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Liver proteomics of gilthead sea bream (*Sparus aurata*) exposed to cold stress

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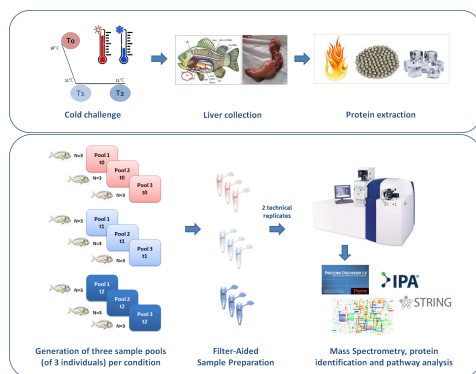
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1 **Liver proteomics of gilthead sea bream (*Sparus aurata*) exposed to cold stress**

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14

15 **Short title: Cold stress and the sea bream liver proteome**

16

17 **Abstract**

18 The gilthead sea bream (*Sparus aurata*, L.) is very sensitive to low temperatures, which induce fasting and  
19 reduced growth performances. There is a strong interest in understanding the impact of cold on fish  
20 metabolism to foster the development and optimization of specific aquaculture practices for the winter  
21 period. In this study, a 8 week feeding trial was carried out on gilthead sea bream juveniles reared in a  
22 Recirculated Aquaculture System (RAS) by applying a temperature ramp in two phases of four weeks each:  
23 a cooling phase from 18°C to 11°C and a cold maintenance phase at 11°C. Liver protein profiles were  
24 evaluated with a shotgun proteomics workflow based on filter-aided sample preparation (FASP) and liquid  
25 chromatography-mass spectrometry (LC-ESI-Q-TOF MS/MS) followed by label-free differential analysis.  
26 Along the whole trial, sea breams underwent several changes in liver protein abundance. These occurred  
27 mostly during the cooling phase when catabolic processes were mainly observed, including protein and lipid  
28 degradation, together with a reduction in protein synthesis and amino acid metabolism. A decrease in protein  
29 mediators of oxidative stress protection was also seen. Liver protein profiles changed less during cold  
30 maintenance, but pathways such as the methionine cycle and sugar metabolism were significantly affected.  
31 These results provide novel insights on the dynamics and extent of the metabolic shift occurring in sea bream  
32 liver with decreasing water temperature, supporting future studies on temperature-adapted feed formulations.  
33 The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the  
34 PRIDE partner repository with the dataset identifier PXD011059.

35

36 **Keywords: cold stress; winter syndrome; gilthead sea bream; liver proteins; methionine; shotgun**  
37 **proteomics.**

38

## 39 1. Introduction

40 Gilthead sea breams (*Sparus aurata*, L.) have their natural habitat in the Mediterranean Sea at water  
41 temperatures ranging seasonally from approximately 26°C in summer to 11-13°C or even lower in winter,  
42 depending on the specific environment. Wild sea breams manage this temperature decrease by migrating to  
43 deeper and warmer water (Davis, 1988). In fact, water temperatures below 13°C lead to both behavioral  
44 (e.g., erratic swimming, voluntary fasting, hyposensitivity to stimuli) and physiological (e.g., impaired  
45 growth, fatty liver, tissue necrosis, infections) stressful changes that can ultimately lead to physiological  
46 dysfunction and death (Contessi et al., 2006; Gallardo et al., 2003; Ibarz et al., 2010b). However, farmed sea  
47 breams living in outdoor tanks and in floating sea cages are unable to avoid this thermal stress, and their  
48 prolonged exposure to temperatures below 13°C causes decrease in activity (Ibarz et al., 2003), growth delay  
49 (Tort et al., 1998), metabolic depression (Ibarz et al., 2018; Sanahuja et al., 2019) and reduced feed  
50 consumption until total fasting when water temperatures fall below 10°C (Ibarz et al., 2010b). Other  
51 physiological alterations include hepatic functionality, with the liver becoming steatotic and whitish due to a  
52 large deposition of lipids, as well as reduced efficiency of adaptive immunity with increased susceptibility to  
53 infections (winter syndrome or winter disease) and alteration of the main redox pathways (Abram et al.,  
54 2017; Ibarz et al., 2007, 2005; Sánchez-Nuño et al., 2018). These phenomena impact farming productions  
55 causing relevant economic losses with a consequent strong interest of fish farmers in finding efficient  
56 strategies for their reduction. One way for compensating thermal stress is represented by enhancing the  
57 nutritional state and metabolism through the use of feeds specifically designed for the colder season, and this  
58 requires understanding the consequences of cold on fish metabolism. To this aim, proteomic, metabolomic  
59 and transcriptomic approaches have been applied to farmed and wild fish biofluids and tissues, such as  
60 serum, liver, muscle and other organs, with differing degrees of success (Addis, 2013; Addis et al., 2010a,  
61 2010b; Alves et al., 2010; Braceland et al., 2013; Brunt et al., 2008; Douxfils et al., 2011; Ghisaura et al.,  
62 2014; Martin et al., 2001; Melis et al., 2017; Mininni et al., 2014; Rodrigues et al., 2012). Proteomics offers  
63 several specific advantages when compared to genomic or transcriptomic strategies, including the ability to  
64 evaluate the actual extent of protein abundance, going beyond the estimates based on gene expression. In  
65 fact, proteins do not always follow a strict relationship with gene transcription but are regulated at the  
66 translational or post-translational level. Liver, the main metabolic organ of the body, has gained the greatest

67 attention in proteomic studies evaluating the influence of farming practices on fish metabolism. Martin and  
68 coworkers (Martin et al., 2001) studied the changes occurring in the liver proteome as a consequence of  
69 different feeding regimens, including dietary plant protein substitution. Liver metabolism is considerably  
70 influenced also by other factors including environmental stress, and might be affected by xenobiotics and  
71 toxins (Addis, 2013; Ghisaura et al., 2014). Numerous research groups focused on the gilthead sea bream  
72 liver proteome to investigate a variety of stressful factors, ranging from handling and crowding (Alves et al.,  
73 2010) to the use of antiparasitic, or antibacterial agents and different environmental pollution (Isani et al.,  
74 2011; Kovacik et al., 2018; Varó et al., 2013). Proteomics has also been used to assess the impact of cold on  
75 fish metabolism (Ibarz et al., 2010a; Parrington and Coward, 2002; Vilhelmsson et al., 2003). However,  
76 these studies applied a gel-based approach (2D-gel electrophoresis, 1D GelC-MS/MS). With the aim of  
77 gathering additional information, we investigated the changes occurring in liver tissue by applying a shotgun  
78 proteomics workflow based on filter-aided sample preparation (FASP), tandem mass spectrometry (MS/MS),  
79 label-free quantitation and, finally, pathway analysis by means of STRING and Ingenuity Pathway Analysis  
80 (IPA). In fact, this approach provides a higher proteome coverage and is less affected by the typical  
81 limitations of gel-based studies, especially when 2D separation is involved (Westermeier et al., 2008), and  
82 additional or complementary information can be obtained in respect to previous studies. Liver proteome  
83 changes were assessed by mimicking the winter challenge conditions in a Recirculated Aquaculture System  
84 (RAS), both immediately after temperature reduction and during cold maintenance.

85

## 86 **2. Materials and Methods**

### 87 **2.1. Experimental design**

88 A detailed description of the trial can be found in our previous work (Melis et al., 2017). For the purposes of  
89 both studies, a total of 60 juvenile gilthead sea breams with an average weight of  $82.0 \pm 4.5$  g were  
90 uniformly distributed in three fiberglass tanks of 550 L with mechanical and biological filtration systems, a  
91 pumping system, a water thermoregulation system, and an automatic control for adjusting and monitoring the  
92 main physicochemical parameters. Fish were first acclimated for two weeks by linearly lowering water  
93 temperature from 20°C to 18°C (t0). Then, temperature was reduced at a rate of approximately 1°C every  
94 Tuesday and Friday until reaching 11°C (cooling phase, t0-t1, 4 weeks). Then, fish were maintained at 11°C

95 (cold maintenance phase, t1-t2) for the same time span (4 weeks). Fish were fed by hand, once a day, an  
96 experimental feed formulation (Aller Aqua, Christiansfeld, Denmark) with 43% protein and 14% fat. Feed  
97 ration was adjusted accounting for fish size, biomass and temperature ( $0.9 \pm 0.03$  g during cooling and  $0.4 \pm$   
98  $0.03$  g during cold maintenance). During the growth trial no mortality occurred, and fish never stopped  
99 eating below 13°C. For the purposes of this study, at the beginning of the trial (t0) and at each time point (t1,  
100 t2), 9 fishes were anesthetized with 1,1,1-trichloro-2-methylpropan-2-ol (2% in marine water) and  
101 transferred in a mixture of marine water and ice (total number of sacrificed subjects = 27). Liver was excised  
102 from each fish, weighed and frozen rapidly in liquid nitrogen in Petri dishes as described by Melis and  
103 coworkers (2017).

104

## 105 **2.2. Protein extraction and quantification**

106 Fish liver protein extraction was performed according to Ghisaura et al. (2016). Briefly, a small portion of  
107 each tissue (100 mg) was placed in a 2 ml Eppendorf safe-lock tube (Eppendorf, Hamburg, Germany) and  
108 immersed at 25% w/v in lysis buffer (7 M urea, 2 M Thiourea, 2% CHAPS) plus protease inhibitor cocktail  
109 (Protease Inhibitor Cocktail for General Use, Sigma-Aldrich, Saint Louis, MO) as indicated in the  
110 manufacturer instructions. Samples were then processed and subjected to three cycles of 5 min at 30  
111 oscillations/s in a TissueLyser mechanical homogenizer (Qiagen, Hilden, Germany). Samples were frozen in  
112 between homogenization cycles to ease tissue disruption and avoid excessive sample heating. Protein  
113 extracts were then centrifuged for 15 min at 18,000 x g at 4°C, quantified with the Pierce 660 nm Protein  
114 Assay Kit (Thermo Scientific - Rockford, IL), evaluated for quality and integrity by SDS-PAGE (data not  
115 shown), and stored at  $-80^{\circ}\text{C}$  until use.

116

## 117 **2.3. Shotgun proteomics**

118 Three protein samples for each time point (t0, t1, t2), each constituted by a biological pool of three fish  
119 liver extracts, were used for shotgun proteomics analysis. Protein extracts (n = 9) were subjected to on-  
120 filter reduction, alkylation, and trypsin digestion according to the filter-aided sample preparation (FASP)  
121 protocol (Wiśniewski et al., 2009), with minor modifications (Ghisaura et al., 2016; Tanca et al., 2013)  
122 using Amicon Ultra-0.5 centrifugal filter units with Ultracel-10 membrane (Millipore, Billerica, MA,

123 USA). Peptide mixture concentration was estimated by using BCA protein assay kit (Thermo Scientific -  
124 Rockford, IL). LC-MS/MS analyses were performed on a Q-TOF hybrid mass spectrometer with a nano  
125 lock Z spray source, coupled on-line with a NanoAcquity chromatography system (Waters) (Pagnozzi et  
126 al., 2014). Two technical replicates were analyzed for each biological pool (total LC-MS/MS runs = 18).  
127 Peptide mixtures were concentrated and washed with an enrichment column and then fractionated over a  
128 250 min gradient on a C18 reverse phase column. The instrument was set up in a data-dependent MS/MS  
129 mode, with a full-scan spectrum followed by tandem mass spectra, selecting peptide ions as the three most  
130 intense peaks of the previous scan. ProteinLynx software (Version 2.2.5), was used to produce the peak  
131 lists as pkl files.

132

#### 133 **2.4. Protein identification and differential proteomic analysis by label-free quantitation**

134 The Q-TOF peak lists were analyzed by Proteome Discoverer software (version 1.4; Thermo Scientific),  
135 after conversion into MGF files. Technical replicates were processed as merged, generating one list of  
136 identified proteins for each biological sample by the Proteome Discoverer Daemon utility. The workflow  
137 was made up of the following nodes (and respective parameters). Spectrum Selector for spectra pre-  
138 processing (precursor mass range: 350–5000 Da; S/N Threshold: 1.5), Sequest-HT as search engine  
139 (Protein Database: Chordata sequences from UniProtKB; Enzyme: Trypsin; Max. missed cleavage sites: 2;  
140 Peptide length range 5–50 amino acids; Max. Delta Cn: 0.05; Precursor mass tolerance: 50 ppm; Fragment  
141 mass tolerance 0.4 Da; Static modification: cysteine carbamidomethylation; Dynamic modification:  
142 methionine oxidation), and Percolator for peptide validation (FDR < 1% based on peptide q-value) (Choi  
143 and Nesvizhskii, 2008; Käll et al., 2009, 2008; Spivak et al., 2009). In order to estimate the extent of  
144 differential protein abundance among sample groups, the Normalized Spectral Abundance Factor (NSAF)  
145 was calculated for each protein according to Zybaylov et al. (Zybaylov et al., 2006) as follows:

146  $NSAF = SAF_i / \sum_{i=1}^N SAF_i$ , where subscript  $i$  indicates a protein identity,  $N$  represents the total number of  
147 proteins, and  $SAF$  is a protein spectral abundance factor (protein spectral counts divided by its length).

148 Finally, the NSAF log ratio ( $R_{NSAF}$ ) was calculated as follows:  $R_{NSAF} = \log_2(NSAF_x + CF)/(NSAF_y + CF)$ ,  
149 where  $NSAF_x$  and  $NSAF_y$  are the summed NSAF values for each protein in sample groups to be compared  
150 ( $x = t1$  or  $t2$ ;  $y = t0$  or  $t1$ , respectively) and  $CF$  is a correction factor, empirically set to 2 (Tanca et al.,



151 2015, 2012) . Statistical significance of differential protein abundance was further assessed by applying the  
152 Student's *t*-test (two-sample comparison,  $p < 0.05$ ) on logarithmic NSAF values, after replacing missing  
153 values with 0.1 (empirically determined as in Zybailov et al., 2006) and corrected by using false discovery  
154 rate (FDR) as a multiple hypothesis testing, with  $FDR < 0.1$  as a threshold limit. Only proteins with  $R_{NSAF} >$   
155  $0.5$  or  $< -0.5$  were considered. The mass spectrometry proteomics data have been deposited to the  
156 ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD011059  
157 (Deutsch et al., 2017; Sanchez et al., 2015; Vizcaíno et al., 2016) and are reported in Ghisaura et al., 2019.

158

### 159 **2.5. Multivariate data statistical analysis**

160 Multivariate statistical data analyses (MVDA) were made using SIMCA-P 13.0 version (Umetrics, Inc.,  
161 Kinnelon, NJ). Prior to analysis, NSAF values were subjected to log transformation and Pareto scaling. For  
162 multivariate preliminary inspection, an unsupervised principal component analysis (PCA) was performed,  
163 followed by supervised OPLS-DA (orthogonal partial-least square discriminant analysis) to display as score  
164 plot the NSAF clustering, according to each temperature variation. Goodness of all MVA models was  
165 evaluated by the cumulative  $R^2(\text{cum})$  and the predictive  $R^2Y(\text{cum})$  and  $Q^2(\text{cum})$  parameters, calculated  
166 according to the cross-validation method. In particular,  $R^2Y(\text{cum})$  is defined as the proportion of variance in  
167 the data explained by the models and indicates the goodness of fit, whereas  $Q^2(\text{cum})$  is defined as the  
168 proportion of variance in the data predictable by the model. Both  $R^2Y(\text{cum})$  and  $Q^2(\text{cum})$  vary between 0  
169 and 1: a good prediction model is indicated by  $Q^2(\text{cum}) > 0.5$ , whereas a  $Q^2(\text{cum}) > 0.8-0.9$  means an  
170 excellent predictive ability of the model; for  $Q^2(\text{cum})$  values close to 0.5, no statistical group separation  
171 between observed clusters was considered (Westerhuis et al., 2008).

172

### 173 **2.6. Pathway analysis**

174 Gene ontology and protein annotations were retrieved from UniProtKB (<http://www.uniprot.org>). The  
175 uncharacterized sequences were blasted on NCBI non-redundant database:  
176 (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to find the homologous proteins. For pathway analysis two online  
177 software packages were used: STRING version 10 (Search Tool for the Retrieval of Interacting  
178 Genes/Proteins; <http://string-db.org>) (Szklarczyk et al., 2017) and the online software package IPA (version

179 9.0; Ingenuity Systems, Redwood City, CA). For STRING, an enrichment analysis was performed with all  
180 differentially expressed proteins, and KEGG pathways, GO Biological Processes and GO Molecular  
181 Functions implemented in the web platform were investigated by using the *Danio rerio* as organism model.  
182 Only the pathways and molecular networks displaying an FDR < 0.05 were considered as significantly  
183 enriched in the protein list and were considered for further analyses. For IPA analysis, since the software  
184 operates on a database built on the literature generated for humans and rodents, fish UniProt IDs were  
185 replaced with the closest mouse (*Mus musculus*) protein equivalents to enable a wider knowledge-based  
186 investigation of pathways as previously described (Addis et al., 2011; Ghisaura et al., 2014; Terova et al.,  
187 2014), being this a larger and better investigated database. The list of protein identifications (IDs), with  
188 their respective  $R_{NSAF}$  and p values (< 0.05) were used for the gene ontology analysis. Diseases and  
189 functions were specifically considered to focus on the physiological and health state of the fish liver during  
190 cold treatment.

191

### 192 3. Results and Discussion

193

#### 194 3.1. Cooling phase

195 A preliminary evaluation of experimental NSAF data with PCA indicated that liver proteomes at t1 were  
196 clearly separated from those at t0 (**Figure S1**). This result was confirmed by a further supervised approach  
197 based on OPLS-DA (**Figure 1a**). Indeed, the OPLS-DA model showed a very good fit parameter ( $R^2Y(cum)$   
198 = 0.819) and a fairly good prediction ability ( $Q^2(cum) = 0.828$ ), as shown by the model validation results  
199 (**Figure 1**).

200 A total of 42 proteins showed statistically significant differences in abundance at t1 vs t0 ( $R_{NSAF} > 0.5$  or <  
201  $-0.5$ ,  $p < 0.05$ , FDR < 0.1) and are listed in **Table 1**. According to STRING, numerous metabolic pathways  
202 were affected, as reported in **Table S1**. KEGG pathways were mainly related to carbon metabolism such as  
203 amino acid metabolism, including the phenylalanine, tyrosine and cysteine and methionine metabolisms.  
204 Other general pathways that encompass metabolic pathways and carbon metabolism were also statistically  
205 significant (FDR < 0.05). Amino acid metabolism plays an important role in fish metabolism for protein  
206 synthesis, glucose formation, and energy. For example, Costas et al, (2011) observed increased levels of

207 amino acids and other metabolites during long term feed deprivation in Senegalese sole fish, and amino acids  
208 are considered the major source of energy in this carnivorous fish species. Feed deprivation led to active  
209 gluconeogenic active processes in the liver supported by proteolysis in 21 days feed-deprived sole,  
210 suggesting that amino acids are employed as a carbon source for gluconeogenesis for the maintenance of  
211 plasma glucose levels. Phenylalanine and tyrosine can influence pigmentation, development, feed intake,  
212 growth performance, immunity, and survival of fish in the natural environment. Tyrosine synthesized from  
213 the essential amino acid phenylalanine is a precursor for important hormones and neurotransmitters,  
214 including thyroid hormones that play an important role next to energy metabolism and protein synthesis  
215 (Jasour et al., 2017; Li et al., 2009).

216 According to IPA, increased proteins were mainly associated to cellular stress and to protein and lipid  
217 degradation processes, while decreased proteins were mostly related to protein synthesis, actin-binding  
218 activity, amino acid metabolism, and protection from oxidative stress (**Table S2**). Several enzymes included  
219 in amino acid metabolism showed significant changes. An increase in fumarylacetoacetase and a reduction in  
220 4-hydroxyphenylpyruvate dioxygenase were observed (**Table 1**), both enzymes being involved in the  
221 catabolism of phenylalanine and tyrosine. Downregulation of phenylalanine and tyrosine catabolism has  
222 been associated to liver damage (Richard et al., 2016), confirming the key role of these amino acids in the  
223 physiological response of sea bream to cold challenge. Catabolism of specific amino acids, including  
224 tyrosine and phenylalanine through homogentisate 1,2-dioxygenase, was also observed to be reduced under  
225 cold stress by Ibarz and coworkers (2010a). This has been associated to the possible entrance of tyrosine and  
226 phenylalanine in the TCA cycle to produce energy. Likely, it has been suggested that high dietary  
227 availability of amino acids, methyl donors (betaine, choline) and cofactors (folate) supports the flux toward  
228 the methionine cycle thus favoring optimal homeostasis, helping fish to cope with exogenous stress and  
229 finally improving feed performance (Richard et al., 2016). Amino acids can be considered an important  
230 glucogenic source in fish. In this regard, high inclusion levels of feathermeal in feed have been correlated  
231 with hepatic levels of leucine, isoleucine, tyrosine, valine, methionine, arginine, and phenylalanine involved  
232 in energy metabolism. Moreover, an increased concentration of these amino acids in the liver was observed  
233 in the same study, indicating their inhibition from entering the TCA metabolic pathway to generate energy  
234 (Jasour et al., 2017). Formimidoyltransferase-cyclodeaminase was also increased. This enzyme is involved in

235 the sub-pathway that synthesizes glutamate by means of histidine degradation and tetrahydrofolate  
236 conversion in the pathway of one carbon metabolism. Thioredoxin and metallothionein were reduced. These  
237 proteins act as primary liver defense under oxidant attack at low temperatures. Their reduction might be  
238 associated to loss of hepatic functionality under cold stress (liver failure), confirming that oxidative stress  
239 and amino acid metabolisms are the pathways mainly affected by cold. Previous results by Ibarz et al.  
240 (2010a) are in line with our findings. In their proteomic study on gilthead sea bream liver under acute cold  
241 challenge, they identified oxidative stress, amino acid metabolisms and carbohydrate metabolism as the most  
242 perturbed pathways.

243 Actin-related proteins such as cofilin-2 were reduced during water cooling. Similarly, Ibarz and coworkers  
244 (2010a) found decreased actin levels in cold-stressed fish. Both alpha and beta tubulins, the components of  
245 cytoskeletal microtubules, were increased, as already observed in previous studies on cold challenged  
246 gilthead sea breams. The same authors suggested that tubulin has a protective effect against cold stress, but  
247 the exact mechanisms are still under study. The evident increase seen in proteasome-associated proteins,  
248 together with the observed changes in cytoskeletal proteins, might suggest the occurrence of tissue  
249 remodeling processes induced by thermal stress. A reduction in heart-like FABP or FABP3 was observed  
250 (**Table 1**). Mininni and coworkers (2014) reported several changes in FABP isoforms according to  
251 transcriptomics, but did not report changes in FABP3.

252 Several “diseases and functions” categories were also significant according to IPA (**Table S2**). These results,  
253 although obtained by comparison of literature based on mouse, support the finding that the most relevant  
254 changes in liver metabolism occurred during the cooling phase. As summarized in **Table S2**, it is worth  
255 noting that several significantly modified proteins were involved in cellular growth and proliferation,  
256 inflammatory response, and infectious diseases. This may suggest some similarity with the well-known  
257 effects of winter syndrome in gilthead sea bream (Ibarz et al., 2010b).

258

### 259 **3.2. Cold maintenance phase**

260 In the cold maintenance phase (constant 11°C, comparison t2 vs t1), liver proteins showed less marked  
261 changes than in the cooling phase. Consequently, the t2 and t1 sample clusters displayed a lower PCA  
262 separation (**Figure S1b**). Quantitative estimation of discriminative values of the model is given by lower

263 Q2(cum) index ( $Q2(\text{cum}) = 0.55$ ), close to the threshold limit for a biological model discrimination  
264 (Westerhuis et al., 2008). Similarly, the goodness of fit of the related OPLS-DA model led to a lower  
265 Q2(cum) value ( $Q2(\text{cum})_{\text{COLDMAINT}} = 0.69$ , **Figure 2**) when compared to the cooling phase ( $Q2(\text{cum})_{\text{COOLING}}$   
266  $= 0.83$ , **Figure 1**). These observations indicate a lower prediction ability of all the MVDA models associated  
267 to the cold maintenance phase vs the cooling phase, and suggest that t2 and t1 (that is, along cold  
268 maintenance) are more similar to each other (**Figure S1b**) than t0 and t1 (beginning and end of cooling  
269 phase).

270 In this case, only 24 significantly differential proteins were identified in all samples ( $R_{\text{NSAF}} > 0.5$  or  $< -0.5$ ,  $p <$   
271  $0.05$ ,  $\text{FDR} < 0.1$ ) and are listed in **Table 2**. Several proteins were associated to the methionine cycle, such as  
272 betaine-homocysteine-S-methyltransferase (BHMT). Interestingly, this protein underwent the most intense  
273 change in abundance observed in the whole study ( $R_{\text{NSAF}} = 2.96$ ). Other proteins with ribosomal activity,  
274 glycolytic and gluconeogenic function, were increased in t2 vs t1, whereas proteins associated to scavenger  
275 activity, migration and cellular organization, protein transport and proteolysis, adenosine and homocysteine  
276 synthesis were decreased (**Table S1**).

277 Enrichment analysis indicated the main KEGG pathways altered during the cold maintenance phase (**Table**  
278 **S1**). Among them, the categories metabolic pathways and glycolysis/gluconeogenesis had three molecules  
279 involved. Amino acid metabolisms, including alanine, aspartate and glutamate metabolism, cysteine and  
280 methionine metabolisms, were also significant, confirming the key role of energy metabolism and of the  
281 methionine cycle. KEGG pathways, GO Biological Processes, and GO Molecular functions are fully detailed  
282 in **Table S1**. Only two diseases and function features were associated to the cold maintenance phase (**Table**  
283 **S2**), supporting the above observations.

284 In a previous investigation on the metabolic response of sea breams to low water temperatures (Melis et al.,  
285 2017), liver gluconeogenesis was more affected than glycolysis during constant cold temperatures. In this  
286 study, an increased abundance of proteins implicated in carbohydrate metabolism was observed in the same  
287 conditions. Also, the observed perturbation of amino acid metabolism during the cold maintenance phase is  
288 confirmed by previous proteomic investigations (Richard et al., 2016). In particular, the methionine cycle  
289 and several related molecules (betaine, choline/phosphocholine and glutathione) were found to be affected  
290 during prolonged exposure to low temperatures. Several scientific reports on cold challenged gilthead sea

291 bream highlight a methionine cycle activation under stress conditions (Ibarz et al., 2010; Mininni et al.,  
292 2014; Richard et al., 2016). Amino acid interconversion and catabolism processes, often resulting in  
293 upregulation of BHMT, are likely triggered during cold stress to prevent hepatic accumulation of cytotoxic  
294 molecules such as homocysteine. However, although fast water temperature decrease such as 20°C to 8°C in  
295 3 days (Ibarz et al., 2010a) can lead to downregulation of BHMT, slower cooling conditions seem to  
296 consistently result in an increased expression. Our previous work (Melis et al., 2017), based on a first gradual  
297 temperature decrease followed by a cold maintenance phase, reached a similar conclusion, i.e. adjustments of  
298 methionine cycle metabolism are activated during prolonged stress while they are not observed during the  
299 first, more acute phases of temperature decrease. In this sense, BHMT appears as a key proteomic regulator  
300 throughout a persistent stressful condition, acting via antioxidant mechanisms by balancing S-adenosyl-  
301 methionine and preventing toxic homocysteine accumulation. The increase in BHMT was also associated to  
302 an unbalanced amino acid composition of diets leading to increased oxidative stress (Ghisaura et al., 2014),  
303 which suggests that BHMT is a good indicator related to general fish homeostasis. The changes in BHMT  
304 levels might therefore be related also to glutathione biosynthesis, reflecting a different extent of oxidative  
305 stress caused on hepatocytes by the different temperatures; more specifically, its increase in the cold  
306 maintenance phase (t2 vs t1) indicates that a higher oxidative stress is exerted by long exposure to winter  
307 temperatures (Ghisaura et al., 2014). Other amino acid degradation pathways and changes in proteins mainly  
308 implicated in stress response and cellular defense were represented by Adenosylhomocysteinase (AHCY or  
309 SAM) that is involved in the superpathway of methionine degradation, as well as in the methionine salvage  
310 pathway; specifically, it catalyzes the reversible hydrolysis of S-adenosylhomocysteine to adenosine and L-  
311 homocysteine and has a central role in the regulation of methyltransferase reactions, important for liver  
312 homeostasis maintenance (Ghisaura et al., 2014). As pointed out by Richard et al., (2016) its deficiency is  
313 usually associated with hepatic damages.

314 Increased purine metabolism also plays a crucial role in facing cold stress. Its involvement was observed in  
315 both muscle and liver of gilthead sea bream during cold maintenance phases by metabolomics (Melis et al.,  
316 2017) and was confirmed here by the observed increase in purine nucleoside phosphorylase-like (PNP). It  
317 was also postulated that the involvement of purine metabolism pathways in cold challenged gilthead sea

318 bream might be somewhat associated to other symptoms of the winter syndrome, such as skin pigmentation  
319 and immune suppression (Melis et al., 2017).

320

### 321 3.3. Overall changes

322 Proteomic profiles at the starting time (t0) were then compared with those at the end of the trial (t2) to gain a  
323 general overview of the changes occurring along prolonged thermal stress. A very clear separation of the two  
324 conditions was highlighted by the PCA score plot (**Figure S1c**) showing a  $Q2(\text{cum}) = 0.68$ . Furthermore,  
325 OPLS-DA score plot and related model validation (**Figure 3**) showed an excellent cluster separation with a  
326  $Q2(\text{cum})$  closer to 0.9 ( $Q2(\text{cum}) = 0.885$ ), which implies a higher goodness of discrimination with respect to  
327 both t1/t0 (**Figure 1**) and t2/t1 (**Figure 2**).

328 In this case, 59 proteins showed differences in abundance ( $R_{\text{NSAF}} > 0.5$  or  $< -0.5$ ,  $p < 0.05$ ,  $\text{FDR} < 0.1$ ) and  
329 are listed in **Table 3**. Proteins related to proteolysis processes, energy conversion, carbohydrate and amino  
330 acid metabolism and mitochondrial activity were increased, while those involved in fatty acid metabolism  
331 and amino acid conversion were decreased, as well as proteins associated to protection from oxidative stress  
332 and purine degradation (**Table S1**). Several stress-driven metabolic changes were observed. Abundance  
333 variations affected proteins involved in the metabolism of key amino acids (histidine, alanine, aspartate and  
334 glutamate; tyrosine, phenylalanine, arginine and proline; cysteine and methionine). Full KEGG pathways,  
335 GO Biological Processes and GO Molecular functions are detailed in **Table S1**. The categories pyruvate  
336 metabolism and starch and sucrose metabolisms were also involved. Moreover, five significantly increased  
337 diseases and functions categories were represented by inflammatory responses of the organism, generation of  
338 reactive oxygen species (and related free radical scavenging functions), liver necrosis, lipid metabolisms and  
339 their oxidation (functional to energy production) (**Table S2**).

340 The analysis of the overall cold challenge confirmed the observations done on the two separate phases  
341 (cooling and cold maintenance). Upon constant temperature decrease, proteins implicated in proteolysis,  
342 lipolysis, glycolysis and glycogenolysis, together with those implicated in amino acid metabolism, increased  
343 in abundance, possibly to intensify energy production during cold stress. In fact, as seen in many studies  
344 (Chang et al., 2018; Ibarz et al., 2010b, 2010a; Richard et al., 2016), there is a strong mobilization of  
345 extrahepatic fat deposits to liver and glycogen reserves. Amino acid degradation pathways (phenylalanine



346 and tyrosine catabolism) and changes in proteins mainly implicated in stress response and cellular defense  
347 were represented by a slightly lowered uricase (**Table 3**), that catalyzes the oxidation of uric acid to 5-  
348 hydroxyisourate and then to allantoin, a degradation product of purine nucleobases. **Table S2** reports  
349 diseases and functions categories affected by cold stress exposure. Cold mainly affected inflammatory and  
350 organ damage processes, oxidative stress response and tyrosine degradation. The involvement of this latter  
351 pathway was recently confirmed by our research group using  $^1\text{H}$  NMR-based metabolic fingerprinting (Melis  
352 et al., 2017) and by Richard and coworkers (Richard et al., 2016) who specifically described phenylalanine  
353 and tyrosine catabolism and their interconversion in the proteomic response of gilthead sea bream to low  
354 temperature.

355

#### 356 **4. Conclusions**

357 The protein makeup of sea bream liver undergoes several changes upon exposure to decreasing water  
358 temperature, suggesting the occurrence of a metabolic shift enabling adaptation to changed environmental  
359 conditions. This shift occurs mainly along temperature lowering in a cold adaptation phase. The maintenance  
360 of low but constant temperatures seems to affect protein levels to a lesser extent, although significant  
361 changes emerge also in this phase, such as in methionine metabolism. Gaining a greater knowledge of sea  
362 bream metabolic changes to cold adaptation might be of use to fish farmers for the development of specific  
363 aquaculture practices aimed at mitigating the negative effects of cold on fish growth, including the design of  
364 novel feed formulations for the winter season.

365

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373 **Table 1**

374

375 Sea bream liver proteins undergoing significant changes during the cooling phase (t1 vs t0).  $R_{NSAF} >$   
376 0.5 or  $< -0.5$ ; p value  $< 0.05$ ; FDR multiple comparison test  $< 0.1$ .

377

Accession number	Protein name	$R_{NSAF} t1/t0$
<i>Increased proteins</i>		
Q4RBW9	Proteasome subunit beta type-2	2.0501
B3F9U6	Hemoglobin beta chain	1.7291
Q1PCB2	Beta globin	1.6826
P86232	Ezrin (Fragments)	1.5108
P11748	Hemoglobin subunit alpha	1.3526
K7GAK5	Tubulin beta-7 chain	0.9969
Q4S3J3	GTP-binding nuclear protein Ran	0.8656
Q91060	Tubulin alpha chain	0.8281
M9P052	Lysosomal acid lipase	0.8276
Q4RVS0	ATP synthase F(0) complex subunit B1, mitochondrial	0.7537
L5M3T4	GTP-binding protein SAR1a	0.707
Q4S798	Nucleolin isoform X2 (Fragment)	0.6757
H2MYW8	Fumarylacetoacetase	0.6599
J7FII7	Glutathione S-transferase (Fragment)	0.6588
G9I0G6	Transferrin	0.6428
S4S3W7	Phosphoglucomutase 1 (Fragment)	0.5869
I3JSE9	Formimidoyltransferase-cyclodeaminase-like	0.5706
G1QD60	H3 histone (Fragment)	0.5705
H2LS09	Nucleolin isoform X1	0.5106
<i>Decreased proteins</i>		
Q0GPQ8	Cytochrome P450 2P11	-0.5457
A0A060VGE8	Cytochrome oxidase subunit II	-0.5506
W5LDH9	Uricase	-0.584
G3PTX7	Endoplasmic reticulum resident protein 27	-0.5926
H0YZD0	Electron transfer flavoprotein subunit alpha, mitochondrial	-0.6356
W5N925	Protein disulfide-isomerase (Fragment)	-0.6434
H2RKV3	Malic enzyme	-0.6595
M4AX90	Peroxisomal 2,4-dienoyl-CoA reductase-like	-0.6643
Q27HS3	Vascular smooth muscle alpha-actin (Fragment)	-0.6784
Q4RKE4	Fatty acid-binding protein, heart-like	-0.7223
Q8JHC5	Metallothionein (Fragment)	-0.7259
F1Q6E1	4-hydroxyphenylpyruvate dioxygenase	-0.7963
A0A060WA9	Adenosylhomocysteinase B	-0.8248
M4VQF0	Glyceraldehyde-3-phosphate dehydrogenase	-0.8662
M4AAN9	Phosphate carrier protein, mitochondrial-like isoform X1	-0.8739
F7DQ24	11-cis retinol dehydrogenase-like	-0.92
F7FYK5	40S ribosomal protein SA-like	-1.0889
A0A060YQH0	Aspartate aminotransferase, cytoplasmic-like	-1.1398
B5X8Y0	Cofilin-2	-1.2505
H2VEH5	Peptidyl-prolyl cis-trans isomerase	-1.2816
B9EN58	Thioredoxin	-1.841
G3HK42	60S ribosomal protein L30	-2.441
G3UYV7	40S ribosomal protein S28 (Fragment)	-2.7022

378

379 **Table 2**  
 380  
 381 Sea bream liver proteins undergoing significant changes during the maintenance phase (t2 vs t1).  
 382  $R_{NSAF} > 0.5$  or  $< -0.5$ ; p value  $< 0.05$ ; FDR multiple comparison test  $< 0.1$ .  
 383

Accession number	Protein name	$R_{NSAF}$ t2/t1
<i>Increased proteins</i>		
I3KAP1	Betaine-homocysteine S-methyltransferase 1-like	2.9636
E9QBF0	Triosephosphate isomerase	1.4764
M7BNB0	60S ribosomal protein L30	1.4457
P61155	40S ribosomal protein S19	1.4016
F6Q602	Probable imidazolonepropionase	1.3571
C1KBH6	Phosphoenolpyruvate carboxykinase	1.3021
H2L7M4	Keratin, type I cytoskeletal 18-like	1.1887
F1QXV8	Phosphoglycerate kinase	1.0446
W5LXZ1	Purine nucleoside phosphorylase-like (Fragment)	0.9842
H2MFC0	Obg-like ATPase 1	0.9796
M4ANE8	Glutamate dehydrogenase, mitochondrial-like	0.766
G3Q9K3	ATP synthase subunit gamma	0.7505
C3KIP4	Myosin light polypeptide	0.7423
B5X124	Deoxyribose-phosphate aldolase	0.6882
I3KYC9	Fumarate hydratase, mitochondrial-like	0.6618
F1R0A9	Glucose-6-phosphate translocase isoform X1	0.5549
<i>Decreased proteins</i>		
M9NZ74	94 kDa glucose-regulated protein	-0.5354
L5M3T4	GTP-binding protein SAR1a	-0.6744
Q4QY80	Elastase 4-like protein (Fragment)	-0.9622
H2UYH6	60S ribosomal protein L6-like	-1.0566
H3CCF6	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial	-1.1152
G5DYL0	Putative s-adenosylhomocysteine hydrolase (Fragment)	-1.294
H7C3T4	Peroxiredoxin-4 (Fragment)	-1.366
P86232	Ezrin	-1.4512

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389  
390**Table 3**Sea bream liver proteins undergoing significant changes along the whole trial (t2 vs t0).  $R_{NSAF} > 0.5$  or  $< -0.5$ ; p value  $< 0.05$ ; FDR multiple comparison test  $< 0.1$ .

Accession number	Protein name	$R_{NSAF}$ t2/t0
<i>Increased proteins</i>		
S7MY91	60S ribosomal protein L12	2.434
Q4RBW9	Proteasome subunit beta type-2	2.1625
Q1PCB2	Beta globin	1.8357
P56251	Hemoglobin subunit beta	1.6591
P11748	Hemoglobin subunit alpha	1.5843
E9QH32	Nucleoside diphosphate kinase	1.5778
M4ABN8	Mitochondrial pyruvate carrier 2-like	1.463
F2YLA1	Transferrin	1.1602
H2V638	Profilin	1.0975
H2U0L4	Mitochondrial 2-oxodicarboxylate carrier	1.0443
H0VMR9	Histone H2B	1.023
K7GAK5	Tubulin beta-7 chain	0.9934
E9QBF0	Triosephosphate isomerase	0.9793
M4AJN9	L-2-hydroxyglutarate dehydrogenase, mitochondrial-like	0.9785
B5XDR2	Inorganic pyrophosphatase 2, mitochondrial	0.7825
H2MWN8	Formimidoyltransferase-cyclodeaminase	0.7612
H2LS09	nucleolin isoform X1	0.7608
H2L7M4	Keratin, type I cytoskeletal 18-like	0.7579
C4PAW7	Microsomal epoxide hydrolase	0.7529
I3KYC9	Fumarate hydratase, mitochondrial-like	0.7459
I3JD93	40S ribosomal protein S25-like	0.718
M4ANE8	Glutamate dehydrogenase, mitochondrial-like	0.7107
A0A060Z139	Ubiquitin-like modifier-activating enzyme 1	0.6808
Q9HAP1	Valosin-containing protein (Fragment)	0.6602
F1R0A9	Glucose-6-phosphate translocase isoform X1	0.6311
H2MNV5	Tubulin beta-1 chain	0.6074
G3Q9Y8	Clathrin heavy chain 1 isoform X2	0.5594
Q4S798	nucleolin isoform X2	0.5317
F6Y7A5	Aldehyde dehydrogenase family 8 member A1 isoformX1	0.5194
K7FFD9	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial	0.5045
<i>Decreased proteins</i>		
E6ZHH2	Catechol-O-methyltransferase domain-containing protein 1	-0.5385
G3NRH9	Transketolase	-0.5529
H2RKV3	Malic enzyme	-0.5545
Q0GPQ8	Cytochrome P450 2P11	-0.5905
E9L835	Beta actin-1	-0.5967
H2L6X3	Urocanate hydratase	-0.6063
G3PTX7	Endoplasmic reticulum resident protein 27	-0.6394
D3TJK0	Alpha-amylase	-0.6405
W5N925	Protein disulfide-isomerase (Fragment)	-0.7119
M4AX90	Peroxisomal 2,4-dienoyl-CoA reductase-like	-0.7159
M9P0A8	Catalase	-0.7396
Q6USB8	Glutathione S-transferase (Fragment)	-0.8419
H2TY77	Carboxypeptidase A1-like	-0.8624
Q4QY80	Elastase 4-like protein (Fragment)	-0.8795

Q8JHC5	Metallothionein (Fragment)	-0.8796
F1Q6E1	4-hydroxyphenylpyruvate dioxygenase	-0.8941
M4AAN9	Phosphate carrier protein, mitochondrial-like isoform X1	-0.9372
K4GAL6	Adenosylhomocysteinase	-0.9525
F7DQ24	11-cis retinol dehydrogenase-like, partial	-0.9856
W5LDH9	Uricase	-1.0609
R0LYE9	Maleylacetoacetate isomerase (Fragment)	-1.0864
F7FYK5	40S ribosomal protein SA-like	-1.1605
B5X3S0	Estradiol 17-beta-dehydrogenase 12-B	-1.1615
M9P0N9	Heart-type fatty acid binding protein	-1.1928
A0A060YQH0	Aspartate aminotransferase, cytoplasmic-like	-1.2149
S9XSM0	Actin, cytoplasmic 2	-1.26
P81399	Fatty acid-binding protein 1, liver	-1.336
H2VEH5	Peptidyl-prolyl cis-trans isomerase	-1.3479
B5X8Y0	Cofilin-2	-1.7414

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391

392 **Figure captions**393 **Fig. 1.**

394 **OPLS-DA score plot based on the NSAF values of liver proteins observed during the cooling phase**  
395 **(t1/t0).** Cross-validation parameters R2X (cum), R2Y (cum) and Q2 (cum) are reported. The ellipse  
396 represents T2 Hotelling's plot with 95% confidence.

397

398 **Fig. 2.**

399 **OPLS-DA score plot based on the NSAF values of liver proteins observed during the cold maintenance**  
400 **phase (t2/t1).** Cross-validation parameters R2X (cum), R2Y (cum) and Q2 (cum) are reported. The ellipse  
401 represents T2 Hotelling's plots with 95% confidence.

402

403 **Fig. 3.**

404 **OPLS-DA score plot based on on the NSAF values of liver proteins undergoing abundance changes**  
405 **along the cooling trial (t2/t0).** Cross-validation parameters R2X (cum), R2Y (cum) and Q2 (cum) are  
406 reported. The ellipse represents T2 Hotelling's plots with 95% confidence.

407

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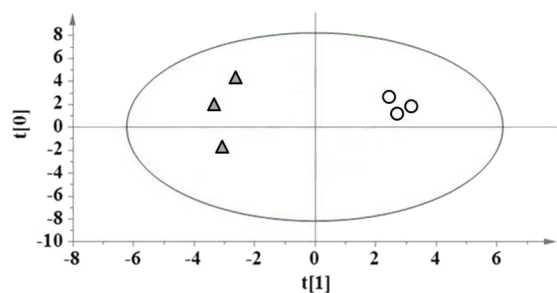


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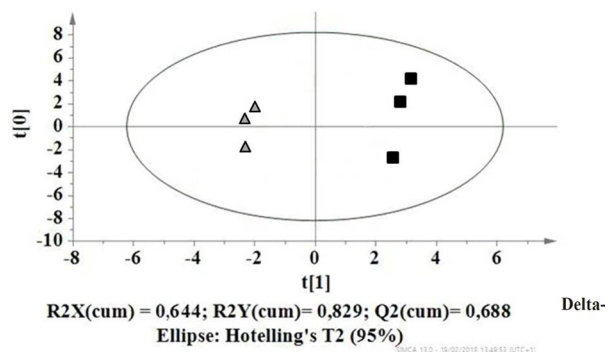
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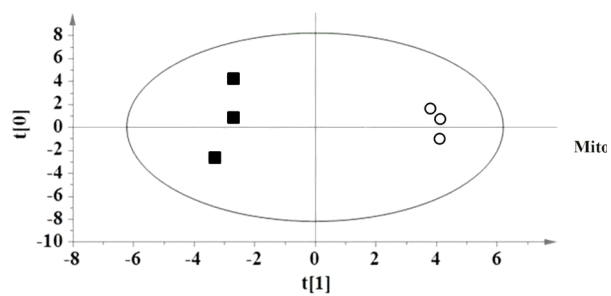
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R2X(cum) = 0,229; R2Y(cum) = 0,822; Q2(cum) = 0,885

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**Highlights**

- ✓ Exposure to cold temperature modifies the sea bream liver protein abundance profile
- ✓ Proteolysis and aminoacid catabolism are most affected during temperature decrease
- ✓ Methionine cycle and sugar metabolism are most affected upon prolonged cold stress
- ✓ Shotgun proteomics complements the data on hepatic metabolism changes following cold stress