Journal of Thermal Biology 82 (2019) 234–241

Contents lists available at ScienceDirect

Journal of Thermal Biology

journal homepage: www.elsevier.com/locate/jtherbio

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

S. Ghisaura[a,1], D. Pagnozzi[a,1], R. Melis[a], G. Biosa[a], H. Slawski[b], S. Uzzau[a,c], R. Anedda[a,**], M.F. Addis[a,d,*]

[a] Porto Conte Ricerche, Tramariglio, Alghero, Italy
[b] Aller Aqua, Christiansfeld, Denmark
[c] Department of Biomedical Sciences, University of Sassari, Italy
[d] Department of Veterinary Medicine, University of Milan, Italy

ARTICLE INFO

Keywords:
Cold stress
Winter syndrome
Gilthead sea bream
Liver proteins
Methionine
Shotgun proteomics

ABSTRACT

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

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD011059.

1. Introduction

Gilthead sea breams (*Sparus aurata, L.*) have their natural habitat in the Mediterranean Sea at water temperatures ranging seasonally from approximately 26 °C in summer to 11–13 °C or even lower in winter, depending on the specific environment. Wild sea breams manage this temperature decrease by migrating to deeper and warmer water (Davis, 1988). In fact, water temperatures below 13 °C lead to both behavioral (e.g., erratic swimming, voluntary fasting, hyposensitivity to stimuli) and physiological (e.g., impaired growth, fatty liver, tissue necrosis, infections) stressful changes that can ultimately lead to physiological dysfunction and death (Contessi et al., 2006; Gallardo et al., 2003; Ibarz et al., 2010b). However, farmed sea breams living in outdoor tanks and in floating sea cages are unable to avoid this thermal stress, and their prolonged exposure to temperatures below 13 °C causes decrease in activity (Ibarz et al., 2003), growth delay (Tort et al., 1998), metabolic depression (Ibarz et al., 2018; Sanahuja et al., 2019) and reduced feed consumption until total fasting when water temperatures fall below 10 °C (Ibarz et al., 2010b). Other physiological alterations include hepatic functionality, with the liver becoming steatosic and whitish due to a large deposition of lipids, as well as reduced efficiency of adaptive immunity with increased susceptibility to infections (winter syndrome or winter disease) and alteration of the main redox pathways (Abram et al., 2017; Ibarz et al., 2007, 2005; Sánchez-Nuño et al., 2018). These phenomena impact farming productions causing relevant economic losses with a consequent strong interest of fish farmers in finding efficient strategies for their reduction. One way for compensating thermal stress is represented by enhancing the nutritional state and metabolism

* Corresponding author. Department of Veterinary Medicine, University of Milan, Italy.
** Corresponding author. Porto Conte Ricerche, Tramariglio, Alghero, Italy.
E-mail addresses: anedda@portocontericerche.it (R. Anedda), filippa.addis@unimi.it (M.F. Addis).
[1] These authors contributed equally.

https://doi.org/10.1016/j.jtherbio.2019.04.005
Received 13 December 2018; Received in revised form 25 February 2019; Accepted 12 April 2019
Available online 17 April 2019
0306-4565/

through the use of feeds specifically designed for the colder season, and this requires understanding the consequences of cold on fish metabolism. To this aim, proteomic, metabolomic and transcriptomic approaches have been applied to farmed and wild fish biofluids and tissues, such as serum, liver, muscle and other organs, with differing degrees of success (Addis, 2013; Addis et al., 2010a, 2010b; Alves et al., 2010; Braceland et al., 2013; Brunt et al., 2008; Douxfils et al., 2011; Ghisaura et al., 2014; Martin et al., 2001; Melis et al., 2017; Mininni et al., 2014; Rodrigues et al., 2012). Proteomics offers several specific advantages when compared to genomic or transcriptomic strategies, including the ability to evaluate the actual extent of protein abundance, going beyond the estimates based on gene expression. In fact, proteins do not always follow a strict relationship with gene transcription but are regulated at the translational or post-translational level. Liver, the main metabolic organ of the body, has gained the greatest attention in proteomic studies evaluating the influence of farming practices on fish metabolism. Martin and coworkers (Martin et al., 2001) studied the changes occurring in the liver proteome as a consequence of different feeding regimens, including dietary plant protein substitution. Liver metabolism is considerably influenced also by other factors including environmental stress, and might be affected by xenobiotics and toxins (Addis, 2013; Ghisaura et al., 2014). Numerous research groups focused on the gilthead sea bream liver proteome to investigate a variety of stressful factors, ranging from handling and crowding (Alves et al., 2010) to the use of antiparasitic, or antibacterial agents and different environmental pollution (Isani et al., 2011; Kovacik et al., 2018; Varó et al., 2013). Proteomics has also been used to assess the impact of cold on fish metabolism (Ibarz et al., 2010a; Parrington and Coward, 2002; Vilhelmsson et al., 2007). However, these studies applied a gel-based approach (2D-gel electrophoresis, 1D GelC-MS/MS). With the aim of gathering additional information, we investigated the changes occurring in liver tissue by applying a shotgun proteomics workflow based on filter-aided sample preparation (FASP), tandem mass spectrometry (MS/MS), label-free quantitation and, finally, pathway analysis by means of STRING and Ingenuity Pathway Analysis (IPA). In fact, this approach provides a higher proteome coverage and is less affected by the typical limitations of gel-based studies, especially when 2D separation is involved (Westermeier et al., 2008), and additional or complementary information can be obtained in respect to previous studies. Liver proteome changes were assessed by mimicking the winter challenge conditions in a Recirculated Aquaculture System (RAS), both immediately after temperature reduction and during cold maintenance.

2. Materials and methods

2.1. Experimental design

A detailed description of the trial can be found in our previous work (Melis et al., 2017). For the purposes of both studies, a total of 60 juvenile gilthead sea breams with an average weight of 82.0 ± 4.5 g were uniformly distributed in three fiberglass tanks of 550 L with mechanical and biological filtration systems, a pumping system, a water thermoregulation system, and an automatic control for adjusting and monitoring the main physicochemical parameters. Fish were first acclimated for two weeks by linearly lowering water temperature from 20 °C to 18 °C (t0). Then, temperature was reduced at a rate of approximately 1 °C every Tuesday and Friday until reaching 11 °C (cooling phase, t0-t1, 4 weeks). Then, fish were maintained at 11 °C (cold maintenance phase, t1-t2) for the same time span (4 weeks). Fish were fed by hand, once a day, an experimental feed formulation (Aller Aqua, Christiansfeld, Denmark) with 43% protein and 14% fat. Feed ration was adjusted accounting for fish size, biomass and temperature (0.9 ± 0.03 g during cooling and 0.4 ± 0.03 g during cold maintenance). During the growth trial no mortality occurred, and fish never stopped eating below 13 °C. For the purposes of this study, at the beginning of the trial (t0) and at each time point (t1, t2), 9 fishes were anesthetized with 1,1,1-trichloro-2-methylpropan-2-ol (2% in marine water) and transferred in a mixture of marine water and ice (total number of sacrificed subjects = 27). Liver was excised from each fish, weighed and frozen rapidly in liquid nitrogen in Petri dishes as described by Melis and coworkers (2017).

2.2. Protein extraction and quantification

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

2.3. Shotgun proteomics

Three protein samples for each time point (t0, t1, t2), each constituted by a biological pool of three fish liver extracts, were used for shotgun proteomics analysis. Protein extracts (n = 9) were subjected to on-filter reduction, alkylation, and trypsin digestion according to the filter-aided sample preparation (FASP) protocol (Wiśniewski et al., 2009), with minor modifications (Ghisaura et al., 2016; Tanca et al., 2013) using Amicon Ultra-0.5 centrifugal filter units with Ultracel-10 membrane (Millipore, Billerica, MA, USA). Peptide mixture concentration was estimated by using BCA protein assay kit (Thermo Scientific - Rockford, IL). LC-MS/MS analyses were performed on a Q-TOF hybrid mass spectrometer with a nano lock Z spray source, coupled online with a NanoAcquity chromatography system (Waters) (Pagnozzi et al., 2014). Two technical replicates were analyzed for each biological pool (total LC-MS/MS runs = 18). Peptide mixtures were concentrated and washed with an enrichment column and then fractionated over a 250 min gradient on a C18 reverse phase column. The instrument was set up in a data-dependent MS/MS mode, with a full-scan spectrum followed by tandem mass spectra, selecting peptide ions as the three most intense peaks of the previous scan. ProteinLynx software (Version 2.2.5), was used to produce the peak lists as pkl files.

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

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

differential protein abundance among sample groups, the Normalized Spectral Abundance Factor (NSAF) was calculated for each protein according to Zybailov et al. (2006) as follows: NSAF = SAFi/$\sum_{i=1}^{N} SAFi$, where subscript i indicates a protein identity, N represents the total number of proteins, and SAF is a protein spectral abundance factor (protein spectral counts divided by its length). Finally, the NSAF log ratio (R_{NSAF}) was calculated as follows: $R_{NSAF} = \log_2(NSAF_x + CF)/(NSAF_y + CF)$, where $NSAF_x$ and $NSAF_y$ are the summed NSAF values for each protein in sample groups to be compared (x = t1 or t2; y = t0 or t1, respectively) and CF is a correction factor, empirically set to 2 (Tanca et al., 2015, 2012). Statistical significance of differential protein abundance was further assessed by applying the Student's *t*-test (two-sample comparison, $p < 0.05$) on logarithmic NSAF values, after replacing missing values with 0.1 (empirically determined as in Zybailov et al., 2006) and corrected by using false discovery rate (FDR) as a multiple hypothesis testing, with FDR < 0.1 as a threshold limit. Only proteins with $R_{NSAF} > 0.5$ or < -0.5 were considered. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD011059 (Deutsch et al., 2017; Sanchez et al., 2015; Vizcaíno et al., 2016) and are reported in Ghisaura et al. (2019).

2.5. Multivariate data statistical analysis

Multivariate statistical data analyses (MVDA) were made using SIMCA-P 13.0 version (Umetrics, Inc., Kinnelon, NJ). Prior to analysis, NSAF values were subjected to log transformation and Pareto scaling. For multivariate preliminary inspection, an unsupervised principal component analysis (PCA) was performed, followed by supervised OPLS-DA (orthogonal partial-least square discriminant analysis) to display as score plot the NSAF clustering, according to each temperature variation. Goodness of all MVA models was evaluated by the cumulative R2(cum) and the predictive R2Y(cum) and Q2(cum) parameters, calculated according to the cross-validation method. In particular, R2Y(cum) is defined as the proportion of variance in the data explained by the models and indicates the goodness of fit, whereas Q2(cum) is defined as the proportion of variance in the data predictable by the model. Both R2Y(cum) and Q2(cum) vary between 0 and 1: a good prediction model is indicated by Q2(cum) > 0.5, whereas a Q2(cum) > 0.8–0.9 means an excellent predictive ability of the model; for Q2(cum) values close to 0.5, no statistical group separation between observed clusters was considered (Westerhuis et al., 2008).

2.6. Pathway analysis

Gene ontology and protein annotations were retrieved from UniProtKB (http://www.uniprot.org). The uncharacterized sequences were blasted on NCBI non-redundant database (http://blast.ncbi.nlm.nih.gov/Blast.cgi): to find the homologous proteins. For pathway analysis two online software packages were used: STRING version 10 (Search Tool for the Retrieval of Interacting Genes/Proteins; http://string-db.org) (Szklarczyk et al., 2017) and the online software package IPA (version 9.0; Ingenuity Systems, Redwood City, CA). For STRING, an enrichment analysis was performed with all differentially expressed proteins, and KEGG pathways, GO Biological Processes and GO Molecular Functions implemented in the web platform were investigated by using the *Danio rerio* as organism model. Only the pathways and molecular networks displaying an FDR < 0.05 were considered as significantly enriched in the protein list and were considered for further analyses. For IPA analysis, since the software operates on a database built on the literature generated for humans and rodents, fish UniProt IDs were replaced with the closest mouse (*Mus musculus*) protein equivalents to enable a wider knowledge-based investigation of pathways as previously described (Addis et al., 2011; Ghisaura et al., 2014; Terova et al., 2014), being this a larger and better investigated database. The list of protein identifications (IDs), with their respective R_{NSAF} and p values (< 0.05) were used for the gene ontology analysis. Diseases and functions were specifically considered to focus on the physiological and health state of the fish liver during cold treatment.

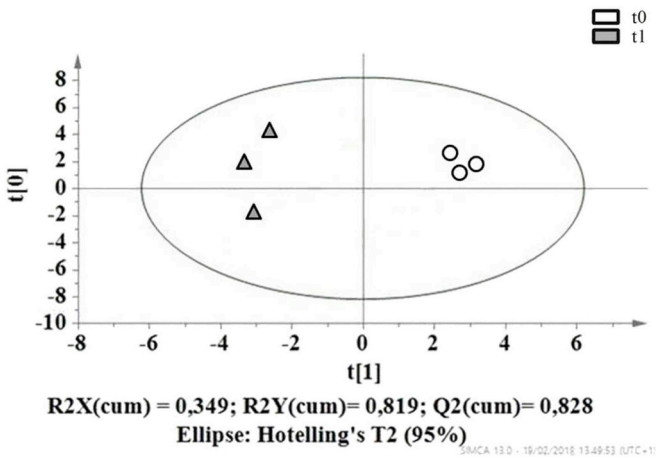

Fig. 1. OPLS-DA score plot based on the NSAF values of liver proteins observed during the cooling phase (t1/t0). Cross-validation parameters R2X (cum), R2Y (cum) and Q2 (cum) are reported. The ellipse represents T2 Hotelling's plot with 95% confidence.

3. Results and discussion

3.1. Cooling phase

A preliminary evaluation of experimental NSAF data with PCA indicated that liver proteomes at t1 were clearly separated from those at t0 (Fig. S1). This result was confirmed by a further supervised approach based on OPLS-DA (Fig. 1a). Indeed, the OPLS-DA model showed a very good fit parameter (R2Y(cum) = 0.819) and a fairly good prediction ability (Q2(cum) = 0.828), as shown by the model validation results (Fig. 1).

A total of 42 proteins showed statistically significant differences in abundance at t1 *vs* t0 ($R_{NSAF} > 0.5$ or < -0.5, $p < 0.05$, FDR < 0.1) and are listed in Table 1. According to STRING, numerous metabolic pathways were affected, as reported in Table S1. KEGG pathways were mainly related to carbon metabolism such as amino acid metabolism, including the phenylalanine, tyrosine and cysteine and methionine metabolisms. Other general pathways that encompass metabolic pathways and carbon metabolism were also statistically significant (FDR < 0.05). Amino acid metabolism plays an important role in fish metabolism for protein synthesis, glucose formation, and energy. For example, Costas et al. (2011) observed increased levels of amino acids and other metabolites during long term feed deprivation in Senegalese sole fish, and amino acids are considered the major source of energy in this carnivorous fish species. Feed deprivation led to active gluconeogenic active processes in the liver supported by proteolysis in 21 days feed-deprived sole, suggesting that amino acids are employed as a carbon source for gluconeogenesis for the maintenance of plasma glucose levels. Phenylalanine and tyrosine can influence pigmentation, development, feed intake, growth performance, immunity, and survival of fish in the natural environment. Tyrosine synthesized from the essential amino acid phenylalanine is a precursor for important hormones and neurotransmitters, including thyroid hormones that play an important role next to energy metabolism and protein synthesis (Jasour et al., 2017; Li et al., 2009).

According to IPA, increased proteins were mainly associated to cellular stress and to protein and lipid degradation processes, while decreased proteins were mostly related to protein synthesis, actin-

Table 1
Sea bream liver proteins undergoing significant changes during the cooling phase (t1 *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} t1/t0
Increased proteins		
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
Decreased proteins		
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

binding activity, amino acid metabolism, and protection from oxidative stress (Table S2). Several enzymes included in amino acid metabolism showed significant changes. An increase in fumarylacetoacetase and a reduction in 4-hydroxyphenylpyruvate dioxygenase were observed (Table 1), both enzymes being involved in the catabolism of phenylalanine and tyrosine. Downregulation of phenylalanine and tyrosine catabolism has been associated to liver damage (Richard et al., 2016), confirming the key role of these amino acids in the physiological response of sea bream to cold challenge. Catabolism of specific amino acids, including tyrosine and phenylalanine through homogentisate 1,2-dioxygenase, was also observed to be reduced under cold stress by Ibarz and coworkers (2010a). This has been associated to the possible entrance of tyrosine and phenylalanine in the TCA cycle to produce energy. Likely, it has been suggested that high dietary availability of amino acids, methyl donors (betaine, choline) and cofactors (folate) supports the flux toward the methionine cycle thus favoring optimal homeostasis, helping fish to cope with exogenous stress and finally improving feed performance (Richard et al., 2016). Amino acids can be considered an important glucogenic source in fish. In this regard, high inclusion levels of feathermeal in feed have been correlated with hepatic levels of leucine, isoleucine, tyrosine, valine, methionine, arginine, and phenylalanine involved in energy metabolism. Moreover, an increased concentration of these amino acids in the liver was observed in the same study, indicating their inhibition from entering the TCA metabolic pathway to generate energy (Jasour et al., 2017). Formimidoyltransferase-cyclodeaminase was also increased. This enzyme is involved in the sub-pathway that synthesizes glutamate by means of histidine degradation and tetrahydrofolate conversion in the pathway of one carbon metabolism. Thioredoxin and metallothionein were reduced. These proteins act as primary liver defense under oxidant attack at low temperatures. Their reduction might be associated to loss of hepatic functionality under cold stress (liver failure), confirming that oxidative stress and amino acid metabolisms are the pathways mainly affected by cold. Previous results by Ibarz et al. (2010a) are in line with our findings. In their proteomic study on gilthead sea bream liver under acute cold challenge, they identified oxidative stress, amino acid metabolisms and carbohydrate metabolism as the most perturbed pathways.

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

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

3.2. Cold maintenance phase

In the cold maintenance phase (constant 11 °C, comparison t2 *vs* t1), liver proteins showed less marked changes than in the cooling phase. Consequently, the t2 and t1 sample clusters displayed a lower PCA separation (Fig. S1b). Quantitative estimation of discriminative values of the model is given by lower Q2(cum) index (Q2(cum) = 0.55), close to the threshold limit for a biological model discrimination (Westerhuis et al., 2008). Similarly, the goodness of fit of the related OPLS-DA model led to a lower Q2(cum) value ($Q2\ (cum)_{COLDMAINT} = 0.69$, Fig. 2) when compared to the cooling phase ($Q2(cum)_{COOLING} = 0.83$, Fig. 1). These observations indicate a lower prediction ability of all the MVDA models associated to the cold maintenance phase *vs* the cooling phase, and suggest that t2 and t1 (that is, along cold maintenance) are more similar to each other (Fig. S1b) than t0 and t1 (beginning and end of cooling phase).

In this case, only 24 significantly differential proteins were identified in all samples ($R_{NSAF} > 0.5$ or < -0.5, $p < 0.05$, FDR < 0.1) and are listed in Table 2. Several proteins were associated to the methionine cycle, such as betaine-homocysteine-S-methyltransferase (BHMT). Interestingly, this protein underwent the most intense change in abundance observed in the whole study ($R_{NSAF} = 2.96$). Other proteins with ribosomal activity, glycolytic and gluconeogenetic function, were increased in t2 vs t1, whereas proteins associated to scavenger

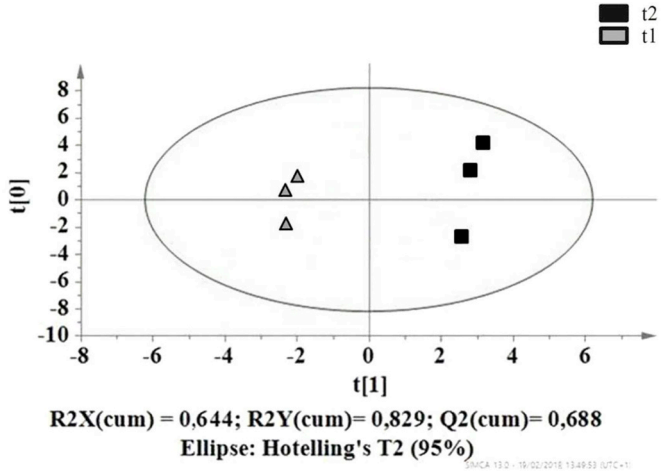

Fig. 2. OPLS-DA score plot based on the NSAF values of liver proteins observed during the cold maintenance phase (t2/t1). Cross-validation parameters R2X (cum), R2Y (cum) and Q2 (cum) are reported. The ellipse represents T2 Hotelling's plots with 95% confidence.

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

Accession number	Protein name	R_{NSAF} t2/t1
Increased proteins		
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
Decreased proteins		
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

activity, migration and cellular organization, protein transport and proteolysis, adenosine and homocysteine synthesis were decreased (Table S1).

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

In a previous investigation on the metabolic response of sea breams to low water temperatures (Melis et al., 2017), liver gluconeogenesis was more affected than glycolysis during constant cold temperatures. In this study, an increased abundance of proteins implicated in carbohydrate metabolism was observed in the same conditions. Also, the observed perturbation of amino acid metabolism during the cold maintenance phase is confirmed by previous proteomic investigations (Richard et al., 2016). In particular, the methionine cycle and several related molecules (betaine, choline/phosphocholine and glutathione) were found to be affected during prolonged exposure to low temperatures. Several scientific reports on cold challenged gilthead sea bream highlight a methionine cycle activation under stress conditions (Ibarz et al., 2010a,b; Mininni et al., 2014; Richard et al., 2016). Amino acid interconversion and catabolism processes, often resulting in upregulation of BHMT, are likely triggered during cold stress to prevent hepatic accumulation of cytotoxic molecules such as homocysteine. However, although fast water temperature decrease such as 20 °C–8 °C in 3 days (Ibarz et al., 2010a) can lead to downregulation of BHMT, slower cooling conditions seem to consistently result in an increased expression. Our previous work (Melis et al., 2017), based on a first gradual temperature decrease followed by a cold maintenance phase, reached a similar conclusion, i.e. adjustments of methionine cycle metabolism are activated during prolonged stress while they are not observed during the first, more acute phases of temperature decrease. In this sense, BHMT appears as a key proteomic regulator throughout a persistent stressful condition, acting via antioxidant mechanisms by balancing S-adenosyl-methionine and preventing toxic homocysteine accumulation. The increase in BHMT was also associated to an unbalanced amino acid composition of diets leading to increased oxidative stress (Ghisaura et al., 2014), which suggests that BHMT is a good indicator related to general fish homeostasis. The changes in BHMT levels might therefore be related also to glutathione biosynthesis, reflecting a different extent of oxidative stress caused on hepatocytes by the different temperatures; more specifically, its increase in the cold maintenance phase (t2 *vs* t1) indicates that a higher oxidative stress is exerted by long exposure to winter temperatures (Ghisaura et al., 2014). Other amino acid degradation pathways and changes in proteins mainly implicated in stress response and cellular defense were represented by Adenosylhomocysteinase (AHCY or SAM) that is involved in the superpathway of methionine degradation, as well as in the methionine salvage pathway; specifically, it catalyzes the reversible hydrolysis of S-adenosylhomocysteine to adenosine and L-homocysteine and has a central role in the regulation of methyltransferase reactions, important for liver homeostasis maintenance (Ghisaura et al., 2014). As pointed out by Richard et al. (2016) its deficiency is usually associated with hepatic damages.

Increased purine metabolism also plays a crucial role in facing cold stress. Its involvement was observed in both muscle and liver of gilthead sea bream during cold maintenance phases by metabolomics (Melis et al., 2017) and was confirmed here by the observed increase in purine nucleoside phosphorylase-like (PNP). It was also postulated that the involvement of purine metabolism pathways in cold challenged gilthead sea bream might be somewhat associated to other symptoms of the winter syndrome, such as skin pigmentation and immune suppression (Melis et al., 2017).

3.3. Overall changes

Proteomic profiles at the starting time (t0) were then compared with those at the end of the trial (t2) to gain a general overview of the changes occurring along prolonged thermal stress. A very clear separation of the two conditions was highlighted by the PCA score plot (Fig. S1c) showing a Q2(cum) = 0.68. Furthermore, OPLS-DA score

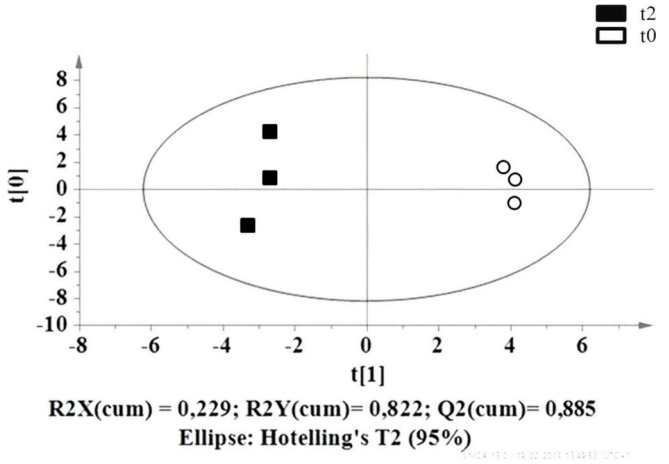

Fig. 3. OPLS-DA score plot based on the NSAF values of liver proteins undergoing abundance changes along the cooling trial (t2/t0). Cross-validation parameters R2X (cum), R2Y (cum) and Q2 (cum) are reported. The ellipse represents T2 Hotelling's plots with 95% confidence.

plot and related model validation (Fig. 3) showed an excellent cluster separation with a Q2(cum) closer to 0.9 (Q2(cum) = 0.885), which implies a higher goodness of discrimination with respect to both t1/t0 (Fig. 1) and t2/t1 (Fig. 2).

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

The analysis of the overall cold challenge confirmed the observations done on the two separate phases (cooling and cold maintenance). Upon constant temperature decrease, proteins implicated in proteolysis, lipolysis, glycolysis and glycogenolysis, together with those implicated in amino acid metabolism, increased in abundance, possibly to intensify energy production during cold stress. In fact, as seen in many studies (Chang et al., 2018; Ibarz et al., 2010b, 2010a; Richard et al., 2016), there is a strong mobilization of extrahepatic fat deposits to liver and glycogen reserves. Amino acid degradation pathways (phenylalanine and tyrosine catabolism) and changes in proteins mainly implicated in stress response and cellular defense were represented by a slightly lowered uricase (Table 3), that catalyzes the oxidation of uric acid to 5-hydroxyisourate and then to allantoin, a degradation product of purine nucleobases. Table S2 reports diseases and functions categories affected

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
Increased proteins		
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
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

by cold stress exposure. Cold mainly affected inflammatory and organ damage processes, oxidative stress response and tyrosine degradation. The involvement of this latter pathway was recently confirmed by our research group using ^{1}H NMR-based metabolic fingerprinting (Melis et al., 2017) and by Richard and coworkers (Richard et al., 2016) who specifically described phenylalanine and tyrosine catabolism and their interconversion in the proteomic response of gilthead sea bream to low temperature.

4. Conclusions

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

Acknowledgements

The PRIDE team is acknowledged for the support for MS data deposition into ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) (identifier PXD011059). This work has been funded by Sardinia Regional Government by means of Sardegna Ricerche (art. 26 L.R. 37/98). The authors are grateful to Dr. Roberto Cappuccinelli for conducting growth trials and Dr. Elia Bonaglini for technical assistance in growth experiments and during sampling. The constant and valuable support of Tonina Roggio is also gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2019.04.005.

References

Abram, Q.H., Dixon, B., Katzenback, B.A., 2017. Impacts of low temperature on the teleost immune system. Biology 6, 39. https://doi.org/10.3390/biology6040039.

Addis, M.F., 2013. Proteomics in foods. In: Toldrá, F., Nollet, L.M.L. (Eds.), Proteomics in Foods: Principles and Applications. Springer US, Boston, MA, pp. 181–203. https://doi.org/10.1007/978-1-4614-5626-1.

Addis, M.F., Cappuccinelli, R., Tedde, V., Pagnozzi, D., Porcu, M.C., Bonaglini, E., Roggio, T., Uzzau, S., 2010a. Proteomic analysis of muscle tissue from gilthead sea bream (Sparus aurata, L.) farmed in offshore floating cages. Aquaculture 309, 245–252. https://doi.org/10.1016/j.aquaculture.2010.08.022.

Addis, M.F., Cappuccinelli, R., Tedde, V., Pagnozzi, D., Viale, I., Meloni, M., Salati, F., Roggio, T., Uzzau, S., 2010b. Influence of Moraxella sp. colonization on the kidney proteome of farmed gilthead sea breams (Sparus aurata, L.). Proteome Sci. 8, 50. https://doi.org/10.1186/1477-5956-8-50.

Addis, M.F., Pisanu, S., Ghisaura, S., Pagnozzi, D., Marogna, G., Tanca, A., Biosa, G., Cacciotto, C., Alberti, A., Pittau, M., Roggio, T., Uzzau, S., 2011. Proteomics and pathway analyses of the milk fat globule in sheep naturally infected by Mycoplasma agalactiae provide indications of the in vivo response of the mammary epithelium to bacterial infection. Infect. Immun. 79, 3833–3845. https://doi.org/10.1128/IAI.00040-11.

Alves, R.N., Cordeiro, O., Silva, T.S., Richard, N., de Vareilles, M., Marino, G., Di Marco, P., Rodrigues, P.M., Conceição, L.E.C., 2010. Metabolic molecular indicators of chronic stress in gilthead seabream (Sparus aurata) using comparative proteomics. Aquaculture 299, 57–66. https://doi.org/10.1016/j.aquaculture.2009.11.014.

Braceland, M., Bickerdike, R., Tinsley, J., Cockerill, D., McLoughlin, M.F., Graham, D.a, Burchmore, R.J., Weir, W., Wallace, C., Eckersall, P.D., 2013. The serum proteome of Atlantic salmon, Salmo salar, during pancreas disease (PD) following infection with salmonid alphavirus subtype 3 (SAV3). J. Proteomics 94, 423–436. https://doi.org/10.1016/j.jprot.2013.10.016.

Brunt, J., Hansen, R., Jamieson, D.J., Austin, B., 2008. Proteomic analysis of rainbow trout (Oncorhynchus mykiss, Walbaum) serum after administration of probiotics in diets. Vet. Immunol. Immunopathol. 121, 199–205. https://doi.org/10.1016/j.vetimm.2007.09.010.

Chang, C.H., Huang, J.J., Yeh, C.Y., Tang, C.H., Hwang, L.Y., Lee, T.H., 2018. Salinity effects on strategies of glycogen utilization in livers of euryhaline milkfish (Chanos chanos) under hypothermal stress. Front. Physiol. 9, 81. https://doi.org/10.3389/fphys.2018.00081.

Choi, H., Nesvizhskii, A.I., 2008. False discovery rates and related statistical concepts in mass spectrometry-based proteomics. J. Proteome Res. 7, 47–50. https://doi.org/10.1021/pr700747q.

Contessi, B., Volpatti, D., Gusmani, L., Galeotti, M., 2006. Evaluation of immunological parameters in farmed gilthead sea bream, Sparus aurata L., before and during outbreaks of "winter syndrome. J. Fish Dis. 29, 683–690. https://doi.org/10.1111/j.1365-2761.2006.00765.x.

Costas, B., Aragão, C., Ruiz-Jarabo, I., Vargas-Chacoff, L., Arjona, F.J., Dinis, M.T., Mancera, J.M., Conceição, L.E.C., 2011. Feed deprivation in Senegalese sole (Solea senegalensis Kaup, 1858) juveniles: effects on blood plasma metabolites and free amino acid levels. Fish Physiol. Biochem. 37, 495–504. https://doi.org/10.1007/s10695-010-9451-2.

Davis, P.S., 1988. Two occurrences of the gilthead, sparus aurata linnaeus 1758, on the coast of northumberland, england. J. Fish Biol. 33 951–951. https://doi.org/10.1111/j.1095-8649.1988.tb05545.x.

Deutsch, E.W., Csordas, A., Sun, Z., Jarnuczak, A., Perez-Riverol, Y., Ternent, T., Campbell, D.S., Bernal-Llinares, M., Okuda, S., Kawano, S., Moritz, R.L., Carver, J.J., Wang, M., Ishihama, Y., Bandeira, N., Hermjakob, H., Vizcaíno, J.A., 2017. The ProteomeXchange consortium in 2017: supporting the cultural change in proteomics public data deposition. Nucleic Acids Res. 45, D1100–D1106. https://doi.org/10.1093/nar/gkw936.

Douxfils, J., Mathieu, C., Mandiki, S.N.M., Milla, S., Henrotte, E., Wang, N., Vandecan, M., Dieu, M., Dauchot, N., Pigneur, L.-M., Li, X., Rougeot, C., Mélard, C., Silvestre, F., Van Doninck, K., Raes, M., Kestemont, P., 2011. Physiological and proteomic evidences that domestication process differentially modulates the immune status of juvenile Eurasian perch (Perca fluviatilis) under chronic confinement stress. Fish Shellfish Immunol. 31, 1113–1121. https://doi.org/10.1016/j.fsi.2011.10.001.

Gallardo, M.Á., Sala-Rabanal, M., Ibarz, A., Padrós, F., Blasco, J., Fernández-Borràs, J., Sánchez, J., 2003. Functional alterations associated with "winter syndrome" in gilthead sea bream (Sparus aurata). Aquaculture 223, 15–27. https://doi.org/10.1016/S0044-8486(03)00164-9.

Ghisaura, S., Anedda, R., Pagnozzi, D., Biosa, G., Spada, S., Bonaglini, E., Cappuccinelli, R., Roggio, T., Uzzau, S., Addis, M.F., 2014. Impact of three commercial feed formulations on farmed gilthead sea bream (Sparus aurata, L.) metabolism as inferred from liver and blood serum proteomics. Proteome Sci. 12, 44. https://doi.org/10.1186/s12953-014-0044-3.

Ghisaura, S., Loi, B., Biosa, G., Baroli, M., Pagnozzi, D., Roggio, T., Uzzau, S., Anedda, R., Addis, M.F., 2016. Proteomic changes occurring along gonad maturation in the edible sea urchin Paracentrotus lividus. J. Proteomics 144, 63–72. https://doi.org/10.1016/j.jprot.2016.05.035.

Ghisaura, S., Melis, R., Biosa, G., Pagnozzi, D., Slawski, H., Uzzau, S., Anedda, R., Addis, M.F., 2019. Liver proteome dataset of Sparus aurata exposed to low temperatures. Data Br.

Ibarz, A., Beltrán, M., Fernández-Borràs, J., Gallardo, M.A., Sánchez, J., Blasco, J., 2007. Alterations in lipid metabolism and use of energy depots of gilthead sea bream (Sparus aurata) at low temperatures. Aquaculture 262, 470–480. https://doi.org/10.1016/j.aquaculture.2006.11.008.

Ibarz, A., Blasco, J., Beltrán, M., Gallardo, M.A., Sánchez, J., Sala, R., Fernández-Borràs, J., 2005. Cold-induced alterations on proximate composition and fatty acid profiles of several tissues in gilthead sea bream (Sparus aurata). Aquaculture 249, 477–486. https://doi.org/10.1016/j.aquaculture.2005.02.056.

Ibarz, A., Fernández-Borràs, J., Blasco, J., Gallardo, M.A., Sánchez, J., 2003. Oxygen consumption and feeding rates of gilthead sea bream (Sparus aurata) reveal lack of acclimation to cold. Fish Physiol. Biochem. 29, 313–321. https://doi.org/10.1007/s10695-004-3321-8.

Ibarz, A., Martín-Pérez, M., Blasco, J., Bellido, D., de Oliveira, E., Fernández-Borràs, J., 2010a. Gilthead sea bream liver proteome altered at low temperatures by oxidative stress. Proteomics 10, 963–975. https://doi.org/10.1002/pmic.200900528.

Ibarz, A., Padrós, F., Gallardo, M.Á., Fernández-Borràs, J., Blasco, J., Tort, L., 2010b. Low-temperature challenges to gilthead sea bream culture: review of cold-induced alterations and "Winter Syndrome. Rev. Fish Biol. Fish. 20, 539–556. https://doi.org/10.1007/s11160-010-9159-5.

Ibarz, A., Sanahuja, I., Özşahniğlu, I., Eroldogan, O., Sánchez-Nuño, S., Blasco, J., Guerreiro, P., Fontanillas, R., Fernández-Borràs, J., 2018. Cold-induced growth arrest in gilthead sea bream Sparus aurata: metabolic reorganisation and recovery. Aquac. Environ. Interact. 10, 511–528. https://doi.org/10.3354/aei00286.

Isani, G., Andreani, G., Carpenè, E., Di Molfetta, S., Eletto, D., Spisni, E., 2011. Effects of waterborne Cu exposure in gilthead sea bream (Sparus aurata): a proteomic approach. Fish Shellfish Immunol. 31, 1051–1058. https://doi.org/10.1016/j.fsi.2011.09.005.

Jasour, M.S., Wagner, L., Sundekilde, U.K., Larsen, B.K., Greco, I., Orlien, V., Olsen, K., Rasmussen, H.T., Hjermitslev, N.H., Hammershøj, M., Dalsgaard, A.J.T., Dalsgaard, T.K., 2017. A comprehensive approach to assess feathermeal as an alternative protein source in aquafeed. J. Agric. Food Chem. 65, 10673–10684. https://doi.org/10.1021/acs.jafc.7b04201.

Käll, L., Storey, J.D., MacCoss, M.J., Noble, W.S., 2008. Assigning significance to peptides identified by Tandem Mass Spectrometry using decoy databases. J. Proteome Res. 7, 29–34. https://doi.org/10.1021/pr700600n.

Käll, L., Storey, J.D., Noble, W.S., 2009. qvality: non-parametric estimation of q-values

and posterior error probabilities. Bioinformatics 25, 964–966. https://doi.org/10.1093/bioinformatics/btp021.

Kovacik, A., Tvrda, E., Miskeje, M., Arvay, J., Tomka, M., Zbynovska, K., Andreji, J., Hleba, L., Kovacikova, E., Fik, M., Cupka, P., Nahacky, J., Massanyi, P., 2018. Trace metals in the freshwater fish Cyprinus carpio: effect to serum biochemistry and oxidative status markers. Biol. Trace Elem. Res. Jul 2, 1–14. https://doi.org/10.1007/s12011-018-1415-x.

Li, P., Mai, K., Trushenski, J., Wu, G., 2009. New developments in fish amino acid nutrition: towards functional and environmentally oriented aquafeeds. Amino Acids 37, 43–53. https://doi.org/10.1007/s00726-008-0171-1.

Martin, S.A.M., Cash, P., Blaney, S., Houlihan, D.F., 2001. Proteome analysis of rainbow trout (Oncorhynchus mykiss) liver proteins during short term starvation. Fish Physiol. Biochem. 24, 259–270. https://doi.org/10.1023/A:1014015530045.

Melis, R., Sanna, R., Braca, A., Bonaglini, E., Cappuccinelli, R., Slawski, H., Roggio, T., Uzzau, S., Anedda, R., 2017. Molecular details on gilthead sea bream (Sparus aurata) sensitivity to low water temperatures from 1H NMR metabolomics. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 204, 129–136. https://doi.org/10.1016/j.cbpa.2016.11.010.

Mininni, A.N., Milan, M., Ferraresso, S., Petochi, T., Di Marco, P., Marino, G., Livi, S., Romualdi, C., Bargelloni, L., Patarnello, T., 2014. Liver transcriptome analysis in gilthead sea bream upon exposure to low temperature. BMC Genomics 15, 765. https://doi.org/10.1186/1471-2164-15-765.

Pagnozzi, D., Biosa, G., Addis, M.F., Mastrandrea, S., Masala, G., Uzzau, S., 2014. An easy and efficient method for native and immunoreactive Echinococcus granulosus antigen 5 enrichment from hydatid cyst fluid. PLoS One 9, e104962. https://doi.org/10.1371/journal.pone.0104962.

Parrington, J., Coward, K., 2002. Use of emerging genomic and proteomic technologies in fish physiology. Aquat. Living Resour. 15, 193–196. https://doi.org/10.1016/S0990-7440(02)01172-5.

Richard, N., Silva, T.S., Wulff, T., Schrama, D., Dias, J.P., Rodrigues, P.M.L., Conceição, L.E.C., 2016. Nutritional mitigation of winter thermal stress in gilthead seabream: associated metabolic pathways and potential indicators of nutritional state. J. Proteomics 142, 1–14. https://doi.org/10.1016/j.jprot.2016.04.037.

Rodrigues, P.M., Silva, T.S., Dias, J., Jessen, F., 2012. Proteomics in aquaculture: applications and trends. J. Proteomics 75, 4325–4345. https://doi.org/10.1016/j.jprot.2012.03.042.

Sanahuja, I., Fernández-Alacid, L., Sánchez-Nuño, S., Ordóñez-Grande, B., Ibarz, A., 2019. Chronic cold stress alters the skin mucus interactome in a temperate fish model. Front. Physiol. 9, 1916. https://doi.org/10.3389/fphys.2018.01916.

Sánchez-Nuño, S., Sanahuja, I., Fernández-Alacid, L., Ordóñez-Grande, B., Fontanillas, R., Fernández-Borràs, J., Blasco, J., Carbonell, T., Ibarz, A., 2018. Redox challenge in a cultured temperate marine species during low temperature and temperature recovery. Front. Physiol. 9, 923. https://doi.org/10.3389/fphys.2018.00923.

Sanchez, A., Reisinger, F., Hermjakob, H., Griss, J., Del-Toro, N., Dianes, J.A., Perez-Riverol, Y., Vizcaíno, J.A., Csordas, A., Eisenacher, M., Xu, Q.-W., Ternent, T., Wang, R., Uszkoreit, J., 2015. PRIDE Inspector Toolsuite: moving toward a universal visualization tool for proteomics data standard formats and quality assessment of ProteomeXchange datasets. Mol. Cell. Proteomics 15, 305–317. https://doi.org/10.1074/mcp.o115.050229.

Spivak, M., Weston, J., Bottou, L., Käll, L., Noble, W.S., 2009. Improvements to the Percolator algorithm for peptide identification from shotgun proteomics data sets. J. Proteome Res. 8, 3737–3745. https://doi.org/10.1021/pr801109k.

Szklarczyk, D., Morris, J.H., Cook, H., Kuhn, M., Wyder, S., Simonovic, M., Santos, A., Doncheva, N.T., Roth, A., Bork, P., Jensen, L.J., von Mering, C., 2017. The STRING database in 2017: quality-controlled protein–protein association networks, made broadly accessible. Nucleic Acids Res. 45, D362–D368. https://doi.org/10.1093/nar/gkw937.

Tanca, A., Biosa, G., Pagnozzi, D., Addis, M.F., Uzzau, S., 2013. Comparison of detergent-based sample preparation workflows for LTQ-Orbitrap analysis of the Escherichia coli proteome. Proteomics 13, 2597–2607. https://doi.org/10.1002/pmic.201200478.

Tanca, A., Pagnozzi, D., Burrai, G.P., Polinas, M., Uzzau, S., Antuofermo, E., Addis, M.F., 2012. Comparability of differential proteomics data generated from paired archival fresh-frozen and formalin-fixed samples by GeLC-MS/MS and spectral counting. J. Proteomics 77, 561–576. https://doi.org/10.1016/j.jprot.2012.09.033.

Tanca, A., Palomba, A., Pisanu, S., Addis, M.F., Uzzau, S., 2015. Enrichment or depletion? The impact of stool pretreatment on metaproteomic characterization of the human gut microbiota. Proteomics 15, 3474–3485. https://doi.org/doi:10.1002/pmic.201400573.

Terova, G., Pisanu, S., Roggio, T., Preziosa, E., Saroglia, M., Addis, M.F., 2014. Proteomic profiling of sea bass muscle by two-dimensional gel electrophoresis and tandem mass spectrometry. Fish Physiol. Biochem. 40, 311–322. https://doi.org/10.1007/s10695-013-9855-x.

Tort, L., Padrós, F., Rotllant, J., Crespo, S., 1998. Winter syndrome in the gilthead sea bream Sparus aurata. Immunological and histopathological features. Fish Shellfish Immunol. 8, 37–47. https://doi.org/10.1006/fsim.1997.0120.

Varó, I., Navarro, J.C., Rigos, G., Del Ramo, J., Calduch-Giner, J.A., Hernández, A., Pertusa, J., Torreblanca, A., 2013. Proteomic evaluation of potentiated sulfa treatment on gilthead sea bream (Sparus aurata L.) liver. Aquaculture 376, 36–44.

Vilhelmsson, O.T., Martin, S.A.M., Poli, B.M., Houlihan, D.F., 2007. Proteomics: methodology and application in fish processing. In: Food Biochemistry and Food Processing, pp. 401–422. https://doi.org/10.1002/9780470277577.ch18.

Vizcaíno, J.A., Csordas, A., Del-Toro, N., Dianes, J.A., Griss, J., Lavidas, I., Mayer, G., Perez-Riverol, Y., Reisinger, F., Ternent, T., Xu, Q.W., Wang, R., Hermjakob, H., 2016. 2016 update of the PRIDE database and its related tools. Nucleic Acids Res. 44, D447–D456. https://doi.org/10.1093/nar/gkv1145.

Westerhuis, J.A., Hoefsloot, H.C.J., Smit, S., Vis, D.J., Smilde, A.K., van Velzen, E.J.J., van Duijnhoven, J.P.M., van Dorsten, F.A., 2008. Assessment of PLSDA cross validation. Metabolomics 4, 81–89. https://doi.org/10.1007/s11306-007-0099-6.

Westermeier, R., Naven, T., Höpker, H.-R., 2008. Proteomics in Practice: A Guide to Successful Experimental Design, second ed. John Wiley & Sons, Ltd.

Wiśniewski, J.R., Zougman, A., Nagaraj, N., Mann, M., 2009. Universal sample preparation method for proteome analysis. Nat. Methods 6, 359–362. https://doi.org/10.1038/nmeth.1322.

Zybailov, B., Mosley, A.L., Sardiu, M.E., Coleman, M.K., Florens, L., Washburn, M.P., 2006. Statistical analysis of membrane proteome expression changes in Saccharomyces cerevisiae. J. Proteome Res. 5, 2339–2347. https://doi.org/10.1021/pr060161n.