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

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ABSTRACT

Two-dimensional difference gel electrophoresis (2D DIGE) was applied to investigate the impact of slaughtering on the *postmortem* 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 *postmortem* 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 favoring AI over AA is observed.

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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 esthetic 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 [1].

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

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[2,3]. Flesh quality in fish is determined by various factors, including husbandry practices, diet, handling at slaughter, the choice of slaughtering method, and *postmortem* handling [4,5]. 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 [6]. Therefore, particular attention should be paid to testing slaughtering methods that allow some degree of *pre-rigor* processing [7].

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 [6]. Some of these killing procedures are not that feasible in commercial situations, requiring high-cost, qualified personnel [8]. 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 [9] 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 [3,10,11] 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* [3]. 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* [12–16]. On the other hand, electrical stunning of Atlantic salmon in water did not accelerate *rigor* development and no injuries were observed in the fish [17].

To date, the influence of slaughtering methods on *postmortem* 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 *postmortem* 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 *premortem* 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.

2. Materials and methods

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 1 m³ with 40 fish per tank and fed Hendrix-Skretting® Power Excel for marine fish. The tanks were connected to a seawater 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. 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 s before death, avoiding aversive behavior.

All fish were considered dead 10 min 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 *postmortem* 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.

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 [18,19]. 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.

2.3. Sample labeling 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 *postmortem* storage at 1°C (T5) were labeled with 400 pmol N-hydroxysuccinimidyl-ester of cyanine dye Cy3 and Cy5 (GE Healthcare), respectively (Table 1). A pooled sample, which served as the internal standard, was labeled with Cy2, as described previously [20]. After quenching with lysine, the labeled proteins were mixed in suitable combinations as summarized in Table 1. IPG buffer (GE Healthcare) and Destreak Rehydration Solution (GE Healthcare) were added to a final volume of 200 μl for each mix.

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 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.

Table 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 sample 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 labeling cyanine used for the sample set.

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

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 *postmortem* storage at 1°C (T5) were evaluated as described previously [20] 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 [20].

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 [21], 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, destained, 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 [20,22].

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 [20]. 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 in-house 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.

3. Results

3.1. Experimental design

In order to compare the modifications introduced in sea bass muscle proteins by different slaughtering methods, a 2D DIGE experiment (Fig. 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 (Fig. 1, rows). In this experiment, samples collected 10 min after the last opercular movement (T0, N=9) and after 5 days of storage at 1 °C (T5, N=9) were evaluated (Fig. 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 (AAT0 vs AIT0 vs SCST0); 2) the muscular proteome between fish slaughtered in three different ways and sampled after 5 days of storage at 1 °C (AAT5 vs AIT5 vs SCST5); and 3) protein degradation after 5 days of *postmortem* conservation at 1 °C (from T0 to T5) in muscles sampled from differently slaughtered fish (AAT0 vs AAT5, AIT0 vs AIT5, and SCST0 vs SCST5).

3.2. Differences in abundance of muscle proteins in sea bass slaughtered with the three procedures: *postmortem* evaluation

To investigate a possible influence of *premortem*/*perimortem* stress on the integrity of proteins in the muscle of fish slaughtered in three different ways (AA, AI, or SCS), the samples taken immediately after death (T0) were analyzed (Fig. 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 (Fig. 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 (Fig. 3, Table 2). The most significant difference in abundance was observed for spots 1, 2, and 3, all identified as nucleoside diphosphate kinase B (NDKB) (Table 3). NDKB consistently decreased in the *perimortem*/*immediate postmortem* period in fish euthanized by asphyxia in comparison 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 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.

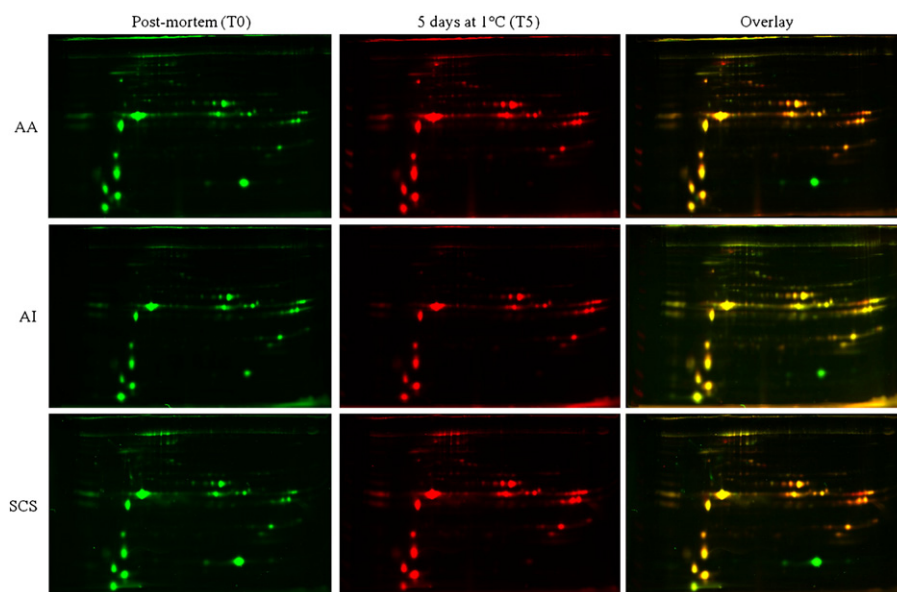


Fig. 1 – Composite image illustrating three representative 2D DIGE gels. The lines report the slaughtering technique used (AA, asphyxia in air; AI, asphyxia in ice; SCS, spinal cord severance). The columns report the total protein profiles observed *post-mortem* (left column, green), after 5 days of 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.

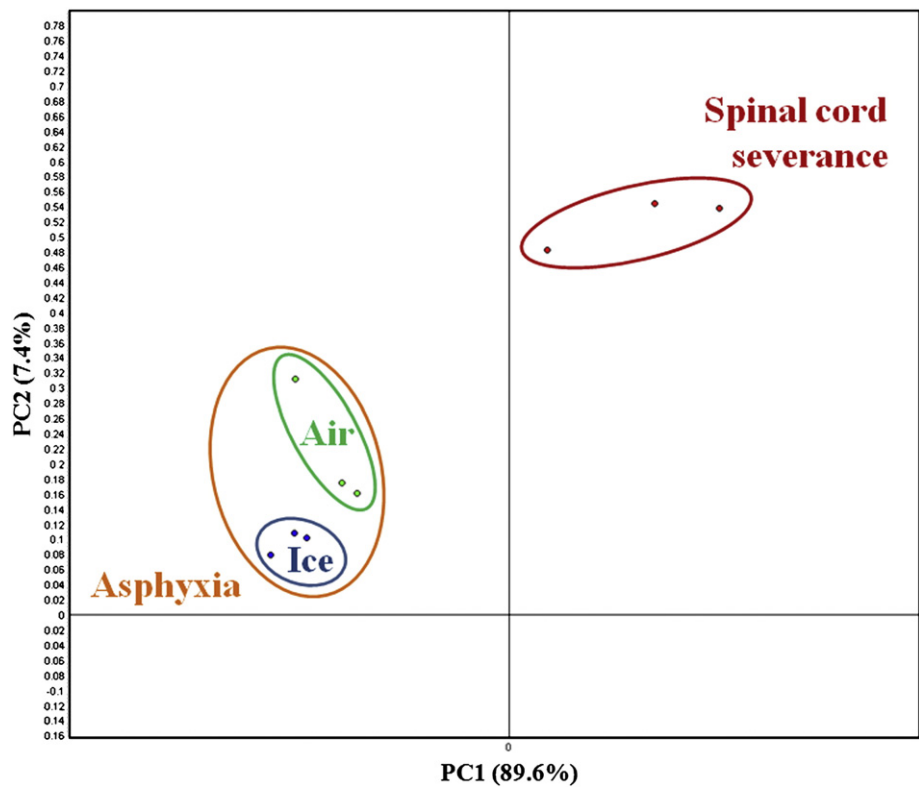


Fig. 2 – Principal components analysis graph indicating the distribution of sample classes into clusters. Each dot represents the variance of the differentially abundant spots in one of the three samples analyzed for each condition. Circles highlight the clustering area corresponding to each slaughtering mode.

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

To investigate a possible influence of the slaughtering method (premortem/perimortem stress) on the postmortem 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 (Fig. 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 3), only two spots identified as

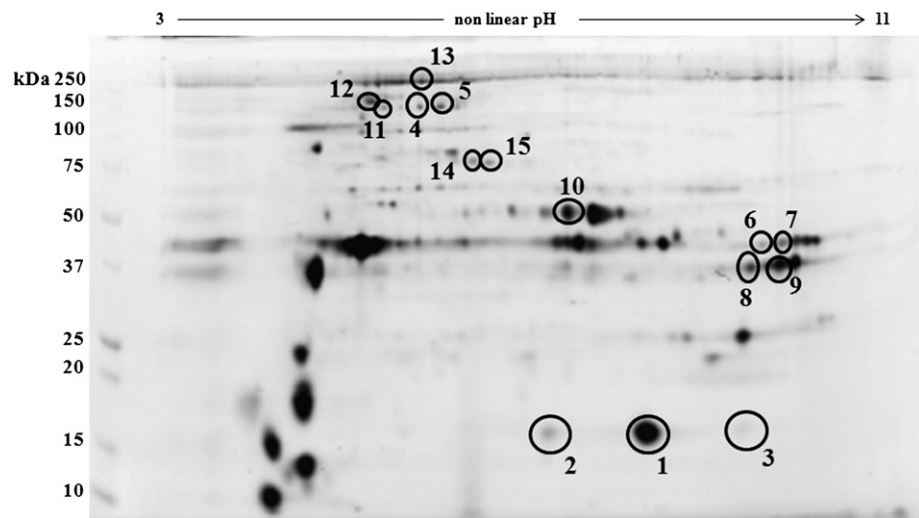


Fig. 3 – Representative map indicating the location of the differential protein spots detected in this study. Protein identification results are reported in Table 2.

Table 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 Fig. 3.

N ^a	Accession ^b	Description	Species	MW ^c	pI ^d	S ^e	%C ^f	UP ^g	Pep ^h	PSM ⁱ
1	295792242	Nucleoside diphosphate kinase B	<i>Epinephelus coioides</i>	16.97	7.29	403.46	59.69	6	6	64
2	229366950	Nucleoside diphosphate kinase B	<i>Anoplopoma fimbria</i>	16.94	6.31	168.97	22.15	1	3	23
3	226441991	Nucleoside diphosphate kinase B	<i>Gillichthys seta</i>	16.98	7.82	315.16	42.45	1	5	45
4	317419297	Myosin-binding protein C, fast-type	<i>Dicentrarchus labrax</i>	124.98	5.63	962.69	20.59	17	17	34
	317420045	Myomesin-1	<i>Dicentrarchus labrax</i>	179.97	6.65	500.44	7.05	8	8	17
5	317419297	Myosin-binding protein C, fast-type	<i>Dicentrarchus labrax</i>	124.98	5.63	234.65	16.78	12	12	25
	317420045	Myomesin-1	<i>Dicentrarchus labrax</i>	222.83	5.79	122.32	2.73	1	5	6
6	295792244	Fructose-bisphosphate aldolase A	<i>Epinephelus coioides</i>	39.63	8.25	186.59	23.69	1	5	9
7	317419331	Fructose-bisphosphate aldolase	<i>Dicentrarchus labrax</i>	39.61	8.32	556.33	23.42	7	7	29
	203480	Creatine kinase	<i>Rattus norvegicus</i>	33.25	7.94	116.11	7.12	2	2	2
8	296785440	Glyceraldehyde 3-phosphate dehydrogenase	<i>Siniperca chuatsi</i>	35.93	8.35	154.85	14.11	2	4	13
9	57791244	Glyceraldehyde-3-phosphate dehydrogenase	<i>Dicentrarchus labrax</i>	35.87	8.44	652.74	34.53	5	8	22
10	37590349	Enolase 1, (alpha)	<i>Danio rerio</i>	47.04	6.58	875.74	28.70	1	11	29
11	226434435	Myosin heavy chain	<i>Takifugu rubripes</i>	221.64	5.65	390.49	8.41	2	14	27
12	90025055	Myosin heavy chain	<i>Dicentrarchus labrax</i>	201.73	5.77	439.65	12.20	2	17	27
13	171702766	Myosin heavy chain	<i>Hippoglossus hippoglossus</i>	26.99	4.90	94.24	40.43	1	5	6
14	21623523	Myosin heavy chain	<i>Oncorhynchus keta</i>	222.01	5.60	154.93	6.04	1	7	10
15	90025055	Myosin heavy chain	<i>Dicentrarchus labrax</i>	201.73	5.77	990.58	11.46	1	17	19

^a Number of spot in Fig. 3.^b NCBI accession number.^c Theoretical molecular weight of full-length protein in kDa.^d Theoretical isoelectric point.^e Mascot score.^f Percent coverage: the minimum coverage of the matched peptide in relation to the full-length sequence.^g Number of unique peptides^h Number of identified peptides.ⁱ Peptide spectra matched.

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 3).

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 postmortem 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 2). Results are illustrated in Table 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 4). The ANOVA interaction 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 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

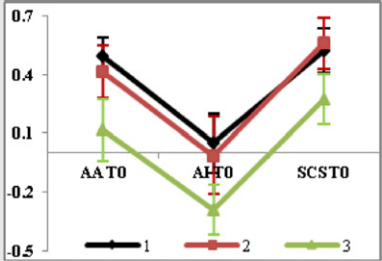
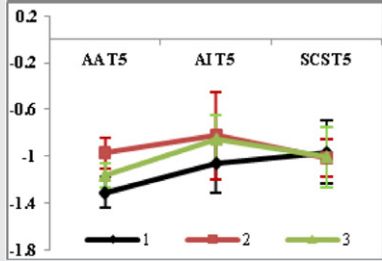
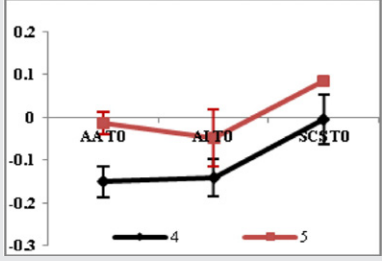
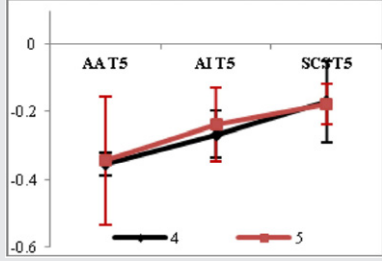
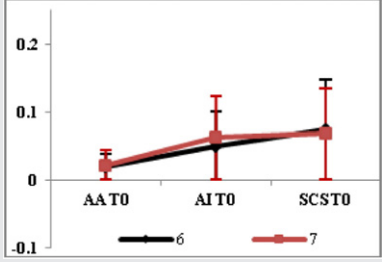
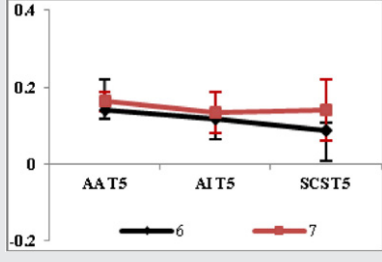
also with this type of analysis, the highest impact seems to be exerted by AA (−49.35), followed by SCS (−37.35), and then by AI (−5.98). In contrast, from the T0 comparisons, AI is the condition most aversive to this protein, followed by AA and SCS. The results obtained for other proteins in the T5/T0 comparison also contrast with the results obtained in the T0 comparison. Clearly, the extensive degradation that occurred in the *perimortem*/immediate *postmortem* 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 *postmortem* or in the first hours of storage can have a significant impact on differential protein abundance and introduce biases on T5/T0 comparisons.

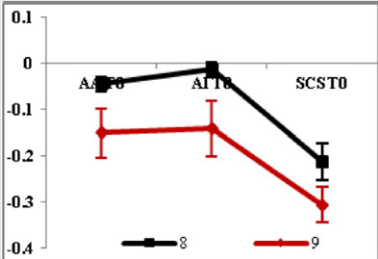
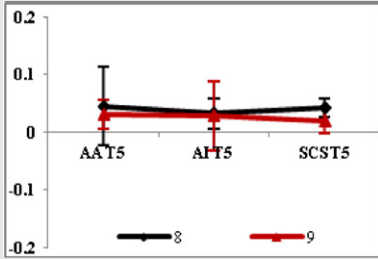
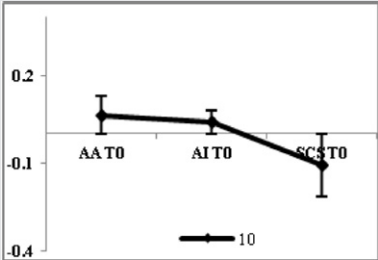
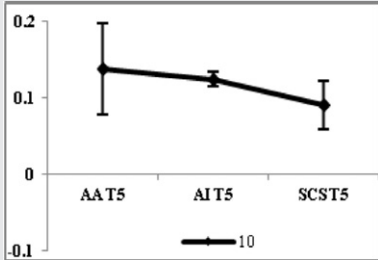
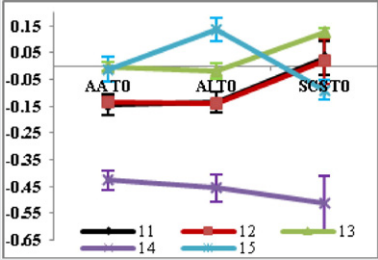
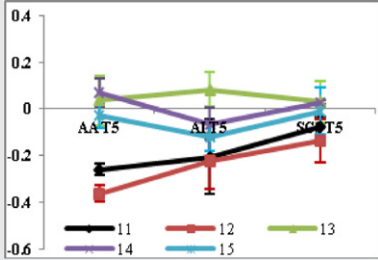
4. Discussion

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 *postmortem* 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

Table 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 ^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	 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
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		
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 5.T-test* = i/a 0.437; i/s 0.016; a/s 0.075	 4.T-test* = i/a 0.124; i/s 0.297; a/s 0.062 5.T-test* = i/a 0.445; i/s 0.540; a/s 0.335
5	Myosin-binding protein C	124.99 5.63	135.62 6.11		
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 7.T-test* = i/a 0.841; i/s 0.016; a/s 0.007	 6.T-test* = i/a 0.662; i/s 0.071; a/s 0.331 7.T-test* = i/a 0.435; i/s 0.917; a/s 0.672
7	Fructose-bisphosphate aldolase	39.62 8.33	41.88 7.76		

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 9.T-test* = i/a 0.847; i/s 0.016; a/s 0.014	 8.T-test* = i/a 0.779; i/s 0.629; a/s 0.941 9.T-test* = i/a 0.953; i/s 0.798; a/s 0.542
9	Glyceraldehyde 3-phosphate dehydrogenase	35.87 8.44	34.21 7.76		
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 12.T-test* = i/a 0.599; i/s 0.032; a/s 0.038 13.T-test* = i/a 0.476; i/s 0.002; a/s 0.0007 14.T-test* = i/a 0.432; i/s 0.232; a/s 0.426 15.T-test* = i/a 0.015; i/s 0.083; a/s 0.002	 11.T-test* = i/a 0.588; i/s 0.253; a/s 0.003 12.T-test* = i/a 0.119; i/s 0.392; a/s 0.017 13.T-test* = i/a 0.556; i/s 0.476; a/s 0.962 14.T-test* = i/a 0.065; i/s 0.078; a/s 0.325 15.T-test* = i/a 0.128; i/s 0.214; a/s 0.779
12	Myosin heavy chain	201.73 5.77	144.59 5.42		
13	Myosin heavy chain	221.47 5.71	149.31 5.59		
14	Myosin heavy chain	222.02 5.60	75.34 6.21		
15	Myosin heavy chain	201.73 5.77	75.35 6.05		

*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.

^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.

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 [23,24]. 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 [20], 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 (Fig. 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 *postmortem* 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 s, 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 *postmortem* 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 3). NDKB is a hexameric complex catalyzing transfer of phosphates from GTP to ADP to form ATP [25]. In some eukaryotes, NDKB is stabilized by nucleotide binding [26]. 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 [3,10,11]. 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 3). In general, SCS 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, AA and AI 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).

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 [20,27,28]. 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 4). As a matter of fact, the occurrence of changes in protein abundance in the *perimortem*/immediate *postmortem* 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*/*postmortem* 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

Table 4 – Comparative degradation trends observed among slaughtering procedures along cold storage (T0 vs T5).

N° spot	Protein	AI	AA	SCS	One-way RMANOVA	Time	Slaughtering	Two-way RMANOVA-interact
		Paired av ratio T5/T0	Paired av ratio T5/T0	Paired av ratio T5/T0		Two-way RMANOVA	Two-way RMANOVA	
Decreased								
1	Nucleoside diphosphate kinase B	−5.98	−49.35	−37.35	1.80E−05	4.90E−07	0.24	0.18
2	Nucleoside diphosphate kinase B	−3.69	−18.82	−15.89	0.00012	5.30E−06	0.51	0.039
3	Nucleoside diphosphate kinase B	−2.71	−11.1	−20.87	0.0008	9.13E−06	0.72	0.059
4	Myosin-binding protein C	−1.46	−2.01	−1.81	8.60E−05	1.30E−05	0.0024	0.23
Increased								
7	Fructose-bisphosphate aldolase A	2.38	2.74	2.09	4.50E−05	2.50E−06	0.02	0.16
15	Myosin heavy chain	2.19	3.13	3.57	3.10E−08	6.40E−10	0.063	0.12
12	Myosin heavy chain	2.18	3.38	3.39	0.00013	9.00E−06	0.023	0.15
13	Myosin heavy chain	2.13	2.28	4.12	0.00079	0.0018	0.00085	0.18
10	Alpha-enolase	1.99	1.83	2.09	4.40E−07	1.50E−08	0.0053	0.091
6	Fructose-bisphosphate aldolase A	1.59	3.44	3.42	2.00E−07	7.90E−09	0.054	0.074
8	Glyceraldehyde-3-phosphate dehydrogenase	1.42	1.52	2.13	1.10E−05	4.90E−06	0.0014	0.00097
9	Glyceraldehyde-3-phosphate dehydrogenase	1.14	1.34	1.99	2.60E−06	1.90E−07	0.0037	0.0083
Paired av ratio: paired average ratio. One-way RMANOVA: one-way repeated measure ANOVA. Two-way RMANOVA: two-way repeated measure ANOVA. Two-way RMANOVA-interact: two-way repeated measure ANOVA-interact. Bold: P≤0.001.								

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 *premortem* 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 [29–31]. 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 [32,33]; and secondly, gentle and rapid killing methods can preserve integrity of muscle proteins, promoting quality and extending shelf-life.

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.

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REFERENCES

- [1] Huss HH. 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; 1988.
- [2] Ribas L, Flos R, Reig L, MacKenzie S, Barton BA, Tort L. Comparison of methods for anaesthetizing Senegal sole (*Solea senegalensis*) before slaughter: Stress responses and final product quality. *Aquaculture* 2007;269:250–8.
- [3] Robb DH, Kestin S, Warriss P. Muscle activity at slaughter: I. Changes in flesh colour and gaping in rainbow trout. *Aquaculture* 2000;182:261–9.
- [4] Ruff N, FitzGerald RD, Cross TF, Teurtrie G, Kerry JP. Slaughtering method and dietary alpha-tocopheryl acetate supplementation affect rigor mortis and fillet shelf-life of turbot *Scophthalmus maximus* L. *Aquaculture Res* 2002;33:703–14.

- [5] Terova G, Preziosa E, Marelli S, Gornati R, Bernardini G, Saroglia M. Applying transcriptomics to better understand the molecular mechanisms underlying fish filet quality. *Food Chem* 2011;124:1268–76.
- [6] Poli BM, Parisi G, Scappini F, Zampacavallo G. Fish welfare and quality as affected by pre-slaughter and slaughter management. *Aquacult Int* 2005;13:29–49.
- [7] Skjervold P. Live-chilling and crowding stress before slaughter of Atlantic salmon (*Salmo salar*). *Aquaculture* 2001;192:265–80.
- [8] Bagni M, Civitareale C, Priori A, Ballerini A. Pre-slaughter crowding stress and killing procedures affecting quality and welfare in sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*). *Aquaculture* 2007;263:52–60.
- [9] Ambroggi F, Sebastio P, Pirazzoli P, Baldrati G. Influenza del sistema di sacrificio su trote iridee di allevamento II: rigor mortis e consistenza del tessuto muscolare *Ind Conserve* 1996;71:157–61.
- [10] Erikson U, Sigholt T, Rustad T, Einarsdottir IE, Jørgensen L. Contribution of bleeding to total handling stress during slaughter of Atlantic salmon. *Aquacult Int* 1999;7:101–15.
- [11] van de Vis H, Kestin S, Robb D, Oehlenschläger J, Lambooi B, Munkner W, et al. Is humane slaughter of fish possible for industry? *Aquaculture Res* 2003;34:211–20.
- [12] Digre H, Erikson U, Misimi E, Lambooi B, Van de Vis H. Electrical stunning of farmed Atlantic cod *Gadus morhua* L.: a comparison of an industrial and experimental method. *Aquaculture Res* 2010;41:1190–202.
- [13] Erikson U, Lambooi B, Digre H, Reimert HGM, Bondø M, Van der Vis H. Conditions for instant electrical stunning of farmed Atlantic cod after de-watering, maintenance of unconsciousness, effects of stress, and fillet quality — a comparison with AQUIS-STM. *Aquaculture* 2012;324–325: 135–44.
- [14] Morzel M, Sohler D, van de Vis H. Evaluation of slaughtering methods for turbot with respect to animal welfare and flesh quality. *J Sci Food Agric* 2002;82:19–28.
- [15] Roth B, Imsland A, Gunnarsson S, Foss A, Schelvis-Smit R. Slaughter quality and rigor contraction in farmed turbot (*Scophthalmus maximus*); a comparison between different stunning methods. *Aquaculture* 2007;272:754–61.
- [16] Scherer R, Augusti PR, Steffens C, Bochi VC, Hecktheuer LH, Lazzari R, et al. Effect of slaughter method on postmortem changes of grass carp (*Ctenopharyngodon idella*) stored in ice. *J Food Sci* 2005;70:C348–53.
- [17] Roth B, Moeller D, Veland JO, Imsland A, Slinde E. The effect of stunning methods on rigor mortis and texture properties of Atlantic salmon (*Salmo salar*). *J Food Sci* 2002;67:1462–6.
- [18] Addis MF, Cappuccinelli R, Tedde V, Pagnozzi D, Porcu MC, Bonaglini E, et al. Proteomic analysis of muscle tissue from gilthead sea bream (*Sparus aurata*, L.) farmed in offshore floating cages. *Aquaculture* 2010;309:245–52.
- [19] Addis MF, Tanca A, Pagnozzi D, Crobu S, Fanciulli G, Cossu-Rocca P, et al. Generation of high-quality protein extracts from formalin-fixed, paraffin-embedded tissues. *Proteomics* 2009;9: 3815–23.
- [20] Terova G, Addis MF, Preziosa E, Pisanu S, Pagnozzi D, Biosi G, et al. Effects of postmortem storage temperature on sea bass (*Dicentrarchus labrax*) muscle protein degradation: analysis by 2-D DIGE and MS. *Proteomics* 2011;11:2901–10.
- [21] Chevallet M, Luche S, Rabilloud T. Silver staining of proteins in polyacrylamide gels. *Nat Protoc* 2006;1:1852–8.
- [22] Addis MF, Tanca A, Pagnozzi D, Rocca S, Uzzau S. 2-D PAGE and MS analysis of proteins from formalin-fixed, paraffin-embedded tissues. *Proteomics* 2009;9:4329–39.
- [23] Lilley KS, Friedman DB. All about DIGE: quantification technology for differential-display 2D-gel proteomics. *Expert Rev Proteomics* 2004;1:401–9.
- [24] Unlü M, Morgan ME, Minden JS. Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. *Electrophoresis* 1997;18:2071–7.
- [25] Berg JM, Tymoczko JL, Stryer L. *Biochemistry*. 5th ed. New York: WH Freeman; 2002.
- [26] Souza TACB, Trindade DM, Tonoli CCC, Santos CR, Ward RJ, Arni RK, et al. Molecular adaptability of nucleoside diphosphate kinase B from trypanosomatid parasites: stability, oligomerization and structural determinants of nucleotide binding. *Mol Biosyst* 2011;7:2189–95.
- [27] Verrez-Bagnis V, Ladrat C, Morzel M, Noël J, Fleurence J. Protein changes in post mortem sea bass (*Dicentrarchus labrax*) muscle monitored by one- and two-dimensional gel electrophoresis. *Electrophoresis* 2001;22:1539–44.
- [28] Jasra SK, Jasra PK, Talesara C. Myofibrillar protein degradation of carp (*Labeo rohita* (Hamilton)) muscle after post-mortem unfrozen and frozen storage. *J Sci Food Agric* 2001;81:519–24.
- [29] Robb DH, Roth B. Brain activity of Atlantic salmon (*Salmo salar*) following electrical stunning using various field strengths and pulse durations. *Aquaculture* 2003;216:363–9.
- [30] Lambooi E, Gerritzen MA, Reimert H, Burggraaf D, van de Vis JW. A humane protocol for electro-stunning and killing of Nile tilapia in fresh water. *Aquaculture* 2008;275:88–95.
- [31] Lambooi E, van de Vis JW, Kuhlmann H, Munkner W, Oehlenschläger J, Kloosterboer RJ, et al. A feasible method for humane slaughter of eel (*Anguilla anguilla* L.): electrical stunning in fresh water prior to gutting. *Aquaculture Res* 2002;33:643–52.
- [32] Council Directive 93/119/EC. On the protection of animals at the time of slaughter or killing. Official Journal of the European Communities No L340/21; 1993.
- [33] Species-specific welfare aspects of the main systems of stunning and killing of farmed seabass and seabream. Scientific Opinion of the Panel on Animal Health and Welfare (Question N° EFSA-Q-2008-441) Adopted on 20th March 2009; 2009.