# Effects of High-Pressure Treatment on the Muscle Proteome of Hake by Bottom-Up Proteomics

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1 **ABSTRACT**

2

3 A Bottom-Up proteomics approach was applied for the study of the effects of
4 high-pressure (HP) treatment on the muscle proteome of fish. The performance of the
5 approach was established for a previous HP treatment (150-450 MPa for 2 min) on
6 frozen (up to 5 months at –10 ºC) European hake (*Merluccius merluccius*). Concerning
7 possible protein biomarkers of quality changes, a significant degradation after applying
8 a pressure ≥ 430 MPa could be observed for phosphoglycerate mutase-1, enolase,
9 creatine kinase, fructose bisphosphate aldolase, triosephosphate isomerase and
10 nucleoside diphosphate kinase; contrary, electrophoretic bands assigned to tropomyosin,
11 glyceraldehyde-3-phosphate dehydrogenase and beta parvalbumin increased their
12 intensity after applying a pressure ≥ 430 MPa. This repository of potential protein
13 biomarkers may be very useful for further HP investigations related to fish quality.

14

15 **KEYWORDS:** Bottom-Up proteomics; frozen hake; high-pressure processing; quality
16 protein biomarkers; fish allergen proteins

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22 **INTRODUCTION**

23

24 High-pressure (HP) processing is a non-thermal and additive-free food
25 preservation technology applied in food industry to inactivate microbial populations and
26 inhibit deteriorative endogenous enzymes, while retaining better sensory quality and
27 nutritional properties.[1, 2] The majority of the applications use moderate to high pressures
28 of 100 to 500 MPa for a holding time of 1-5 min. The effects of the HP treatment on
29 microorganisms have been studied for years, showing that yeasts and molds are more
30 pressure-sensitive than vegetative bacterial cells and that bacterial spores are pressure-
31 resistant.[3] Furthermore, activity of deteriorative endogenous enzymes that degrade food,
32 as lipases, phospholipases, trimethylamine oxide demethylase, peroxidases, and
33 lipoxygenases, has shown to be reduced as a result of HP treatment.[4] HP can reduce the
34 use of food additives, improve digestibility and potentially reduce allergenicity.[5, 6]
35 Consequently, HP processed foodstuffs present an improvement in food quality, safety
36 and an extension of shelf-life.

37 HP treatment can give rise to structural changes on proteins, which alter food
38 proteins conformation and activity. The effects on proteins structure can be summarized
39 as promoting oligomers dissociation, monomers denaturation, changes on active sites
40 and the occurrence of non-enzymatic post-translational modifications (nePTMs).[7] While
41 covalent bonds are not broken by HP treatment, HP can trigger the formation of new
42 disulfide bonds.[8] Additionally, weak hydrogen, ionic and hydrophobic bounds tend to
43 cause irreversible aggregation and gelation by modifying the enzymes active site and
44 masking the access of epitopes.[9] The intensity and reversibility of these effects are
45 strongly dependent of the strength of the HP conditions.

46 Proteomics as the discipline for the large-scale analysis of proteins of a
47 particular biological system at a particular time, has greatly contributed for the
48 assessment of quality, safety and bioactivity of seafood products.[10-12] Using a Bottom-
49 Up proteomics workflow, proteins are commonly separated by gel electrophoresis,
50 converted into peptides using enzymes (such as trypsin), and the resulting peptides are
51 analyzed by tandem mass spectrometry (MS/MS). Using different search engines, like
52 Sequest and Mascot, fragmentation spectra are assigned to putative peptide sequences
53 and the assignments are then validated with programs like PeptideProphet or
54 Percolator.[13] Different food proteomics topics have been successfully implemented,
55 such as seafood authentication, allergen detection and microorganism contamination.[14-
56 16] Despite this outstanding potential of proteomics tools, quality changes evaluation
57 during storage or processing, as for example after HP treatment, is still scarce to date.
58 Concerning fish species, Ortea et al. (2010) studied the HP effect on the sarcoplasmic
59 fraction of chilled coho salmon.[17] These authors verified that a protein identified by
60 MS/MS as phosphoglycerate mutase was found to decrease its content when fish was
61 treated with a pressure ≥ 170 MPa. Besides, protein separation by one-dimensional (1-
62 D) SDS-PAGE and identification by MS/MS showed a higher stability of myofibrillar
63 proteins (150-450 MPa) compared to sarcoplasmic ones when a fatty fish species was
64 studied (Atlantic mackerel, *Scomber scombrus*).[18] More recently, HP effect (150-450
65 MPa) on sarcoplasmic proteins of a frozen half-fat fish species (horse mackerel,
66 *Trachurus trachurus*) was evaluated, and a 450 MPa treatment caused a significant
67 degradation of phosphoglycerate mutase 1 and 2, glycogen phosphorylase, pyruvate
68 kinase, beta-enolase and triosephosphate isomerase.[19] Furthermore, protein bands
69 assigned to tropomyosin, troponin T and parvalbumin beta increased their presence at

70 450 MPa treatment.[19] However, research focused on the HP effects on a low-fat fish
71 species has not been performed to date.

72 European hake (*Merluccius merluccius*) is a lean white fish species belonging to
73 the Merlucciidae family. It has shown significant quality losses during frozen storage
74 due to trimethylamine oxide breakdown into dimethylamine and formaldehyde, two
75 highly deteriorative compounds. Consequently, great attention is being devoted by
76 manufacturers to find preservative technological treatments to inhibit its quality loss.
77 Previous investigations evaluated HP pre-treatments (range 150-450 MPa) on physical
78 properties (color and texture) of hake, during frozen storage at –10 ºC for 5 months,
79 showing functional properties improvement of frozen hake pre-treated at 300 MPa.[20]

80 In the present work, the effect of a previous HP treatment (150-450 MPa for 2
81 min) on frozen (up to 5 months at –10 ºC) European hake was studied for the first time
82 by Bottom-Up proteomics. For this purpose, image analysis of SDS-PAGE profiles,
83 protein identification by MS/MS analysis and database searching were performed for
84 both sarcoplasmic and myofibrillar proteins. From frozen hake previously submitted to
85 HP processing, several protein biomarkers of HP treatments were selected.

86

87

88 **MATERIALS AND METHODS**

89

90 **Chemicals and reagents**

91 Bicinchoninic acid (BCA), dithiothreitol (DTT), sodium dodecyl sulphate
92 (SDS), Tris-HCl, and the protease inhibitor phenylmethylsulphonyl fluoride (PMSF)
93 were purchased from Sigma (St. Louis, MO, USA). Ammonium persulphate (APS),
94 bromophenol blue and N,N,N',N'-tetramethylethylenediamine (TEMED) were
95 purchased from GE Healthcare Science (Uppsala, Sweden). Acrylamide and bis N,N'-
96 methylene-bis-acrylamide were obtained from Bio-rad (Hercules, CA, USA). Glycerol
97 was obtained from Merck (Darmstadt, Germany). Sequencing grade porcine trypsin was
98 purchased from Promega (Madison, WI, USA). All other chemicals were
99 reagent/analytical grade and water was purified using a Milli-Q system (Millipore,
100 Billerica, MA, USA).

101

102 **Raw fish, processing, storage and sampling**

103 European hake (102 individuals) were collected in the Vigo harbor (North-
104 Western Spain) and transported under ice to the laboratory. Three hake individuals were
105 placed in flexible 20/70 polyamide/polyethylene (PA/PE) bags and vacuum sealed at
106 150 mbar (Plásticos Macar – Indústria de Plásticos Lda. Palmeira, Portugal). The length
107 and weight of the specimens ranged 27.5-29.5 cm and 180-205 g, respectively.

108 HP treatments at 150, 170, 300, 430 and 450 MPa were performed in University
109 of Aveiro in a 55-L high pressure unit (WAVE 6000/55 HT; NC Hyperbaric, Burgos,
110 Spain) during 2 min pressure holding time (Figure 1).[20] Water applied as the
111 pressurizing medium at 3 MPa $s^{-1}$ yielded 54, 63, 100, 115 and 121 s as the come-up
112 times for the 150, 170, 300, 430 and 450 MPa treatments, respectively, while

113 decompression time was less than 3 s. During HP treatment, the temperature was
114 controlled at 20 ºC using a thermostatic bath connected to the HP equipment.
115 After HP treatments, hake individuals were frozen at –20 ºC for 48 hours and
116 then stored at –10 ºC being samples analyzed after 0, 10, 75, 140 and 150 days
117 according to the following design of pressure-level/frozen-storage-time treatments
118 (Figure 1):[20] T1 (0.1 MPa/0 days; fish without HP processing, submitted to the freezing
119 step but without frozen storage; i.e., freezing-step control), T2 (300 MPa/0 days; fish
120 submitted to a 300-MPa processing and freezing step, but without the frozen storage),
121 T3 (0.1 MPa/10 days; fish without HP processing and submitted to freezing step and a
122 10-day frozen storage), T4 (170 MPa/10 days; fish submitted to a 170-MPa processing,
123 freezing step and a 10-day frozen storage), T5 (430 MPa/10 days; fish submitted to a
124 430-MPa processing, freezing step and a 10-day frozen storage), T6 (0.1 MPa/75 days;
125 fish without HP processing and submitted to freezing step and a 75-day frozen storage),
126 T7 (150 MPa/75 days; fish submitted to a 150-MPa processing, freezing step and a 75-
127 day frozen storage), T8 (300 MPa/75 days; fish submitted to a 300-MPa processing,
128 freezing step and a 75-day frozen storage), T9 (450 MPa/75 days; fish submitted to a
129 450-MPa processing, freezing step and a 75-day frozen storage), T10 (0.1 MPa/140
130 days; fish without HP processing and submitted to freezing step and a 140-day frozen
131 storage), T11 (170 MPa/140 days; fish submitted to a 170-MPa processing, freezing
132 step and a 140-day frozen storage), T12 (430 MPa/140 days; fish submitted to a 430-
133 MPa processing, freezing step and a 140-day frozen storage), T13 (0.1 MPa/150 days;
134 fish without HP processing and submitted to freezing step and a 150-day frozen storage)
135 and T14 (300 MPa/150 days; fish submitted to a 300-MPa processing, freezing step and
136 a 150-day frozen storage).[20]

137 Three replicates (n=3) for each treatment (T0 to T14) were analyzed
138 independently. Each analysis was based on the corresponding protein extract from the
139 fish white muscle pooled from three individual fish. A frozen storage temperature (–10
140 ºC) higher than commercial practice (–18 ºC) was chosen as an accelerated test
141 condition to better estimate the extent of the pressure level and frozen storage effects on
142 frozen hake proteins.
143
144 **Extraction of sarcoplasmic proteins**
145 Sarcoplasmic proteins extraction was prepared as described previously by
146 Carrera et al. (2007).[21] Briefly, 0.5 g of hake white muscle were homogenized in 4 mL
147 of 10 mM Tris-HCl buffer, pH 7.2, supplemented with 5 mM protease inhibitor PMSF,
148 during 2 min using an Ultra-Turrax homogenizer (IKA-Werke, Staufen, Germany).
149 Then, fish muscle extracts were centrifuged at 40,000 ×g for 20 min at 4 ºC (J221-M
150 centrifuge; Beckman, Palo Alto, CA, USA). The supernatants containing the
151 sarcoplasmic proteins were recovered and stored at –80 ºC until used. Protein pellets
152 were then used for the myofibrillar protein extraction.
153 Protein concentration in the sarcoplasmic protein extracts was determined by the
154 bicinchoninic acid (BCA) method (Sigma Chemical Co., USA).
155
156 **Extraction of myofibrillar proteins**
157 Protein pellets were recovered by homogenization with 2 mL of 60 mM Tris-
158 HCl, pH 7.5, supplemented with 2.5% denaturing agent SDS and 5 mM DTT using an
159 Ultra-Turrax homogenizer for 30 s. Then, samples were centrifuged at 40,000 ×g for 5
160 min at 4 ºC (Avanti centrifuge J-25I, Beckman Coulter, Palo Alto, CA, USA).
161 Supernatants were recovered and stored at –80 ºC. The protein pellets were subjected to

162 a second protein extraction, with homogenization and centrifugation carried out alike.
163 Both supernatants were mixed and stored at –80 ºC until used.

164 Protein concentration in the myofibrillar protein extracts was determined after
165 sample precipitation by the methanol/chloroform protocol and then quantified with
166 BCA.

167

168 **SDS-polyacrylamide gel electrophoresis**

169 Sarcoplasmic or myofibrillar proteins were separated on 12% (v/v)
170 polyacrylamide gels (acrylamide/N,N'-ethylene-bis-acrylamide, 200:1) with a stacking
171 gel of 4% polyacrylamide. A total of 25 µg of proteins in Laemmli buffer were boiled
172 for 5 min at 100 ºC and separated per well in a Mini-PROTEAN 3 cell (Bio-Rad,
173 Hercules, CA, USA). The running buffer consisted of an aqueous solution, composed
174 by 1.44% (w/v) glycine, 0.67% Tris-base, and 0.1% SDS. Running conditions were 80
175 V for the first 20 min and then 120 V until the end of the electrophoresis.

176 Gels were stained overnight with the Coomassie dye PhastGel Blue R-350 (GE
177 Healthcare, Uppsala, Sweden). Scanned Coomassie-stained gels were analysed by
178 means of the 1-D gel electrophoresis analysis software LabImage 1D (Kapelan Bio-
179 Imaging Solutions, Halle, Germany) using the optical intensity (band volume) to
180 quantify protein level and the rubber band method as the background correction method.

181

182 ***In-gel* protein digestion with trypsin**

183 After selection of the protein bands of interest, these were excised from the 1-D
184 gel electrophoresis, taking care to maximize the protein-to-gel ratio and were *in-gel*
185 reduced, alkylated and digested with trypsin as previously described Jensen et al.
186 (1999).[22] Briefly, protein bands were washed with ultrapure LC-MS water (Water

187 Optima, Fisher Chemical, CA, USA) and dehydrated with acetonitrile (Fisher Chemical,
188 CA, USA). Afterwards, the pieces were dried in a vacuum centrifuge and rehydrated for
189 30 min on ice with a solution of sequencing-grade porcine trypsin (Promega, Madison,
190 WI, USA) in 50 mM ammonium bicarbonate buffer, pH 8.0 and then digested overnight
191 at 37 ºC.
192

193 **LC-MS/MS analysis in a linear-trap-quadrupole (LTQ)-Orbitrap-Elite mass**
194 **spectrometer**

195 Peptides were acidified with formic acid, cleaned on a $C_{18}$ MicroSpin$^{TM}$ column
196 (The Nest Group, South-borough, MA) and analyzed by liquid chromatography-tandem
197 mass spectrometry (LC-MS/MS) using a Proxeon EASY-nLC II liquid chromatography
198 system (Thermo Fisher Scientific, San Jose, CA, US) coupled to a LTQ-Orbitrap Elite
199 mass spectrometer (Thermo Fisher Scientific).[23] Peptide separation (1 µg) was done on
200 a RP column (EASY-Spray column, 50 cm x 75 µm ID, PepMap C18, 2 µm particles,
201 100 Å pore size, Thermo Fisher Scientific) with a 10-mm pre-column (Accucore XL
202 C18, Thermo Fisher Scientific) using 0.1% formic acid (mobile phase A) and 98%
203 acetonitrile (98% ACN) with 0.1% formic acid (mobile phase B). A 120 min of linear
204 gradient from 5 to 35% B, at a flow of 300 nL $min^{-1}$ was used. A spray voltage of 1.95
205 kV and a capillary temperature of 230 ºC were used for ionization. The peptides were
206 analyzed in positive mode (1 µscan; 400-1600 amu), followed by 10 data-dependent
207 collision-induced dissociation (CID) MS/MS scans (1 µscans), using a normalized
208 collision energy of 35% and an isolation width of 3 amu. Dynamic exclusion for 30 s
209 after the second fragmentation event was applied and unassigned charged ions were
210 excluded from the analysis.
211

212 **Processing of the mass spectrometry data**

213 All the MS/MS spectra were analyzed using SEQUEST-HT (Proteome
214 Discoverer 2.1 package, Thermo Scientific) against the Teleostei UniProt/TrEMBL
215 database (release 2017_03; 1,363,430 entries). The following restrictions were used:
216 tryptic cleavage with up to 2 missed cleavage sites and tolerances of 0.8 Da for parent
217 ions and 0.6 Da for MS/MS fragment ions. Carbamidomethylation of Cys (C*) was
218 considered as a fixed modification. The permissible variable modifications were:
219 methionine oxidation (Mox) and acetylation of the N-terminus of the protein (N-Acyl).
220 The results were subjected to statistical analysis with the Percolator algorithm to keep a
221 false discovery rate (FDR) below 1%. In addition, some contaminated peptides from
222 other bands were eliminated.

223

224 **Statistical analysis**

225 The data of 1-D gel electrophoresis analysis were subjected to statistical analysis
226 by principal component analysis (PCA) using the statistical package R version 3.4.1
227 available in Internet (http://www.r-project.org). PCA results were grouped against the
228 non-HP-treated sample according to the PC1 and PC2 results. Heat map analysis and
229 hierarchical clustering were conducted using the function *heatmap.2* on R, using the
230 Euclidean distance and the complete linkage method.

231

232 **RESULTS AND DISCUSSION**

233

234 **Effects of HP treatment on the sarcoplasmic protein fraction by Bottom-Up**
235 **proteomics**

236 Information related to this protein fraction is shown in Figures 2 (SDS-PAGE
237 profiles) and 3 (effect of HP treatment for each of the individual sarcoplasmic proteins),
238 while Table 1 summarizes the list of sarcoplasmic protein bands (S1-S19) analyzed by
239 LC-MS/MS and identified by SEQUEST-HT. Complete lists of identified peptides and
240 the corresponding proteins are presented in tables included in the Supplementary Data
241 1.

242 Skeletal myosin heavy chain (118.71 kDa; protein band S1) showed an
243 important reduction when hake specimens were submitted to relatively high HP levels
244 (300 to 450 MPa) and an increase after 140 and 150 days of storage time (Figure 3a).
245 This protein is also a myofibrillar protein with a reduced stability at low ionic strength.
246 It is involved in muscle contraction and has previously shown to become insoluble with
247 the application of a pressure level $\geq$ 300 MPa.[24]

248 Glycogen phosphorylase (95.41 kDa; protein band S2) demonstrated a
249 remarkable reduction at 450 MPa and an increase after 140 and 150 days of storage time
250 (Figure 3b). This enzyme participates in the carbohydrate metabolism and its stability
251 was found to be correlated with firmness evaluation of rainbow trout.[25] These results are
252 in accordance with previous observations in medium-fat horse mackerel species.[18, 19]

253 Phosphoglycerate mutase-1 (67.5 kDa; protein band S3) is an enzyme, which
254 catalyzes the eight step of glycolysis and the results showed also an important reduction
255 of this protein band for high pressure conditions (300 to 450 MPa) (Figure 3c).

256 Pyruvate kinase (55.82 kDa; protein band S4) catalyzes the last step of
257 glycolysis; in agreement with Figure 3d, a relevant reduction of this protein at pressure
258 ≥ 300 MPa was verified. This phenomenon was also observed in medium-fat horse
259 mackerel.[19] Besides, De Felice et al. (1999) have demonstrated that the enzymatic
260 activity of this protein was irreversibly inhibited at 350 MPa due to the formation of
261 intramolecular disulfide bonds.[26]

262 Enolase (isoform beta, muscle; 44.80 kDa; protein band S5) is a metalloenzyme
263 responsible for the penultimate step of the glycolysis and is also considered a potential
264 fish allergen in cod, salmon and tuna species.[27] Beta-enolase belongs to the TIM barrel
265 proteins that consists of two domains with several alpha helices and beta sheets that
266 forms a complex with two $Mg^{2+}$. Interestingly, a noteworthy complete reduction of this
267 protein was noticed in hake treated with the two highest HP conditions (430 and 450
268 MPa) independently of the storage time (Figure 3e). Previous publications on frozen
269 medium-fat horse mackerel species showed that pressure levels of 300-450 MPa
270 reduced the beta-enolase presence.[18, 19] A total of twenty-three fish beta-enolase proteins
271 are included in the Allergome database (www.allergome.org/) (i.e. Gad m 2, Sal s 2,
272 Thu a 2). Immunoglobulin E (IgE) reaction to beta-enolases was found in 62.9% of the
273 fish sensitive patients. Beta-enolases are more allergenic to adults than to children.[27]
274 This work demonstrates that hake beta-enolases are very sensitive to the effect of high-
275 pressure treatment (≥ 430 MPa) (Figure 3e).

276 Creatine kinase (42.02 kDa; protein band S6) is an energy buffer protein that
277 enhances the skeletal muscle contractility. It is a homooctamer and each monomer
278 consists of a small alpha-helical domain and a large domain containing an eight-
279 stranded antiparallel beta-sheet flanked by seven alpha-helices. In addition, this protein
280 is considered a potential fish allergen in tuna species.[28] Results of the HP treatment in

281 hake showed a complete creatine kinase reduction at the two highest HP conditions (430
282 and 450 MPa) (Figure 3f). Previous publications on medium fat-fish showed that HP
283 treatment at 300-450 MPa reduced creatine kinase presence.[18, 19] The stability of this
284 protein was also found to be correlated with firmness of rainbow trout.[25] Twenty-five
285 fish creatine kinases are included in the Allergome database (i.e. Cyp c CK, Dan re CK,
286 Gad m CK, Sal s CK).[29] As is demonstrated in the present work the stability of hake
287 creatine kinase is very low when a pressure treatment ≥ 430 MPa is applied (Figure 3f).

288 Fructose bisphosphate aldolase (39.54 kDa; protein band S7), which is involved
289 in gluconeogenesis and glycolysis, is also considered a potential fish allergen in cod,
290 salmon and tuna species.[27] Results performed on hake specimens showed a complete
291 reduction of this protein at the highest HP conditions (430 and 450 MPa) (Figure 3g),
292 being this result similar to those of a previous work in frozen medium-fat horse
293 mackerel, which showed degradation of this enzyme at pressure levels of 300-450
294 MPa.[18, 19] Ten aldolase fish allergens are deposited in the Allergome database (i.e. Gad
295 m 3, Sal s 3, Thu a 3) and are more allergenic to adults than to children.[27] Aldolase
296 belongs to the TIM barrel proteins and IgE reaction to fructose bisphosphate aldolase
297 was found in 50.0% of fish sensitive patients. The results of the present work showed
298 that the stability of hake fructose bisphosphate aldolase is very low, since it is
299 completely absent when pressure ≥ 430 MPa is applied (Figure 3g).

300 Glyceraldehyde-3-phosphate dehydrogenase (35.98 kDa; protein band S8) is an
301 enzyme that catalyzes the sixth step of glycolysis. The results did not show relevant
302 differences on the band volume of this protein at the different HP conditions tested; as
303 an exception, a slight increase in its intensity was detected after applying a pressure ≥
304 430 MPa (Figure 3h).

305 Tropomyosin (32.70 kDa; protein band S9) plays a pivotal role in the regulation
306 of muscle contraction and is also considered a potential fish allergen in tilapia species.[30]
307 Tropomyosin is an alpha-helical, linear structure protein from the cell cytoskeleton.
308 Results showed an increase of this protein at the two highest pressure conditions (430
309 and 450 MPa) (Figure 3i). Previous studies on medium fat-fish species showed also an
310 increase after applying a 450 MPa processing.[19] Tropomyosin (32.70 kDa) was
311 described as a fish allergen in few patients so far and several fish tropomyosin allergens
312 are registered in the Allergome database (i.e. Gad m 4, Sal s 4, Ore m 4).[30] In this work,
313 a remarkable stability of hake tropomyosins to the different HP treatments was found,
314 and additionally, an increased presence after applying a pressure ≥ 430 MPa was
315 detected (Figure 3i).

316 Troponin T (29.70 kDa; protein band S10) is part of the troponin complex and is
317 responsible for muscle contraction. Results showed an increase of this protein band at
318 the two highest pressure levels (430 and 450 MPa) (Figure 3j). Muscle troponin T also
319 increased its presence intensity after a 450 MPa treatment in frozen medium-fat horse
320 mackerel.[19]

321 Phosphoglycerate mutase-2 (27.59 kDa; protein band S11) is an isozyme that
322 catalyzes the eighth step of glycolysis. The results showed a complete degradation of
323 this protein band for the two highest pressure intensities (430 to 450 MPa) (Figure 3k).
324 In previous results, the presence of this protein was also found to decrease when chilled
325 fatty salmon was treated with a pressure ≥ 170 MPa.[17] In addition, after a 3-month
326 storage, Atlantic mackerel samples treated at 150 MPa revealed the disappearance of
327 this protein.[19] Besides, in medium fat-fishes, this protein demonstrated a strong
328 reduction at 300-450 MPa.[19]

329 Triosephosphate isomerase (25.36 kDa; protein band S12) demonstrated an
330 important reduction at the two highest pressure conditions (430-450 MPa) (Figure 3l).
331 This enzyme plays an important role in glycolysis and is essential for efficient energy
332 production. Previous results in frozen medium-fat horse mackerel species demonstrated
333 an important reduction after a 450 MPa treatment.[19]

334 Myosin light chain 1 (22.14 kDa; protein band S13) is a polypeptide subunit of
335 myosin, involved in muscle contraction. Results showed a remarkable increase of this
336 band at the two highest pressure conditions (430 and 450 MPa) (Figure 3m).

337 Troponin I (20.90 kDa, protein band S14) and troponin C (18.35 kDa, protein
338 band S15) are part of the troponin protein complex, being the latter responsible for
339 binding calcium to activate muscle contraction. Results did not show relevant
340 differences of the volume band of protein band S14 at the different HP conditions
341 (Figure 3n), except for a remarkable increase of the protein band S15 at the highest HP
342 conditions (430 and 450 MPa; Figure 3o).

343 Nucleoside diphosphate kinase (16.94 kDa; protein band S16) is an enzyme
344 required for the synthesis of nucleoside triphosphates. Thirteen different isoforms
345 belonging to the Merlucciidae family were *de novo* mass spectrometry sequenced.[21] The
346 present results on hake showed a relevant reduction of this protein at the two highest
347 pressure levels (430 and 450 MPa) (Figure 3p).

348 Myosin light chain 2 (15.11 kDa, protein band S17) and myosin light chain 3
349 (14.23 kDa; protein band S18) are polypeptide subunits of myosin complex, the first
350 being involved in muscle contraction. Results showed a remarkable increase of the
351 corresponding bands at 300/450 MPa (Figure 3q - S17) and at day 150 of storage for
352 300 MPa (Figure 3r - S18), respectively.

353 Parvalbumin beta (11.08 kDa; protein band S19) is considered the major fish
354 allergen.[31] A total of 25 different parvalbumin isoforms belonging to the Merlucciidae
355 family were completely *de novo* mass spectrometry sequenced.[32] Results showed an
356 increase of this protein at the two highest pressure conditions (430 and 450 MPa)
357 (Figure 3s). Previous publications on medium-fat fish also reported an important
358 increase at 450 MPa.[19] According to their structure, they are members of the EF-hand
359 protein family, composed by a peptide helix/peptide loop/peptide helix-sequence. Fish
360 parvalbumins have three EF-hand motifs but only two are functional to bind $Ca^{2+}$ or
361 $Mg^{2+}$ ions. These proteins are thermostable proteins that present high stability towards
362 food processing and enzymatic digestion.[33] In the current study, it was also found that
363 hake parvalbumins present a remarkable stability to HP treatments and even showed an
364 increase in their intensity after applying pressure ≥ 430 MPa (Figure 3s). These results
365 might be used for the development of further allergenic studies using different hake HP
366 treatments and sera from parvalbumin allergic patients.
367
368 **Effects of HP treatment on the myofibrillar protein fraction by Bottom-Up**
369 **proteomics**
370 Information related to this protein fraction is shown in Figures 4 (SDS-PAGE
371 profiles) and 5 (effect of HP treatment on each individual protein), while Table 1
372 summarizes the list of myofibrillar protein bands (M1-M9) analyzed by LC-MS/MS and
373 identified by SEQUEST-HT. The complete lists of identified peptides and the
374 corresponding proteins are presented in Tables in the Supplementary Data 2.
375 The results showed that myofibrillar proteins are more stable under the current
376 pressurization treatments (150-450 MPa) than sarcoplasmic ones when a lean fish (i.e.,

377 hake) was studied. Similar results were obtained in another study when myofibrillar
378 proteins were studied in fatty fish species.[18, 19]

379 Protein band M1 assigned to fast skeletal myosin heavy chain (118.71 kDa),
380 glycogen phosphorylase (95.41 kDa, protein band M2), actin (alpha skeletal muscle,
381 42.50 kDa, protein band M3), tropomyosin (32.70 kDa, protein band M4) and troponin
382 C (18.35 kDa, protein band M8) did not reveal important differences in the protein
383 profiles after the different HP treatments and frozen storage times (Figure 5a-5d and
384 5h).

385 Reduction of the bands volume was detected in various cases. This concerns
386 protein band M5 (29.70 kDa; troponin T) at 300-450 MPa treatment (Figure 5e), protein
387 band M7 (20.90 kDa, troponin I) at the highest HP condition (450 MPa) (Figure 5g) and
388 protein band M6 (15.11 kDa, myosin light chain 2) at the highest-pressure conditions
389 (430-450 MPa) (Figure 5i). On the other hand, myosin light chain 3 (14.23 kDa)
390 showed a presence increase at day 140 of storage for 430 MPa (Figure 3f).

391

392 **Protein biomarkers of HP treatments**

393 According to the present results, to select specific protein biomarkers of HP
394 treatments, sarcoplasmic proteins were found as the most adequate fraction to be
395 investigated, because more protein differences in terms of protein band intensity were
396 found under the current pressurization treatments (150-450 MPa).

397 Figure 6 shows the results of principal component analysis (PCA) for the
398 sarcoplasmic proteins. In the present work, the PCA plot (73%) corroborated the clear
399 differentiation of the samples according to the HP treatment in three different clusters:
400 low (≤ 170 MPa), medium (300 MPa) and high (≥ 430 MPa) HP treatments. These

401 results supported that the most important parameter to differentiate the samples is the
402 pressure level independently of the storage time.

403 Figure 7 shows the heatmap that groups the results according to the similarity of
404 the band volume of the sarcoplasmic proteins identified by Bottom-Up proteomics (S1-
405 S19) (rows) and the HP treatment (columns). The legend color bar indicates the band
406 volume values, showing high-intensity (red), low-intensity (green) and without-intensity
407 (grey). The R program estimated a threshold of 0.15. Based on the results of the
408 heatmap, three different clusters were identified in the X axis. Cluster 1 groups the
409 samples treated with the higher pressure levels (≥ 430 MPa), cluster 2 groups the
410 samples treated with a medium pressure level (300 MPa) and cluster 3 groups the
411 samples treated with a lower pressure level (≤ 170 MPa).

412 Within cluster 1 (≥ 430 MPa), specific top protein biomarkers can be proposed
413 as presenting a significant reduction in the exclusive band volume, as creatine kinase,
414 beta-enolase, fructose bisphosphate aldolase, phosphoglycerate mutase-1,
415 triosephosphate isomerase and nucleoside diphosphate kinase. Conversely, the
416 application of a relatively high pressure (≥ 430 MPa) allowed identifying the top
417 exclusive protein biomarkers that presented a significant increase only in this cluster,
418 such as tropomyosin, beta parvalbumin and glyceraldehyde-3-phosphate
419 dehydrogenase.

420 Regarding exclusive protein biomarkers that presented a significant reduction in
421 the band volume when a 300-MPa treatment was applied (cluster 2), pyruvate kinase,
422 phosphoglycerate mutase-2, glycogen phosphorylase and myosin heavy chain were
423 selected. The top protein biomarkers that presented a significant increase after a
424 pressure treatment ≥ 300 MPa were troponin I and parvalbumin beta.

425 Finally, in cluster 3 (≤ 170 MPa), the top exclusive protein biomarkers that
426 presented a significant presence in the band volume were pyruvate kinase,
427 phosphoglycerate mutase-2 and glycogen phosphorylase.
428

429 **Final statements**

430 For the first time, Bottom-Up proteomics was applied to study the effects of a
431 previous HP treatment (150-450 MPa for 2 min) on frozen (up to 5 months at –10 ºC)
432 European hake. HP treatment using pressures of 150-170 MPa showed not altering
433 effects in the protein abundance. Protein biomarkers of HP treatments were selected as
434 phosphoglycerate mutase-1, beta-enolase, creatine kinase, fructose bisphosphate
435 aldolase, triosephosphate isomerase and nucleoside diphosphate kinase, which showed a
436 significant degradation after applying a pressure ≥ 430 MPa. Conversely, protein
437 biomarkers assigned to tropomyosin and glyceraldehyde-3-phosphate dehydrogenase
438 increased their intensity in the electrophoretic profiles after applying a pressure ≥ 430
439 MPa. This repository of protein biomarkers will be very useful for further HP
440 investigations related to fish quality.
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570

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578

579 **CONFLICT OF INTEREST**

580 The authors have declared no conflict of interest.

581

582 **ETHICAL STATEMENT**

583 No ethics approval was necessary because the present manuscript does not
584 include studies with living bodies.

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587

588 **FIGURE CAPTIONS**

589

590 **Figure 1:** Experimental workflow used in the present work.

591

592 **Figure 2:** SDS-PAGE profiles of the sarcoplasmic protein fraction of hake samples
593 treated with different high-pressure levels and subsequently stored at –10ºC for
594 different storage times: profiles for 170, 300 and 430 MPa and stored for 0, 10
595 and 140 days, replicates (a) and (b); and profiles for 150, 300 and 450 MPa and
596 stored for 0, 75 and 150 days, replicates (c) and (d). At each sampling time,
597 comparison to control profile (i.e., 0.1-MPa samples) is included. MW denotes
598 molecular weight.

599

600 **Figure 3:** Profiles of the band volume for each sarcoplasmic protein of hake samples
601 treated with different high-pressure levels (150, 170, 300, 430, and 450 MPa)
602 and subsequently stored at –10 ºC for 0, 10, 75, 140 or 150 days. At each
603 sampling time, comparison to control profile (i.e., 0.1-MPa samples) is included.

604

605 **Figure 4:** SDS-PAGE profiles of the myofibrillar protein fraction of hake samples
606 treated with different high-pressure levels and subsequently stored at –10ºC for
607 different storage times: profiles for 170, 300 and 430 MPa and stored for 0, 10
608 and 140 days, replicates (a) and (b); and profiles for 150, 300 and 450 MPa and
609 stored for 0, 75 and 150 days, replicates (c) and (d). At each sampling time,
610 comparison to control profile (i.e., 0.1-MPa samples) is included. MW denotes
611 molecular weight.

612

613 **Figure 5:** Profiles of the band volume for each myofibrillar protein of hake samples
614 treated with different high-pressure levels (150, 170, 300, 430, and 450 MPa)
615 and subsequently stored at –10 ºC for 0, 10, 75, 140 or 150 days. At each
616 sampling time, comparison to control profile (i.e., 0.1-MPa samples) is included.
617
618 **Figure 6:** PCA plot of the sarcoplasmic proteins treated with different high-pressure
619 levels (150, 170, 300, 430, and 450 MPa) and subsequent storage at –10 ºC for 0,
620 10, 75, 140 or 150 days.
621
622 **Figure 7:** Heatmap of the Euclidean hierarchical clustering of the sarcoplasmic proteins
623 treated with different high-pressure levels (150, 170, 300, 430, 450 MPa) and
624 subsequently stored at –10 ºC for 0, 10, 75, 140 or 150 days.
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634 **SUPPORTING INFORMATION**

635

636 **SUPPLEMENTARY DATA 1:** Complete list of identified peptides and proteins
637 analyzed by LC-MS/MS and identified by SEQUEST-HT of the sarcoplasmic
638 protein bands (S1-S19) of hake.

639

640 **SUPPLEMENTARY DATA 2:** Complete list of identified peptides and proteins
641 analyzed by LC-MS/MS and identified by SEQUEST-HT of the myofibrillar
642 protein bands (M1-M9) of hake.

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654 **Figure 1**

655

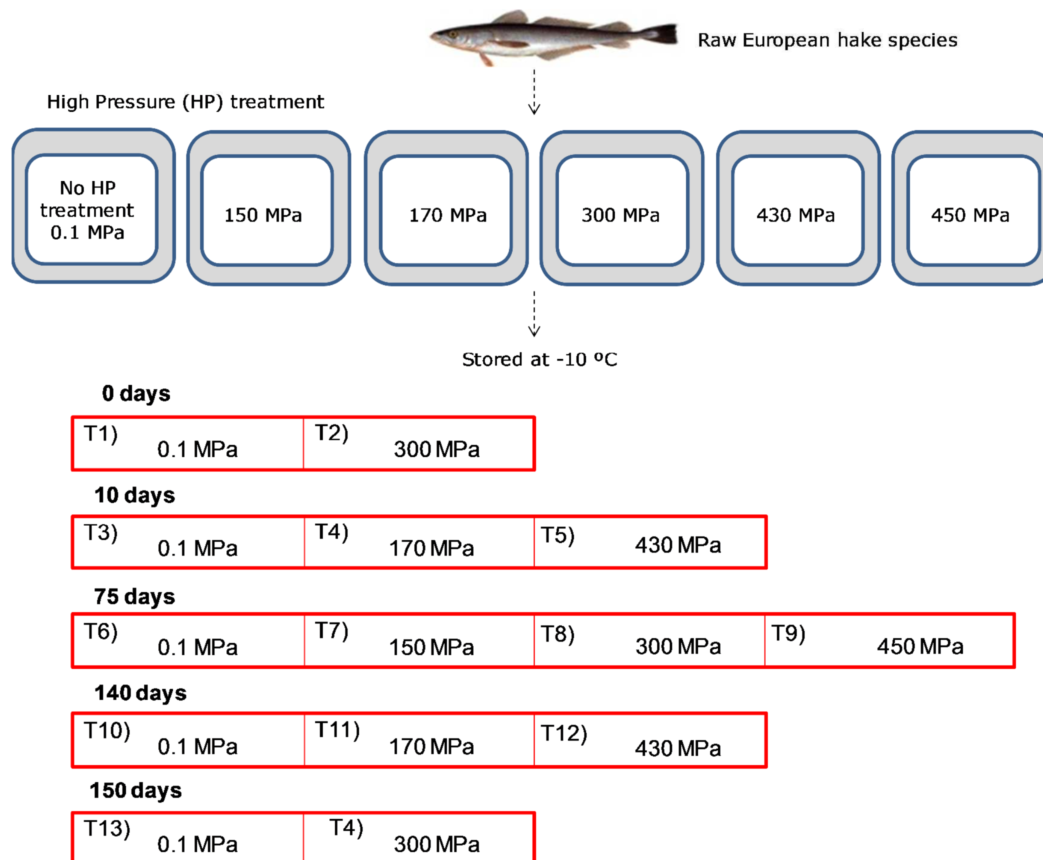

Figure 2

**Sarcoplasmic proteins**

a)
Day 0 0 10 10 10 140 140 140
HP (MPa) 0.1 0.1 300 0.1 170 430 0.1 170 430 MW (kDa)
S1 S2 S3 S4 S5 S6 S7 S8, S9 S10 S11 S12 S13 S14 S15 S16 S17 S18 S19
100 70 30 15

b)
Day 0 0 10 10 10 140 140 140
HP (MPa) 0.1 0.1 300 0.1 170 430 0.1 170 430 MW (kDa)
200 150 120 100 85 70 60 50 40 30 25 20 15 10

c)
Day 0 0 75 75 75 75 150 150
HP (MPa) 0.1 0.1 300 0.1 150 300 450 0.1 300 MW (kDa)
100 70 30 15

d)
Day 0 0 75 75 75 75 150 150
HP (MPa) 0.1 0.1 300 0.1 150 300 450 0.1 300 MW (kDa)
200 150 120 100 85 70 60 50 40 30 25 20 15 10

**Figure 3**

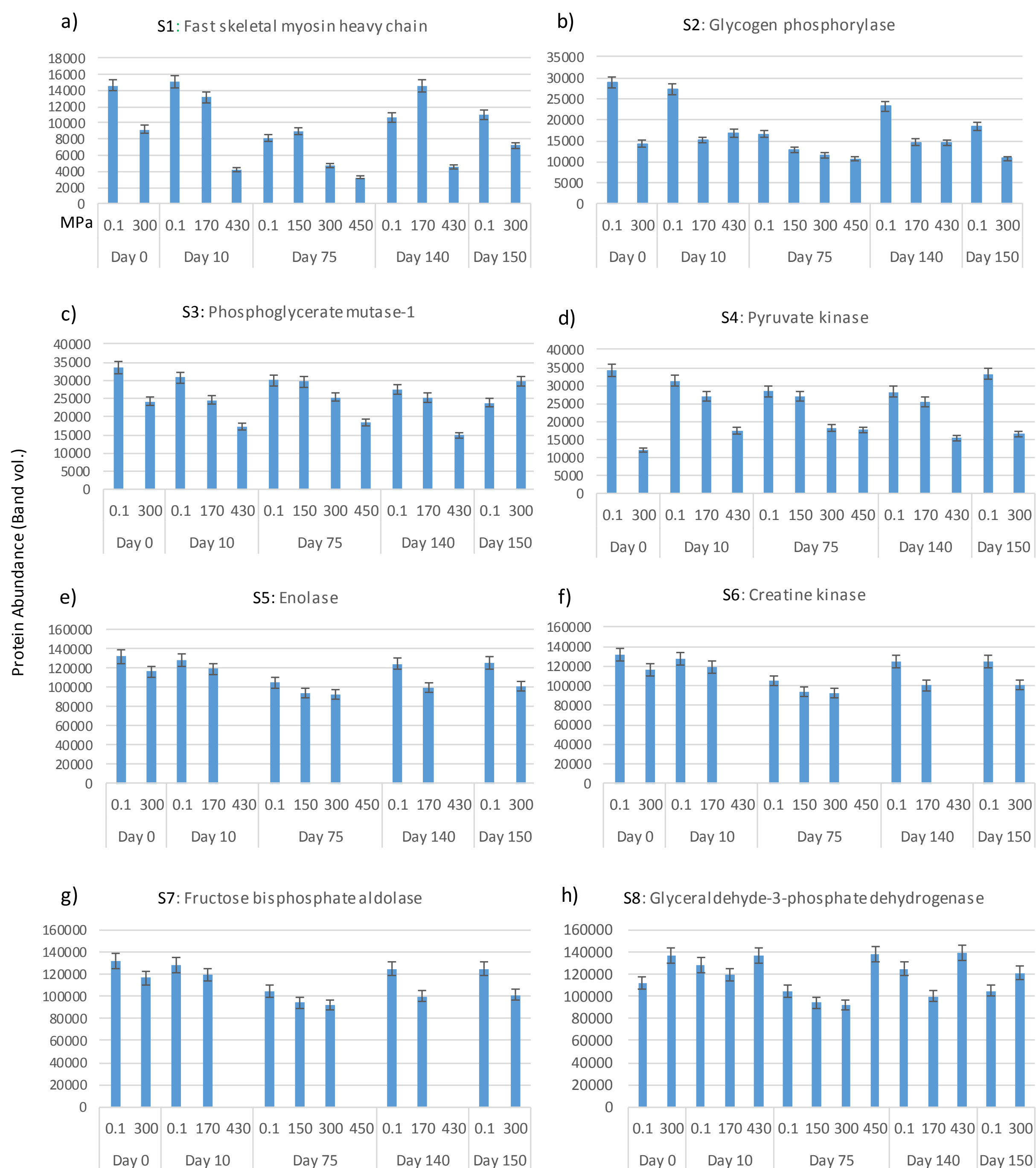

**Figure 3.** Continuation

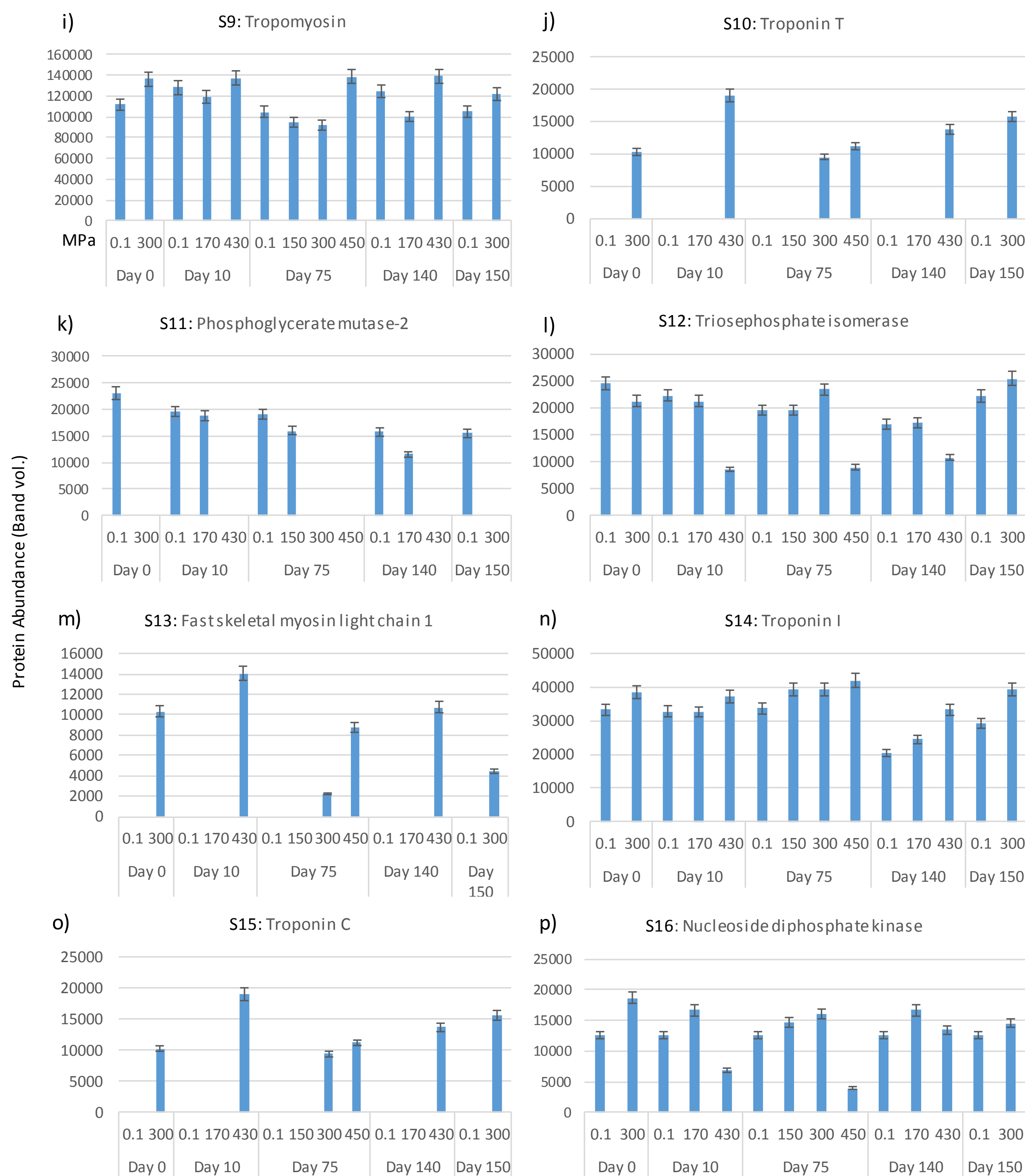

**Figure 3.** Continuation.

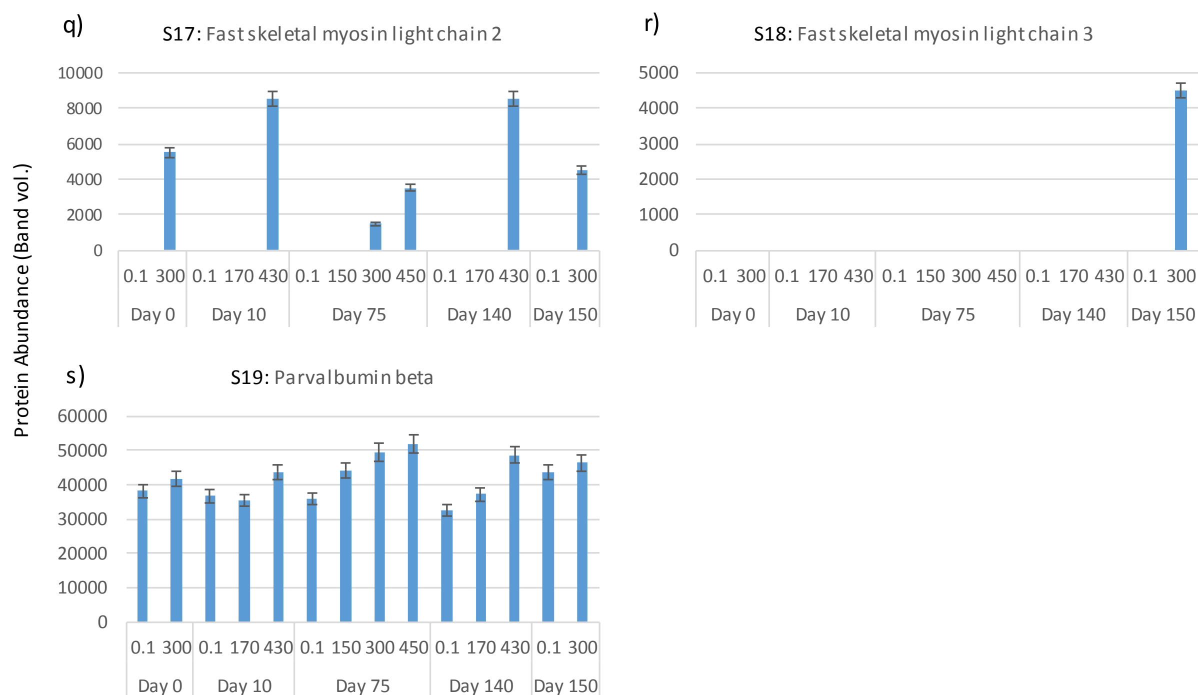

Figure 4

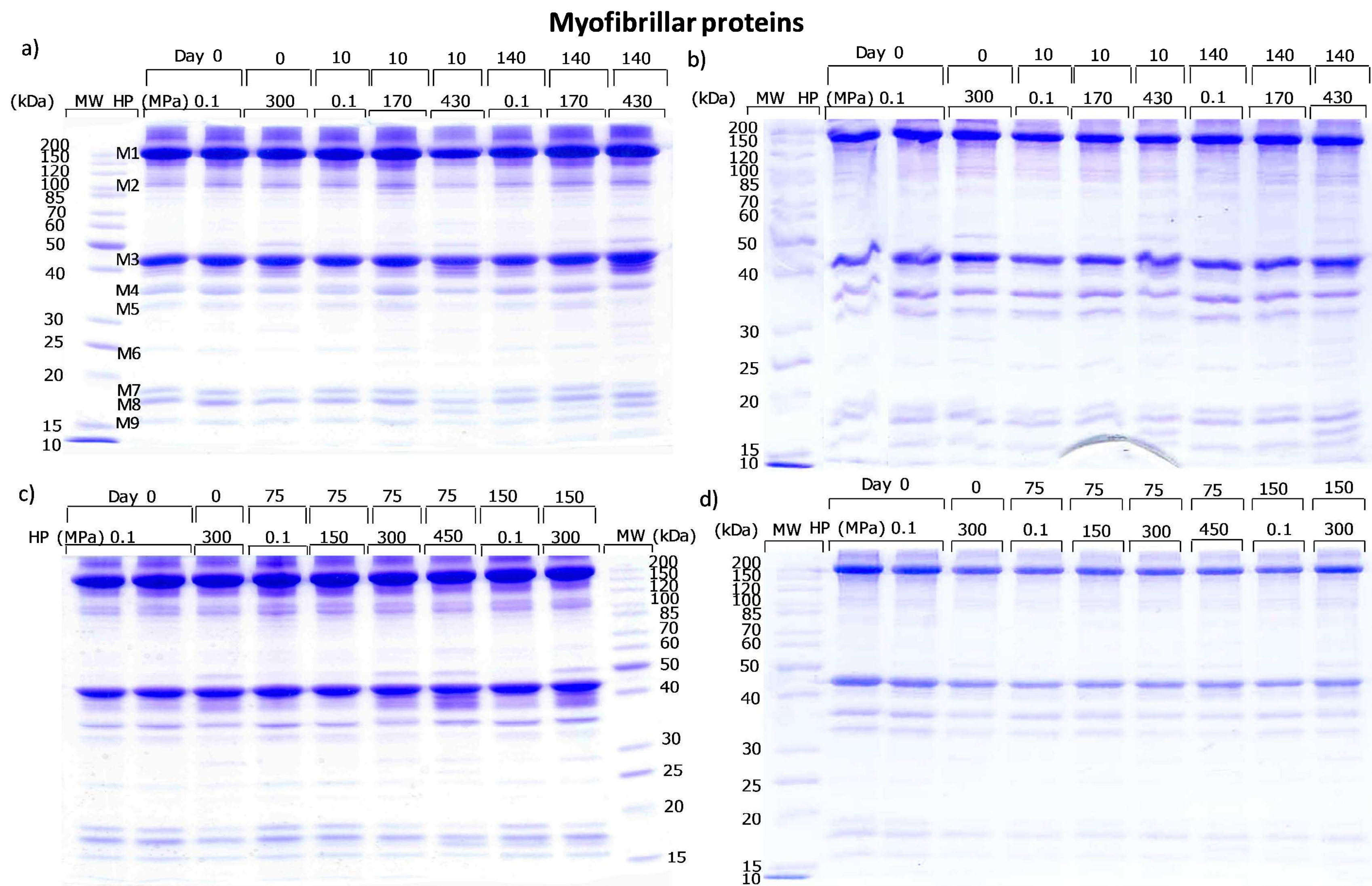

**Figure 5**

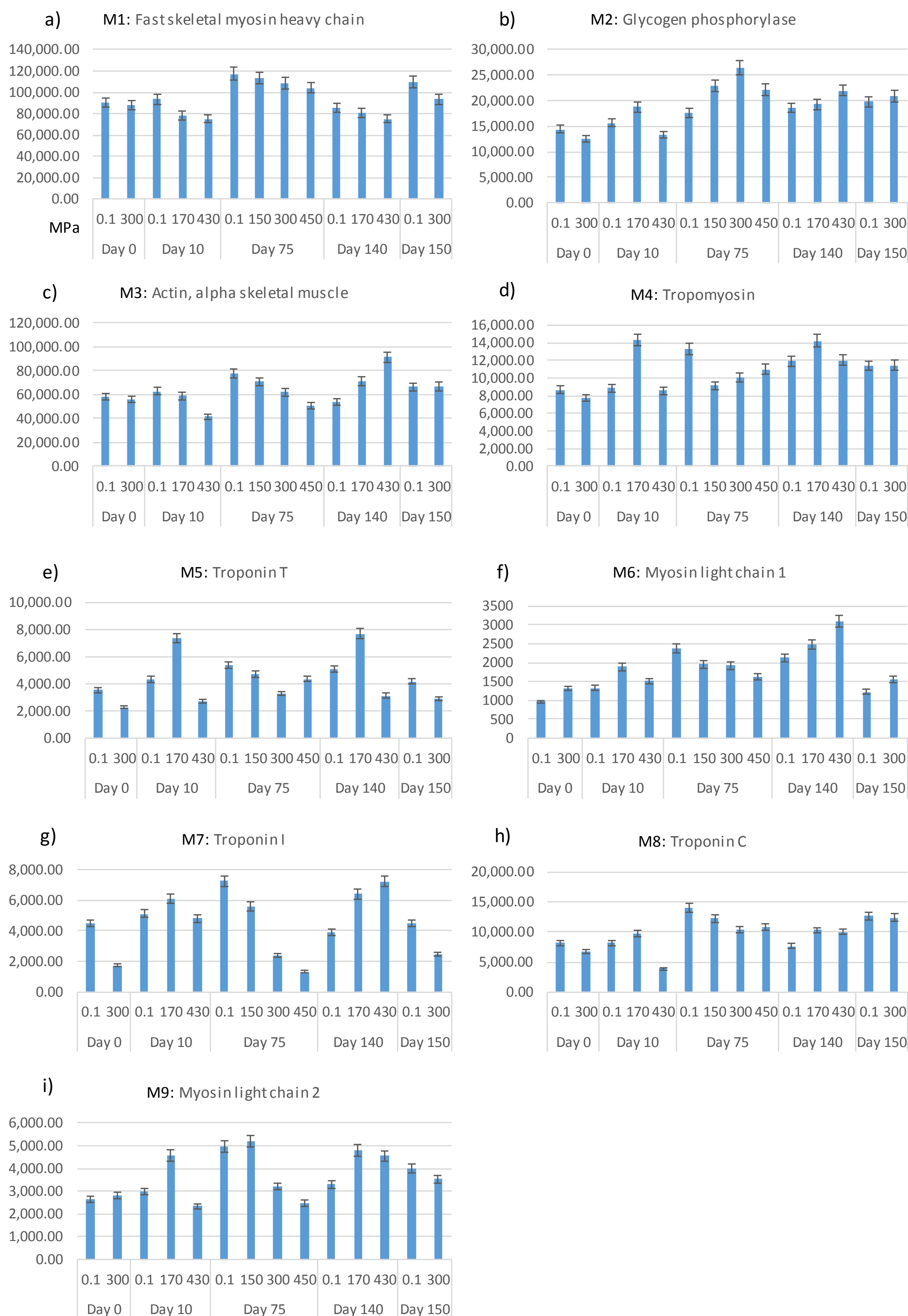

**Figure 6**

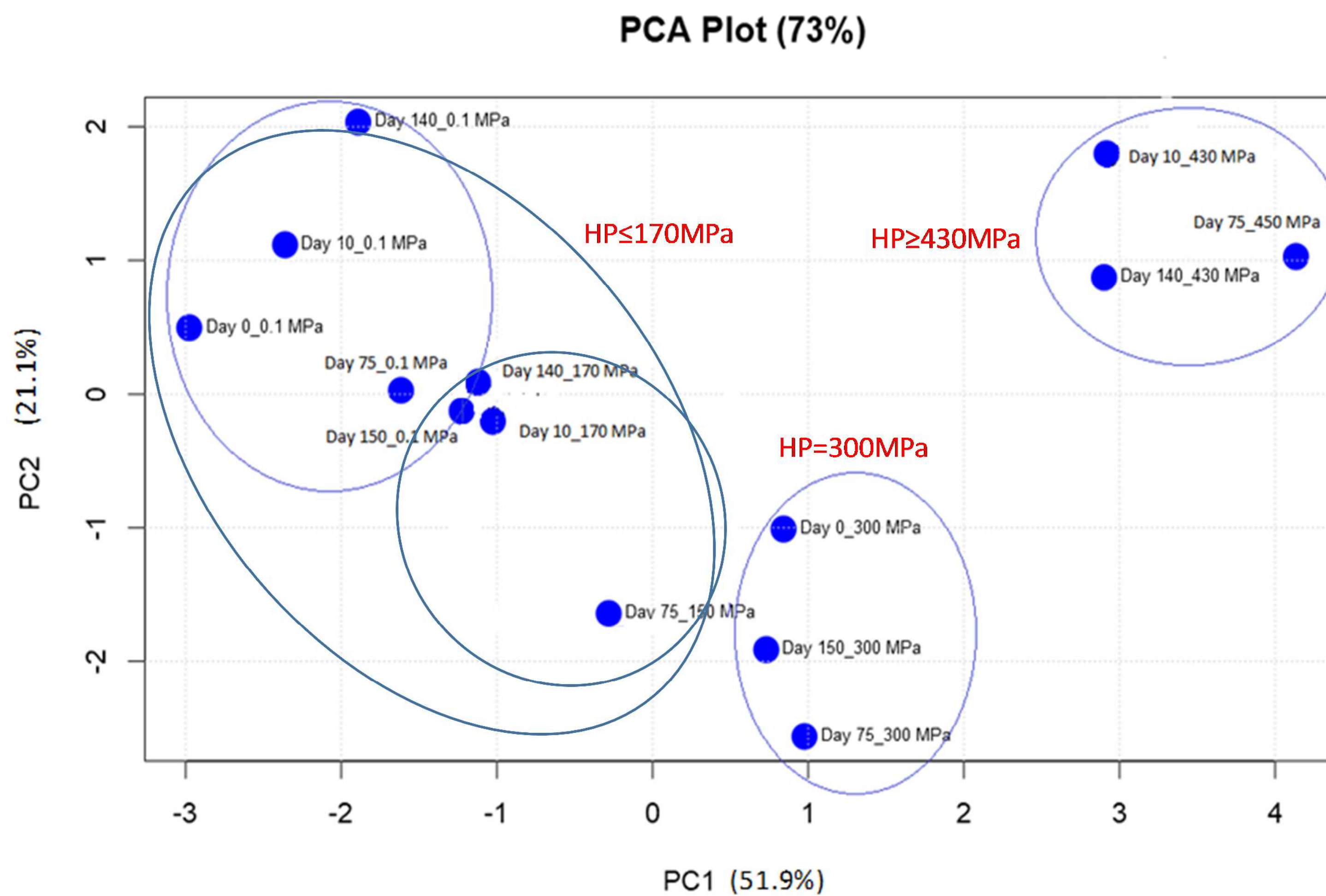

**Figure 7**

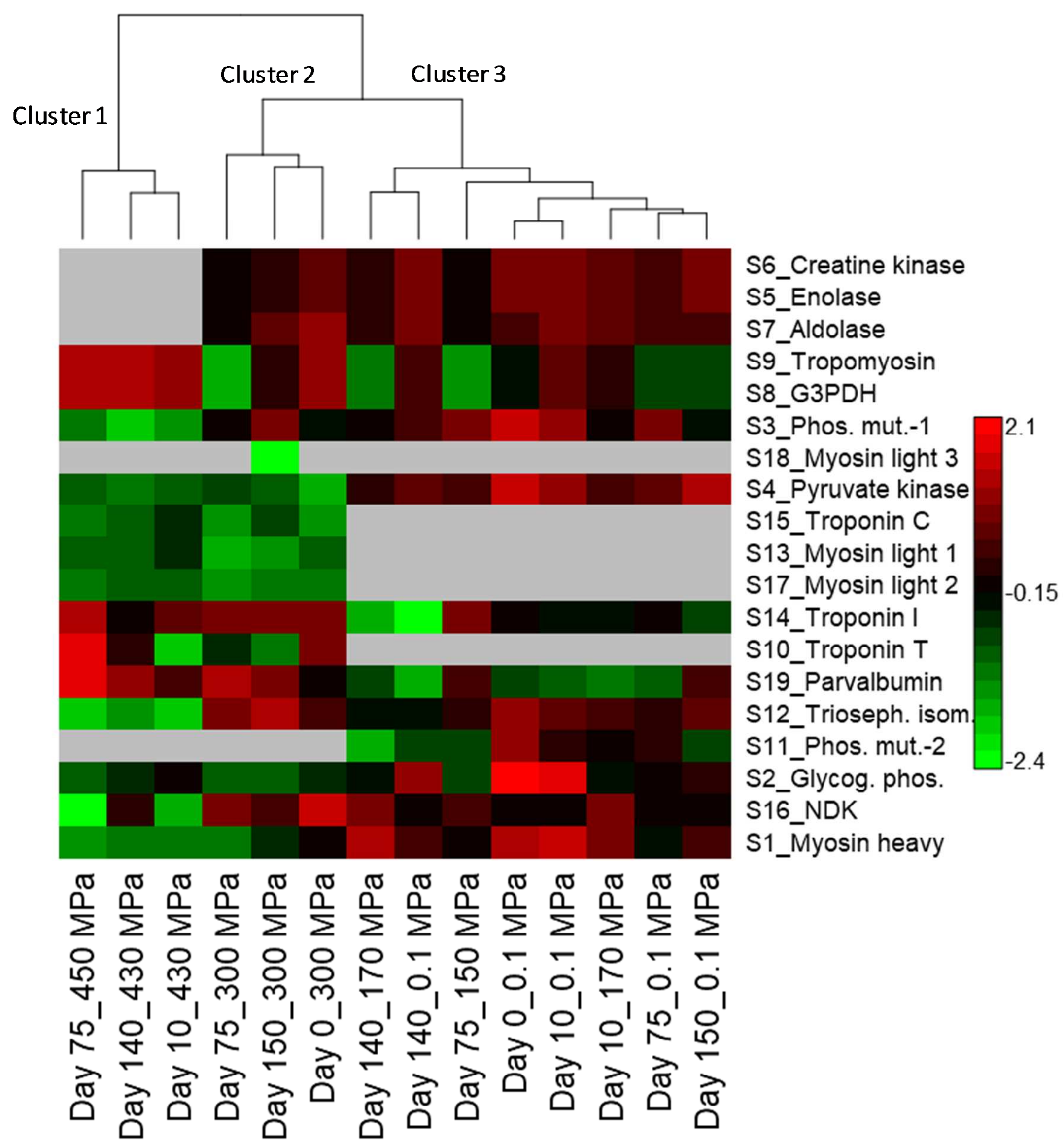

**Table 1:** Sarcoplasmic (S) and myofibrillar (M) protein bands identified by Bottom-Up proteomics.

| Sarcoplasmic Band ID | MW obs./theor. (kDa) | Protein Description | UniProtKB Accession | Gene | Peptide Counts | Non-redundant Peptides | Sequence Coverage (%) |
|---|---|---|---|---|---|---|---|
| S1 | 118.71/128.60 | Fast skeletal myosin heavy chain | Q8JIV5 | myhz2 | 13 | 7 | 32.1 |
| S2 | 95.41/97.092 | Glycogen phosphorylase | P11217 | pygma | 8 | 3 | 3.1 |
| S3 | 67.43/68.52 | Phosphoglycerate mutase-1 | Q7SZR4 | pgam1a | 46 | 10 | 8.27 |
| S4 | 55.82/58.11 | Pyruvate kinase | Q6NXD1 | pkma | 4 | 4 | 5.6 |
| S5 | 44.80/47.43 | Enolase (isoform beta, muscle) | Q568G3 | eno3 | 66 | 16 | 27.80 |
| S6 | 42.02/42.82 | Creatine kinase | Q90X19 | ckma | 55 | 10 | 14.2 |
| S7 | 39.54/39.74 | Fructose-bisphosphate aldolase | Q803Q7 | aldoaa | 12 | 5 | 10.71 |
| S8 | 35.98/35.78 | Glyceraldehyde-3-phosphate dehydrogenase | Q5XJ10 | gapdh | 21 | 12 | 15.31 |
| S9 | 32.70/32.72 | Tropomyosin | P13104 | tpma | 65 | 24 | 26.05 |
| S10 | 29.70/29.35 | Troponin T | B7ZVL6 | tnnt3b | 62 | 12 | 8.16 |
| S11 | 27.59/28.82 | Phosphoglycerate mutase-2 | Q7T3G4 | pgam2 | 42 | 6 | 12.55 |
| S12 | 25.36/26.85 | Triosephosphate isomerase | Q1MTI4 | tpi1a | 23 | 9 | 14.92 |
| S13 | 22.14/21.03 | Fast skeletal myosin light chain 1 | Q6P0G6 | myl1 | 24 | 5 | 8.421 |
| S14 | 20.90/18.95 | Troponin I | Q8QG69 | tnni2a | 7 | 3 | 8.772 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| S15 | 18.35/18.21 | Troponin C | Q9I8U8 | tnnc2 | 49 | 9 | 7.5 |
| S16 | 16.94/16.99 | Nucleoside diphosphate kinase | Q9DFL9 | ndk | 102 | 8 | 11.41 |
| S17 | 15.11/16.62 | Fast skeletal myosin light chain 2 | I6R7V1 | myl2b | 18 | 5 | 12.94 |
| S18 | 14.23/16.50 | Fast skeletal myosin light chain 3 | Q9IB40 | mylz3 | 70 | 9 | 28.38 |
| S19 | 11.08/11.29 | Parvalbumin beta | P02620 | N/A | 94 | 65 | 66.67 |
| | | | | | | | |
| **Myofibrillar Band ID** | **MW obs./theor. (kDa)** | **Protein Description** | **UniProtKB Accession** | **Gene** | **Peptide Counts** | **Non-redundant Peptides** | **Sequence Coverage (%)** |
| M1 | 118.71/128.60 | Fast skeletal myosin heavy chain | Q8JIV5 | myhz2 | 62 | 55 | 32.10 |
| M2 | 95.41/97.092 | Glycogen phosphorylase | P11217 | pygma | 4 | 2 | 1.425 |
| M3 | 42.50/42.02 | Actin, alpha skeletal muscle | F1QUN8 | acta1a | 33 | 28 | 45.36 |
| M4 | 32.70/32.72 | Tropomyosin alpha-1 chain | P13104 | tpma | 33 | 16 | 11.97 |
| M5 | 29.70/29.35 | Troponin T | B7ZVL6 | tnnt3b | 38 | 5 | 8.16 |
| M6 | 22.14/21.03 | Myosin light chain 1 | Q6P0G6 | myl1 | 8 | 4 | 8.42 |
| M7 | 20.90/18.95 | Troponin I | Q8QG69 | tnni2a | 7 | 3 | 8.77 |
| M8 | 18.35/18.21 | Troponin C | Q9I8U8 | tnnc2 | 37 | 7 | 7.5 |
| M9 | 15.03/16.62 | Myosin light chain 2 | I6R7V1 | myl2b | 11 | 5 | 12.94 |

**TOC graphic:**