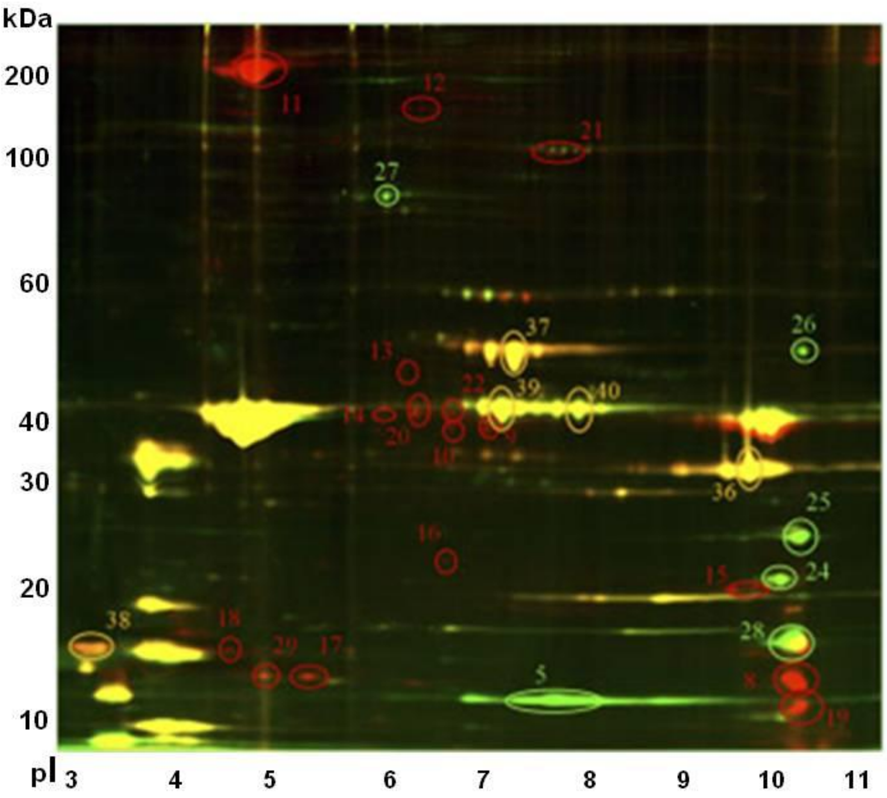

Figure 4.2 - Overlay 2-D DIGE profile of protein patterns corresponding to sea bass muscle at death (T0) and after 5 days of *post mortem* storage at 18°C ($T5_{18}$). Equal amounts (60 mg) of two pools of proteins extracted from T0 (*n*=3) and ($T5_{18}$) (*n*=3) samples (green and red signals, respectively), together with a standard mix of equimolar amounts of the six aforementioned biological replicates, were loaded on the same gel. Proteins undergoing a significant variation in abundance (42-fold change) are numbered, circled, and indicated in red (increase) or green (decrease). The resulting overlapping colors (from red to green, through shades of yellow) reflect the expression of each protein along the time scale of investigation.

This, together with their detection in different areas of the 2-D DIGE gel for samples at T0 and $T5_{18}$ (Figure 4.2), at lower molecular weights (5.57 and 5.63 versus 222 kDa, respectively), and at higher isoelectric points (8.24 and 6.05 versus 5.6, respectively) than expected (Table 4.4), indicates that they are likely fragments of a protein with a high molecular weight, as sequence coverage is calculated relative to the full-length protein. In particular, the concentration of the MHC fragment (spot 19) increased, thereby implying an MHC breakdown concurrent with 5-day storage of sea bass muscle at 18°C. The increase seen in MLC 2 (spots 17 and 29) could also be

indicative of myosin degradation. Interestingly, in the study of Kjærsgard et al. (2003), MLC 2 was found to increase about threefold during ice storage of cod.

A similar study on *post mortem* storage of pig for 72 h showed the concentration of MLC 2 to increase about fivefold during *post mortem* storage (Lametsch et al., 2003).

On the other hand, the abundance of several spots decreased significantly during storage at 181C. In particular, six spots showed high fold change values and were subjected to MS analysis. Namely, protein identities were NDP kinase B (spot 5), phosphoglycerate mutase 2, elongation factor 1-a, MHC, and troponin I (spots 24 and 25, 26, 27, and 28, respectively). As opposite to spots showing a strong increase upon storage, all spots showing a statistically significant decrease upon storage did all produce protein identifications compatible with the molecular weights predicted from their sequences.

4.3.3 Influence of *post mortem* storage temperature on muscle protein profile

When observing proteins that decreased during storage, a consistency of data between the two storage times could be seen. In order to evaluate the differences in protein degradation occurring upon storage at two different temperatures, and to pinpoint possible markers of cold storage by means of an overall comparison, a 2-D DIGE analysis was performed on sea bass muscle after 5 days of *post mort*em storage at 1 and 18°C (Figure 4.3). As a result, several protein spots that differed significantly in intensity were detected (Table 4.5).

Among others, some spots showed interesting increasing trends, such as spots 8 and 19, which as discussed above, did likely correspond to degradation fragments of GAPDH and MHC, respectively (fold change), as well as those corresponding to MLC 2 (spots 17 and 29). Conversely, spots identified as phosphoglycerate mutase 2 (spot 25), desmin (spot 34), myosin-binding protein C (spot 12), and MHC (27) showed a stronger degradation upon storage at 18°C when compared with storage at 1°C ($p<0.05$).

Interestingly, almost all of the spots with a decreasing trend provided MS identifications compatible with the expected molecular weight of the protein. The only protein identified among spots of decreasing abundance that did not match the expected molecular weight was MHC in spot 27 (96 versus 222 kDa, and pI 6.05 versus 5.63); however, it should be noted that MHC was detected at the same position in the map generated from samples at T0, as well as in fresh sea bream muscle (Addis et al., 2009), and might justify this apparent discrepancy.

Spot no.	Protein name	Average ratio $T5_{18}/T0$	1-ANOVA
8	Glyceraldehyde-3-phosphate dehydrogenase	47.01	0.0021
11	Myosin heavy chain	13.88	0.004
12	Myosin-binding protein C	8.44	0.0021
15	Triosephosphate isomerise B	7.22	0.0081
17	Myosin light chain 2	6.06	0.019
18	Eukaryotic translation initiation factor 5A-1	5.22	0.012
19	Myosin heavy chain	5.14	0.031
20	Enolase	4.19	0.071
21	Glycogen phosphorylase	3.48	0.012
22	Enolase	3.1	0.045
29	Myosin light chain 2	2.27	0.012
5	Nucleoside diohosophate kinase B	-15.52	0.033
24	Phosphoglycerate mutase-2	-3.84	0.0057
25	Phosphoglycerate mutase-2	-3.21	0.011
26	Elongation factor 1-α	-3.6	0.0059
27	Myosin heavy chain	-3.07	0.017
28	Troponin I	-2.66	0.0046

Table 4.3 - List of protein spots undergoing significant variation in abundance (<2-fold change) after 5 days of *post mortem* storage at 18°C ($T5_{18}$) with respect to the time of death (T0). Difference were determined by one-way analysis of variance (1-ANOVA) ($p<0.05$).

In addition, the comparative analysis showed that sea bass myosin may degrade even at low temperatures of storage. Similarly, Wang et al. (2009) found MHC breakdown in myofibrillar proteins of cod stored at temperatures between 0 and 6°C. With respect to deterioration processes, proteome analysis has also been reported for shrimps (Martinez et al., 2001). In this study, proteome analysis of Arctic (Pandalus borealis) and tropical shrimps (Penaeus japonicus and Penaeus monodon) revealed differences in *post mortem* degradation of muscle proteins between cold-water and warm-water species.

Muscle proteases seemed to be inhibited during storage in the tropical species, but not in the Arctic species; such a result was tentatively attributed to a significantly higher difference between living and storage temperatures in the case of tropical shrimps. Other studies in fish have well established that the stability of myofibrillar proteins differs *post mortem*, depending on the habitat temperature of the species. During iced and frozen storage, the thermal characteristics of myosin subunits deteriorate faster in cold- than in warm-water fish (Davies et al., 1994; Hastings et al., 1985).

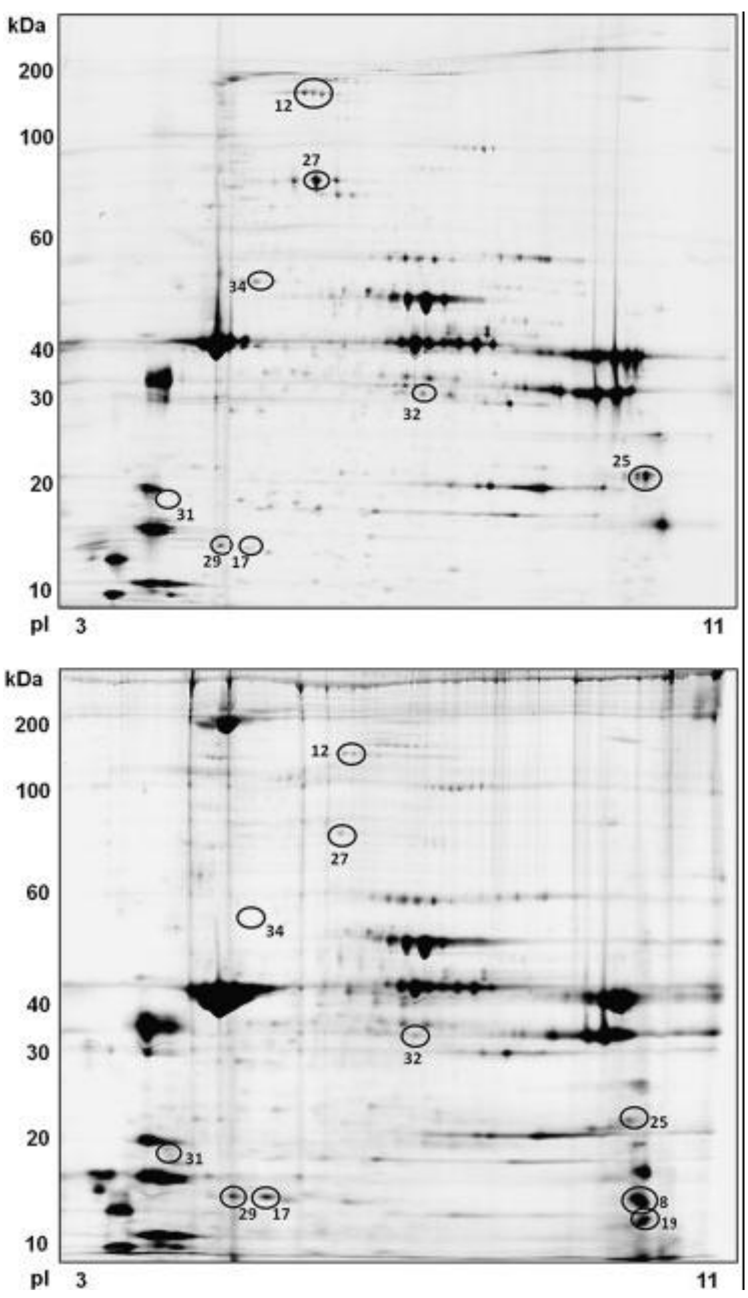

Figure 4.3 - Protein profiles of 5 days *post mortem* protein extracts from sea bass muscle stored at 1°C ($T5_1$) (top) and stored at 18°C ($T5_{18}$) (bottom). Proteins undergoing a significant variation in abundance (42-fold change) are numbered and circled.

Spot	Identified protein	Acc. No.[a]	Species	Theoretical Mr/pI[b]	Observed Mr/pI[c]	Score[d]	Q.M.[e]	%C[f]
1	Fructose-bisphosphate aldolase A	P04075	*H.sapiens*	39.851/8.30	46.00/7.66	58	3	5
2	Fructose-bisphosphate aldolase A	P04075	*H.sapiens*	39.851/8.30	46.00/7.76	251	11	13
3	Glycogen phosphorylase	P11217	*H.sapiens*	97.487/6.57	107.00/7.26	498	17	10
4	Phosphoglucomutase-1	Q08DP0	*B. taurus*	61.836/6.36	67.00/7.12	582	18	7
5	Nucleoside diphosphate kinase B	P85292	*M. magellanicus*	14.280/5.70	15.00/7.17	406	13	23
6	Fatty acid-binding protein	Q4TZH2	*B. grunniens*	14.827/6.73	17.00/4.56	33	1	5
7	Parvalbumin	B6UV97	*H. molitrix*	11.624/4.46	13.00/4.0	189	7	36
8	Glyceraldehyde-3-phosphate dehydrogenase	Q9PTW5	*P. olivaceus*	36.190/8.70	18.00/8.23	254	4	13
9	Unidentified							
10	Unidentified							
11	Myosin heavy chain	D5JZI9	*D. labrax*	202.759/5.61	187.00/5.39	2032	71	17
12	Myosin-binding protein C	Q90688	*G. gallus*	143.054/5.96	141.00/6.08	87	2	1
13	Unidentified							
14	Unidentified							
15	Triosephosphate isomerase B	Q90XG0	*D. rerio*	27.096/6.45	28.00/8.00	392	13	29
16	Unidentified							
17	Myosin light chain 2	Q9IB31	*P. argentata*	19.153/4.73	17.00/5.45	385	9	67
18	Eukaryotic translation initiation factor 5A-1	Q6EWQ7	*B. taurus*	17.049/5.08	19.00/5.40	55	2	5
19	Myosin heavy chain	Q9IBD7	*S. dumerili*	222.534/5.57	13.00/8.24	134	3	2
20	Enolase	Q6PC12	*D. rerio*	47.481/6.16	45.00/6.27	995	44	35
21	Glycogen phosphorylase	P11217	*H.sapiens*	97.487/6.57	107.00/7.22	498	17	10
22	Enolase	Q6TH14	*D. rerio*	47.841/6.25	45.00/6.30	508	34	20
24	Phosphoglycerate mutase 2	Q32HV0	*B. taurus*	28.838/8.99	29.00/8.14	56	3	31
25	Phosphoglycerate mutase 2	Q32KV0	*B. taurus*	28.838/8.99	36.00/8.20	56	3	6
26	Elongation factor 1-alpha	P68103	*B. taurus*	50.451/9.10	60.00/8.21	295	27	18
27	Myosin heavy chain	P13538	*G.gallus*	223.976/5.63	96.00/6.05	40	2	0
28	Fast skeletal muscle troponin I	P48788	*H. sapiens*	21.496/8.87	22.00/8.23	95	7	4
29	Myosin light chain 2	Q9DEA8	*T. chalcogramma*	19.152/5.73	19.00/5.20	708	31	2
34	Desmin	O62654	*B.taurus*	53.556/5.21	61.00/5.62	84	3	5
35	Fructose-bisphosphate aldolase A	Q803Q7	*D. rerio*	40.228/8.35	45.00/8.10	596	20	37
36	Glyceraldehyde-3-phosphate dehydrogenase	Q5I1Z5	*D. labrax*	36.041/8.56	40.00/8.01	449	24	48
37	Alpha-enolase	P06733	*H. sapiens*	47.481/6.25	54.00/6.98	651	23	28
38	Myosin light chain 2	Q9IB25	*T. trachurus*	19.136/4.71	17.00/3.91	803	301	84
39	Creatine kinase M-type	C7ASM1	*S. chuatsi*	43.123/6.32	46.00/6.90	685	29	33
40	Creatine kinase M-type	C1BIK3	*O. mordax*	42.991/6.32	47.00/7.24	286	13	22

[a] Accession number.
[b] Theoretical molecular weight/isoelectric point of full-length protein.
[c] Molecular weight/isoelectric point of full-length protein calculated upon calibration of electrophoretic gels.
[d] Score: probability score in Mascot program (the probability that the observed match between the experimental data and mass values calculated from a candidate peptide sequence is a random event.
[e] Queries Matched; indicates the number of matched peptides in database search.
[f] Percent coverage: the minimum coverage of the matched peptide in relation to the full-length sequence.

4.4 - Tandem mass spectrometry protein identifications

Spot no.	Protein name	Average ratio $T5_1/T5_{18}$	1-ANOVA
8	Glyceraldehyde-3-phosphate dehydrogenase	> 60	< 0.05
19	Myosin heavy chain	> 60	< 0.05
17	Myosin light chain 2	56.56	0.009
29	Myosin light chain 2	51.16	0.013
12	Myosin-binding protein C	23.21	0.007
27	Myosin heavy chain	-5.23	0.015
34	Desmin	-3.36	0.022
25	Phosphoglycerate mutase 2	-2.24	0.044

Table 4.5 - List of protein spots undergoing significant variation in abundance (<2-fold change) after 5 days of *post mortem* storage at 1°C with respect to those stored at 18°C . Difference were determined by one-way analysis of variance (1-ANOVA) ($p<0.05$).

The myofibrillar packaging protein desmin (spot 34) was found to strongly decrease after 5 days of storage at 18°C, although a decrease was also observed upon storage at 18°C.

Desmin is the predominant protein of the intermediate cytoskeleton of mature muscle filaments (Greaser et al., 1991), being thus essential for maintaining myofibril, myofiber, and whole muscle tissue structural and functional integrity (Capetanaki et al., 1997).

Owing to its role in maintaining the integrity of the muscle cell, desmin degradation is believed to have a significant impact on meat quality (Taylor et al., 1995; Huff-Lonergan et al., 1996). Abundant studies have reported *post mortem* proteolysis of desmin during aging of muscle in pork (Christensen et al., 2004; Kristensen et al., 2001), in beef (Taylor et al., 1995; Huff-Lonergan et al., 1996; Takahashi et al., 1996), and in fish (Campus et al., 2010; Seki et al., 1984).

The most striking observation of this study was the consistent decrease in abundance of NDP kinase B observed after 5 days of storage at both temperatures. When comparing the abundance decrease of NDP kinase B at $T5_1$ with that at $T5_{18}$, it can be clearly observed how variation of this proteins is strongly susceptible to the storage time and occurs very rapidly independent of the storage temperature.

Indeed, the average ratio of NDP kinase B did not differ significantly between $T5_1$ (-20.13) and $T5_{18}$ (-15.52), and after 5 days of storage the protein was almost undetectable in both maps. In a study published in 2001, Verrez-Bagnis et al. reported the *post mortem* evolution of protein patterns in farmed sea bass muscle. The protein pattern was monitored by SDS-PAGE and by 2-DE after 0, 2, 4, and 6 days of cold storage.

SDS-PAGE revealed the gradual disappearance of a protein band of 16 kDa after fish death. The loss of this polypeptide was related to the time of *post mortem* storage; 94% of degradation was observed after 96 h. This is in agreement with our results, which highlight a steep decrease in NDP kinase B with storage time. However, the identity of the 16-kDa sarcoplasmic protein described by Verrez-Bagnis et al. (2001) remained unknown as it did not significantly match any known proteins present in the protein databases of that year.

We hypothesize that the NDP kinase B identified in our study might correspond to the spot reported by Verrez-Bagnis et al. (2001). This suggests that NDP kinase B may represent a possible biomarker for fish freshness.

4.4 Concluding remarks

In conclusion, the results of the present study demonstrated a relatively more stable protein pattern in sea bass muscle as compared with mammalian muscle. This indicates that sea bass muscle proteins might be proteolysed to a relatively less degree. The greatest alterations in sea bass filet protein composition were ascribed to the 18°C storage, and distinct changes appeared after 5 days of storage at this temperature.

Although to a lesser extent, also storage at 1°C introduced modifications in the total protein profile of sea bass muscle, especially concerning NDP kinase B, for which a fast and significant decrease was observed at both storage temperatures. The results presented here provide important indications on the events occurring in sea bass muscle proteins during storage and provide an important basis for future studies aimed to determine how these changes are related to quality traits of fish filet.

Chapter 5

2D DIGE/MS to investigate the impact of slaughtering techniques on *post mortem* integrity of fish filet proteins

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Keywords:

Post mortem
sea bass
muscle
slaughtering
protein degradation
NDKB

ABSTRACT

Two-dimensional difference gel electrophoresis (2D DIGE) was applied to investigate the impact of slaughtering on the *post mortem* integrity of muscle tissue proteins in European sea bass (*Dicentrarchus labrax*). Three different slaughtering techniques were evaluated: asphyxia in air (AA), asphyxia in ice (AI), and spinal cord severance (SCS). Principal components analysis (PCA) revealed a significant divergence of SCS samples, whereas AA and AI samples, although grouped separately, were less divergent and could be included in a single asphyxia cluster. In terms of single proteins, the most significant impact was seen on nucleoside diphosphate kinase B, which was consistently less affected when fish were slaughtered by SCS as compared to asphyxia. Integrity of the sarcomeric proteins myosin heavy chain and myosin binding protein C and of the cytosolic proteins fructose biphosphate aldolase, glyceraldehyde 3-phosphate dehydrogenase, and enolase 1 was also better preserved upon SCS slaughtering. Most interestingly, the influence on muscle protein integrity could be detected since the early *post mortem* phase. In conclusion, slaughtering by SCS preserves protein integrity better than death by asphyxia, either in ice or in air. Both asphyxia conditions are comparably more adverse than SCS to muscle protein integrity, although a general trend favouring AI over AA is observed.

5.1 Introduction

The word “quality” is used widely and with many meanings. In the fishing industry the term "quality fish" often relates to expensive species or to the size of fish. Most often, however, quality is synonymous with aesthetic appearance and freshness and refers to the extent of spoilage of the fish.

To government authorities, which are mainly concerned with possible health hazards, good quality means the absence of harmful agents such as parasites, chemicals, or pathogenic organisms (Huss et al., 1988). Nowadays, fish flesh quality is an indispensable factor for marketing. It is interesting to note that quality and welfare issues are intrinsically linked as there is evidence that inadequate fish husbandry produces lower meat quality (Ribas et al., 2007; Robb et al., 2000).

Flesh quality in fish is determined by various factors, including husbandry practices, diet, handling at slaughter, the choice of slaughtering method, and *post mortem* handling (Ruff et al., 2002; Terova et al., 2010). Nevertheless, if procedures before slaughter and the slaughtering are carried out without taking care to avoid severe stress conditions to the animal, these can strongly influence flesh quality and the subsequent changes during storage of the final product (Poli et al., 2005). Therefore, particular attention should be paid to testing slaughtering methods that allow some degree of *prerigor* processing (Skjervold, 2001).

A variety of slaughtering techniques are applied in aquaculture, each one inducing a different stress level. The slaughtering methods commonly used in European fish farms are death by asphyxia in air (AA), asphyxia in ice slurry (AI), electrical stunning, carbon dioxide narcosis, knocking and percussive stunning, and spiking (Poli et al., 2005). Some of these killing procedures are not that feasible in commercial situations, requiring high-cost, qualified personnel (Ambroggi et al., 1996). As a matter of fact, in most commercial fish farms in Italy, sea bass is killed by AI.

Numerous studies have focused on the impact of different slaughtering methods on fish flesh quality. In particular, Ambroggi (1996) showed the influence of four different slaughtering methods (percussive stunning, asphyxia, carbon dioxide narcotization, and electrocution) on *rigor mortis* and glycogen consumption in *Oncorhynchus mykiss*. Several other studies (Robb et al., 2000; Erikson et al., 1999; van de Vis et al., 2003) focused on the relation between slaughtering method and ATP depletion since the latter is strictly related to some important indicators of freshness, such as *rigor mortis*, muscle firmness, and odor. Choosing the most suitable slaughtering method is an important step for assuring good quality of fish products. It is known that physical activity at or before slaughter affects the flesh quality of fish. Greater activity before death leads to a more rapid increase in muscle pH, resulting in shorter times to onset of rigor (Robb et al., 2000). An animal that struggles at slaughter goes into *rigor* very rapidly, whereas minimal stress at slaughter ensures minimal physical damage to fish. However, delayed onset of *rigor* is not, by definition, an indicator of correct handling or treatment, as electrical stunning, even though consciousness is lost immediately, may promote onset of *rigor* (Digre et al., 2010; Erikson et al., 2012; Morzel et al., 2002; Scherer et al., 2005). On the other hand, electrical

stunning of Atlantic salmon in water did not accelerate rigor development and no injuries were observed in the fish (Roth et al., 2002).

To date, the influence of slaughtering methods on *post mortem* protein degradation in fish has not yet been subjected to a detailed investigation. Therefore, the aim of this study was to investigate the effects of different slaughtering methods, namely, AA, AI, and spinal cord severance (SCS), on *post mortem* proteome changes in European sea bass (*Dicentrarchus labrax*) and, hence, on fish muscle quality. These methods were chosen because death by AI is commonly used in fish farms in Italy, whereas death by SCS is a quick method of killing with relatively low stress and *pre mortem* muscle activity when compared to AI and AA, and is representative of other methods aimed to avoid animal stress. The extent of protein degradation was assessed by 2D DIGE as a tool by which abundance of protein spots resulting from degradation or chemical modification of muscle proteins can be visualized and compared.

5.2 Materials and methods

5.2.1 Fish samples

The experiment was carried out at the Department of Biotechnology and Life Sciences of the University of Insubria (Varese, Italy). Sea bass (*Dicentrarchus labrax*) of about 150 g were reared into three tanks of 1m3 with 40 fish per tank and fed Hendrix-Skretting® Power Excel feed for marine fish. The tanks were connected to a sea water recirculation system under the following water conditions: temperature 20±2°C, pH 7, total ammonia < 0.2mg/L, and dissolved oxygen over 99% of the saturation. After 2 days of starvation, nine fish were removed from water and slaughtered in the three following different ways:

Asphyxia in air (AA). After being taken out of the water, three of the nine fish were left in the open air, thus preventing oxygen exchange with the environment, whereby the animals suffocated. As this method is extremely aversive to fish, the animals showed long, violent escape behavior as demonstrated by excessive exercise.

Asphyxia in ice (AI). Another three of the nine fish were immersed in ice-water slurry (ice was mixed with seawater in a 3:1 ratio). The temperature of the slurry was maintained constant during the process by adding ice if necessary. As fish were sedated by ice they showed a short escape response evidenced by agitated swimming behavior.

Spinal cord severance (SCS): The remaining fish were taken out of water and immediately euthanized by severing the spinal cord. As fish were euthanized by three operators, they remained out of water for no more than 10 seconds before death, avoiding aversive behavior.
All fish were considered dead ten minutes after the last opercula movements (respiration/gill movements) were observed. After death, all fish were stored ungutted for up to 5 days in a refrigerator at 1°C. The first tissue specimens were harvested at the time of death (T0 = 10 min after the last opercula movements were observed) by taking a fragment of white muscle tissue from the lateral-dorsal muscle quadrant (epiaxial) of each fish. The next sampling was performed similarly. After 5 days of *post mortem* storage at 1°C. The 18 muscle fragments taken in this way were immediately frozen at -80°C and stored at this temperature until the proteomic analysis. All procedures were approved by the Animal Care Committee of the University of Insubria and conducted according to the guidelines of the Italian Committee on Animal Care.

5.2.2 Protein extraction

Proteomic experiments were carried out at the Proteomics Laboratory of Porto Conte Ricerche (Tramariglio, Alghero). Proteins were extracted from frozen muscle tissues using a TissueLyser mechanical homogenizer (Qiagen, Hilden, Germany) as reported previously (Addis et al., 2010; Addis et al., 2009). Briefly, a small portion (50 mg) of minced frozen fish muscle was placed in a 2 mL Eppendorf safe-lock tube (Eppendorf, Hamburg, Germany) containing lysis buffer (8 M urea, 2% CHAPS, and 0.5% IPG buffer; GE Healthcare, Little Chalfont, UK) at a 5% w/v ratio. Each sample was then processed with a TissueLyser mechanical homogenizer. All extracts were clarified by centrifugation, quantified with the 2D Quant kit (GE Healthcare), tested for quality and quantity by SDS-PAGE, and stored at -80°C until analysis.

5.2.3 Sample labelling with DIGE fluorophores

Sixty micrograms of protein extracted from each of the nine muscle specimens sampled at the time of death (T0), and from the other nine sampled after 5 days of *post mortem* storage at 1°C (T5) were labelled with 400 pmol N-hydroxysuccinimidyl-ester of cyanine dye Cy3 and Cy5 (GE Healthcare), respectively (Table 5.1). A pooled sample, which served as the internal standard, was labelled with Cy2, as described previously (Terova et al., 2011). After quenching with lysine, the labelled proteins were mixed in suitable combinations as summarized in Table 5.1. IPG buffer

(GEHealthcare) and Destreak Rehydration Solution (GE Healthcare) were added to a final volume of 200 µl for each mix.

Gel	Cy2	Cy3	Cy5
A	Int. standard	AA T0_1	AA T5_1
B	Int. standard	AA T0_2	AA T5_2
C	Int. standard	AA T0_3	AA T5_3
D	Int. standard	SCS T0_1	SCS T5_1
E	Int. standard	SCS T0_2	SCS T5_2
F	Int. standard	SCS T0_3	SCS T5_3
G	Int. standard	AI T0_1	AI T5_1
H	Int. standard	AI T0_2	AI T5_2
I	Int. standard	AI T0_3	AI T5_3

Table 5.1 - Design of the 2D DIGE experiment. Letters indicate the different gels composing the experiment, and each line indicates the samples that were loaded in the gel. Int. standard refers to the samples pool used as an internal reference for software analysis. AA, AI, and SCS indicate the slaughtering mode (asphyxia in air, asphyxia in ice, and spinal cord severance, respectively). T0 and T5 refer to the time of sampling, and the number refers to the biological replicate (fish number). Cy2, Cy3, and Cy5 indicate the labelling cyanine used for the sample set.

5.2.4 2D gel electrophoresis

First-dimension IsoElectric Focusing (IEF) was performed using 11-cm IPG strips (pH 3-10 NL; BioRad Laboratories, Hercules, CA). The labeled sample mixtures prepared as shown in Table 5.1 were applied onto the strips by overnight passive rehydration at room temperature. The strips were focused on an IPGphor equipped with the Ettan™ IPGphor™3 loading manifold (GE Healthcare) at 20°C for a total of about 30,000 Vh. After IEF, the strips were equilibrated, reduced, and alkylated by sequential incubation in 2% DTT and 2.5% iodoacetamide in 50 mM Tris-HCl (pH 8.8), 6 M urea, 20% glycerol, and 2% SDS, for 10 min each. The second-dimension SDS-polyacrylamide gel electrophoresis was conducted on precast AnykDa Criterion gels (BioRad) in a Dodeca Cell (BioRad) until the tracking dye reached the bottom of the gel.

5.2.5 Image analysis and statistical processing of data

After 2D electrophoresis, gels were scanned on a Typhoon Trio+ image scanner (GE Healthcare), transferred to the ImageQuant V5.2 software package (GE Healthcare), and exported to the DeCyder Batch Processor and DIA (Differential-In gel Analysis) modules (GE Healthcare). The results related to fish slaughtered in three different ways (AI, AA, and SCS) and sampled at T0 and at 5 days of *post mortem* storage at 1°C (T5) were evaluated as described previously (Terova et al., 2011) with minor modifications. Briefly, statistical analysis was performed with the DeCyder-BVA (Biological Variation Analysis, v.6.5) module by applying the false discovery rate (FDR) to minimize false positives. A q-value of 0.05 was specified as the lowest FDR. Data were normalized for computing the fold changes. Internal comparison of T0 and T5 samples among the three slaughtering conditions were performed according to the log standardized abundance, and mean, SD, and *t*-test were calculated. Principal components analysis was performed by the EDA module. Comparison of T5 vs T0 samples for time and slaughtering procedure was performed first by one-way ANOVA, and then by two-way ANOVA by setting time and slaughtering as conditions ($p<0.001$), with the BVA module of the DeCyder software. At the end of the analysis process, protein spots with a statistically significant variation and with >2 fold volume difference were selected for analysis by tandem mass spectrometry. The electrophoretic gels were calibrated for protein molecular weight (Mr) and isoelectric point (pI) in order to assign the correct pI and Mr to the differentially abundant spots detected by differential image analysis, as described previously (Terova et al., 2011).

5.2.6 Tandem mass spectrometry analysis.

Preparative 2D PAGE gels were set up by loading 200 µg of protein extract into 3-10 NL, 11-cm IPG strips (BioRad), which were then focused and subjected to 2-DE electrophoresis as described above. All blue molecular weight markers (BioRad) were also loaded for calibrating molecular weights across the gel. The gel was subjected to mass-compatible silver staining (Chevallet et al., 2006), digitalized by scanning with an ImageScanner II (GE Healthcare), and matched to the DIGE gel images generated at T0 for the three slaughtering procedures using the software ImageMaster Platinum 6.0.1, in order to track the spots to be excised for protein identification. Matched spots of interest were manually excised from the gels, distained, and subjected to O/N tryptic digestion. Peptide mixtures were then collected by squeezing with acetonitrile and centrifugation, then acidified, dried, resuspended in formic acid, and stored at -20°C (Terova et al., 2011; Chevallet et al., 2006).

LC-MS/MS analyses were performed on a XCT Ultra 6340 ion trap equipped with a 1200 HPLC system and a chip cube (Agilent Technologies, Palo Alto, CA), as described (Terova et al., 2011). Briefly, samples were concentrated and desalted on an enrichment column with formic acid, and peptides were fractionated on a C18 reverse-phase column directly coupled to a nanospray source. Data Analysis software, provided by the manufacturers, was used to analyze MS/MS spectra and to generate a peak list which was analyzed by Proteome Discoverer (version 1.3, Thermo Scientific) using an inhouse Mascot server (version 2.3, Matrix Science) for protein identification in the updated NCBI database, employing the Chordata (vertebrates and relatives) taxonomy and the following search parameters: precursor mass tolerance 300 ppm; fragment mass tolerance 0.6 Da; charge state +2, +3, and +4; enzyme trypsin; two missed cleavages; cysteine carbamidomethylation as static modification; and N-terminal glutamine conversion to pyroglutamic acid and methionine oxidation as dynamic modifications.

5.3 Results

5.3.1 Experimental design

In order to compare the modifications introduced in sea bass muscle proteins by different slaughtering methods, a 2D DIGE experiment (Figure 5.1) was carried out on sea bass muscle samples collected from a total of nine animals, three of which were killed by AA, three by AI, and three by SCS (Figure 5.1, rows). In this experiment, samples collected 10 minutes after the last opercular movement (T0, N=9) and after 5 days of storage at 1°C (T5, N=9) were evaluated (Figure 5.1, columns). This experimental design enabled three types of comparisons: 1) the muscular proteome between fish slaughtered in three different ways and sampled after death (AA_{T0} vs AI_{T0} vs SCS_{T0}); 2) the muscular proteome between fish slaughtered in three different ways and sampled after 5 days of storage at 1°C (AA_{T5} vs AI_{T5} vs SCS_{T5}); and 3) protein degradation after 5 days of *post mortem* conservation at 1°C (from T0 to T5) in muscles sampled from differently slaughtered fish (AA_{T0} vs $AA_{T}5$, AI_{T0} vs AI_{T5}, and SCS_{T0} vs SCS_{T5}).

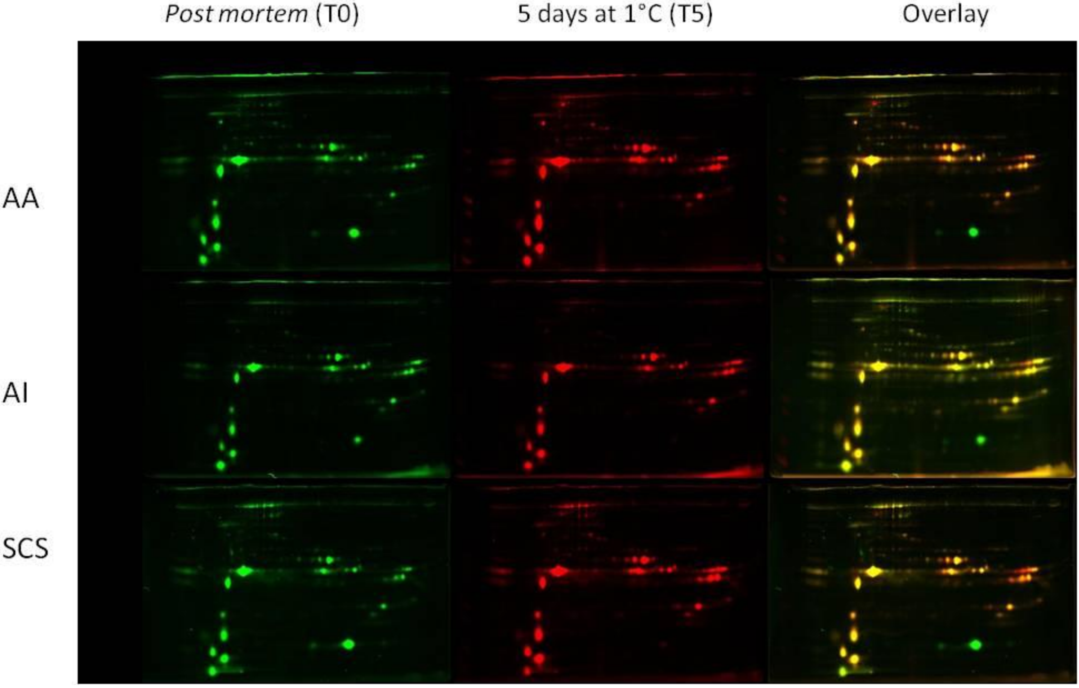

Figure 5.1 - Composite image illustrating three representative 2D DIGE gels. The lines report the slaughtering technique used (AA, asphyxia in air; AI, asphyxia in ice; SCS, spinal cord severance). The columns report the total protein profiles observed *post mortem* (left column, green), after 5 days of storage at 1°C (middle column, red), and the composite overlay image generated by the superimposition of the other two (yellow). From left to right: 3-10 non linear pI gradient; from top to bottom: 250-10 kDa range.

5.3.2 Differences in abundance of muscle proteins in sea bass slaughtered with the three procedures; *post mortem* evaluation.

To investigate a possible influence of *pre mortem/perimortem* stress on the integrity of proteins in muscle of fish slaughtered in three different ways (AA, AI, or SCS), the samples taken immediately after death (T0) were analyzed (Figure 5.1, left column). Statistically significant differences in protein abundance profiles were detected between the three slaughtering procedures. The principal components analysis (PCA) performed on all differential protein spots ($p<0.05$) generated three separate clusters (Figure 5.2). In particular, a significant divergence was observed for samples generated by the SCS method whereas the AA and AI clusters were less divergent and could be grouped as a single asphyxia cluster. A differential analysis of protein spots was therefore carried out using their logarithmic standardized abundance. A total of 15 significantly different spots were detected and subjected to protein identification by MS/MS (Figure 5.3, Table 5.2). The most significant difference in abundance was observed for spots 1, 2, and 3, all identified as nucleoside diphosphate kinase B (NDKB) (Table 5.3). NDKB consistently

decreased in the perimortem/immediate *post mortem* period in fish euthanized by asphyxia in comparison

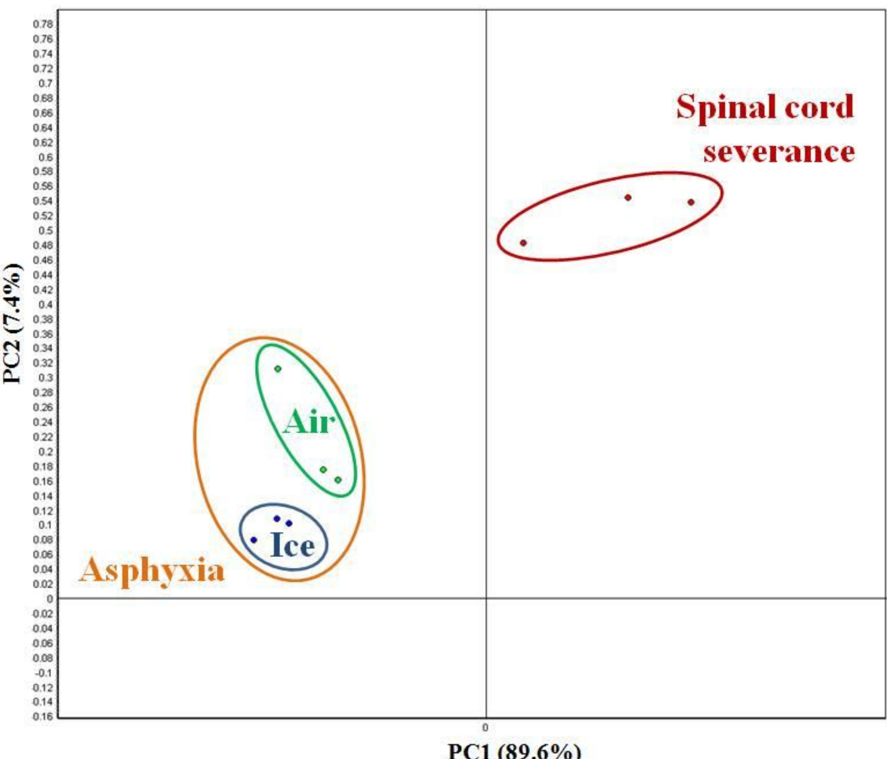

Figure 5.2 - Principal components analysis graph indicating the distribution of sample classes into clusters. Each dot represents the variance of the differentially abundant spots in one of the three samples analyzed for each condition. Circles highlight the clustering area corresponding to each slaughtering mode.

to SCS, whereas in asphyxiated fish the decrease in abundance was more pronounced for AI ($p<0.05$). Significant changes were also observed for myosin binding protein C (MbpC), fructose-bisphosphate aldolase (FBA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), enolase, and myosin heavy chain (Mhc) (Table 5.3, spots 4-15). For these proteins, statistically significant differences were observed only when comparing SCS to asphyxia (AA or AI); conversely, no significant differences were observed between fish killed by asphyxia "in air" or "in ice" conditions.

5. 3.3 Differences in abundance of muscle proteins in sea bass slaughtered with the three procedures; evaluation after 5 days of *post mortem* storage at 1°C.

To investigate a possible influence of the slaughtering method (*pre mortem/peri mortem* stress) on the *post mortem* degradation of proteins in muscle of fish slaughtered in three different ways (AA, AI, or SCS), an analysis of samples taken after

5 days of storage at 1°C was carried out (Figure 5.1, middle column). In contrast to that observed for T0 samples, only minimal differences in abundance were found among muscle protein profiles corresponding to different slaughtering techniques. In fact, although slight differences were observed for the same proteins that changed at T0 (Table 5.3), only two spots identified as

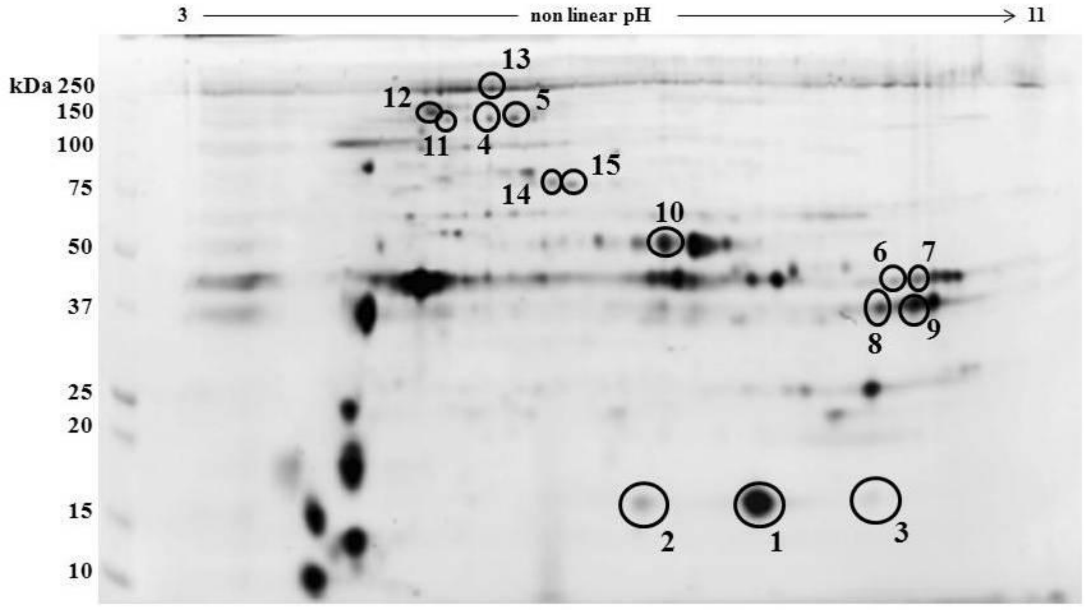

Figure 5.3 - Representative map indicating the location of the differential protein spots detected in this study. Protein identification results are reported in Table 5.2.

Mhc produced statistically significant values (11, 12). Consistently to that observed for T0, statistical significance was obtained only when comparing SCS to asphyxia, either in ice or air. Although a general trend favoring AI could be observed for these two spots, the difference between the two asphyxia conditions was not statistically significant (Table 5.3).

5.3.4 Differences in abundance of muscle proteins in sea bass slaughtered with the three procedures; comparative evaluation of samplings at the time of death and after 5 days of *post mortem* storage at 1°C.

The protein profiles obtained at T0 for each slaughtering method were compared with the respective profiles at T5, producing 12 differentially expressed spots that were identified by MS/MS (Table 5.2).

Results are illustrated in Table 5.4. The statistical significance of the fold change obtained for each differentially expressed spot in the three slaughtering procedures was assessed by one-way ANOVA. Then, a two-way ANOVA was applied to evaluation of the variables “time” and “slaughtering”. As a result, the variation in protein abundance appeared to be significantly

dependent from the storage time, whereas slaughtering mode was statistically significant only for Mhc (spot 13, Table 5.4). The ANOVA interact test did also point out an interesting behavior for a spot identified as GAPDH, by highlighting a possible additive effect of slaughtering and time (spot 8, Table 5.4).

In general, however, the results produced by the T5/T0 comparison do not entirely agree with the observations reported above. In fact, although NDKB shows the highest degradation also with this type of analysis, the highest impact seems to be exerted by AA (-49.35), followed by SCS (-37.35), and then by AI (-5.98). In contrast, from the T0 comparisons, AI is the condition most aversive to this protein, followed by AA and SCS. The results obtained for other proteins in the T5/T0 comparison also contrast with the results obtained in the T0 comparison. Clearly, the extensive degradation that occurred in the *peri mortem*/immediate *post mortem* time period introduces a bias in this comparison, since abundance at the hypothetical “starting point” of this experiment was already changed as a result of the damage to muscle proteins in the early *post-mortem* events. This indicates that care must be taken when performing these types of studies since degradation occurring immediately *post mortem* or in the first hours of storage can have a significant impact on differential protein abundance and introduce biases on T5/T0 comparisons.

5.4 Discussion

In this work, 2D DIGE and mass spectrometry were applied to investigate the degradation differences introduced in sea bass muscle proteins by three different slaughtering techniques, AA, AI, and SCS, both immediately after death and after 5 days of *post mortem* storage at 1°C.

The 2D DIGE approach is ideal for this type of proteomic investigation. In fact, changes can be detected in molecular weight or in isoelectric point due to degradation, to the occurrence of chemical changes in lateral side chains, or to loss of post-translational modifications. Moreover, the ability to run the study samples in the same gel and against an internal standard reinforces the robustness of the differential analysis (Lilley et al., 2004; Ünlü et al., 1997). In our opinion, these applications fully exploit the strengths still offered by traditional gel-based proteomics. The strategy was successfully applied in a previous study investigating the impact of storage time and temperature on fish muscle protein integrity (Terova et al., 2011), providing results that are in agreement with those reported here. The first and most significant finding from this work is the clear impact of the slaughtering technique on muscle protein degradation. In fact, the PCA analysis performed at T0 on the total protein profiles reveals a clear separation of the sample group corresponding to SCS (Figure 5.2), while the two asphyxia conditions, although forming two

separate groups, are not clearly different from each other and can be grouped in a single cluster. When comparing the logarithmic standard abundance for spots above the significance threshold, less protein degradation is clearly observed in fish slaughtered by severing the spinal cord rather than by asphyxia, either in ice or in air. Most importantly, the impact of the slaughtering technique is clear and significant from the early *post mortem* period.

SCS represented a less stressful slaughtering method as fish were euthanized by different operators immediately after being collected and were out of the water for no more than 10 seconds, reducing as much as possible aversive behavior. In contrast, asphyxia was extremely adverse to fish, who showed prolonged violent escape behavior in the form of intense exercise. Such prolonged muscular activity in the *premortem* phase likely caused a higher degradation rate of muscle proteins in the *perimortem* and early *post mortem* phases. The impact of the perimortem phase on proteins can be significant, especially when considering that, as stated in the 2009 EFSA report on stunning and killing of seabream and sea bass, it may take 70 min before behavioral responses to administered stimuli in asphyxiated sea bass are lost. Moreover, chilling of fish may prolong the period of consciousness, compared to asphyxia in air.

Concerning single proteins, the most significant and dramatic change in abundance was observed for all three spots belonging to NDKB (Table 5.3). NDKB is an hexameric complex catalyzing transfer of phosphates from GTP to ADP to form ATP (Berg et al., 2002). In some eukaryotes, NDKB is stabilized by nucleotide binding (Souza et al., 2011). Although this has not been investigated for fish NDKB, the reason for this sudden and massive decrease in the abundance of this protein might be explained by the ATP depletion occurring as a consequence of the intense *perimortem* muscular activity. In fact, the relation between slaughtering method and ATP depletion and its impact on onset and duration of *rigor mortis* are well known (Robb et al., 2000; Erikson et al., 1999; van de Vis et al., 2003). The consumption of muscle energy reserves might cause the massive dephosphorylation of nucleotides, leading to conformational changes in NDKB that make it less stable and more sensitive to degradation. SCS is the least stressful and most sudden type of death among the three investigated and thus does not deplete muscle ATP reserves as dramatically, which might favor a longer stability of this protein. Surprisingly, however, AI impacted NDKB stability more than AA. One might expect that AA, causing a more intense muscular activity before death, should have a higher impact on NDKB. However, hypothermia might play a role by impacting on the time to death and/or by introducing a further stress on fish.

Conversely, cytoskeletal protein integrity seems to benefit from asphyxia in ice. In fact, although not statistically significant, when observing the fate followed by MbpC (spots 4, 5) and by the three Mhc spots at higher molecular weight (spots 11, 12, 13), a general trend favoring AI over AA

can be seen (Table 5.3). In general, SCS samples displayed a higher abundance of protein spots, with p*I*/M*r* values similar to those expected, indicating that these were likely intact proteins. In this case, higher abundance indicates preservation of protein integrity. On the other hand, AA and AI samples displayed a higher abundance of proteins, with p*I*/M*r* values quite different from those expected, likely indicating the appearance of degradation products (lower M*r*) or of chemical transformations (different p*I*).

When T5 profiles were compared, only two spots showed statistically significant differences, also in this case favoring SCS over AA and AI. Both spots were identified as Mhc and were localized at high molecular weight. Mhc is an abundant and large protein and one of the main constituents of the sarcomere, the contractile unit of skeletal muscle. As such, the degradation process might take longer to complete, both due to the protein dimensions and to its release from the supramolecular contractile structure. Long-term degradation of Mhc has been reported by other authors; they observed that Mhc is fairly stable when fish muscle is stored in ice for a prolonged period (Terova et al., 2011; Verrez-Bagnis et al., 2001; Jasra et al., 2001). This longer degradation kinetics may make it possible to detect a residual effect of the slaughtering technique even after 5 days of storage in refrigerated conditions.

As a further analysis, we assessed the informative value of comparing the magnitude of changes occurring between T0 and T5 within a slaughtering technique as a measure for comparatively assessing intensity of the degradation process among slaughtering procedures (Table 5.4). As a matter of fact, the occurrence of changes in protein abundance in the *perimortem*/immediate *post mortem* period actually changes the “starting point” abundance of proteins, which is then reflected in the relative abundance ratio seen for the different slaughtering procedures. In fact, the 2D DIGE analysis combined with the DeCyder processing of data takes into consideration the fold change undergone by protein spots between the two conditions under examination. When part of the degradation has already occurred at the starting point of the analysis (T0), the T5/T0 fold change of proteins undergoing degradation in the immediate *perimortem/post mortem* time frame is paradoxically lower for the proteins most affected by degradation, since the starting point is an already reduced spot. This carries with itself an error which is translated in the statistical analysis, and unfortunately introduces a bias that can lead to misinterpretation of the statistical results. It is advised that, when such studies are performed with comparative purposes, the profiles at T0 be compared first among the slaughtering conditions in order to assess if significant abundance changes have already occurred in the total protein profiles.

The results obtained in this work have manifold practical implications. First, a correlation has been demonstrated between several proteins with spoilage processes and the killing method. For

example, NDKB and Mhc may provide tools for monitoring fish quality, freshness, and the maintenance of continuity in the cold storage chain along shelf-life.

Secondly, it is clearly demonstrated that the stress suffered at slaughter has a profound impact on the integrity of muscle proteins, and that a sudden death can impact favorably on shelf-life of the final product. This is important, even when considering that SCS is not practical when large-scale catches are to be performed, such as from sea cages or inland plants; in fact, other methods can be implemented to avoid *pre mortem* stress, and many are already in place. For example, various studies show that fish can be instantaneously stunned by application of an electrical current of sufficient strength to induce immediate unconsciousness (Robb et al., 2003, Lambooij et al., 2008; Lambooij et al., 2002). Adopting easily applied methods to reduce the impact of stress at slaughter is therefore recommended, since gentle and rapid killing methods can have a significant impact on fish quality by preserving integrity of muscle proteins and therefore extending shelf-life. The adoption of easily applicable methods to reduce the impact of stress at slaughter is therefore recommended for several reasons: firstly, the avoidance of general discomfort is being encouraged by EU directives, also for fish (Council Directive 93/119/EC; EFSA, 2009); and secondly, gentle and rapid killing methods can preserve integrity of muscle proteins, promoting quality and extending shelf-life.

5.5 Conclusions

In conclusion, slaughtering by spinal cord severance has a lower impact on muscle proteins than does death by asphyxia, either in ice or in air. These results further encourage the adoption of humane slaughtering techniques, which eliminate avoidable suffering and enable a better preservation of fish muscle protein integrity.

N[a]	Accession[b]	Description	Species	Score[c]	Std. Ab[d]	UP[e]	Pep[f]	PSM[g]
1	295792242	**Nucleoside diphosphate kinase B**	*Epinephelus coioides*	995.26	59.73	2	7	64
2	229366950	**Nucleoside diphosphate kinase B**	*Anoplopoma fimbria*	373.74	22.15	1	3	23
3	226441991	**Nucleoside diphosphate kinase B**	*Gillichthys seta*	949.69	42.45	1	5	45
4	317419297	**Myosin-binding protein C, fast-type**	*Dicentrarchus labrax*	962.69	20.59	17	17	34
5	317419297	**Myosin-binding protein C, fast-type**	*Dicentrarchus labrax*	234.65	16.78	12	12	25
6	295792244	**Fructose-bisphosphate aldolase A**	*Epinephelus coioides*	186.59	23.69	1	5	9
7	317419331	**Fructose-bisphosphate aldolase**	*Dicentrarchus labrax*	958.21	31.40	8	11	53
8	296785440	**Glyceraldehyde 3-phosphate dehydrogenase**	*Siniperca chuatsi*	154.85	14.11	2	4	13
9	57791244	**Glyceraldehyde-3-phosphate dehydrogenase**	*Dicentrarchus labrax*	686.50	33.63	5	8	26
10	37590349	**Enolase 1, (alpha)**	*Danio rerio*	994.85	28.70	3	10	50
11	226434435	**Myosin heavy chain**	*Takifugu rubripes*	390.49	8.41	2	14	27
12	90025055	**Myosin heavy chain**	*Dicentrarchus labrax*	439.65	12.20	2	17	27
13	171702766	**Myosin heavy chain**	*Coryphaenoides yaquinae*	94.24	40.43	1	5	6
14	21623523	**Myosin heavy chain**	*Oncorhynchus keta*	154.93	6.04	1	7	10
15	90025055	**Myosin heavy chain**	*Dicentrarchus labrax*	481.41	12.37	1	18	21
16	33186832	**Desmin**	*Takifugu rubripes*	102.35	5.76	2	2	2
17	33186832	**Desmin**	*Takifugu rubripes*	114.10	4.21	2	2	6
18	209902355	**Parvalbumin**	*Hypophthalmichthys molitrix*	33.00	5.00	1	1	5

[a]Number of spot in figure map and spots
[b]NCBI Accession number
[c]Mascot score
[d]Logarithmic standardized abundance at T0 as calculated by DeCyder analysis
[e]Percent coverage
[f]Number of identified peptides
[g]Peptide spectra matched

Table 5.2 - Identification results obtained by MS/MS analysis on spots showing statistically significant differences in abundance among the different experimental conditions. Spot numbers refer to those indicated in Figure 5.3.

N[a]	Description	Th. Mr/pI[b]	Obs. Mr/pI[c]	Log Std abundance at T0[d]	Log Std abundance at T5[e]
1	**Nucleoside diphosphate kinase B**	16.98 7.30	13.95 7.27		
2	**Nucleoside diphosphate kinase B**	16.95 6.32	13.95 6.59		
3	**Nucleoside diphosphate kinase B**	15.67 7.83	13.95 7.62		
				1.T-test*= **i/a 0.013; i/s 0.013;** a/s 0.754 **2**.T-test*= **i/a 0.036; i/s 0.014;** a/s 0.247 **3**.T-test*= **i/a 0.026; i/s 0.006;** a/s 0.249	**1**.T-test*= i/a 0.554; i/s 0.833; a/s 0.121 **2**.T-test*= i/a 0.555; i/s 0.561; a/s 0.765 **3**.T-test*= i/a 0.263; i/s 0.673; a/s 0.382

N[a]	Description	Th. Mr/pI[b]	Obs. Mr/pI[c]	Log Std abundance at T0[d]	Log Std abundance at T5[e]
4	**Myosin-binding protein C**	124.99 5.63	135.62 6.09	**4.**T-test*= i/a 0.788; **i/s 0.030**; **a/s 0.020**	**4.**T-test*= i/a 0.124; i/s 0.297; a/s 0.062
5	**Myosin-binding protein C**	124.99 5.63	135.62 6.11	**5.**T-test*= i/a 0.437; **i/s 0.016**; a/s 0.075	**5.**T-test*= i/a 0.445; i/s 0.540; a/s 0.335
6	**Fructose-bisphosphate aldolase**	39.63 8.25	41.88 7.66	**6.**T-test*= i/a 0.090; **i/s 0.030; a/s 0.024**	**6.**T-test*= i/a 0.662; i/s 0.071; a/s 0.331
7	**Fructose-bisphosphate aldolase**	39.62 8.33	41.88 7.76	**7.**T-test*= i/a 0.841; **i/s 0.016; a/s 0.007**	**7.**T-test*= i/a 0.435; i/s 0.917; a/s 0.672
8	**Glyceraldehyde 3-phosphate dehydrogenase**	35.94 8.35	34.21 7.64	**8.**T-test*= i/a 0.073; **i/s 0.001; a/s 0.002**	**8.**T-test*= i/a 0.779; i/s 0.629; a/s 0.941
9	**Glyceraldehyde 3-phosphate dehydrogenase**	35.87 8.44	34.21 7.76	**9.**T-test*= i/a 0.847; **i/s 0.016; a/s 0.014**	**9.**T-test*= i/a 0.953; i/s 0.798; a/s 0.542
10	**Enolase 1 (alpha)**	47.04 6.58	50.92 6.61	**10.**T-test*= i/a 0.473; **i/s 0.030; a/s 0.006**	**10.**T-test*= i/a 0.706; i/s 0.153; a/s 0.287
11	**Myosin heavy chain**	221.65 5.66	139.30 5.40	**11.**T-test*= i/a 0.811; **i/s 0.017; a/s 0.015**	**11.**T-test*= i/a 0.588; i/s 0.253; **a/s 0.003**
12	**Myosin heavy chain**	201.73 5.77	144.59 5.42	**12.**T-test*= i/a 0.599; **i/s 0.032; a/s 0.038**	**12.**T-test*= i/a 0.119; i/s 0.392; **a/s 0.017**
13	**Myosin heavy chain**	221.47 5.71	149.31 5.59	**13.**T-test*= i/a 0.476; **i/s 0.002; a/s 0.0007**	**13.**T-test*= i/a 0.556; i/s 0.476; a/s 0.962
14	**Myosin heavy chain**	222.02 5.60	75.34 6.21	**14.**T-test*= i/a 0.432; i/s 0.232; a/s 0.426	**14.**T-test*= i/a 0.065; i/s 0.078; a/s 0.325
15	**Myosin heavy chain**	201.73 5.77	75.35 6.05	**15.**T-test*= i/a **0.015**; i/s 0.083; **a/s 0.002**	**15.**T-test*= i/a 0.128; i/s 0.214; a/s 0.779

[a]Number of spot in figure map and spots
[b]Theoretical pI and Mr values for the protein sequence matched
[c]Observed pI and Mw as determined by gel calibration
[d]Logarithmic standardized abundance at T0 as calculated by DeCyder analysis
[e]Logarithmic standardized abundance at T5 as calculated by DeCyder analysis

*i/a; asphyxia in ice vs asphyxia in air; i/s; asphyxia in ice vs spinal cord severance; a/s; asphyxia in air vs spinal cord severance. Statistically significant values are indicated in bold.

Table 5.3 - Composite table summarizing protein abundance trends observed at T0 (left) and at T5 (right) for the three slaughtering procedures evaluated in this study. Spots corresponding to the same protein are grouped into one graph, and are indicated by different colors.

N°	Protein	AA	AI	SCS	AA	AI	SCS
		Paired Av Ratio T5/T0	Paired Av Ratio T5/T0	Paired Av Ratio T5/T0	Paired T-test	Paired T-test	Paired T-test
	Decreased						
1	**Nucleoside diphosphate kinase B**	**-49.35**	**-5.98**	**-37.35**	**0.0085**	**0.0482**	**0.0310**
2	**Nucleoside diphosphate kinase B**	**-18.82**	**-3.69**	**-15.89**	**0.0088**	**0.0433**	**0.0310**
3	**Nucleoside diphosphate kinase B**	**-11.1**	**-2.71**	**-20.87**	**0.0140**	**0.0332**	**0.0490**
17	**Desmin**	**-1.99**	**-2.14**	-1.42	**0.0330**	**0.0320**	0.1800
14	**Myosin heavy chain**	-1.03	**-1.99**	-1.1	0.1800	**0.0050**	0.3600
16	**Desmin**	-1.7	**-1.99**	-1.32	**0.0270**	**0.0390**	0.2100
18	**Parvalbumin**	-1.57	**-1.99**	-1.76	0.1000	**0.0480**	0.1300
5	**Myosin-binding protein C**	**-1.99**	-1.55	**-2.15**	**0.0310**	**0.0460**	**0.0470**
4	**Myosin-binding protein C**	**-2.01**	-1.46	-1.81	**0.0480**	0.0600	0.0930
	Increased						
7	**Fructose-bisphosphate aldolase A**	**2.74**	**2.38**	**2.09**	**0.0085**	**0.0028**	**0.0330**
15	**Myosin heavy chain**	**3.13**	**2.19**	**3.57**	**0.0088**	**0.0052**	**0.0450**
12	**Myosin heavy chain**	**3.38**	**2.18**	**3.39**	**0.0088**	**0.0028**	0.0710
13	**Myosin heavy chain**	**2.28**	**2.13**	**4.12**	**0.0085**	**0.0170**	0.0670
11	**Myosin heavy chain**	**3.41**	**2.04**	**3.36**	**0.0120**	**0.0028**	0.1370
10	**Enolase 1 (alpha)**	1.83	**1.99**	**2.09**	**0.0190**	**0.0036**	**0.0490**
6	**Fructose-bisphosphate aldolase A**	**3.44**	**1.59**	**3.42**	**0.0085**	**0.0550**	**0.0310**
8	**Glyceraldehyde-3-phosphate dehydrogenase**	1.52	1.42	**2.13**	**0.0140**	**0.0270**	**0.0390**
9	**Glyceraldehyde-3-phosphate dehydrogenase**	1.34	1.14	**1.99**	**0.0150**	**0.0420**	**0.0370**

Table 5.4 - Comparative degradation trends observed among slaughtering procedures along cold storage (T0 vs T5).

Chapter 6

Proteomic Profiling of Sea Bass Muscle by Two-Dimensional Gel Electrophoresis and Tandem Mass Spectrometry

This report is in press at *Aquaculture*

ABSTRACT

Consumers' demands for seafood products have increased in the recent years, and this situation has led to the need to guarantee their safety, traceability, authenticity, and health benefits. The application of proteomics to aquaculture may play a key role in the development of new strategies enabling to gather novel and more detailed information on the quality of seafood products. In this study, the proteome profile of European sea bass (*Dicentrarchus labrax*) muscle was analyzed using two-dimensional electrophoresis (2-DE) and tandem mass spectrometry (MS/MS) with the aim of providing a more detailed characterization of its specific protein expression profile. A highly populated and well resolved 2-DE map of the sea bass muscle tissue was generated, and the corresponding protein identity was provided for a total of 54 abundant protein spots. Upon Ingenuity Pathway Analysis, the proteins mapped in the sea bass muscle profile were mostly related to glycolysis and to the muscle myofibril structure, together with other biological activities crucial to fish muscle metabolism and contraction, and therefore to fish locomotor performance. The data presented in this work provide important and novel information on the sea bass muscle tissue-specific protein expression, which can be useful for future studies aimed to improve seafood traceability, food safety/risk management and authentication analysis.

Keywords:

European sea bass proteome
MS/MS analysis
2-DE
Muscle
aquaculture

6.1 Introduction

The rapid growth in world population has contributed to increase the fish demand for human nutrition. The latest data on the world fisheries and aquaculture supply report about 142 million tons of fish provided in 2008. Of these, 115 million tons were used as human food, corresponding

to an estimated apparent per capita supply of about 17 kg (live weight equivalent), which is an all-time high (FAO, 2010). Aquaculture is becoming an increasingly important source of fish and shellfish available for human consumption (Zhou et al., 2012). The growing demand for fish from consumers has opened new markets and has increased the circulation and distribution of fish products, but, at the same time, has caused the development of fraudulent practices, which damage the health of consumers and cause economic loss to the community, as well as to companies and operators of the sector. In this context, food traceability main drivers are food safety/risk management and authentication to prevent fraudulent labeling and to certify the origin of products in the market. To prevent economic frauds and health hazards, the European Union (Council Regulation (EC) No. 104/2000) establishes that fish products can enter the commercial circuit only if the commercial name, method of production and capture area are clearly indicated on the label (Civera, 2003). Traceability regulations have the potential to reduce fraud issues, and the information reported on labels can potentially decrease product adulteration as well as provide indications on quality of the seafood product through all stages of production, processing and distribution. As a consequence of this, the development of robust analytical techniques aimed at achieving a precise identification and characterization of fish species in both raw and processed fish material is gaining increasing importance (Piñeiro et al., 1999). There are several methods suitable for species identification in seafood, or for determining the life-history of the product, which are needed in order to verify the traceability documentation of the product and to detect fraud (Martinez et al., 2003). Along with molecular techniques, such as DNA-based tags, developed for species identification in seafood, and other ones ranging from the simple techniques of PCR-fragment size determination (Perez et al., 2004) to PCR-RFLP (restriction fragment length polymorphism) (Perez et al., 2005) to direct sequencing of target DNA fragments (Toffoli et al., 2008), proteomic technologies are proving to be powerful tools in this area, particularly for addressing questions on the health status of the organism, stresses or contamination levels at the place of breeding, and *post mortem* treatment (Martinez and Friis 2004). Most of the proteomic studies have been carried out in zebrafish (*Danio rerio*) as a model organism for biological research, but the application of proteomic technologies to species with commercial interest is gradually increasing. However, proteomic studies in fish are still limited by the fact that attaining a high identification rate is problematic due to the relative paucity of available protein sequence data for these animals (Sveinsdottir and Gudmundsottir, 2011), although the availability of muscle proteomics data would benefit many aspects including shelf-life, traceability and authentication (Piňeiro et al., 2003; Vilhelmsson et al., 2005; Fornè et al., 2010). These aspects are of increasing importance, both economically and from a public health standpoint. In addition, the application of proteomics to aquaculture can play a key role in the

development of new breeding strategies, in the preservation of biodiversity, and in the reduction of the environmental impact (Monti et al., 2005). Thus, proteome analysis is an important addition to studies based on mRNA detection to increase our understanding of the cellular responses to environmental stimuli (Sveinsdottir and Gudmundsottir, 2011; Terova et al., 2011).

Two very recent reports by Terova et al., 2011 and Addis et al., 2012, exploited proteomic techniques (2D-DIGE followed by MS/MS identification of differential protein spots) to assess the effects of storage temperature and slaughter methods in the European sea bass muscle tissue. Here, we report a detailed proteome map of sea bass skeletal muscle tissue obtained by 2-D electrophoresis (2-DE) and tandem mass spectrometry (MS/MS) analysis. The work described here provides a reference proteome map of the sea bass muscle for use in traceability and species authenticity studies, and as a reference for identification of possible freshness and quality markers. Using these methods, a detailed characterization of the sea bass muscle proteome was obtained, focused mostly on proteins relevant to the normal protein composition of the sea bass muscle. In addition, a study on the predicted biochemical pathway components was carried out by means of Ingenuity Pathway Analysis.

6.2 Materials and methods

6.2.1 Samples

Fish rearing and sample preparation was carried out at the Department of Biotechnology and Life Sciences (DBSV) of the University of Insubria (Varese, Italy). One month before slaughter, sea bass (*Dicentrarchus labrax*) were randomly distributed into three tanks of $1m^3$ each, 40 fish for tank, and fed Hendrix-Skretting® Power Excel feed for marine fish. The tanks were connected to a sea water recirculation system under the following water conditions: temperature 20±2°C, pH 7, total ammonia < 0.2mg/L, and dissolved oxygen over 99% of the saturation. At the start of the experiment and after 4 days starvation, fish were removed from water and sacrificed. Fish were stored ungutted in a refrigerator at 1°C. All muscle fragment taken in this ways were immediately frozen at -80°C and stored at this temperature until the molecular biology analysis. All procedures were approved by the Animal Care Committee of the University of Insubria and conducted according to the guidelines of the Italian Committee on Animal Care.

6.2.2 Protein extraction

Proteomic experiments were carried out at the Proteomic Laboratory of Porto Conte Ricerche (Tramariglio, Alghero, Italy). Proteins were extracted from frozen muscle tissues using a TissueLyser mechanical homogenizer (Qiagen, Hilden, Germany). For extraction, a small portion (50 mg) of frozen fish muscle was minced with a sterile scalpel, placed in a 2-mL Eppendorf safe-lock tube (Eppendorf, Hamburg, Germany), and then immersed in lysis buffer (8 M urea, 2% CHAPS, and 0.5% IPG buffer; GE Healthcare, Little Chalfont, UK) at a 5% w/v ratio. Three cycles of 1.5 min at 30 cycles/s in the TissueLyser mechanical homogenizer were employed for each sample. All extracts were then clarified for 15 min at 12,000 x g at 4°C, quantified by the Bradford method (Bradford, 1971), tested for quality and quantity by SDS-PAGE, and then stored at -80°C until the moment of analysis.

6.2.2.1 2D gel electrophoresis

First-dimension IsoElectric Focusing (IEF) was performed using 24 cm precast IPG strips (pH 3 to 11, nonlinear (NL); GE Healthcare). A total of 450 µl of protein preparation were applied onto the strips by passive rehydration overnight at room temperature. Three strips (three experimental replicates) were run together in a IPGphor equipped with the Ettan™ IPGphor™3 loading manifold (GE Healthcare). The strips were focused at 20°C for a total of about 90,000 Vh. After IEF, the strips were sequentially incubated in a freshly prepared solution of 1% dithiothreitol (DTT) and 2% iodoacetamide in 50 mM Tris-HCl (pH 8.8), 6 M urea, 20% glycerol, and 2% sodium dodecyl sulfate (SDS) for 10 min. The second-dimension SDS-polyacrylamide gel electrophoresis was conducted on 8% to 16.5% polyacrylamide gradient gels, using an Ettan™ DALT*twelve* electrophoresis system (GE Healthcare), for 30 min at 5 W/gel and then for 5 h at 17 W, at 25°C.

6.2.2.2 Protein identification

After electrophoresis, the gel slab was fixed in 50% methanol, 10% acetic acid in water for 30 min. It was then washed for 15 min with 5% methanol in water and additionally for 15 min for 3 times with water to remove the remaining acid. The gel was sensitized by 15 min incubation in 120mg/L sodium thiosulfate, and it was then rinsed with three changes of distilled water for 30 sec each. After rinsing, the gel was submerged in chilled 2g/L silver nitrate solution and incubated for about 25 min. After incubation, the silver nitrate was discarded, and the gel slab was washed with three changes of distilled water for 1min each and then developed in 30 g/L sodium carbonate (37% formaldehyde in water and 0,2% sodium thiosulfate) with intensive shaking. After the desired intensity of staining was achieved, the reaction was stopped with 14g/L of EDTA

(Ethylenediaminetetraacetic acid, anhydrous, Sigma) and was stored in distilled water at 4°C until analyzed.

6.2.2.3 Image digitalization and analysis

Stained gels were digitalized with an Image Scanner (GE Healthcare), and images were processed with ImageMaster Platinum 6.0 (GE Healthcare), using the standard software workflow for spot detection and enumeration. All the software steps were manually verified in order to eliminate artifact spots, split spots, and missed spots. For gel analysis, %Vol values of spots were used, intended as arbitrary units assigned by the software. A differential analysis was carried out among replicate gels in order to estimate the repeatability of the muscle tissue map. Isoelectric point and molecular weight values were validated by calibration with internal standards as described previously (Terova et al., 2011; Addis et al., 2012).

6.2.3 Spot picking and in situ tryptic digestion.

For protein identification, preparative 2D-PAGE gels were set up by overnight rehydration loading of 300 µg of protein extract into 3-11 NL 24 cm IPG strips. Strips were then focused and subjected to second dimension electrophoresis as described above. After electrophoresis, the gel slab was subjected to mass-compatible silver staining. Visible protein spots of interest were manually excised from the gels, destained with 15 mM $K_3Fe(CN)_6$ in 50 mM $Na_2S_2O_3$, washed with water, and then stored in acetonitrile. The spots were then subjected to an O/N tryptic digestion at 37°C in 50 mM $(NH_4)HCO_3$, pH 8.0, by using between 40 and 100 ng of trypsin, depending on spot intensity. Peptide mixtures were then collected by squeezing with acetonitrile, followed by centrifugation. Peptides were subsequently acidified with 20% TFA, dried in SpeedVac® (Eppendorf), resuspended in formic acid 0.2%, and stored at -20°C.

6.2.4 Tandem mass spectrometry analysis.

LC-MS/MS analysis was performed on a XCT Ultra 6340 ion trap equipped with a 1200 HPLC system and a chip cube (Agilent Technologies, Palo Alto, CA). After loading, samples were concentrated and desalted at 4 µl/min on a 40 nL enrichment column (75 µm x 43 mm, Agilent Technologies chip), with 0.2% formic acid. Peptides were then fractionated on a C_{18} reverse-phase

capillary column at flow rate of 300nl/min, with a linear gradient of eluent B (0.2% formic acid in 95% acetonitrile) in A (0.2% formic acid in 2% acetonitrile) from 3 to 60% in 20 min. ESI parameters were as follows: Capillary voltage 1730 V; dry gas (N_2), 5.00 L/min; dry temperature, 325 °C; trap drive, 100; skimmer 30 V; lens 1, -5.00 V; octopole RF amplitude, 200 Vpp; capillary exit, 90 V. The ion trap mass spectrometer was operated in positive ion mode. Trap ICC smart target was 300000 units and maximal accumulation time was 100 ms. MS/MS was operated at a fragmentation amplitude of 1.3 V, and threshold ABS was 6000 units. Scan speed was 8,100 uma/sec in MS and 26,000 uma/sec in MS/MS scans. Peptide analysis was performed scanning from m/z 250 to m/z 2200 in AutoMS (n) precursor selection mode of the three most intense ions (fragmentation mass range from 100 to 2200 m/z). Dynamic exclusion was used to acquire a more complete survey of the peptides by automatic recognition and temporary exclusion (0.15 min) of ions from which definitive mass spectral data had previously acquired. Data Analysis software, provided by the manufacturers, was used to analyze MS/MS spectra and to generate a peak list which was introduced in the in-house Mascot MS/MS ion search software (Version 2.3, Matrix Science, Boston, MA) for protein identification in NCBI database using the Chordata (vertebrates and relatives) taxonomy. Search parameters were as follows: peptide tolerance 300 ppm, MS/MS tolerance 0.6 Da, charge state +2, +3 and +4, enzyme trypsin, allowing 2 missed cleavage.

6.2.5 Data and network pathway analysis

The list of protein identifications was imported in the online software package IPA (Ingenuity Systems, Redwood City, CA), and network analyses were performed by substituting UniProt IDs with the UniProt ID (Uniprot Consortium, 2008) for the closest human protein equivalent in order to enable the best exploitation of the knowledge-based IPA software, version 12718793, updated May 2012.

6.3. Results and discussion

6.6.3.1 MS/MS and data analysis

The 2DE reference map of sea bass (*Dicentrarchus labrax*) muscle is presented in this work (Figure 6.1). 2DE enabled the separation of proteins over the entire pH 3-11 range, and comprised proteins between 10 and 250 kDa. The experimental intra-sample variability was very low, as determined by analyzing changes in the volume of protein spots on three 2D gels from the same sample. Observation of multiple repeats of 2D gels showed that spot locations and intensities

were highly similar between gels from the same sample. For identification, the most abundant single spot of the isoelectric series was excised, as described previously (Terova et al., 2011; Addis et al., 2012).

The proteins identified in this work covered a molecular masses (Mr) ranging from 13.9 to 193 kDa, and isoelectric points (pI) ranging from 3.91 to 8.23. Approximately 72% of the total proteins identified in sea bass muscle tissue were acidic proteins, having theoretical pI values ranging from 4 to 7, whereas 28% o f the total proteins identified were basic proteins, having theoretical pI values ranging from 7 to 9 (Figure 6.2B). In general, there was a good correlation between observed and theoretical Mr values of the identified proteins (Table 6.1).

Protein identifications were classified according to their cellular localization and biological function. As a result, over half (60%) of the total proteins analyzed were found to be localized in the cytoplasm, while 16% were localized in the nucleus, and 14% in filaments (Figure 6.2D). About 51 % of the total identified proteins were classified as enzymes involved in glycolytic processes (Figure 6.2A), while 25% of the proteins identified were structural proteins, followed by binding proteins with 7%, such as calcium ion binding proteins and ATP binding proteins, and a minor constituent being transport and biosynthesis proteins (Figure 6.2A). Among the enzymes, kinases-transferases were the most frequently identified functional protein class; in fact, 20% of the total identified proteins were categorized in this protein category.

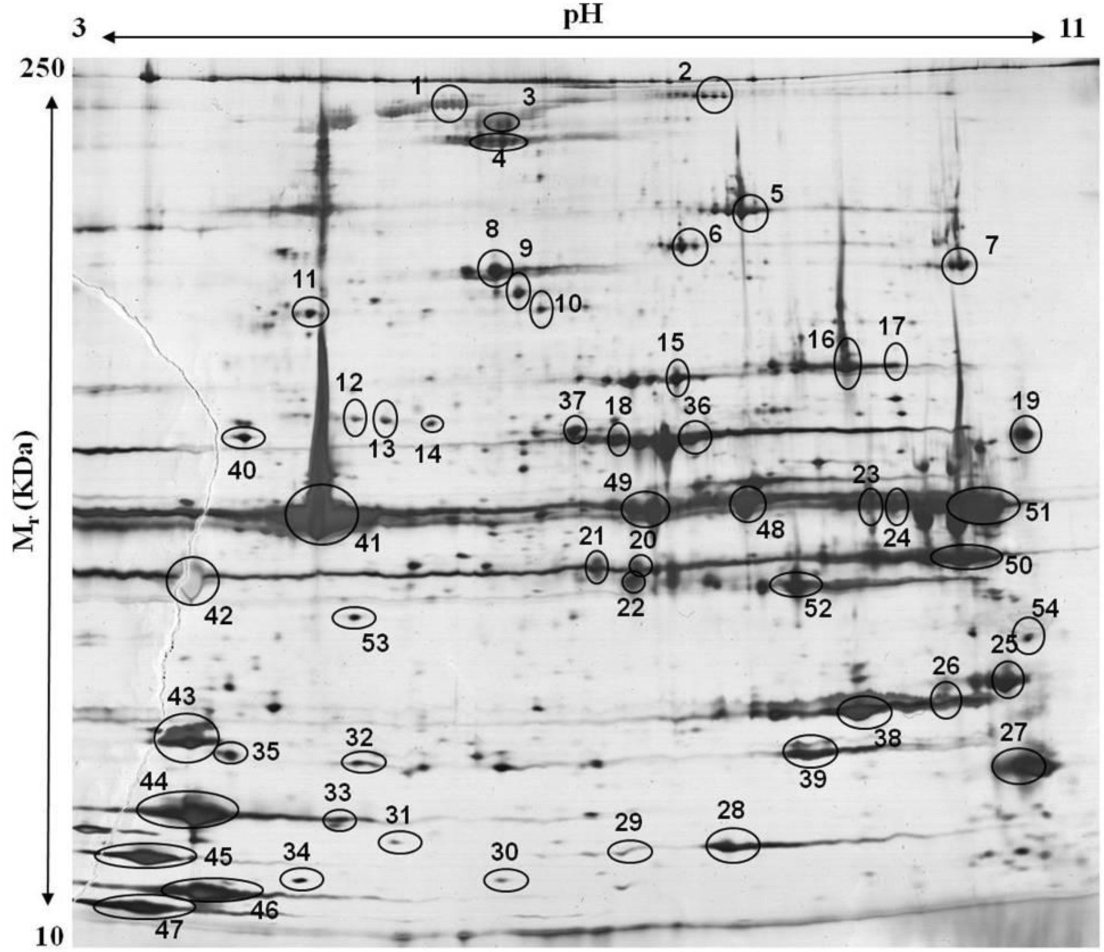

Figure 6.1 - Two-dimensional gel reference map of sea bass *Dicentrarchus labrax* skeletal muscle. 2DE was performed using a pH a range 3-11 in the first dimension. The protein loading was 500 µg and the gel was stained using the silver staining procedure. Fifty-four spots were identified and analyzed for mass spectrometry (MS/MS).

These enzymes included kinases, aldolases, dehydrogenases, isomerases, mutases, enolases, and others. Six proteins (11%) belonged to the enolase family. Five proteins (9%) were classified as dehydrogenases, and other five proteins (9%) were classified as isomerases. A series of glycolytic enzymes were identified in this study. These proteins were glycerol-3-phosphate dehydrogenase, phosphoglucomutase-1, pyruvate kinase, enolase, aldolase (also known as fructose-biphosphate aldolase A), glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate mutase 2, phosphoglycerate mutase 1, nucleoside diphosphate kinase B, lactoylglutathione lyase.

In addition to sarcomeric proteins, myofibrillar or structural proteins made up a significant group of the proteins identified in sea bass muscle. There were a total of seven different myofibrillar protein detected, including actin, troponin I, desmin, M-protein, myosin heavy chain, myosin light chain, myosin light chain 2. Other than these, minor proteins such as elongation factor 1-alpha,

parvalbumin, fatty acid binding protein, eukaryotic translation initiation factor 5a-1, heat shock 70 kDa protein, and others, (as listed in Table 6.1) were also identified.

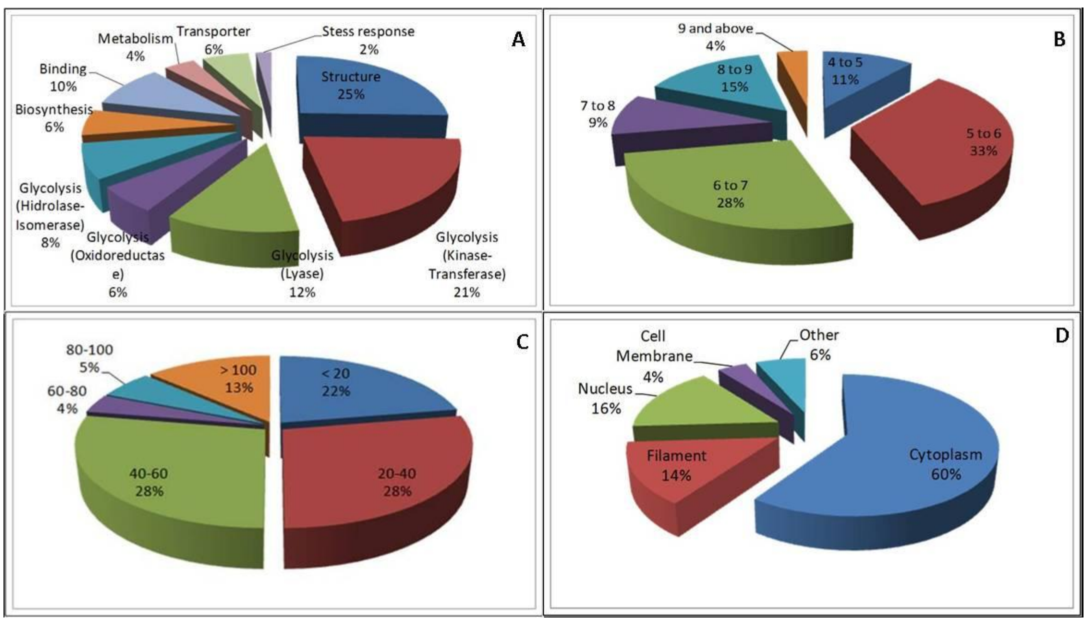

Figure 6.2 - Pie chart distribution of protein identities based on (a) function of the proteins, (b) pI of the protein, (c) molecualr weight, and (d) location of the proteins.

6.3.2 Network pathway analysis

Analysis of the muscle proteins for biological pathways based on the Ingenuity Pathways Knowledge Base software (Ingenuity® Systems; www.ingenuity.com) selected 33 protein identities out of the 54 identified (Supplement Table S61). To these proteins, several others were associated by the knowledge-based software to the networks, and the sum of interactions among these proteins is graphically illustrated in Figure 6.3. Three protein networks were identified with high significance: organ morphology, skeletal and muscular system development and function, carbohydrate metabolism (1); cell-to-cell signaling and interaction, cell-mediated immune response, and cellular movement (2); and cell morphology, developmental disorder, ophthalmic disease (3) (Table 6.2). Fifteen protein identities, namely, LDHA, PKM2, AK1, ENO1, GAPDH, CKM, DES, FABP3, PFKM, EEF1A1, ATP5E, myosin, MYL6, MYL7, and MYH2 were found to be involved in network 1 (Figure 6.3, A, and Supplement Table S61). Eleven protein identities, namely MYOM2, ALDOA, AMPD3, MUSK, NME2, PVALB, MYBPC3, HSPA2, TPI1, GPD1, and GLO1 were found to be involved in network 2 (Figure 6.3, B, and Supplement Table S61). Seven proteins, namely MDH1, TNNI2, EIF5A, PYGM, PGAM2, PGM1 and TPM1 were found to be involved in network 3 (Figure 6.3, C, and Supplement Table S61).

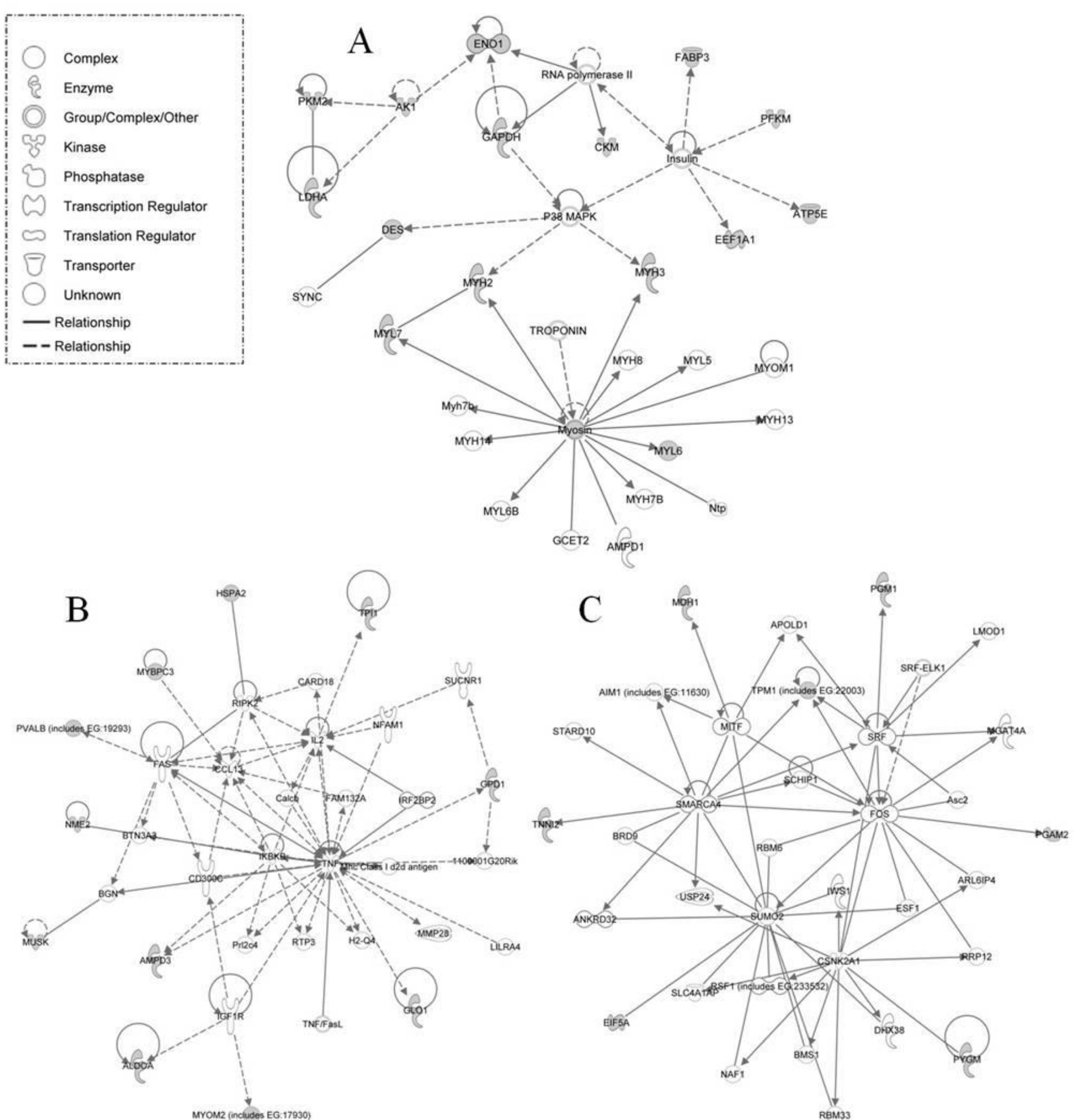

Figure 6.3 - Top networks obtained by Ingenuity Pathway Analysis of all sea bass muscle proteins examined in this study (Ingenuity® Systems; www.ingenuity.com). Three protein networks were obtained with the following associated network functions: A, organ morphology, skeletal and muscular system development and function, carbohydrate metabolism; B, cell-to-cell signalling and interaction, cell-mediated immune response, cellular movement; C, cell morphology, developmental disorder, ophthalmic disease. Shaded proteins are those used for analysis; white proteins are those identified by the software as significantly associated to the network according to the knowledge-based attribution.

The top biological functions identified, both in terms of molecular and cellular functions and of physiological functions were consistent with the tissue under examination. In fact, the highest score was attributed to Carbohydrate Metabolism with 14 protein identities (p-value 1.08E-11 to 4.88E-02) (Table 6.2, Supplement Table 6S2), followed by Nucleic Acid Metabolism (p-value 6.43E-07 to 2.15E-02) and Small Molecule Metabolism (p-value 6.43E-07 to 4.88E-02), and by Molecular Transport (p-value 6.82E-05 to 4.88E-02) (Table 6.2, Supplement Table 6.S2), all fundamental for

the energy consumption requirements of the metabolically active muscle. Moreover, the highest number of identified proteins was found in Small Molecule Metabolism.

Top Networks		
Associated Network Functions		**Score**
Organ Morphology, Skeletal and Muscular System Development and Function, Carbohydrate Metabolism		37
Cell-To-Cell Signaling and Interaction, Cell-mediated Immune Response.		25
Cellular Movement Cell Morphology, Developmental Disorder, Ophthalmic Disease		14
Top Biological Functions		
Name	**p-value**	**Molecules**
Molecular and Cellular Functions		
Carbohydrate Metabolism	1.08E-11 - 4.88E-02	14
Nucleic Acid Metabolism	6.43E-07 - 2.15E-02	14
Small Molecule Metabolism	6.43E-07 - 4.88E-02	21
Cell Signaling	6.82E-05 - 3.63E-02	7
Molecular Transport	6.82E-05 - 4.88E-02	13
Physiological System Development and Function		
Organ Morphology	8.25E-10 - 3.21E-02	16
Skeletal and Muscular System Development and Function	8.25E-10 - 4.67E-02	16
Cardiovascular System Development and Function	3.35E-06 - 5.00E-02	9
Tissue Development	9.54E-05 - 5.00E-02	10
Tissue Morphology	6.85E-04 - 3.42E-02	4
Diseases and Disorders		
Neurological Disease	2.97E-08 - 3.00E-02	15
Skeletal and Muscular Disorders	1.32E-07 - 4.88E-02	18
Hereditary Disorder	2.48E-07 - 1.94E-02	20
Cardiovascular Disease	7.20E-06 - 4.98E-02	6
Developmental Disorder	7.20E-06 - 4.88E-02	11
Top Canonical Pathways		
Name	**p-value**	**Ratio**
Glycolysis/Gluconeogenesis	1.49E-13	9/130 (0.069)
Calcium Signaling	1.96E-06	6/210 (0.029)
Pyruvate Metabolism	1.53E-05	4/139 (0.029)
Pentose Phosphate Pathway	3.64E-05	3/80 (0.038)
Inositol Metabolism	6.82E-05	2/18 (0.111)

Table 6.2 - Results of the Ingenuity Pathway Analysis concerning the top networks, biological functions and canonical pathways obtained upon analysis of all the sea bass muscle protein identities considered in this study.

Concerning Physiological System Development and Function, Organ Morphology (p-value 8.25E-10 to 3.21E-02), strictly followed by Skeletal and Muscular Development and Function (p-value 8.25E-10 to 4.67E-02), showed the highest significance, consistently with the presence of protein constituents of the muscle tissue contractile units. In addition, these two categories show a large number of proteins identified (Table 6.2, Supplement Table S6.2). Still, Cardiovascular System Development and Function (p-value 3.35E-06 to 5.00E-02) was also identified due to the extreme

similarity between the two striated muscles. Finally, Tissue Development and Tissue Morphology were also found as significant (p-value 9.54E-05 to 5.00E-02 and 6.85-04 to 3.42E-02, respectively) (Table 6.2, Supplement Table S6.2).

In keeping with these observations, the five top canonical pathways were Glycolysis/Gluconeogenesis, Calcium Signalling, Pyruvate Metabolism, Pentose Phosphate pathway and Inositol Metabolism. Diseases and disorders significantly associated were also evaluated, and these were also consistent with the tissue under examination. Even the finding of the association with neurological diseases, although apparently inconsistent, was due to muscle proteins associated with movement disorders.

In this study, we report the 2D PAGE proteome profile of normal adult European sea bass muscle tissue in normal conditions, with the indication of the protein identity corresponding to 54 abundant spots. These proteins are responsible for various functions, including carbohydrate metabolism, skeletal and muscular system development, molecular transport, tissue development and neurological disease. Most of the protein identities obtained in this study take part in the glycolytic metabolism which, being contraction one of the main activities of fish muscle tissue, is essential for the locomotory power of fish together with sarcoplasmic proteins.

This study provides novel information on the muscle tissue-specific protein expression in the European sea bass, which could be useful for future studies in the fields of seafood traceability, food safety/risk management and authentication analysis.

spot. no.	Identified Protein	Accession no.	Symbol	Species	Location	Biological function	Score	% Coverage	Q. matched	% Volume	pI/M_r Theorical (KDa)	pI/M_r Observed (KDa)
1	**Myosin heavy chain**	Q90339	MHC	*C. carpio*	Cytoplasm Thick filament	Muscle contraction	285	11	26	0.554	5.57/222.318	5.80/191
2	**M-protein**	O55124	Myom2	*M.musculus*	Cytoskeleton	Muscle contraction	48	0	1	0.321	5.51/165.956	6.55/193
3	**Myosin heavy chain**	Q90339	MHC	*C. carpio*	Cytoplasm Thick filament	Muscle contraction	38	11	1	0.374	5.57/222.318	5.82/163
4	**Myosin-binding protein C**	Q90688	MYBPC3	*G. gallus*	Thick filament	Cell adhesion	87	1	2	0.432	5.96/143.054	5.98/142
5	**Glycogen phosphorylase**	P11217	PYGM	*H. sapiens*	Cytoplasm	Carbohydrate metabolism, Glycogen metabolism	498	10	17	0.160	6.57/97.487	7.26/107
6	**AMP deaminase 3**	Q01432	AMPD3	*H. sapiens*	Cytoplasm	Nucleotide metabolism	138	4	9	0.045	6.51/89.269	7.06/101
7	**6-phosphofructokinase**	Q0IIG5	PFKM	*B. taurus*	Cytoplasm	Glycolysis	227	7	8	0.044	8.56/86.095	8.02/93
8	**Myosin heavy chain**	P13538	MHC	*G. gallus*	Cytoplasm Thick filament	Muscle contraction	40	0	2	0.446	6.05/223.976	6.05/96
9	**Myosin heavy chain**	P13538	MHC	*G. gallus*	Cytoplasm Thick filament	Muscle contraction	40	0	2	0.059	5.63/223.976	6.16/88
10	**Myosin heavy chain**	P13538	MHC	*G. gallus*	Cytoplasm Thick filament	Muscle contraction	49	1	2	0.095	5.63/223.976	6.28/85
11	**Heat shock 70 kDa protein**	P08106	HSPA2	*G. gallus*	Cell surface	Stress response	216	11	11	0.106	5.53/69.936	5.33/86
12	**Desmin**	O62654	DES	*B. taurus*	Cytoplasm Intermediate filament	Muscle protein	84	5	3	0.052	5.21/53.556	5.53/61
13	**Desmin**	Q05AI8	desmb	*D. rerio*	Cytoplasm Intermediate filament	Muscle protein	85	7	6	0.126	5.36/54.298	5.62/61
14	**Glycerol-3-phosphate Dehydrogenase**	Q5XIZ6	gpd1l	*D. rerio*	Cytoplasm	Glycolysis (Oxidoreductase)	135	5	7	0.111	5.45/38.944	5.73/61
15	**Phosphoglucomutase-1**	Q08DP0	PGM1	*B. taurus*	Cytoplasm	Glycolysis (Isomerase)	582	7	18	0.245	6.36/61.836	7.04/66
16	**Pyruvate kinase isozyme**	P00548	PKM2	*G. gallus*	Cytoplasm	Glycolysis (Kinase Transferase)	546	13	33	0.379	7.29/58.434	7.63/70
17	**Pyruvate kinase**	Q8QGU8	PKM2	*T. rubripes*	Cytoplasm	Glycolysis (Kinase Transferase)	294	15	12	0.073	7.96/58.572	7.90/71
18	**Alpha -enolase**	Q9XSJ4	ENO1	*B. taurus*	Cell membrane, Cytoplasm, Membrane	Glycolysis (Lyase)	320	14	17	0.992	6.63/47.639	6.70/55
19	**Elongation factor 1α**	P68103	EEF1A1	*B. taurus*	Cytoplasm Nucleus	Protein biosynthesis	295	18	27	0.068	9.10/50.451	8.21/60
20	**Elongation factor 1α**	P13549	eef1as	*X. laevis*	Cytoplasm Nucleus	Protein biosynthesis	202	16	15	0.141	9.10/50.524	6.81/41
21	**Malate dehydrogenase**	Q5ZME2	MDH1	*G. gallus*	Cytoplasm	Tricarboxylic acid cycle (Oxidoreductase)	124	9	5	0.135	6.92/36.748	6.57/42
22	**Creatine kinase M-type**	Q9XSC6	CKM	*B. taurus*	Cytoplasm	Glycolysis (Kinase Transferase)	71	3	1	0.062	6.63/43.190	6.78/40
23	**Fructose-bisphosphate aldolase A**	P04075	ALDOA	*H. sapiens*	Cytoplasm	Glycolysis (Lyase)	58	5	3	0.164	8.30/39.851	7.76/46
24	**Glyceraldehyde-3-phosphate dehydrogenase**	Q5R2J2	GAPDH	*T. sinensis*	Cytoplasm Nucleus	Apoptosis, Glycolysis (Oxidoreductase)	251	13	11	0.341	8.70/36.049	7.84/46
25	**Phosphoglycerate mutase 2**	Q32KV0	PGAM2	*B. taurus*	Cytoplasm	Glycolysis (Hydrolase Isomerase)	56	6	3	0.736	8.99/28.838	8.23/35

spot. no.	Identified Protein	Accession no.	Symbol	Species	Location	Biological function	Score	% Coverage	Q. matched	% Volume	pI/M_r Theorical (KDa)	pI/M_r Observed (KDa)
26	**Triosephosphate isomerase B**	Q90XG0	tpi1b	*D. rerio*	Cytoplasm	Glycolysis (Isomerase)	392	29	13	0.215	6.45/27.096	8.10/34.5
27	**Troponin I**	P48788	TNNI2	*H. sapiens*	Cytoplasm Nucleus	Muscle protein	95	4	7	1.196	8.87/21.496	8.14/29
28	**Nucleoside diphosphate kinase B (Fragments)**	P85292	NME2	*M. magellanicus*	Nucleus	Glycolysis (Kinase Transferase)	406	23	13	0.403	5.70/14.280	7.17/15
29	**Nucleoside diphosphate kinase B (Fragments)**	P85292	NME2	*M. magellanicus*	Nucleus	Glycolysis (Kinase, Transferase)	265	13	4	0.013	5.70/14.280	6.64/15
30	**Nucleoside diphosphate kinase B (Fragments)**	P85292	NME2	*M. magellanicus*	Nucleus	Glycolysis (Kinase Transferase)	345	23	10	0.026	5.70/14.280	6.36/13.9
31	**Nucleoside diphosphate kinase B (Fragments)**	Q2EN76	NME2	*S. scrofa*	Nucleus	Glycolysis (Kinase Transferase)	84	7	2	0.110	7.77/17.279	5.89/15
32	**Nucleoside diphosphate kinase B (Fragments)**	Q2EN76	NME2	*S. scrofa*	Nucleus	Glycolysis (Kinase Transferase)	39	7	1		7.77/17.279	5.89/25
33	**Eukaryotic translation initiation factor 5A-1**	Q6EWQ7	EIF5A	*B. taurus*	Cytoplasm, Endoplasmic reticulum, Membrane, Nuclear pore complex, Nucleus	Protein biosynthesis, Protein transport, Translocation, Transport, mRNA transport	55	5	2	0.180	5.08/17.049	5.40/19
34	**Fatty acid-binding protein**	Q4TZH2	FABP3	*B. grunniens*	Cytoplasm	Transporter	33	5	1	0.341	6.73/14.827	4.56/17
35	**Lactoylglutathione lyase**	Q9CPU0	Glo1	*M. musculus*	Cytoplasm	Glycolysis (Lyase)	42	7	5	0.150	5.24/20.967	4.92/26
36	**Alpha-enolase**	P06733	ENO1	*H. sapiens*	Cell membrane, Cytoplasm, Membrane, Nucleus	Glycolysis (Lyase)	651	18	23	0.205	7.01/47.481	7.11/56
37	**Alpha -enolase**	P19140	ENO1	*A. platyrhynchos*	Cell membrane, Cytoplasm, Membrane, Nucleus	Glycolysis (Lyase)	921	26	27	0.301	6.37/47.609	6.45/56
38	**Triosephosphate isomerase B**	Q90XG0	tpi1b	*D. rerio*	Cytoplasm	Glycolysis (Isomerase)	1430	36	41	0.858	6.45/27.096	7.73/26
39	**Adenylate kinase isoenzyme 1**	P12115	ak1	*C. carpio*	Cytoplasm	Glycolysis (Kinase, Transferase)	426	36	39	0.372	6.64/21.532	6.92/25
40	**F1 ATP synthase beta subunit**	C1J0J0	ATP5E	*G. seta*	Cytoplasm	ATP synthesis, Hydrogen ion transport, Rule Base , Ion transport, Transporter	372	26	14	0.067	5.15/53.949	5.11/52
41	**Actin**	Q7T2J3	ACT	*C. carpio*	Cytoplasm	ATP binding	940	66	47	9.545	5.22/42.276	5.39/44
42	**Tropomyosin α-1 chain**	P84335	TPM1	*L. aurata*	Cytoplasm, Cytoskeleton	Actin-binding	957	70	37	3.694	4.69/32.767	4.66/40
43	**Myosin-light chain 1**	Q90W41	mlc1	*S. japonicus*	Cytoplasm Thick filament	Muscle contraction	375	58	13	1.555	4.70/21.343	4.52/26
44	**Myosin regulatory light chain 2**	P02609	MYLPF	*G. gallus*	Cytoplasm	Muscle protein	129	20	6	3.016	4.77/18.941	4.57/19
45	**Myosin light chain 2**	Q9IB25	mlc2	*T. trachurus*	Cytoplasm	Muscle protein	803	84	31	1.164	4.71/19.136	3.91/17
46	**Parvalbumin**	C0LEL4	PVALB	*B. saida*	Cytoplasm	Calcium ion binding	108	17	3	2.164	4.46/11.678	4.35/15
47	**Parvalbumin**	B6UV97	PVALB	*H. molitrix*	Cytoplasm	Calcium ion binding	189	36	7	0.517	4.46/11.624	3.98/14
48	**Muscle-type creatine kinase**	C7ASM1	CKM	*S. chuatsi*	Cytoplasm	Glycolysis (Kinase, Transferase)	685	33	29	2.675	6.41/43.123	7.24/47

spot. no.	Identified Protein	Accession no.	Symbol	Species	Location	Biological function	Score	% Coverage	Q. matched	% Volume	pI/M_r Theorical (KDa)	pI/M_r Observed (KDa)
49	**Creatine kinase M-type**	C1BIK3	CKM	*O. mordax*	Cytoplasm	Glycolysis (Kinase, Transferase)	286	22	13	4.323	6.32/42.991	6.90/46
50	**Glyceraldehde-3-phosphate dehydrogenase**	Q5I1Z5	GAPDH	*D. labrax*	Cytoplasm	Glycolysis (Oxidoreductase)	449	48	24	5.329	8.56/36.041	8.01/40
51	**Fructose-bisphosphate aldolase A**	Q803Q7	aldoaa	*D. rerio*	Cytoplasm	Glycolysis (Lyase)	568	40	34	5.501	8.50/40.087	7.97/45
52	**Lactate dehydrogenase-A**	Q7T3M3	LDH-A	*C. caudalis*	Cytoplasm	Glycolysis (Oxidoreductase)	131	12	5	0.313	6.92/36.392	7.42/39
53	**Capping protein (actin filament) muscle Z-line beta**	B5DFX6	capzb	*S. salar*	Cytoplasm	actin cytoskeleton organization	121	14	6	0.067	5.39/31.202	5.49/37
54	**Phosphoglycerate mutase 2**	Q32KV0	PGAM2	*B. taurus*	Cytoplasm	Glycolysis (Hydrolase Isomerase)	56	6	3	0.174	8.20/8.99	8.20/36

Table 6.1 - List of 54 protein identities from the European sea bass muscle based on mass spectrometry (MS/MS) analysis. Proteins were submitted to UniProt database and accession numbers were obtained. The protein identities are listed according to their accession number obtained from the UniProt database, gene name, symbol, species, location, biological function, score, percentage overage, q. matched, percentage volume, pI/Mr theorical and pI/Mr observed.

S. nr.	*Molecules in networks*	*Score*	*Focus molecules*	*Top functions*
1	**LDHA, PKM2, AK1, ENO1, GAPDH, CKM,** RNA Polimerase II, MAPK, **DES,** SYNC, Insulin, **FABP3, PFKM, EEF1A1, ATP5E,** MYH3, TROPONIN, **MYOSIN,** MYH8, MYL5, MYOM1, MYH13, **MYL6,** Ntp, MYH7B, AMPD1, GCET2, MYL6B, MYH1, MYH7B, **MYL7, MYH2**	37	15	Organ morphology, skeletal and muscular system development and function, carbohydrate metabolism
2	**MYOM2,** IGF1R, **ALDOA, AMPD3, MUSK,** BGN, BTN3A3, **NME2,** FAS, **PVALB**, **MYBPC3,** RIPK2, **HSPA2, TPI1,** CARD18, IL2, CCL13, FAM132A, IRF2BP2, NFAM1, SUCNR1, **GPD1,** 10001G20Rix, MHC class I, LILR4, MMP28, H2-P4, **GLO1,** TNF/FasL, RTP8, Pr12cA, IKBKB, CD300C, **TNF**	25	11	Cell-to-cell signaling and interaction, cell-mediated immune response, and cellular movement
3	**MDH1,** AIM2, STARD10, **TNNI2,** BRD9, ANKRD3L, **EIF5A,** SLC4A1AP, NAF1, BMS1, RBM33, DHX38, **PYGM,** RRP12, ARL61P4, **PGAM2,** Asc2, MGAT4A, LMOD1, **PGM1,** APOLD1, MITF, **TPM1,** SCHIP1, SRF, FOS, RBM6, SUMO2, IWS1, ESF1, CSNK2A1, RSF1, USP24, SMARCA4, USP24	14	7	Cell morphology, developmental disorder, ophthalmic disease

Supplementary Table S6.1 - The protein identities involved in various pathways based on network analysis are shown. Proteins identified in this study are indicated in bold.

Molecules in Biological Functions			
Molecular and Cellular Functions	*Focus molecules*	*p-value*	*Top Biological Functions*
ALDOA, CKM, EEF1A1, ENO1, FABP3, GAPDH, GLO1, GPD1, PFKM, PGAM2, PGM1, PKM2, PYGM, TPI1	14	1.08E-11 - 4.88E-02	Carbohydrate metabolism
AK1, ALDOA, AMPD3, ATP5E, CKM, DES, EEF1A1, ENO1, GAPDH, GPD1, LDHA, MDH1, NME2, PKM2	14	6.43E-07 - 2.15E-02	Nucleic acid metabolism
AK1, ALDOA, AMPD3, ATP5E, CKM, DES, EEF1A1, ENO1, FABP3, GAPDH, GPD1, LDHA, MDH1, NME2, PFKM, PKM2, PGAM2, PGM1, PYGM, PVALB, TPI1	21	6.43E-07 - 4.88E-02	Small molecule metabolism
CKM, EEF1A1, MUSK, MYBPC3, NME2, PKM2, PVALB	7	6.82E-05 - 3.63E-02	Cell signaling
AK1, ALDOA, CKM, EEF1A1, EIF5A, ENO1, GAPDH, FABP3, LDHA, NME2, PGAM2, PKM2, PVALB	13	6.82E-05 - 4.88E-02	Molecular Transport
Physiological System Development and Function	*Focus molecules*	*p-value*	*Top Biological Functions*
ALDOA, CKM, DES, ENO1, FABP3, MYBPC3, MYH2, MYL6, MYL7, MYOM2, PGAM2, PFKM, PKM2, PVALB, TNNI2, TPM1	16	8.25E-10 - 3.21E-02	Organ morphology
AK1, ALDOA, CKM, DES, MYBPC3, MYH2, MYH3, MYL6, MYOM2, MUSK, PFKM, PGAM2, PKM2, PVALB, TNNI2, TPM1	16	8.25E-10 - 4.67E-02	Skeletal and muscular system development and function
CKM, DES, ENO1, FABP3, MYBPC3, MYL7, PFKM, PVALB, TPM	9	3.35E-06 - 5.00E-02	Cardiovascular system development and function
DES, LDHA, MYBPC3, MYH2, MYL6, MUSK, PFKM, PGAM2, PKM2, TPM1	10	9.54E-05 - 5.00E-02	Tissue development
DES, ENO1, MYBPC3, TPM1	4	6.85E-04 - 3.42E-02	Tissue morphology
Diseases and Disorders	*Focus molecules*	*p-value*	*Top Biological Functions*
AK1, EEF1A1, FABP3, GAPDH, GPD1, LDHA, MDH1, MYOM2, MUSK, PFKM, PGAM2, PKM2, PVALB, TNNI2, TPI1	15	2.97E-08 - 3.00E-02	Neurological diseases
AK1, DES, EEF1A1, GAPDH, GPD1, LDHA, MDH1, MYBPC3, MYH2, MYH3, MYOM2, MUSK, PFKM, PGAM2, PKM2, PVALB, TNNI2, TPI1	18	1.32E-07 - 4.88E-02	Skeletal and muscular disorders
AK1, ALDOA, DES, EEF1A1, ENO1,	20	2.48E-07 - 1.94E-02	Hereditary disorder

GAPDH, GPD1, LDHA, MYBPC3, MYH2, MYH3, MYOM2, PFKM, PYGM, PGAM2, PKM2, PVALB, TNNI2, TPI1, TPM1			
CKM, DES, FABP3, MYBPC3, PFKM, TPM1	6	7.20E-06 - 4.98E-02	Cardiovascular diseases
ALDOA, CKM, DES, FABP3, GPD1, MUSK, MYBPC3, MYH3, PFKM, PYGM, TPM1	11	7.20E-06 - 4.88E-02	Developmental disorder

Supplementary Table S6.2. The protein identities involved in various pathways based on Top Biological Functions analysis are shown.

Chapter 7

Discussion

Changes that occur after death in fish muscle have an important impact on the aquaculture industry due to the significant effect on the quality of the filet and subsequent acceptance by consumers. Although some technique is available to evaluate quality and freshness in the fish, these are not entirely objective, but rather they rely on human judgment and proper training of panels.

The objectives of the present Thesis were to understand which different methods slaughtering and storage temperatures can influence more on the quality of the filet using different molecular methods of analysis and to identify possible markers of freshness.

Afterwards, the integrity of total RNA was analyzed in 210 fragments of muscle tissue, collected from 30 sea bass slaughtered by using three different sacrifice techniques, and subsequently stored for 5 days *postmortem* at two different temperatures: 1°C and 18°C. The obtained RQI values by analysis of electrophoresis capillaries were compared, total RNA extracted from samples stored at 18°C had, as expected, degraded more than RNA extracted from samples stored at 1°C, without showing any significant slaughtering method-dependent degradation rates. A possible explanation for the slow RNA degradation at 1°C is the minor activity of ribonucleases, enzymes responsible for degrading total RNA at temperatures around ro.
In samples stored at 1°C, the group of fish that died from asphyxia in air showed a significant reduction in the RQI value with respect to the other two groups sacrificed by hypothermia and by severing the spinal cord from 24 h up to 5 days *post mortem*. The exhausting muscular activity of fish slaughtered by asphyxia in air certainly leads to a rapid decline in ATP reserves, with lactic acid formation and a consequent decrease in pH, which contributes to the rapid RNA degradation. It may also be assumed that asphyxia promotes the formation of free radicals during the *premortem* phase, which contributes to the increasing rate of degradation of total RNA.

In the light of these results, a new experiment was planned, with the aim of identifying potential biochemical markers using 2D-DIGE and mass spectrometry (Chapter 5 and 6). Given the recognized importance of storage conditions for *post mortem* deterioration of fish muscle, in the chapter 5 has focused on the storage temperature, which is considered the factor with the strongest impact in this process. In general, the 2-D profile of fish muscle after 5 days of *post mortem* storage at 1°C appeared well preserved, and the extent of degradation was minimal. Identification of some spot, in particular nucleoside diphosphate kinase B, showed the highest abundance decrease during storage at 1°C, with an average ratio of -20.13 after 5 days of *post mortem.* The observation of an increasing spot upon storage might appear surprising. It should be taken into account, however, that the apparent increase in the abundance of a spot in the 2-D map can depend on protein modifications occurring as a result of *post mortem* biochemical processes, such as dephosphorylation, oxidation, or loss of charged amino acid side chains, leading to a shift in the isoelectric point. This might be the case of proteins for which an increase in only one spot of the isoelectric series was detected. As an example, for fructose bisphosphate aldolase A, glycogen phosphorylase, and phosphoglucomutase-1 the appearance of 'laterally shifted' spots is evident when examining the DIGE overlay patterns.

In order to investigate the trend in protein degradation at higher storage temperatures, a similar analysis of the protein profiles of sea bass muscle after 5 days of *post mortem* storage at 18°C was also performed. As expected, a higher number of spots showed a significant quantitative variation compared with at the time of death samples. When subjected to MS identification, many of these spots did not produce statistically significant results. This might be an indication that this constellation of spots might be represented by a mixture of proteolytic fragments derived from the most abundant muscle proteins, being each present at amounts too low to enable MS identification with the instrumentation used in this work. Other differentially abundant spots produced MS identifications corresponding to proteins having higher molecular weights than those calculated based on spot location, suggesting that they were fragments of larger proteins. Among these, glyceraldehyde-3-phosphate dehydrogenase and MHC were identified in two strongly increased spots in the basic, low–molecular-weight region, in agreement with proteolytic events occurring during storage. These proteins are among the most abundant protein components of striated fish muscle.

Finally, in order to evaluate the differences in protein degradation occurring upon storage at two different temperatures, and to pinpoint possible markers of cold storage by

means of an overall comparison, a 2-D DIGE analysis was performed on sea bass muscle after 5 days of *post mortem* storage at 1°C and 18°C. As a result, several protein spots that differed significantly in intensity were detected. Among others, some spots showed interesting increasing trends, such as GAPDH and MHC, respectively (fold change), as well as those corresponding to MLC 2. Interestingly, almost all of the spots with a decreasing trend provided MS identifications compatible with the expected molecular weight of the protein. The only protein identified among spots of decreasing abundance that did not match the expected molecular weight was MHC; however, it should be noted that MHC was detected at the same position in the map generated from samples at T0, and might justify this apparent discrepancy. The most striking observation of this study was the consistent decrease in abundance of NDP kinase B observed after 5 days of storage at both temperatures. When comparing the abundance decrease of NDP kinase B at 1°C with that at 18°C, it can be clearly observed how variation of this proteins is strongly susceptible to the storage time and occurs very rapidly independent of the storage temperature. This suggests that NDP kinase B may represent a possible biomarker for fish freshness.

Another study (Chapter 5) was applied to investigate the degradation differences introduced in sea bass muscle proteins by three different slaughtering techniques, death by asphyxiation, death from hypothermia and death by severing the spinal cord, both immediately after death and after 5 days of *post mortem* storage at 1°C. The 2D DIGE approach is ideal for this type of proteomic investigation. In fact, changes can be detected in molecular weight or in isoelectric point due to degradation, to the occurrence of chemical changes in lateral side chains, or to loss of post-translational modifications. Moreover, the ability to run the study samples in the same gel and against an internal standard reinforces the robustness of the differential analysis (Lilley et al., 2004; Ünlü et al., 1997). The first and most significant finding from this work is the clear impact of the slaughtering technique on muscle protein degradation. In fact, the PCA analysis performed at the time of death on the total protein profiles reveals a clear separation of the sample group corresponding to group death by severing the spinal cord, while the two asphyxia conditions, although forming two separate groups, are not clearly different from each other and can be grouped in a single cluster. When comparing the logarithmic standard abundance for spots above the significance threshold, less protein degradation is clearly observed in fish slaughtered by severing the spinal cord rather than by asphyxia, either in ice or in air. Most importantly, the impact of the slaughtering technique is clear and significant from the early *post mortem* period. The death by severing the spinal cord represented a less stressful slaughtering method as fish were euthanized by different operators immediately after being collected and

were out of the water for no more than 10 seconds, reducing as much as possible aversive behavior. In contrast, asphyxia was extremely adverse to fish, which showed prolonged violent escape behavior in the form of intense exercise. Such prolonged muscular activity in the *premortem* phase likely caused a higher degradation rate of muscle proteins in the *perimortem* and early *post mortem* phases.

Concerning single proteins, the most significant and dramatic change in abundance was observed for all three spots belonging to NDKB. NDKB is an hexameric complex catalyzing transfer of phosphates from GTP to ADP to form ATP (Berg et al., 2002). In some eukaryotes, NDKB is stabilized by nucleotide binding (Souza et al., 2011). Although this has not been investigated for fish NDKB, the reason for this sudden and massive decrease in the abundance of this protein might be explained by the ATP depletion occurring as a consequence of the intense *perimortem* muscular activity. In fact, the relation between slaughtering method and ATP depletion and its impact on onset and duration of rigor mortis are well known (Robb et al., 2000; Erikson et al., 2006; van de Vis et al., 2003). The consumption of muscle energy reserves might cause the massive dephosphorylation of nucleotides, leading to conformational changes in NDKB that make it less stable and more sensitive to degradation. The death by severing the spinal cord is the least stressful and most sudden type of death among the three investigated and thus does not deplete muscle ATP reserves as dramatically, which might favour a longer stability of this protein. Surprisingly, however, death from hypothermia impacted NDKB stability more than death by asphyxiation. One might expect that death by asphyxiation, causing a more intense muscular activity before death, should have a higher impact on NDKB. However, hypothermia might play a role by impacting on the time to death and/or by introducing a further stress on fish. Conversely, cytoskeletal protein integrity seems to benefit from asphyxia in ice. In fact, although not statistically significant, when observing the fate followed by MbpC and by the three Mhc spots at higher molecular weight, a general trend favoring death from hypothermia over death by asphyxiation can be seen. In general, death by severing the spinal cord samples displayed a higher abundance of protein spots, with pI/Mr values similar to those expected, indicating that these were likely intact proteins. In this case, higher abundance indicates preservation of protein integrity. On the other hand, death by asphyxiation and death from hypothermia samples displayed a higher abundance of proteins, with pI/Mr values quite different from those expected, likely indicating the appearance of degradation products (lower *Mr*) or of chemical transformations (different *pI*).

As a further analysis, we wanted to compare the magnitude of changes occurring between at the time of death and after 5 days *post mortem* within a slaughtering technique as a measure for comparatively assessing intensity of the degradation process among slaughtering procedures. As a matter of fact, the occurrence of changes in protein abundance in the *perimortem*/immediate *post mortem* period actually changes the “starting point” abundance of proteins, which is then reflected in the relative abundance ratio seen for the different slaughtering procedures. When part of the degradation has already occurred at the starting point of the analysis (at the time of death), after 5 days *post mortem* and at the time of death ratio fold change of proteins undergoing degradation in the immediate *peri mortem/post mortem* time frame is paradoxically lower for the proteins most affected by degradation, since the starting point is an already reduced spot. This carries with itself an error which is translated in the statistical analysis, and unfortunately introduces a bias that can lead to misinterpretation of the statistical results. It is advised that, when such studies are performed with comparative purposes, the profiles at the time of death be compared first among the slaughtering conditions in order to assess if significant abundance changes have already occurred in the total protein profiles.

Finally, as the last objective was realized a 2D map of an European sea bass muscle tissue in normal conditions, indentifying 54 abundant spots. Analysis by Ingenuity Pathways Knowledge Base has identified various functions in the proteins, including carbohydrate metabolism, skeletal and muscular system development, molecular transport, tissue development and neurological disease. Most of the protein identities obtained in this study take part in the glycolytic metabolism which, being contraction one of the main activities of fish muscle tissue, is essential for the locomotory power of fish together with sarcoplasmic proteins.

Future studies

Seafood quality is determined by complex interactions of biological and environmental factors, but is often classified according to the appearance of the raw material, like texture, colour and taste. There has been an interest in finding alternative rapid methods for this task. The use of alternative rapid methods for fish grading in fish production does not only improve scientific interest or technical performance but can add value for both procedures and consumers in the fish processing chain (Amerongen et al., 2007).

This Thesis has important indications on the events occurring in sea bass muscle proteins during storage and slaughtering methods and then provides an important basis for future studies aimed to determine how these changes are related to quality traits of fish filet.

One way to identified new molecular markers for quality traits is to use experimental designs, where genetically well defined groups of animals are analysed, and preferentially, the influence of single genes could be characteristic in detail. Although the different slaughter methods and their effect on welfare (Lambooij et al., 2002a,b,c; Robb et al., 2003, 2002; Roth et al., 2007a) and the flesh quality are fairly documented, the practical knowledge and documentation of how humane slaughter methods affect the fish welfare and the quality parameters and then composure of the protein profile are scarce. The need for large scale experiments is required to reveal a more realistic relationship between slaughter methods and product quality as there might be a discrepancy between the experimental and commercial conditions, whereas commercial handling and processing methods might overshadow positive and negative effects of the existing pre slaughter and stunning methods. Therefore, future studies could investigate the effects of methods less stressful for the animal in combination with pre-slaughter conditions, using technique as such proteomic to identify or confirm markers of freshness.

Finally, it is appropriate to compare different maps of the muscular profile of sea bass coming from different parts of Europe to be valuable also in characterising the protein components of complex foods, which traditionally has been difficult to control. Proteomics will also be useful for developing analytic assays for authenticity of food products, like the verification of an animals origin, or related to whether regulations and food regulations and food policies has keep kept, e.g.., related to on animal transport (Bendixen, 2005).

Chapter 8

General Conclusions

The results of this Thesis indicate that, storage conditions are important for *post mortem* deterioration of fish muscle, and temperature is one of the factors with the strongest impact on this process.

Storage temperature negatively affected the integrity of total RNA (higher degradation at 18 °C). However, the RNA degradation was a slow process under the conditions investigated and, and the transcripts of μ-calpain and cathepsin L were present for up to 5 days *post mortem* in the muscle of sea bass stored at either 1 °C or 18 °C.

The greatest alterations in sea bass filet protein composition were observed upon *post mortem* storage at 18°C, with distinct changes appearing in the 2-D protein profile after 5 days of storage at this temperature. Degradation of the myofibrillar protein myosin heavy chain and of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase, among the most abundant muscle proteins, was clearly observed upon storage at higher temperatures. One of the most interesting observations was the rapid and significant decrease in the abundance of nucleoside diphosphate kinase B and phosphoglycerate mutase 2, which was observed also at low storage temperatures and appeared to be temperature-independent. NDP kinase B could be thus a potential biochemical marker enabling the evaluation of fish filet freshness quality.

Pre-slaughter and slaughter stressful practices can have an important effect on the flesh quality in fish. Stress suffered at slaughter has a negative impact on the *post mortem* integrity of muscle proteins. Therefore, the reduction of the stress at slaughter might be a satisfactory strategy for both animal welfare and product quality.

References

Abbas KA, Mohamed A, Jamilah B, Ebrahimian M (2008). A review on correlations between fish freshness and pH during cold storage. American Journal of Biochemistry and Biotechnology 4 (4):416-421.

Acerete L, Balasch JC, Espinosa E, Josa A, Tort L (2004). Physiological responses in Eurasian perch (*Perca fluviatilis*. L.) subjected to stress by transport and handling. Aquaculture 237, 167–178.

Addis MF, Cappuccinelli R, Tedde V, Pagnozzi D, Porcu MC, Bonaglini E, Roggio T, Uzzau S (2010). Proteomic analysis of muscle tissue from gilthead sea bream (*Sparus aurata*, L.) farmed in offshore floating cages. Aquaculture 309:245–52.

Addis MF, Pisanu S, Preziosa E, Pagnozzi D, Bernardini G, Roggio T, Uzzau S, Saroglia M, Terova G (2012). 2D DIGE/MS to investigate the impact of slaughtering techniques on postmortem integrity of fish filet proteins. Journal of Proteomics 75, 3654- 3664.

Addis MF, Tanca A, Pagnozzi D, Crobu S, Fanciulli G, Cossu-Rocca P, Uzzau S (2009). Generation of high-quality protein extracts from formalin-fixed, paraffin-embedded tissues. Proteomics 9, 3815-23.

Addis MF, Tanca A, Pagnozzi D, Rocca S, Uzzau S (2009). 2-D PAGE and MS analysis of proteins from formalin-fixed, paraffin-embedded tissues. Proteomics 9, 4329-39.

Alban A, David SO, Bjorkesten L, Andersson C, Sloge E, Lewis S, Currie I (2003). A novel experimental design for comparative two-dimensional gel analysis: two-dimensional difference gel electrophoresis incorporating a pooled internal standard. Proteomics 3, 36-44.

Alves RN, Cordeiro O, Silva TS, Richard N, de Vareilles M, Marino G, Di Marco P, Rodrigues PM, Conceição LEC (2010). Metabolic molecular indicators of chronic stress in gilthead sea bream (*Sparus aurata*) using comparative proteomics. Aquaculture 299, 57–66.

Amarita F (2007). Methods for Microbiological quality assessment of seafood. http://valid.aztionline.com/index.php?idioma=en&seccion=6&ctipo=2&contenido=320&menu=136&submenu=142

Ambroggi F, Sebastio P, Pirazzoli P, Baldrati G (1996). Influenza del sistema di sacrificazione su trote iridee di allevamento. II: rigor mortis e consistenza del tessuto muscolare. Industria Conserve. 71 (N.D.) 157-161.

Amerongen AV, Barug D, Lauwaars M (2007). Rapid Methods for food quality determination. Wabeninggen Academic Publishers, Netherlands.

and slaughter management. Aquaculture International 13, 29–49.

Ando M, Toyohara H, Sakaguchi M (1993). Post mortem tenderization of rainbow trout muscle caused by the disintegration of collagen fibres in the pericellular connective tissue. Nippon Suisan Gakkaishi 58, 567-570.

Arthur JSC, Elce JS, Hegadorn C, Williams K, Green PA (2000). Disruption of the murine calpain small subunit gene, capn4: Calpain is essential for embryonic development but not for cell growth and division. Molecular and Cellular Biology 20, 4474–4481.

Astier C, Labbe JP, Roustan C, Benyamin Y (1991). Sarcomeric disorganization in post-mortem fish muscles. Comporative Biochemistry and Physiology B 100B, 459–465.

Ayala MD, Albors OL, Blanco A, Alcazar AG, Abellan E, Zarzosa GR, Gill F (2005). Structural and ultrastructural changes on muscle tissue of sea bass, *Dicentrarchus labrax* L., after cooking and freezing. Aquaculture 250, 215-231.

Bagni M, Civitareale C, Priori A, Ballerini A (2007). Pre-slaughter crowding stress and killing procedures affecting quality and welfare in sea bass (*Dicentrarchus labrax*) and sea bream (Sparus aurata). Aquaculture 263, 52-60.

Bagni M, Civitareale C, Priori A, Ballerini A, Finoia M, Brambilla G, Marino G (2007). Pre-slaughter crowding stress and killing procedures affecting quality and welfare in sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*). Aquaculture 263,52–60

Bahar B, Monahan FJ, Moloney AP, Schmidt O, MacHugh DE, Sweeney T (2007). Long-term stability of RNA in post-mortem bovine skeletal muscle, liver and subcutaneous adipose tissues. BMC Molecular Biology, 8, 108.

Bahuaud D, Mørkøre T, Østbye TK, Veiseth-Kent E, Thomassen MS, Ofstad R (2010). Muscle structure responses and lysosomal cathepsins B and L in farmed Atlantic salmon (Salmo salar L.) pre- and post-rigor fillets exposed to short and long-term crowding stress. Food Chemistry, 118, 602–615.

Barton AJL, Pearson RCA, Najlerahim A, Harrison PJ (1993). Pre-and postmortem influences on brain RNA. Journal of Neurochemistry, 61, 1–11. Carnevali O, Cionna C, Tosti L, Cerda J, Gioacchini G, (2008). Changes in cathepsin gene expression and relative enzymatic activity during gilthead sea bream oogenesis. Molecular Reproduction and Development 75(1), 97–104.

Bendixen E (2005). The use of proteomics in meat science. Meat Science 71, 138-149.

Berg JM, Tymoczko JL, Stryer L. Biochemistry, 5th ed., W H Freeman, New York, (2002).

Bisogni CA, Ryan GJ, Regenstein JM (1987). What if fish quality? Can we incorporate consumer perceptions? IN Kramer DE, Liston J (Eds.), Seafood quality determination (pp.547-563). Amsterdam: Elsevier Science Publishers BV.

Botta JR (1995). Evaluation of seafood freshness quality. New York: VCH Publishers Inc,

Boyd NS, Wilson ND, Jerret AR, Hall BI (1984). Effects of brain destruction on post-harvest muscle metabolism in the fish kahawai (*Arripis trutta*). Journal of Food Science 49, 177–179.

Bremner HA (2000). Toward practical definitions of quality for food science. Critical reviews in Food Science and Nutrition 40, 83-90.

Busconi L, Folco EJ, Martone CB, Sanchez JJ (1989). Post mortem changes in cytoskeletal elements of fish muscle. Journal of Food Biochemistry 13, 443–451.

Campus M, Addis MF, Cappuccinelli R, Porcu MC Pretti L, Tedde V, Secchi N, Stara G, Roggio T (2010) Stress relaxation behaviour and structural changes of muscle tissues from Gilthead Sea Bream (*Sparus aurata* L.) following high pressure treatment. Journal of Food Engineering 96, 192–198.

Capetanaki Y, Milner DJ, Weitzer G (1997). Desmin in muscle formation and maintenance: knockouts and consequences. Cell Structure and Function 22, 103–116.

Chéret R, Delbarre-Ladrat C, de Lamballerie-Anton M, Verrez-Bagnis V (2007). Calpain and cathepsin activities in post mortem fish and meat muscles. Food Chemistry, 101, 1474–1479.

Chevalier F (2010). Highlights on the capacities of ‘‘Gel-based’’ proteomics. Proteome Science 8, 23.

Chevallet M, Luche S, Rabilloud T (2006). Silver staining of proteins in polyacrylamide gels. Nature Protocols 1, 1852-8.

Christensen M, Henckel P, Purslow PP (2004). Effect of muscle type on the rate of post-mortem proteolysis in pig. Meat Science 66, 595–601.

Civera T (2003). Species identification and safety of fish products. Veterinary Research Communications 27, 481-489.

Conte FS (2004). Stress and the welfare of cultured fish. Applied Animal Behaviour Science 86, 205-23.

Council Directive 93/119/EC (1993). On the protection of animals at the time of slaughter or killing. Official Journal of the European Communities No 1.340/21.

Council Regulation (EC) 104/2000 of 17 December 1999 on the common organization of the markets in fishery and aquaculture products. Official Journal L17, 21.014.2000, 22-52.

Davies JR, Ledward DA, Bardsley RG, Poulter RG (1994). Species dependence of fish myosin stability to heat and frozen storage. International Journal of Food Science and Technology 29, 287–301.

Delbarre-Ladrat C, Chéret R, Taylor R, Verrez-Bagnis V (2006). Trends in post mortem aging in fish: understanding of proteolysis and disorganization of the myofibrillar structure. Critical Reviews in Food Science and Nutrition 46, 409–421.

Diana JS (2009). Aquaculture Production and Biodiversity Conservetion. BioScience, 59, 27-38.

Digre H, Erikson U, Misimi E, Lambooij B, Van deVis H (2010). Electrical stunning of farmed Atlantic cod *Gadus morhua* L.: a comparison of an industrial and experimental method. Aquaculture Res 41, 1190-1202.

EFSA (2009). Scientific opinion of the panel on animal health and welfare on a request from the European Commission on welfare aspect of the main systems of stunning and killing of farmed seabass and seabream. The EFSA Journal 1010, 1–52.

Erikson U, Hultmann L, and Steen JE (2006). Live chilling of Atlantic salmon (Salmo salar) combined withmild carbon dioxide anaesthesia. I. Establishing a method for large-scale processing of farmed fish. Aquaculture 252, 183-98.

Erikson U, Lambooij B, Digre H, Reimert HGM, Bondø M, Van der Vis H (2012). Conditions for instant electrical stunning of farmed Atlantic cod after de-watering, maintenance of

unconsciousness, effects of stress, and fillet quality - A comparison with AQUI-S™. Aquaculture 324-325:135–144.

Erikson U, Sigholt T, Rustad T, Einarsdottir IE, Jørgensen L (1999). Contribution of bleeding to total handling stress during slaughter of Atlantic salmon. Aquaculture International 7, 101-15.

European Food Safety Authority, (2004). Scientific report of the Scientific Panel on Animal Health and Welfareon a request from the Commission related to welfare aspects of animal stunning and killing methods, p178. www.efsa.europa.eu/ cs/BlobServer/Scientific _Opinion/opinion_ahaw_02 _ej45_ stunning _report_v2_en1,1.pdf?ssbinary=true. Accessed November 4, 2008.

FAO (2002). World agriculture: towards 2015/2030. Food and Agriculture Organization of the United Nations. www.fao.org/docrep/004/y3557e/y3557e10.htm#q. Accessed August 20, 2008.

FAO (2002). World agriculture: towards 2015/2030. Food and Agriculture Organization of the United Nations. www.fao.org/docrep/004/y3557e/y3557e10.htm#q. Accessed August 20, 2008.

FAO (2009). World review of fisheries and aquaculture. The state of world fisheries and aquaculture. ftp://ftp.fao.org/docrep/fao/011/i0250e/i0250e.pdf

FAO 2005-2010. Cultured Aquatic Species Information Programme. Tect by Bagni M. In: FAO Fisheries and Aquaculture Department (Online). Rome. Updated 6 April 2005. (Cited 30 September 2010). http://www.fao.org/fishery/culturedspecies/Dicentrarchus_labrax/en.

FAO Fisheries and Aquaculture Department (2010). Food and agriculture organization of the united nations. The State of World Fisheries and Aquaculture http://www.fao.org

Fitzpatrick R, Casey OM, Morris D, Smith T, Powell R, Sreenan JM (2002). Post-mortem stability of RNA isolated from bovine reproductive tissues. Biochimica et Biophysica Acta 1574, 10–14.

Fontanesi L, Colombo M, Beretti F, Russo V (2008). Evaluation of postmortem stability of porcine skeletal muscle RNA. Meat Science 80, 1345–1351.

Food and Agriculture Organization of the United Nations (2006). Nearly half of all fish eaten today farmed, not caught. FAONewsroom. www.fao.org/newsroom/en/news/2006/1000383/index.html. Accessed August 20, 2008.

Food and Agriculture Organization of the United Nations (2008). Appendix I – Fish and fishery products- apparent consumption. ftp://ftp.fao.org/fi/stat/summery/appIybc.pdf. Accessed September 18, 2008.

Fornè I, Abiàn J, Cerdà J (2010). Fish proteome analysis: model organism and non-sequenced species. Proteomics 10, 858-872.

Geesink G, Morton J, Kent M, Bickerstaffe R (2000). Partial purification and characterization of Chinook salmon (Oncorhynchus tshawytscha) calpains and an evaluation of their role in post mortem proteolysis. Journal of Food Science 65, 1318–1324.

Gemmell B (2001). Managing agricultural resources for biodiversity conservation: a guide to best practices. Draft version. UNEP/UNDP GEF Biodiversity Planning Support Program.

Gharbi S, Gaffney P, Yang A, Zvelebil MJ, Cramer R, Waterfield MD, Timms JF (2002). Evaluation of two-dimensional different gel electrophoresis for proteomic expression analysis of a model breast cancer cell system. Molecular & Cellular Proteomics 1,91-98.

Goll D, Dayton W, Singh I, Robson R (1991). Studies on alphaactinin/ actin interaction in the Z-disk by using calpain. The Journal of Biological Chemistry 266, 8501–8510.

Gonzalez EG, Krey G, Espiñeira M, Diez A. Puyet A, Bautista JM (2010). Population proteomics of the European Hake (Merluccius merluccius). Journal of Proteome Research 9, 6392–6404.

Greaser M (1991). An overview of the muscle cell cytoskeleton. Annual Reciprocal Meat Conference of the American Meat Science Association 44, 1–5.

Grigorakis K, Alexis MN (2005). Effects of fasting on the meat quality and deposition of commercial-size farmed gilthead sea bream (*Sparus aurata*, L) different dietary regimes. Aquaculture Nutrition 11, 341-344.

Guhaniyogi J, Brewer G (2001). Regulation of mRNA stability in mammalian cells. Gene 265, 11–23.

Han JZ, Wang YB (2008). Proteomics: present and future in food science and technology. Trends in Food Science & Technology 19, 26-30.

Harvey DJ (2004) U.S. seafood market shifts to aquaculture. U.S. Department of Agriculture Economic Research Service, AmberWaves, April. www.ers.usda.gov/AmberWaves/april04/Findings/USSeafood.htm. Accessed August 20, 2008.

HAS (2005). Humane harvesting of salmon and trout: Guidance notes No. 5. (Wheathamstead, U.K.: Humane Slaughter Association).

Hastings RJ, Rodger GW, Park R, Matthews AD, Anderson EM (1985). Differential scanning calorimetry of fish muscle: the effect of processing and species variation. Journal of Food Science 50, 503–510.

Huff-Lonergan E, Mitsushahi T, Beekman DD, Parrish FC, Olson DG, Robson RM (1996). Proteolysis of specific muscle structural proteins by calpains at low pH and temperature is similar to degradation in post mortem bovine muscle. Journal of Animal Science 74, 993–1008.

Huss HH (1995). Quality and quality changes in fresh fish. FAO Fisheries Technical Paper 348, Rome, FAO.

Huss HH (1988). Fresh fish-quality and quality changes- Chapter 5. Assessment of fish quality. Food & Agriculture Org. A training manual prepared for the FAO/DANIDA training programme on fish technology and quality control.

Inoue H, Kimura A, Tuji T (2002). Degradation profile of mRNA in a dead rat body: Basic semi-quantification study. Forensic Science International 130, 127–132.

Istvan ES (2002). Structural mechanism for statin inhibit ion of 3-hydrox-3-methylglutaryl coenzyme A redcutase. American Heart Journal 144, S27-S32

Iwamoto M, Yamanka H, Watabe S, Hashimoto K (1987). Effect of storage temperature on rigor mortis and ATP degradation in plaice (*Paralichthys olivaceus*) muscle. Journal of Food Science 52, 1514-1517.

Izquierdo MS, Montero D, Robaina L, Caballero MJ, Rosenlund G, Gines R (2005). Alterations in fillet fatty acid profile and flesh quality in gilthead seabream (*Sparus aurata*) fed vegetable oils for a long term period. Recovery of fatty acid profile fish oil feeding. Aquaculture 250, 431-444.

Jasra SK, Jasra PK, Talesara C (2001). Myofibrillar protein degradation of carp (*Labeo rohita* (Hamilton) muscle after post-mortem unfrozen and frozen storage. Journal of the Science of Food and Agriculture 81, 519-24.

Jekely G, Friedrich P (1999). The evolution of the calpain family as reflected in paralogous chromosome regions. Journal of Molecular Evolution, 49, 272–281.

Jerrett AR, Holland AJ (1998). Rigor tension development in excised “rested”, “partially exercised” and “exhausted” Chinook salmon white muscle. Journal of Food Science 63, 48-52.

Johnson SA, Morgan DG, Finch CE (1986). Extensive postmortem stability of RNA from rat and human brain. Journal of Neuroscience Research 16, 267–280.

Johnston IA (1999). Muscle development and growth: potential implication for flesh quality in fish. Aquaculture 177, 99-115.

Kestin S, Wotton SB, Adams S (1995). The effect of CO_2, concussion or electrical stunning of rainbow trout (*Oncorhynchus mykiss*) on fish welfare. In: Svennevig N and Krogdahl Å (eds.), Quality in Aquaculture, Special Publication 23 (Gent, Belgium: European Aquaculture Society, pp. 380-1).

Kestin SC, Van de Vis JW, and Robb DHF (2002). Protocol for assessing brain function in fish and the effectiveness of methods used to stun and kill them. The Veterinary Record 150:302-07.

Kislinger T, Cox B, Kannan A, Chung C, Hu P, Ignatchenko A Scott MS, Gramolini AO, Morris Q, Hallett MT, Rossant J, Hughes TR, Frey B, Emili A (2006). Global survey of organ and organelle protein expression in mouse: combined proteomic and transcriptomic profiling. Cell 125, 173-186.

Kjæersgard IVH, Jessen F (2003). Proteome analysis elucidating post mortem changes in cod (Gadus morhua) muscle proteins. Journal of the Science of Food and Agriculture 51, 3985–3991.

Kjærsgard IVH, Nørrelykke MR, Jessen F (2006). Changes in cord muscle proteins during frozen storage revealed by proteome analysis and multivariate data analysis. Proteomics 2006 6, 1606-1618.

Kristensen L, Purslow PP (2001). The effect of ageing on the water-holding capacity of pork: role of cytoskeletal proteins. Meat Science 58, 17–23.

Kwon JY, Prat F, Randall C, Tyler CR (2001). Molecular characterization of putative yolk processing enzymes and their expression during oogenesis and embryogenesis in rainbow trout (*Oncorhynchus mykiss*). Biology of Reproduction 65(6), 1701–1709.

Lambooij E, Gerritzen MA, Reimert H, Burggraaf D, van de Vis JW (2008). A humane protocol for electro-stunning and killing of Nile tilapia in fresh water. Aquaculture 275:88-95.

Lambooij E, Kloosterboer RJ, Pieterse C, Gerritzen MA, van de Vis JW (2003). Stunning of farmed African catfish (*Clarias gariepinus*) using a captive needle pistol; assessment of welfare aspects. Aquaculture Research 34,1353-58.

Lambooij E, van de Vis JW, Kloosterboer RJ, Pieterse C (2002). Welfare aspects of live chilling and freezing of farmed eels (*Anguilla anguilla* L.): neurological and behavioral assessment. Aquaculture 210, 159-169.

Lambooij E, van de Vis JW, Kuhlmann H, Munkner W, Oehlenschlager J, Kloosterboer RJ, Pieterse C (2002). A feasible method for humane slaughter of eel (*Anguilla anguilla* L.): electrical stunning in fresh water prior to gutting. Aquaculture Res 33, 643-52.

Lametsch R, Karlsson A, Rosenvold K, Andersen H, Roepstorff P, Bendixen E (2003). Post mortem proteome changes of porcine muscle related to tenderness. Journal of Agricultural and Food Chemistry 51, 6992–6997.

Lane A, Charles J (2005). Defining Indicators for Sustainable Aquaculture Development in Europea. A multi-stakeholder workshop held in Oostende, Belgium (21-23).

Leonard S, Logel J, Luthman D, Casanova M, Kirch D, Freedman R (1993). Biological stability of mRNA isolated from human postmortem brain collections. Biological Psychiatry, 33, 456–466.

Lilley KS, Friedman DB (2004). All about DIGE: quantification technology for differential-display 2D-gel proteomics. Expert Review of Proteomics 1,401-9.

Lines JA, Robb DH, Kestin SC, Crook SC, Benson T (2003). Electric stunning: a humane slaughter method for trout. Aquacultural Engineering 28,141-54.

Lull ME, Carkaci-Salli N, Freeman WM, Myers JL, Midgley FM, Thomas NJ, Kimatian SJ, Vrana KE, Undar A (2008). Plasma biomarkers in pediatric patients undergoing cardiopulmonary bypass. Pediatric Research 63, 638-644.

Luten JB, Oehlenschläger J, Olafsdóttir G (2003). Quality of fish from catch to consumer: Labbelling, Monitoring and Traceability. Wageningen Academic Pub, 2003 - 456

Malik KJ, Chen CD, Olsen TW (2003). Stability of RNA from the retina and retinal pigment epithelium in a porcine model simulating human eye bank conditions. Investigative Opthalmology and Visual Science 44, 2730–2735.

Marchuk L, Sciore P, Reno C, Frank CB, Hart DA (1998). Post-mortem stability of total RNA isolated from rabbit ligament, tendon and cartilage. Biochimica et Biophysica Acta 1379, 171–177.

Marouga R, David S, Hawkins E (2005). The development of the DIGE system: 2-D fluorescence difference gel analysis technology. Analytical and Bioanalytical Chemistry 382, 669–678.

Martinez I, Aursand M, Erikson U, Singstadb, TE, Veliyulinb E, van der Zwaagb C (2003). Destructive and non-destructive analytical techniques for authentication and composition analyses of foodstuffs. Trends Food Sci.Technol.14, 489-498.

Martinez I, Friis T J, Careche M (2001). Postmortem muscle protein degradation during ice-storage of Arctic (*Pandalus borealis*) and tropical (*Penaeus japonicus* and *Penaeus monodon*) shrimps: a comparative electrophoretic and immunological study. Journal of the Science of Food and Agriculture 81, 1199–1208.

Martinez I, Friis TJ (2004). Application of proteome analysis to seafood authentication. Proteomics 4, 347-354.

Martinsdóttir E, Luten JB, Schelvis-Smit AAM, Hylding G (2003). Development of QIM – past and future. (Ed) Luten JB, Oehlenschläger J, Olafsdòttir G. Quality of fish from catch to consumer: labelling monitoring and traceability pp265-272.

Marx H, Brunner B, Weinzierl W, Hoffman R, Stolle A (1997). Methods of stunning freshwater fish: impact on meat quality and aspects of animal welfare. Zeitschrift fur Lebenschmittel Untersuchung und Forschung 204: 282–286.

McLean L, Young IS, Doherty MK, Robertson DHL Cossins AR, Gracey AY, Beynon RJ, Whitfield PD (2007). Global cooling: cold acclimation and the expression of soluble proteins in carp skeletal muscle. Proteomics 7, 2667-2681.

Mochizuki S, Sato A (1994). Effects of various killing procedures and storage temperatures on post-mortem changes in the muscle of horse mackerel. Nippon Suisan Gakkaishi 60 (1), 125–130.

Monti G, De Napoli L, Mainolfi P, Barone R, Guida M, Marino G, Amoresano A (2005) . Monitoring food quality by microfluidic electrophoresis, gas chromatography, and mass spectrometry techniques: effects of aquaculture on the sea bass (*Dicentrarchus labrax*). Analytical Chemistry 77, 2587-2594.

Morris PC, Beattie C, Elder B, Finlay J, Gallimore P, Jewison W, Lee D, Mackenzie K, McKinney R, Sinnott R, Smart A, Weir M (2005). Application of a low oil pre-harvest diet to manipulate the composition and quality of Atlantic salmon, *Salmo salar* L. Aquaculture 244, 187-201.

Morrisson E, Mielche M, Purslow PP (1998). Immunolocalisation of intermediate filaments proteins in porcine meat. Fiber type and muscle-specificity variations during conditioning. Meat Science 50, 91–104.

Morzel M, Sohier D, van de Vis H (2002). Evaluation of slaughtering methods for turbot with respect to animal welfare and flesh quality. Journal of the Science of Food Agriculture 82, 19–28.

Mourente G, Bell JG (2006). Partial replacement of dietary fish oil with blends of vegetable oils (rapeseed, linseed and palm oils) in diets for European sea bass (Dicentrarchus labrax L.) over a long term growth study: effects on muscle and liver fatty acid composition and effectiveness of a fish oil finishing diet. Comparative Biochemistry and Physiology 145B, 389-399.

Nielsen J (1995). Sensory methods. In Huss HH. Editor. Quality and quality changes in fresh fish. Rome, FAO Fisheries Technical Paper, No 348. p 130-139.

Ocaño-Higuera VM, Marquez-Rìos E, Canizales-Dàvila M, Castillo-Yàñez FJ,Pacheco-Aguilar R, Lugo-Sànchez ME, Garcìa-Orozco KD, Graciano-Verdugo AZ (2009). Postmortem changes in cazon fish muscle stored on ice. Food Chemistry 116, 933–938.

Oehlenschlager J (1992). Evaluation of some well established and some underrated indices for the determination of freshness and/or spoilage of ice stored wet fish. Quality assurance in Food industry. pp 339-358.

Oehlenschlager J, Sörensen NK (1998). Criteria of seafood freshness and quality aspects. In methods to determine the freshness of fish in research and industry. Proceeding of the final meeting of the concerted action AIR CT94-2283, Nantes (pp.30-35). Paris: Institut International du Froid.

Ogata H, Aranishi F, Hara K, Osatomi K, Ishihara T (1998). Proteolytic degradation of myofibrillar components by carp cathepsin L. Journal of the science of food and agriculture 74 (4).

Olafsdottir G, Martinsdottir E, Dalgaard P, Najensen B, Undeland I, Mackie IM, Henehen G, Nielesen and Nilsen (1997). Method to evaluate fish freshness in research and industry. Trends in Food Science & Technology 8, 258-265.

Olafsdottir G, Nesvadba P, Di Natale C, Careche M, Oehlenschläger J, Tryggvadóttir SV, Schubring R, Kroeger M, Heia K, Esaiassen M, Macagnano A, Jørgensen BM (2004). Multisensor for fish quality determination. Trends in Food Science & Technology 15, 86-93.

Ottera H, Roth B, Torrissen OJ (2001). Do killing methods affect the quality of Atlantic Salmon? In: Kestin, S.C., Warriss, P.D. (Eds.), Farmed Fish Quality. Blackwell Science Ltd, Oxford, p. 398–399.

Ozogul F, Kuley E, Ozogul Y (2007). Sensory, chimica and microbiological quality parameters in sea bream (*Sparus aurata*) stored in ice or wrapped in cling film or in aluminium foil at 2±1 C. International Journal of Food Science & Technoloty 42, 903-909.

Pankhurst NW, Sharples DF (1992). Effects of capture and confinement on plasma cortisol concentrations in the snapper, *Pagrus auratus*. Australian Journal of Marine and Freshwater Research 43: 345–356.

Papa I, Alvarez C, Verrez-Bagnis V, Fleurence J, Benyamin Y (1996). Post mortem release of fish white muscle alpha-actinin as a marker of disorganization. Journal of the Science of Food and Agriculture 72, 63–70.

Papa I, Taylor RG, Astier C, Ventre F, Lebart MC, Roustan C, Ouali A, Benyamin Y (1997). Dystrophin cleavage and sarcolemma detachment are early post mortem changes on bass (Dicentrarchus labrax) white muscle. Journal of Food Science 62, 917–921.

Parisi G, Mecatti M, Lupi P, Zampacavallo G, Poli BM (2001). Fish welfare and quality: experimental results on rearing and harvesting practices. In: Proceedings of the Third Congress of the European Society for Agricultural and Food Ethics (EURSAFE 2001) ‘‘Food Safety, Food Quality and Food Ethics’’, 3–5 October 2001, Florence, Italy, pp. 337–340.

Parrington G, Coward K (2002). Use of emerging genomic and proteomic technologies in fish physiology. Aquatic Living Resources 15, 193–196.

Perez J, Alvarez P, Martinez JL (2005). Genetic identification of hake and megrim eggs in formaldehyde-fixed plankton samples. Ices Journal of Marine Science 62, 908-914.

Perez J, Garcia-Vazquez E (2004). Genetic identification of nine hake species for detection of commercial fraud. Journal of Food Protect 67, 2792-2796.

Periago MJ, Ayala MD, Lopez-Albors O, Abdel I, Martínez C, García-Alcazar A, Rosa G, Gill F (2005). Muscle cellularity and flesh quality of wild and farmed sea bass, *Dicentrarchus labrax* L. Aquaculture 249, 175-188.

Pickering T (2011). Aquaculture and biodiversity developing principles for aquaculture of introduced species. Seventh Heads of Fisheries Meeting, 28 Feb-4 March 2011, Noumea; New Caledonia.

Piñeiro C, Barros-Velázquez J, Sotelo CG, Gallardo JM (1999). The use of two-dimensional electrophoresis in the characterization of the water-soluble protein fraction of commercial flat fish species. Zeitschrift für Lebensmitteluntersuchung und -Forschung A 208, 342-348.

Piňeiro C, Barros-Velàzquez J, Vàzquez J Figueras A, Gallardo JM (2003). Proteomics as a toll for the investigation of seafood and other marine products. Journal of Proteomic Research 2, 127-135.

Poli BM, Parisi G, Scappini F, Zampacavallo G (2005). Fish welfare and quality as affected by pre-slaughter and slaughter management. Aquaculture International 13,29-49.

Poli BM, Scappini F, Parisi G, Zampacavallo G, Mecatti M, Lupi P, Mosconi G, Giorgi G, Vigiani V (2004). "Traditional and innovative stunning slaughtering methods for European seabass compared by the complex of the assessed behavioural, plasmatic and tissue stress and quality indexes at death and during shelf life." In Procedingns of the 34th WEFTA Conference 2004, Lubeck, Germany. www.wefta.org/file_DIR/03-11-2008_12-39-10_96_WEFTA_Proceedings%20Lubeck%202004.pdf.

Pottinger TG (2001). Effects of husbandry stress on flesh quality indicators in fish. In: Kestin SC, Wariss, P.D. (eds.), Farmed Fish Quality Fishing News Books, Oxford, pp. 145–160.

Raatikainen O, Reinikainen V, Minkkinen P, Ritvanen T, Muje P, Pursiainen J, Hiltunen T, Hyvonen P, Von Wright A, Reinikainen S (2005). Multivariate modeling of fish freshness index based on ion mobility spectrometry measurement. Analytica Chimica Acta 544, 128-134.

Rémignon H, Mollette C, Babile R, Fernandez X (2006). Current advances in proteomic analysis and its use for the resolution of poultry neat quality problems. World's Poultry Science Journal 62, 123-130.

Ribas L, Flos R, Reig L, MacKenzie S, Barton BA, Tort L (2007). Comparison of methods for anaesthetizing Senegal sole (*Solea senegalensis*) before slaughter: Stress responses and final product quality. Aquaculture 269, 250-8.

Robb DFH, Kestin SC (2002). Methods used to kill fish: field observations and literature reviewed. Animal Welfare 11, 269-282.

Robb DH, Kestin S, Warriss P (2000). Muscle activity at slaughter: I. Changes in flesh colour and gaping in rainbow trout. Aquaculture 182, 261-69.

Robb DH, Roth B (2003). Brain activity of Atlantic salmon (*Salmo salar*) following electrical stunning using various field strengths and pulse durations. Aquaculture 216, 363-69.

Robb DHF (2001). The relationship between killing methods and quality. In Farmed Fish Quality (Ed. By SC Kestin and PD Warriss), pp. 220-233. Blackwell Science Ltd., Oxford, UK.

Robb DHF, Arrison PD (2000). Muscle activity at slaughter: I. Changes flesh colour and gaping in rainbow trout. Aquaculture 182, 261-269.

Robb DHF, Kestin SC (2002). Methods used to kill fish: field observations and literature reviewed. Animal Welfare 11, 269-82.

Robb DHF, O'Callaghan M, Lines JA, Kestin SC (2002). Electrical stunning of rainbow trout (Oncorhynchus mykiss): factors that affect stun duration. Aquaculture 205, 359-71.

Robb DHF, Wotton SB, McKinstry JL, Sørensen NK, Kestin SC (2000). Commercial slaughter methods used on Atlantic salmon: determination of the onset brain failure by electroencephalography. Veterinary Record 147, 298-303).

Robb DHF, Wotton SB, McKinstry JL, Sørensen NK, Kestin SC (2000). Commercial slaughter methods used on Atlantic salmon: determination of the onset of brain failure by electroencephalography. The Veterinary Record 147, 298-303.

Rodrigues PM, Silva TS, Dias J, Jessen F (2012) PROTEOMICS in aquaculture: Applications and trends. Journal of Proteomics 75, 4325-4345.

Ross BM, Knowler JT, McCulloch J (1992). On the stability of messenger RNA and ribosomal RNA in the brains of control human subjects and patients with Alzheimer's disease. Journal of Neurochemistry 58, 1810–1819.

Roth B, Imsland A, Gunnarsson S, Foss A, Schelvis-Smit R (2007). Slaughter quality and rigor contraction in farmed turbot (*Scophthalmus maximus*); a comparison between different stunning methods. Aquaculture 272, 754–761.

Roth B, Moeller D, Veland JO, Imsland A, and Slinde E (2002). The effect of stunning methods on rigor mortis and texture properties of Atlantic salmon (*Salmo salar*). Journal of Food Science 67, 1462-66.

Roth B, Slinde E, and Robb DHF (2006). Field evaluation of live chilling with CO_2 on stunning Atlantic salmon (Salmo salar) and the subsequent effect on quality. Aquaculture Research 37, 799-804.

Roth B, Slinde E, Arildsen J (2006). Pre or post mortem muscle activity in Atlantic salmon (Salmo salar). The effect on rigor mortis and the physical properties of flesh. Aquaculture 257, 504–510.

Ruff N, FitzGerald RD, Cross TF, Teurtrie G, Kerry JP (2002). Slaughter method and dietary α-tocopheryl acetate supplementation affect rigor mortis and fillet shelf-life of turbot Scophthalmus maximus L. Aquaculture Research 33, 703-714.

Salem M, Nath J, Rexroad CE, Killefer J, Yao J (2005). Identification and molecular characterization of the rainbow trout calpains (Capn1 and Capn2): Their expression in muscle wasting during starvation. Comparative Biochemistry and Physiology. B-Biochemistry and Molecular Biology 140B, 63–71.

Sambrook J, Russell DW (2001). Molecular cloning: A laboratory manual. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press.

Scherer R, Augusti PR, Steffens C, Bochi VC, Hecktheuer LH, Lazzari R, Rodunz-Neto J, Pomblum SCG, Emanuelli T (2005). Effect of slaughter method on Postmortem changes of grass carp (*Ctenopharyngodon idella*) stored in ice. Journal of Food Science 70, C348-C353.

Scherer R, Daniel AP, Augusti PR, Lazzari R, Lima RL, Fries LLM, Radunz Neto J, Emanuelli T (2004). Effect of Chlorinated ice on chemical and microbiological features of grass carp (*Ctenopharyngodon idella*) flesh. Ciėncia e Tecnologia de Alimentos 24, 680-684.

Scientific Opinion of the Panel on Animal Health and Welfare. Species-specific welfare aspects of the main systems of stunning and killing of farmed seabass and seabream. (Question N° EFSA-Q-2008-441) Adopted on 20th March 2009.

Seear PJ, Sweeney GE (2008). Stability of RNA isolated from post-mortem tissues of Atlantic salmon (*Salmo salar* L.). Fish Physiology and Biochemistry, 34, 19–24.

Seki N, Tsuchiya H (1991). Extensive changes during storage in carp myofibrillar proteins in relation to fragmentation. Bulletin of Nippon Suisan Gakkaishi 57,927-933.

Seki N, Watanabe T (1984). Connectin content and its postmortem changes in fish muscle. The Journal of Biochemistry 95, 1161–1167.

Shephard KL (1994). Functions for fish mucus. Reviews in Fish Biology and Fisheries 4, 401-29.

Sigholt Erikson U, Rustad TM, Johansen TS (1997). Handling stress and storage temperature affect meat quality of farm-raised Atlantic salmon. Journal of Food Science 62, 898-906.

Sikorski ZE, Kolokowska A, Sun Pan B (1990). Post-harvest biochemical and microbial changes. In: Seafood: Resources, Nutritional Composition and Preservation (ed. By ZE Sikorski), pp 55-75. CRC Press, Boca Raton, FL, USA.

Skjervold P (2001). Live-chilling and crowding stress before slaughter of Atlantic salmon (Salmo salar). Aquaculture 192, 265-80.

Skjervold PO, Fjæra SO, Østby PB, and Einen Olai (2001). Live-chilling and crowding stress before slaughterof Atlantic salmon (*Salmo salar*). Aquaculture 192:265-80.

Skjervold PO, Fjæra SO, Østby PB (1999). Rigor in Atlantic salmon as affected crowding stress prior to chilling before slaughter. Aquaculture 175, 93-101.

Southgate P, Wall T (2001). Welfare of farmed fish at slaughter. In Practice 23:277-84.

Souza TACB, Trindade DM, Tonoli CCC, Santos CR, Ward RJ, Arni RK, Oliveira AH, Murakami MT (2011). Molecular adaptability of nucleoside diphosphate kinase B from trypanosomatid parasites: stability, oligomerization and structural determinants of nucleotide binding. Molecular BioSystems 7, 2189-95.

Stasyk T, Schiefermeier N, Skortsov S, Zwierzina H Peränen J, Bonn GK, Huber LA (2007). Identification of endosomal pidermal growth factor receptor signalling targets by functional organelle proteomics. Molecular & Cellular Proteomics 6,908-922.

Sveinsdottir H, Gudmundsottir A (2011). Proteome analysis of abundant proteins in early Atlantic cod (*Gadus morhua*) larvae. Icelandic Agricultural Science 24, 23-31.

Takahashi K (1996). Structural weakening of skeletal muscle tissue during post mortem ageing of meat: the non-enzymatic mechanism of meat tenderization. Meat Science 43, S67–S80.

Tang W, Deng Z, Oses-Prieto JA, Suzuki N, Xhu S, Zhang X, Burlingame AL, W ZY (2008). Proteomic studies of brassinosteroid signal transduction using prefactionation and 2D DIGE. Molecular & Cellular Proteomics 7,728-738.

Taylor RG, Geesink GH, Thompson VS., Koohmaraie M, Goll DE (1995). Is Z-disk degradation responsible for post mortem tenderization? Journal of Animal Science 73, 1351–1367.

Terova G, Preziosa E, Marelli S, Gornati R, Bernardini G, Saroglia M (2011). Applying transcriptomics to better understand the molecular mechanisms underlying fish filet quality. Food Chemistry 124, 1268-76.

Terova G, Addis MF, Preziosa E, Pisanu S, Pagnozzi D, Biosa G, Gornati R, Bernardini G, Roggio T, Saroglia M (2011). Effect of post mortem storage temperature on sea bass (Dicentrarchus labrax) muscle protein degradation: analysis by 2-D DIGE and mass spectrometry. Proteomics 11, 2901-2010.

The UniProt Consortium (2008). The universal protein resource (UniProt). Nucleic Acids Research 36, D190-D195.

Thomas PM, Pankhurst NW, Bremner HA (1999). The effect of stress and exercise on post-mortem biochemistry of Atlantic salmon and rainbow trout. Journal of Fish biology 54, 1177-1196.

Timms JF, Cramer R (2008). Difference gel electrophoresis. Proteomics 8, 4886-4897.

Tingaud-Sequeira A, Cerda J (2007). Phylogenetic relationships and gene expression pattern of three different cathepsin L (Ctsl) isoforms in zebrafish: Ctsl is the putative yolk processing enzyme. Gene, 386, 98–106.

Tobiassen T, Sørensen NK (1999). Influence of killing methods on time of death of Atlantic salmon (Salmo salar) and rainbow trout (Salmo gairdneri) as measured by behavioural indices of sensibility and reflexes. In: Proceedings of the ‘‘Aquaculture Europe 1999’’, EAS Special Publication n. 27, p. 244

Toffoli D, Hrbek T, de Araujo MLG, De Almeida, MP (2008). A test of the utility of DNA Barcoding in the radiation of the freshwater stingray genus Potamotrygon (*Potamotrygonidae, Myliobatiformes*). Genetics and Molecular Biology 31, 324-336.

Tsuchiya H, Seki N (1991). Action of calpain on alpha-actinin within and isolated from carp myofibrils. Nippon Suisan Gakkaishi 57, 1133–1139.

Tsunemoto K, Osatomi K, Nozaki Y, Hara K, Ishihara T (2004). Molecular characterization of cathepsin L from hepatopancreas of carp (*Cyprinus carpio*). Comparative Biochemistry and Physiology. Part B-Biochemistry and Molecular Biology 137B, 107–114.

Unlü M, Morgan ME, Minden JS (1997). Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis 18, 2071–2077.

Van de Vis H, Kestin S, Robb D, Oehlenschlager J, Lambooij B, Münkner W, Holmer Kuhlmann H, Kloosterboer K, Tejada M, Huidobro A, Otterå H, Roth B, Sørensen NK, Akse L, Byrne H, Nesvadba P (2003). Is humane slaughter of fish possible for industry? Aquaculture Research 34, 211-20.

Vercauteren FGG, Arckens L, Quirion R (2006). Application and current challenges of proteomic approaches, focusing on two-dimensional electrophoresis. Amino Acids 31, 485-488.

Verres Bagnis V, Ladrat C, Noelle J, Fleurence J (2002). In vitro proteolysis myofibrillar and sarcoplasmic proteins of European sea bass (*Dicentrarchus labrax* L.) by endogenous m-calpain. Journal of the Science of Food and Agriculture 82, 1256-1262.

Verrez-Bagnis V, Ladrat C, Morzel M, Noël J, Fleurence J (2001). Protein changes in post mortem sea bass (*Dicentrarchus labrax*) muscle monitored by one- and two-dimensional gel electrophoresis. Electrophoresis 221539-44.

Verrez-Bagnis V, Noel J, Sautereau C, Fleurence J (1999). Desmin degradation in post mortem fish muscle. Journal of Food Science 64, 240–242.

Vilhelmsson OT, Martin SAM, Poli BM, Houlihan DF (2005). Proteomics: methodology and application in fish processing. In: Hui, Y.H. (Ed.), Food Biochemistry and Food processing, Blackwell Publishing, Ames, Iowa, pp. 401–417.

Von Eggeling F, Gawriljuk A, Fiedler W, Ernest G, Claussen U, Klose J, Römer I (2001). Fluorescent dual colour D-protein gel electrophoresis for rapid detection of differences in

protein pattern with standard image analysis software. International of Journal Molecular Medicine 8, 373-377.

Walker E, McNicol AM (1992). In situ hybridization demonstrates the stability of mRNA in post-mortem rat tissues. Journal of Pathology 168, 67–73.

Wang PA, Martinez I, Olsen RZL, (2009). Myosin heavy chain degradation during post mortem storage of Atlantic cod (Gadus morhua L.). Food Chemistry 115, 1228–1233.

Warren CM, Arteaga GM, Rajan S, Ahmed RP, Wieczorek DF, Solaro RJ (2008). Use of 2D DIGE analysis reveals altered phosphorylation in a tropomyosin mutant (Glu54Lys) linked to dilated cardiomyopathy. Proteomics 8, 100-15.

Wetzel DM, Bohn MC, Hamill RW, (1994). Post-mortem stability of mRNA for glucocorticoid and mineralocorticoid receptor in rodent brain. Brain Research 649, 117–121.

World Health Organization (2007). Global and regional food consumption patterns and trends, section 3.5, availability and consumption of fish. Website address: www.who.int/nutrition/topics/3_foodconsumption/en/index5.html.

Yamanoue M, Takahashi K (1988). Effect of paratropomyosin on the increase in sarcomere length of rigor-shortened skeletal muscles. Journal of Biochemistry 103, 843-847.

Yeh HY, Klesius PH (2009). Channel catfish, Ictalurus punctatus, cysteine proteinases: Cloning, characterization and expression of cathepsin H and L. Fish & Shellfish Immunology 26, 332–338.

Yue S, An HSUS Report: The Welfare of Farmed Fish at Slaughter. The Humane Society of the United States. www.hsus.org/farm/resources/research/welfare/fish_and_pain.html.

Zhou X, Ding Y, Wang Y (2012). Proteomic: present and future in fish, shellfish and seafood. Aquaculture 4, 11–20.