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Oxidative attack during temperature fluctuation challenge compromises liver protein homeostasis of a temperate fish model

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Abstract

Seasonal variations in water temperature are a natural stressor of temperate fish that affect growth performance and metabolism globally. Gilthead sea bream is one of the most economically interesting species in the Mediterranean; but its liver metabolism is affected by the cold season. However, the effects of cold on protein turnover mechanisms have hardly been studied. Here, we study the relationship between liver oxidative status and protein homeostasis pathways during a 50-day low temperature period at 14°C, and subsequent recovery at two times: 7 days (early recovery) and 30 days (late recovery). Liver redox status was determined by measuring oxidised lipids and proteins, the glutathione redox cycle and major antioxidant enzymes activities. Protein turnover was analysed via liver protein expression of HSP70 and HSP90; proteasome 26S subunits and polyubiquitination, as markers of the ubiquitin–proteasome system (UPS); and cathepsin D, as a lysosomal protease. Low temperature exposure depressed antioxidant enzyme activities, affecting the glutathione redox cycle and reducing total glutathione levels. Both the UPS and lysosomal pathways were also depressed and consequently, oxidised protein accumulated in liver. Interestingly, both protein oxidation and polyubiquitination tagging depended on protein molecular weight. Despite all these alterations, temperature recovery reverted most consequences of the cold at different rates: with delayed recovery of total glutathione levels and oxidised protein degradation with respect to enzyme activities recovery. All these findings demonstrate that protein liver homeostasis is compromised after chronic cold exposure and could be the cause of liver affectations reported in aquaculture of temperate fish.

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Introduction

Protein homoeostasis is the fundamental condition which allows the preservation of all the functional cellular proteins in both terrestrial and marine habitats. In mammals, all cellular proteins undergo continuous synthesis and degradation thereby maintaining a functional proteome and allowing rapid changes in levels of specific proteins for regulatory purposes (Kaushik and Cuervo 2012). However, different intrinsic and extrinsic factors continuously damage proteins, mainly causing protein oxidation by oxidative stress (Chondrogianni et al. 2014). Thus, the study of affectation (enzymatic and non-enzymatic) of the antioxidant system by stressor conditions is crucial to understand protein susceptibility to oxidation. According to Benarroch (2011), in response to protein damage and/or misfolding due to oxidative stress or redox shift, heat shock proteins (HSPs) are initially activated because of their chaperone activity (Feder and Hofmann 1999; Iwama et al. 2004). Nevertheless, protein oxidation is very common and there are many potential sources of damage; and since the refolding capacity of HSPs is limited, proteins need to be repaired in order to avoid diseases and cellular death (Kassahn et al. 2009; Kaushik and Cuervo 2012; Chondrogianni et al. 2014). Protein degradation is a major intracellular function which is crucial for protein turnover and cellular viability. The ubiquitin-proteasome system (UPS) and the lysosome are two of the main proteolytic systems (Jung et al. 2009; Shang and Taylor 2011; Turk et al. 2012; Ciechanover 2012; Chondrogianni et al. 2014). The proteasome 26S is a large protein complex located in cytoplasm and nucleus. This complex is formed by a catalytic central structure (20S) with multiple protease functions, capped by two regulatory subunits (19S) that selectively control the entry of proteins (Glickman and Ciechanover 2002; Jung et al. 2009). To be recognised prior to entry in proteasome, proteins are tagged by ubiquitin: a small protein (Peters et al. 1994). The lysosomal system is considered to be a cellular organelle; it is also associated with autophagy processes (Mizushima 2007) and has an elevated non-specific protein degradation capacity as a result of the combined random and limited action of various proteases, with the cathepsin family being one of the most important in mammals (Kaushik and Cuervo 2012; Chondrogianni et al. 2014) and also in fish (Salmerón et al. 2015). If protein damage exceeds cellular repair capacity or major protein degradation pathways are affected, accumulation of large molecular protein aggregates and impaired protein functions have been reported which can compromise cell viability and lead to cell death (Chondrogianni et al. 2014; Galluzzi et al. 2016).

Fish, as poikilotherms, cannot regulate their body temperature and low seawater temperatures in winter can have negative effects on fish physiology. As a result, there is a

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significant reduction in aquaculture production of temperate fish, since fish growth is arrested in winter, and this could represent a critical production bottleneck and a threat to sustainability. Gilthead sea bream (*Sparus aurata*), a prominent species in Mediterranean aquaculture production, is an eurythermal fish species that tolerates temperatures as low as 5°C (Ravagnan 1978; Barnabé 1990). However, it is one of the marine species that is characterized by the cold growth arrest, resulting in a halt in aquaculture production for 3-4 months (reviewed in Ibarz et al. 2010b). Liver is one of the main targets of this cold challenge. Specifically it is being affected by lipid accumulation (Gallardo et al. 2003; Ibarz et al. 2007a) and oxidant insult (Viarengo et al. 1995; Abele and Puntarulo 2004; Ibarz et al. 2010a; Feidantsis et al. 2018; Sánchez-Nuño et al. 2018a), exhibiting a lack of capacity to compensate intermediary metabolism to low temperatures (Ibarz et al. 2003, 2007a, b; Sánchez-Nuño et al. 2018a). Whereas metabolic response of liver to cold have been extensively studied, it is little known regarding to protein refolding and degradation pathways in the liver of *Sparus aurata* during exposure to low temperature and subsequent recovery.

Recent studies indicate the presence of increased levels of liver lipoperoxidation and nitric oxide, as well as an increase of proteolysis after exposure to 8°C for just 15 days (Ibarz et al. 2010a). In seasonal studies in outdoor tanks with flow-through seawater (Richard et al. 2016) reported that the liver proteome intracellular oxidative stress is affected by the winter season (low temperature) in relation to disruption of endoplasmic reticulum protein folding. Moreover, transcriptome analysis revealed oxidative stress and protein denaturation in the liver leading to cell death after 21 days at 6.8°C (Mininni et al. 2014). However, few data exist on protein turnover in liver related to temperature challenges in gilthead sea bream. Kyprianou et al. (2010) reported increased HSP70 and HSP90 protein expression levels in liver after 10 days of *Sparus aurata* exposure to temperatures below 18°C. In a seasonal study in sea cages, Feidantsis et al. 2013 reported elevated heat shock response (HSR) after kinases (MAPK) and activation of mitogen-activated protein kinases during warming in spring. Regarding degradation pathways, Feidantsis et al. 2018 observed a gradual increase in ubiquitin conjugates when increasing water temperature and two peaks in the autophagy pathways at the lowest and highest water temperatures during seasonal temperature variations. Regarding to protein turnover, the vast majority of studies are performed in muscle, due to the interests of the farming industry. Salmerón et al. (2015) reported that in gilthead sea bream the main proteolytic mechanisms are regulated in a coordinate fashion during ontogeny and this controls muscle growth. Moreover, the diet composition and the feeding rate can modulate their expression. In rainbow trout (*Oncorhynchus mykiss*), fasting

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studies evidenced increased proteasome activity (Salem et al. 2007), whereas the mRNA expression of several UPS members was lower on re-feeding (Rescan et al. 2007; Seiliez et al. 2008). Also, mRNA expression of cathepsins B, D and S increased and decreased, during fasting and refeeding respectively (Rescan et al. 2007).

At view of the scarce information available on liver protein homeostasis in temperate fish in response to natural low temperatures, in the present study we aimed to determine the relationship between proteins damaged by oxidative stress and protein homeostasis pathways in liver during low temperatures and posterior recovery using a temperate fish model: the gilthead sea bream. To achieve this, we focused on: 1) the oxidative stress response, determining liver levels of oxidised lipids and oxidised proteins; 2) antioxidant liver capacity, measuring total glutathione (tGSH) as well as the oxidised and reduced forms (GSH and GSSG, respectively), and the activities of antioxidant enzyme superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GR); 3) refolding capacity, determining liver protein expression of HSP70, HSP90; and 4) the most relevant proteolytic pathways, such as the UPS, determining liver protein expression of 19S and 20S proteasome subunits as well as the polyubiquitination levels, and the liver lysosomal degradation by determining cathepsin D abundance.

Material and Methods

Animal maintenance and experimental procedure

Gilthead sea bream, 145 g average body weight, from a local fish farm were acclimated indoors at the *Centre d'Aquicultura* (CA-IRTA, Sant Carles de la Ràpita, Tarragona, Spain) at 22°C for two weeks using a standard commercial fish feed (Skretting_ARC). Following this period, the fish were randomly distributed in three 500 L fiberglass tanks (30 fish per tank) in a water recirculating system IRTAmar™, with monitoring of solid and biological filters, water temperature, and oxygen concentration. Furthermore, throughout the experimental period, nitrite, nitrate and ammonia concentrations were maintained at their initial values. Feed was automatically delivered for 30 min during each feeding session, at 8:00 am and 4:00 pm. The diet was provided by Skretting Aquaculture Research Centre (ARC) and contained 47% of crude protein with a 16% lipid fraction. Satiation was ensured throughout the experimental period by calculating estimated daily feed intake and allowing a ration 20% above this value. Feeding rate was recorded daily, and uneaten feed was collected daily 30 min after each meal ration following the specifications of IRTAmarTM's system, dried for 24h at 80°C and subsequently weighed to calculate actual feed intake.

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The experimental period (115 days) comprised three thermal periods: pre-cold (PC), during which the fish were maintained at 22°C for 30 days; cold (C), during which the temperature was cooled to 14°C over five days and then maintained at this temperature for a further 45 days (making a total of 50 days, including the cooling down period); and recovery, during which time the fish were warmed from 14°C to 22°C over five days. During recovery, two sampling points were established: early recovery (ER) on day 7 after starting recovery and late recovery (LR) on day 35. The low temperature of 14°C has been selected due to below it a threshold of cold tolerance has been described for gilthead sea bream (revised in Ibarz et al. 2010b).

Sampling

At the end of each period, the fish were fasted overnight before sampling. Nine fish (3 per replication tank) were captured at random and anesthetised with 2-phenoxyethanol (100 ppm) diluted in seawater. Body weight and length were measured, blood samples were taken from the caudal vessels, using EDTA-Li as an anticoagulant, and the fish were killed by severing the spinal cord. Plasma was obtained by centrifuging the blood at 13,000 g at 4°C for 5 min, and then kept at -80°C until analysis. Livers were removed and frozen in liquid nitrogen and kept at -80°C until homogenisation. The experimental procedures complied with the Guidelines of the Council of the European Union (86 / 609 / EU), the Spanish Government (RD 1201 / 2005) and the University of Barcelona (Spain) for the use of laboratory animals.

Redox Metabolites

Lipid peroxidation was analysed using the thiobarbituric reactive species (TBARS) assay and quantified by determining the concentration of the end reactive product malondialdehyde (MDA), according to Sánchez-Nuño et al. 2018b. Briefly, liver was homogenised in 10% (w/v) RIPA solution (50 M Tris (pH 7.4), 1% Triton X-100, 150 mM NaCl, 5 M NaF, 0.1% sodium dodecyl sulphate and 1% sodium deoxycholate) containing a commercial protease inhibitor cocktail. Suspensions were centrifuged at 2,000 g for 5 min, and the resulting pellet was discarded. Plasma samples for TBARS assays were pre-diluted in PBS (10% w/v). The formation of the MDA-TBA adduct was fluorometrically measured at an excitation wavelength of 515 nm and an emission wavelength of 550 nm. The calibration curve was determined with tetraethoxypropane (Slikker et al. 2001). Values are expressed as MDA in nanomoles per gram of total lipids in the plasma or per mg of fresh liver weight.

Advanced oxidised protein products (AOPPs) in liver homogenates were assayed using a modified version of Witko-Sarsat et al. 2003 adapted for fish in Sánchez-Nuño et al. 2018b.

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For sample preparation, the tissue was homogenised in PBS (10% w/v) and centrifuged at 5,000 g at 4°C for 10 min. Before testing, the liver samples were pre-diluted in PBS until a maximum concentration of 25 mg of protein per mL was obtained. AOPP formation was spectrophotometrically measured at 340 nm, involving a standard calibration curve using 100 µL of chloramine-T solution (0–100 µmol/L). AOPP concentration was expressed as chloramine-T nanomoles per mg of protein in liver.

Glutathione

Hepatic levels of reduced glutathione (GSH) and oxidised glutathione (GSSG) were determined using the procedure described by Hissin and Hilf 1976 and adapted for gilthead sea bream (Sánchez-Nuño et al. 2018b). Liver samples were homogenised in cold buffer containing 5 mM phosphate–EDTA buffer (pH 8.0) and 25% HPO_3 (3.75:1). The homogenates were ultracentrifuged at 100,000 g at 4°C for 30 min and the resulting supernatants were used to determine GSH and GSSG concentrations, using the fluorescent probe o-phthalaldehyde (OPA). GSSG was previously incubated with N-ethylmaleimide to avoid interference with GSH complexes. After 15 min of incubation, fluorescence was determined at an emission wavelength of 420 nm (excitation at 350 nm) and data are shown as GSH nanomoles or GSSG nanomoles per g of fresh weight and as total glutathione (tGSH = GSH + GSSG) and glutathione power (GSH/GSSG ratio).

Protein determination

The total amounts of proteins in liver were determined using the Bradford and Williams protein assay (Bradford and Williams 1976) adapted for a 96-well plate, with the Bradford reagent obtained from Bio-Rad (CA, USA) and using albumin to derive the standard curve.

Redox enzyme activities

Liver samples for enzyme analysis were pulverised in liquid nitrogen and homogenised with a 1:9 (w/v) cold buffer (100 mM Tris-HCl, 0.1 mM EDTA and 0.1% Triton X-100 (v:v), pH 7.8). The homogenates were centrifuged at 25,000 g at 4°C for 30 min. After centrifugation, supernatants containing the protein extract were aliquoted and kept at -80°C until analysis.

Superoxide dismutase (SOD, EC 1.15.1.1) hepatic activity was determined using the enzymatic method of McCord and Fridovich 1969 adapted for fish (Furné et al. 2009). A diluted aliquot was mixed with 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 1 mM cytochrome C, 1 mM xanthine, 0.5 IU/ml xanthine oxidase and sodium hydrosulphite. Enzyme activity was determined by the increasing optical density at 550 nm over subsequent

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180 s periods. Arbitrary units were defined as one activity unit corresponding to the amount of enzyme required to produce 50% inhibition of the ferricytochrome-C reduction rate.

Catalase (CAT, EC 1.11.1.6) hepatic activity was analysed following Aebi 1984 with minor modifications, measuring the decrease in the optical density of hydrogen peroxide (H_2O_2) at 240 nm. The medium used for reading CAT activity was 50 mM potassium phosphate buffer, pH 7.0, and a fresh solution of 10.6 mM H_2O_2.

Glutathione peroxidase (GPX, EC 1.11.1.9) activity was assayed by measuring the oxidation of NADPH at 340 nm (Bell et al. 1985). Measurements were performed in 50 mM potassium phosphate buffer (pH 7.2) containing 1 mM EDTA, 2 mM sodium azide, 0.5-1 U/ml glutathione reductase, 2 mM reduced glutathione and 0.1 mM NADPH.

Glutathione reductase (GR, EC 1.8.1.7) activity was measured by analysing NADPH oxidation at 340 nm (Carlberg and Mannervik 1985). Assays were performed in 0.1 M potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 0.63 mM NADPH and 3.25 mM oxidised glutathione.

All enzymatic analysis was performed in 96-well microplates at 25°C ± 0.5°C using a Tecan M200 spectrophotometer (Tecan Trading AG, Switzerland). All reagents, substrates, coenzymes and purified enzymes were from Sigma (USA) and Bio-Rad Laboratories, Inc. (USA). Except for SOD (as explained above), the enzymatic activities are shown as IU (CAT) or mIU (GPX and GR) per g of fresh liver weight, where one unit is defined as the amount of enzyme required to transform 1 µmol of the substrate per minute, under the assay conditions.

Western-Blot analyses

Whole liver cell lysates were prepared by homogenising the livers in: 50 mM Tris (pH 7.5), 1 mM EDTA, 5 mM MgCl2, 150 mM NaCl, supplemented with DTT, and protease inhibitors (final concentrations were 10 µg/mL aprotinin, 2 µg/mL pepstatin, 2 µg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The samples were then centrifuged at 12,000 g for 10 min and the protein concentration measured in the supernatant. Supernatants were treated with Laemmli loading buffer and 34 µg of proteins resolved on SDS-polyacrylamide (10%) gels and transferred to nitrocellulose. Membranes were then blocked for 1 h or overnight (depending on the antibody affinity) with 4% non-fat dry milk (BioRad) in Tris-buffered saline (TBS) (pH 7.4) containing 0.05% (w/v) Tween 20 (TTBS). Membranes were washed three times in TTBS and probed overnight or for 1 hour depending on the following primary antibodies: anti-HSP90, anti-HSP70, anti-4-hydroxynonenal or 4-HNE (Novus Biologicals), anti-ubiquitin (Sigma-

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Aldrich), anti-cathepsin D (Santa Cruz Biotechnology), anti-Rpt1 from 19S, and 20S proteasome core subunits (α5/α7, β1, β5, β5i, and β7) (Enzo life sciences). Detection was performed with anti-IgG-HRP for mouse, rabbit and goat (Santa Cruz Biotechnology). The blots were visualised with enhanced chemiluminescence (Bio-Rad) and scanned on a Fujifilm LAS-3000 Imager (Fujifilm Corporation, Tokyo, Japan). Digital images were quantified using Quantity One software (BioRad) and normalised by the total amount of protein detected by Ponceau S staining (Sigma-Aldrich).

Statistical analysis

Statistical differences between the different temperature periods for each diet were analysed by one-way nested analysis of variance (ANOVA), with tank as the random factor to test for any possible tank effect. When there was no tank effect, ANOVA followed by Tukey's or Dunnett's post-hoc test was performed when variances were uniform or not, respectively. To compare diets within each period, Student's t-tests were performed. Differences were considered statistically significant when the p values were less than 0.05. The Shapiro-Wilk test was used to ensure a normal distribution of the data; while the uniformity of the variances was determined by Levene's test. All statistical analysis was undertaken using commercial software (PASW version 20.0, SPSS Inc., Chicago, IL, USA).

Results

Effects of temperature fluctuation on body parameters

Body weight, body length and condition factor, as well as liver weight and hepatosomatic index (HSI) are summarised in Table 1. Body weight showed no differences between C sampling (day 80) and PC (day 30), thus evidencing cold-induced growth arrest. Concomitantly, higher liver weight and higher HSI were present during sustained cold. Both growth arrest and the higher HSI reverted at late recovery (LR).

Effects of temperature fluctuation on redox status

Oxidised lipids, oxidised proteins (AOPP), total glutathione (tGSH), reduced glutathione (GSH) and oxidised glutathione (GSSG), as well as the GSH/GSSG ratio, are also recorded in Table 1. Accumulation of conjugated proteins with 4-HNE as the major biomarker relating lipoperoxidation and protein oxidation, were also analysed by Western blot during the temperature fluctuation (Fig. 1A and Fig. 1B). Chronic low temperatures reduced total glutathione and unbalanced the glutathione redox ratio. Whereas cold exposure diminished oxidised lipids, oxidised proteins accumulated, significantly increasing 50% with respect to PC

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values (Table 1). The early days of temperature restoration to 22°C did not produce a glutathione ratio recovery, while higher hepatic AOPP levels were maintained and 4-HNE protein conjugates accumulated (Fig. 1A). During LR (tested after 30 days of recovery) only the glutathione ratio remained unbalanced. From Figure 1B, it is evident that the 4-HNE adduct accumulation pattern was not homogenously distributed, showing two major groups of proteins for HNE conjugation: between 37 and 50 kDa, and between 75 and 100 kDa. As the fate of 4-HNE protein conjugates is preferably the lysosomal degradation pathway, cathepsin-D abundance levels were also analysed across the temperature fluctuation (Fig. 1C). In response to low temperatures, cathepsin-D protein expression was drastically reduced and this was not reverted in ER. Moreover, this could be related to the increased HNE protein conjugate damage in this period. After 30 days of recovery, liver cathepsin-D protein expression had returned to PC values.

Antioxidant enzyme activities (SOD, CAT, GR and GPX) in response to temperature changes are shown in Figure 2. As a result of chronic thermal stress, CAT and GR maximal activities were reduced two folds. However, SOD did not change and only GPX increased to compensate for the low temperatures. Together with the lower amounts of tGSH, these results indicate that cold exposure reduced antioxidant protection in liver. Temperature recovery gradually restored CAT activity, but GR remained at cold levels and SOD activity was surprisingly reduced by 40%. As a result, the GR/GPX ratio, an indication of glutathione redox cycle quality, was reduced by half at low temperatures and did not recover after rewarming. These results suggest a major alteration due to low temperatures in the glutathione redox cycle, affecting the related enzymes GR and GPX, and resulting in a lower presence of total glutathione (GSH+GSSG). Although the amount of tGSH recovered during recovery, enzyme activities were not restored and both the GR/GPX and GSH/GSSG ratios were altered.

Liver protein expression of turnover-related pathways during thermal fluctuation

In this study, HSP70 and HSP90 liver accumulation were determined as they are related to refolding proteins with chaperone activity (Fig. 3A and 3B); the proteasome 19S regulatory (Rpt1-19S) and proteasome 20S (Core 20S) catalytic subunits were also determined as proteasomal activity (Fig. 3C and 3D). Chronic cold exposure did not modify HSP accumulation or proteasomal 19S or 20S subunit abundance, indicating the lack of adaptation (as they are not up-regulated) of chaperone protection and protein proteasomal turnover, respectively. Moreover, the ratio of 19S/20S (Fig. 3E), an indicator of adequate proteasome conformation, changed from 2 to 4-4.5 as a result of the thermal challenge, due to the reduction of 20S

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protein expression. These cold effects later reverted, but the ER period was not enough to achieve PC values. Figure 4 shows the polyubiquitination tagging of liver proteins through the thermal challenge. We determined the presence of 12 main polyubiquitinated bands which were present in all animals and samplings, to be further analysed. Whereas total levels of polyubiquitination (Fig. 4A) did not change during the temperature fluctuations, three specifics bands showed significant differences in their polyubiquitin-labelling. A high-molecular-weight band at 150 kDa showed double the amount of ubiquitination labelling at low temperatures, with respect to PC conditions (Fig. 4B). Two main bands of around 40 kDa and 30 kDa molecular weight showed lower labelling at low temperatures (Fig. 4C and 4D, respectively). ER did not revert these changes in the ubiquitination of these specific bands; and at LR, only the high-molecular-weight band showed a reversal of the cold affectation, while the 40 kDa and 30 kDa bands did not recover PC labelling values.

Discussion

Effects of sustained low temperatures on liver protein homeostasis

Redox status is defined as the balance between oxidants (or pro-oxidants) and antioxidants. When this equilibrium is disrupted, tilting the equilibrium toward an oxidised state, oxidative stress is produced. In the Figure 5 we have summarised all the observed alterations on liver redox balance of gilthead sea bream submitted to the thermal fluctuation resembling to natural cold season. An acute cold challenge in temperate fish could produce increased amounts of liver TBARS (Ibarz et al. 2010b) or increased liver AOPPs (Sánchez-Nuño et al. 2018b). The results concerning the impacts of low temperatures agree with previous results indicating accumulation of AOPPs in liver of mammals and fish after long term exposure at cold (Selman et al. 2002; Carbonell et al. 2016). Moreover, HNE appears to be the most toxic product of lipid peroxidation reported in mammals (Esterbauer and Cheeseman 1990; Pryor and Porter 1990), damaging proteins by adding covalent adducts and accelerating protein aggregation (Perluigi et al. 2012; Chondrogianni et al. 2014; Carbonell et al. 2016). Despite depression of the proteolytic systems, the accumulation of 4HNE cross-linked proteins does not seem to increase after 50 days of exposure to 14°C. In order to maintain low levels of oxidants, both enzymatic and non-enzymatic antioxidants are present in the liver of fish (Martínez-Álvarez et al. 2005). In vertebrates, the glutathione redox cycle is the main endogenous non-enzymatic antioxidant (Masella et al. 2005). In our study, tGSH was reduced 20% during exposure to cold, mainly due to a fall in reduced glutathione (GSH), as previously reported for gilthead sea bream (Sánchez-Nuño et al. 2018b). The activities of main

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antioxidant enzymes (SOD, CAT, GR and GPX) once again evidenced the lack of adaptation of liver enzyme activities, with CAT and GR being reduced by half whereas SOD and GPX did not increase. Lower levels of both non-enzymatic (glutathione) and enzymatic antioxidants during low temperatures diminished liver antioxidant defences and would be the origin of the accumulation of oxidised proteins in gilthead sea bream liver during cold exposure (see Figure 5).

As proteins are very sensitive to oxidation, several cellular pathways contribute in the repair and elimination of damaged proteins and thus prevent their accumulation and aggregation (Berlett and Stadtman 1997). The aggregation of proteins is associated with major pathologies that can lead to cell death. One of the first mechanisms to cope with protein damage is binding with HSPs. Few data exist on liver HSP protein expression in fish species, possibly due to the lower hepatic accumulation of these chaperones (mainly HSP70) compared with other tissues such as muscles and gills (Place and Hofmann 2005; Feidantsis et al. 2013). Our results showed that the exposure to 14°C did not result in changes in HSP70 or HSP90 liver abundance, thereby coinciding with a seasonal study performed in sea cages by Feidantsis et al. (2013), where no increase in either chaperone as reported, and there were even lower levels of HSP90. According to cold-induced hepatic depression (reviewed in Ibarz et al. 2010b), unaltered levels of chaperones would imply lower HSP refolding capacity or at least slow refolding activity.

When a protein is irremediably damaged, its fate is to be recycled via UPS degradation pathways, or to be removed/autophaged via a lysosomal degradation process. Protein degradation via UPS involves two discrete and successive steps (also included in the summary Figure 5 for this model of fish temperate species): tagging of the substrate protein by the covalent attachment of multiple ubiquitin molecules, and the subsequent degradation of the tagged protein by the 26S proteasome, composed of the catalytic 20S core and the 19S regulator (Hershko and Ciechanover 1998). The capacity to remove damaged proteins by the proteasome may prevent oxidative stress and it has been suggested that this is also part of the antioxidant defences (Jung et al. 2009). Moreover, in fish inhabiting permanently cold marine environments, this enzymatic complex could play a key role in antioxidant defence systems (Gogliettino et al. 2016). Here, we analysed both polyubiquitination and the function of the proteasomal subunits 20S and 19S in gilthead sea bream in response to low temperatures. With regard to specific degradation via the proteasomal complex, we determined that the classical value for the ratio of 19S/20S subunits of 2, forming the 26S proteasome (Peters et al. 1994), was also present in gilthead sea bream; but this was drastically modified in response to

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cold due to the reduced amount of the catalytic subunit 20S. Gogliettino et al. (2016) performed a comparative analysis in a variety of tissues collected from an Antarctic fish, *T. bernacchii* and a temperate fish, *Dicentrarchus labrax*, reporting increased efficiency of the degradation machinery in the cold-adapted fish. This was not the case of gilthead sea bream exposed to low temperatures, which manifested lower antioxidant capacity, higher oxidant attack on liver proteins, a lack of acclimation response of HSP chaperone activity and affected proteasome 26S formation. From our results it seems that the total amount of liver polyubiquitinated proteins, which are in the way to be drive to the proteasome degradation system, was not altered after 50 days of cold exposure. This result would be in agreement with those obtained from cultured sea bream during the cold season (Feidantsis et al. 2018). Elsewhere, it has been reported that marine fish adapted to polar temperatures exhibit high levels of ubiquitin-conjugated proteins (Todgham et al. 2007). In order to embrace the real scope of this process, we analysed the most important individual polyubiquitinated bands for the first time in fish. In view of our results, a different pattern of ubiquitination appears to exist in liver proteins in response to chronic low temperatures depending on the molecular weight, with high-molecular-weight proteins (150 kDa) being more polyubiquitinated. In contrast, low-molecular-weight proteins were significantly less tagged under cold conditions. To our knowledge, no literature on fish has addressed specific ubiquitination of proteome bands, and we believe that further studies are necessary to elucidate the relevance of higher labelling of higher-molecular-weight proteins. In mammals there is controversy concerning the relationship between protein mass and the rate of turnover; classically, it was suggested that high-molecular-weight proteins tended to be turned over more rapidly than low-molecular-weight proteins (Dice and Goldberg 1975). However, recent studies using dynamic labelling techniques, such as SILAC, have suggested that the degree of disorder in a protein is a dominant determinant of protein stability (Belle et al. 2006; Doherty et al. 2009).

Finally, in this study we analysed the lysosomal degradation pathways via cathepsin D protein expression: an aspartic peptidase classed in the pepsin family of proteases and considered a major lysosomal endopeptidase in the liver of many species, including fish (Brooks et al. 1997). The aggregation of proteins as a result of the accumulation of cross-linked 4-HNE proteins cannot be degraded through the UPS. In fact, some mammal pathologies are a consequence of the inhibition of the proteasome due to accumulation of 4-HNE protein aggregates. Due to its unlimited proteolytic capacity, the lysosome could even eliminate protein aggregates (Chondrogianni et al. 2014). Our results showed a drastic reduction in cathepsin D abundance in liver at the end of the cold period. In fish, the vast majority of

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studies of the lysosomal pathway have focused on muscle tissue and mostly study fasting conditions. Salmerón et al. (2015) in gilthead sea bream and Cassidy et al. 2018 in Artic charr reported higher expression of distinct cathepsins under fasting, including cathepsin D in muscle; while Guderley et al. (2003) reported higher cathepsin D enzymatic activity in cod and also in liver under fasting. Although, the induced cold temperatures provoked lower food intake in gilthead sea bream, even fasting below 13°C (reviewed in Ibarz et al. 2010b), the current study demonstrated that low temperatures drastically reduce cathepsin D activity and consequently, this liver proteolytic pathway.

Effects of temperature recovery on liver protein homeostasis

Previous studies in gilthead sea bream have highlighted the importance of focusing on the recovery mechanisms after the cold season (Tort et al. 2004; Ibarz et al. 2007a, 2010b). In previous work, we demonstrated that whereas liver intermediary metabolism is restored after the cold season (Sánchez-Nuño et al. 2018a), the recovery of antioxidant enzymatic mechanisms after 30 days at 22°C was delayed and glutathione redox status was highly affected (Sánchez-Nuño et al. 2018b). Coinciding with those previous results of ours, after 30 days of recovery, the antioxidant enzymatic activities were only partially restored, evidencing a delayed recovery (lower SOD and GR activities). The GR/GPX ratio remained altered as did the GSH/GSSG glutathione ratio, confirming the unbalanced situation and severe affectation of the glutathione redox cycle, despite the better recovery of tGSH and GSH reported in (Sánchez-Nuño et al. 2018b). These data confirm the delayed and incomplete recovery of liver antioxidant defences, and the susceptibility to oxidative attack during ER.

Regarding protein homeostasis, all the mechanisms seemed to have recovered at the end of recovery period. Since HSP70 and HSP90 abundance were not modified by cold, we assumed that their activity would only depend on temperature during warming. Curiously, the HSP family was first described as being protective against thermal stress due to a temperature increase. In our study, warming from 14°C to 22°C did not increase liver chaperones. In contrast, the red muscle, white muscle and hearth of gilthead sea bream under indoor conditions showed higher HSP70 and HSP90 accumulation at higher temperatures (Feidantsis et al. 2009); while in a seasonal experiment under aquaculture conditions, they reached their highest levels in early spring or peaked in May after the temperature increased (Feidantsis et al. 2013). However, the reduction of oxidised proteins seems to be time dependent, with seven days insufficient to revert the cold oxidative attack and damaged protein accumulation (both AOPPs and 4-HNE) in liver. In consonance with this, ubiquitin-tagged proteins were not

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observed to change during ER with respect C. At the end of the recovery time, ubiquitin tagging of high-molecular-weight proteins reverted, but fewer low-molecular-weight proteins were still destined to be recycled. Meanwhile Feidantsis et al. 2018, in a seasonal trial in cages, reported a gradual increase of total ubiquitin conjugates from April to June in heart and liver due to increased metabolic activity. Again, more studies will be necessary to understand better the specific pattern of polyubiquitination with respect to protein molecular weight. At each thermal period, the 4-HNE oxidised proteins were accumulated between 50 and 40 kDa and also between 100 and 75 kDa, suggesting a binding pattern of these adducts cross linked with proteins. Surprisingly, although it is not degraded by the proteasome, the 4-HNE weight distribution pattern coincides with the ubiquitinated bands that showed thermal differences. Aldehydic products such as MDA and HNE are relatively stable and are capable of roaming freely and attacking molecules, e.g. DNA, proteins, lipids far from their origin (Weber et al. 2013). In mammals, it has been reported that these aldehydic fragments modify proteins altering their function and they are also considered as cytotoxic second messengers of oxidative stress, which makes them highly utilised biomarkers in biological research (Uchida 2003; Zarkovic 2003). In fish, knowledge in this field is extremely limited and further studies are needed to confirm our preliminary results regarding cold alterations and which proteins are the main targets of oxidative attack. In parallel, proteasomal 20S subunit protein expression was depressed at ER and recovered at LR, restoring liver recycling capacity via the proteasome pathway and maybe reverting AOPP accumulation. Few data exist on proteasome protein expression in liver during warming in fish. Dorts et al. (2012) reported in sculpin fish, *Cottus gobio*, increased proteasome activity in liver and gill after exposure to elevated temperature and Lamarre et al. (2010) observed increased activity in liver of the wolffish (*Anarhichas minor)* acclimated to high temperatures. Finally, the lysosomal activity was also restored later (LR), with cathepsin D abundance recovering to PC values, probably enhancing the proteolysis and degradation of 4-HNE protein aggregates, as the reduction in 4-HNE labelled proteins evidenced at LR. In agreement with this, Feidantsis et al. (2018) reported that liver lysosomal autophagy activities follow seasonal dynamics, with the highest values in June after the temperature recovery. All these findings suggest that protein homeostasis is highly affected after cold, but this would be a transient condition for gilthead sea bream. However, the disturbances in protein turnover could affect the key functions of liver after the cold season and could be the basis of liver-associated pathologies in gilthead sea bream aquaculture (reviewed in Ibarz et al. 2010b).

Conclusions

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This study provides evidence in fish for the first time that the early stages of temperature warming after an extended period at low temperatures is a critical moment for liver protein homeostasis in temperate fish species (summarised in Figure 5). After metabolic cold depression, the glutathione redox cycle and both the proteasome and lysosomal proteolytic pathways were strongly compromised. As a consequence, liver accumulated oxidised proteins and protein aggregation (AOPPs and 4-HNE respectively). However, after 30 days of increased temperatures, proteolysis was recovering, reverting the cold-altered condition in liver (details in the graphical abstract). Surprisingly, the ubiquitination pattern showed dependence on molecular weight, thus being a potential marker for further studies to better understand protein half-life, the protein oxidation pattern or the tendencies of selective and controlled proteasome proteolysis under thermal fluctuations. Current work contributes to expanding the scarce knowledge in temperate fish species of the protein repairing/protection machinery and protein turnover in liver. All these results could improve our capacity to cope with the unproductive winter period in aquaculture conditions.

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Table 1. Morphometric parameters and liver redox metabolites of juvenile gilthead sea bream throughout temperature fluctuation.

	Pre-Cold (Day 30)	Cold (Day 80)	Early Recovery (Day 87)	Late Recovery (Day 115)
Morphometric parameters				
Body Weight	179.4 ± 5.5^{a}	205.2 ± 5.9^{ab}	216.3 ± 9.1^{b}	271.8 ± 8.5^{c}
Body Length	18.1 ± 0.1^{a}	19.6 ± 0.3^{b}	19.5 ± 0.2^{b}	21.4 ± 0.2^{c}
Condition Factor	3.0 ± 0.1^{a}	2.7 ± 0.1^{b}	2.9 ± 0.1^{ab}	2.8 ± 0.1^{ab}
Hepatosomatic Index	1.8 ± 0.2^{a}	2.4 ± 0.1^{b}	2.4 ± 0.1^{b}	1.2 ± 0.1^{c}
Liver redox metabolites				
Oxidised lipids	10.1 ± 2.8^{ab}	4.4 ± 0.7^{a}	4.9 ± 1.7^{a}	15.0 ± 4.1^{b}
Oxidised proteins	3.0 ± 0.2^{a}	4.6 ± 0.3^{b}	4.9 ± 0.8^{b}	2.6 ± 0.9^{ab}
Total GSH	1316.9 ± 87.2^{a}	975.3 ± 54.1^{b}	1134.3 ± 56.6^{ab}	1243.3 ± 52.8^{a}
GSH	1125.2 ± 72.1^{a}	855.0 ± 57.8^{b}	940.3 ± 47.1^{ab}	1025.4 ± 49.6^{ab}
GSSG	191.7 ± 18.5^{ab}	169.9 ± 13.3^{a}	194.0 ± 15.2^{ab}	217.8 ± 11.4^{b}
GSH/GSSG	6.1 ± 0.4^{a}	5.1 ± 0.3^{b}	5.0 ± 0.3^{b}	4.8 ± 0.3^{b}

Data are expressed as Mean ± S.E.M.

Significant groups ($p<0.05$, One-Way ANOVA) were indicated by different letters Growth parameters: Body weight (g), Body length (cm), Condition Factor (Body weight/Body lenght3x100), HSI = Hepatosomatic Index (Liver weight/Body weight);

Liver redox metabolites: Oxidised lipids (nmols MDA/mg fw), oxidised proteins (nmols chloramine-T/mg fw), total glutathione, GSH and GSSG (nmols/mg fw), GSH/GSSG (arbitrary units).

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Figure Legends

Figure 1. 4-HNE-protein conjugated labelling and Cathepsin D expression. Western blot was normalised by the total protein amount with Ponceau staining. Data are shown as arbitrary units as mean ± standard error of mean. A) Total 4-HNE-protein labelling, B) Western blot example of 4-HNE labelling distribution in MWs ranges. C) Cathepsin-D liver expression; Pre-Cold (PC), data obtained from day 30 of the pre-cold period at 22°C; Cold (C), data obtained after 50 days at 14°C (day 80); Early and Late Recovery (ER and LR respectively), data obtained 7 and 35 days after temperature restoration to 22°C (day 87 and 115), respectively. Significant groups ($p<0.05$, One-Way ANOVA) were indicated by different letters.

Figure 2. Activity of redox-associated enzymes throughout the study period: A) SOD, B) CAT, C) GR, D) GPX, and E) GR/GPX ratio). Values are mean ± standard error of the mean. PRE-COLD, data obtained from day 30 of the pre-cold period at 22°C; COLD, data obtained after 50 days at 14°C (day 80); EARLY and LATE RECOVERY, data obtained 7 and 35 days after temperature restoration to 22°C (day 87 and 115), respectively. Significant groups ($p<0.05$, One-Way ANOVA) were indicated by different letters.

Figure 3. Expression of chaperone HSPs, and ubiquitin-proteasome system (UPS). Western blot was normalised by the total protein amount with Ponceau staining. Data are shown as arbitrary units as mean ± standard error of mean. A) HSP70; B) HSP90; C) Rpt1 19S proteasome regulatory subunit; D) 20S proteasome core; E) 19S/20S ratio. Pre-Cold (PC), data obtained from day 30 of the pre-cold period at 22°C; Cold (C), data obtained after 50 days at 14°C (day 80); Early and Late Recovery (ER and LR respectively), data obtained 7 and 35 days after temperature restoration to 22°C (day 87 and 115), respectively. Significant groups ($p<0.05$, One-Way ANOVA) were indicated by different letters.

Figure 4. Expression of protein ubiquitination labelling in liver during thermal fluctuation. Total ubiquitination as well as the 12 major bands were analysed (arrows indicate the analysed bands). Total ubiquitin and each selected band of the Western blot were normalised by the total protein amount with Ponceau staining. Data are shown as arbitrary units as mean ± standard error of mean. A) Total ubiquitin; B) Ubiquitinated 150KDa band; C) Ubiquitinated 40KDa band; D) Ubiquitinated 30KDa band. Pre-Cold (PC), data obtained from day 30 of the pre-cold period at 22°C; Cold (C), data obtained after 50 days at 14°C (day 80); Early and Late Recovery (ER and LR respectively), data obtained 7 and 35 days after temperature restoration to 22°C (day 87 and 115), respectively. Significant groups ($p<0.05$, One-Way ANOVA) were indicated by different letters.

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Figure 5. Summary of liver redox balance of gilthead sea bream submitted to low temperatures and recovery challenge. Explanation is provided in the Discussion section.

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Highlights

- Antioxidants defenses and glutathione levels were compromised after 50 days at 14°C
- Temperature recovery highly depressed proteasome 26S and lysosome pathways
- Depressed proteolytic pathways in liver allowed oxidized protein accumulation
- After 30 days of temperature recovery, proteolytic pathways expression was restored
- Ubiquitination patterns were molecular weight dependent at every thermal period

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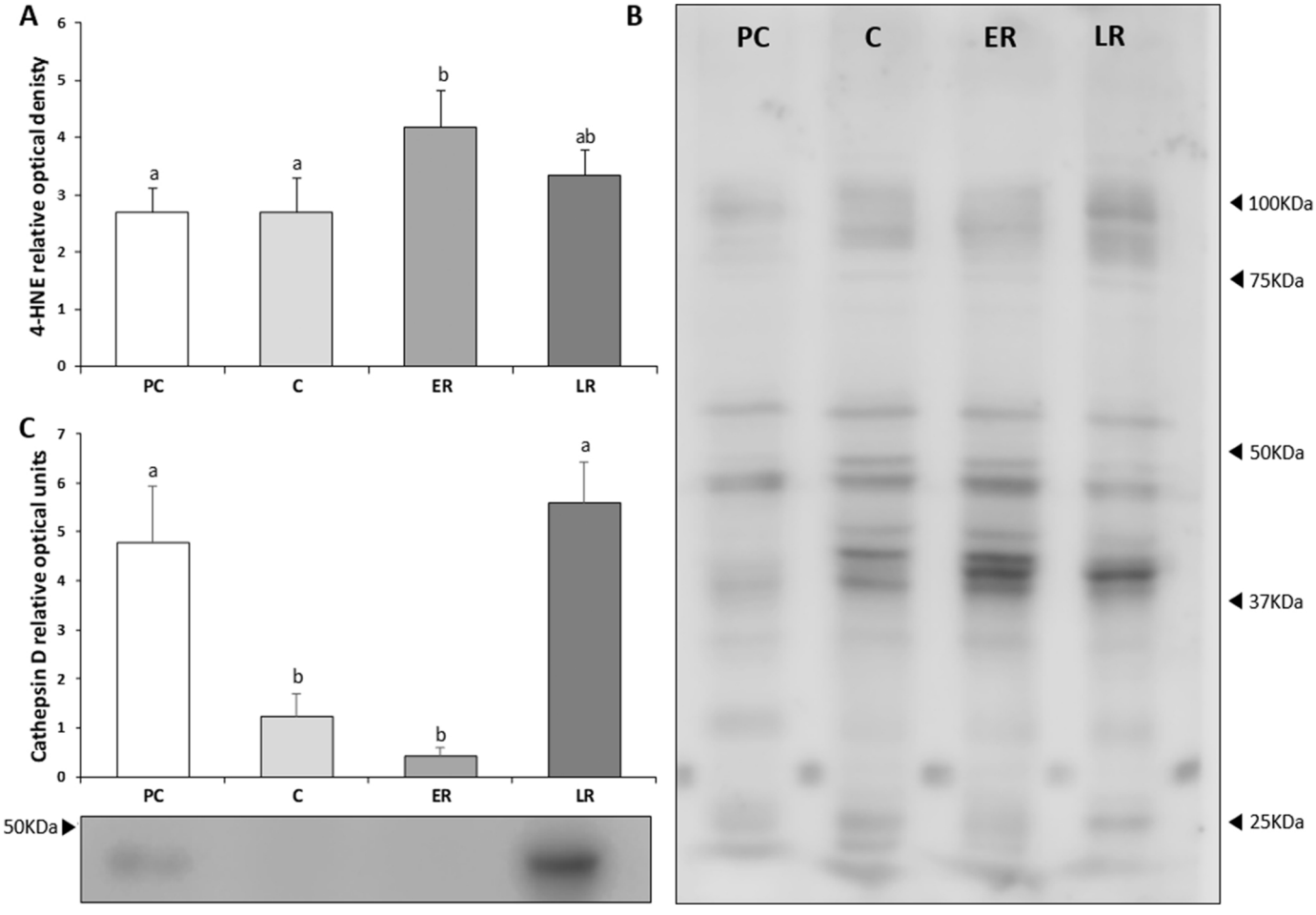
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Figure 1

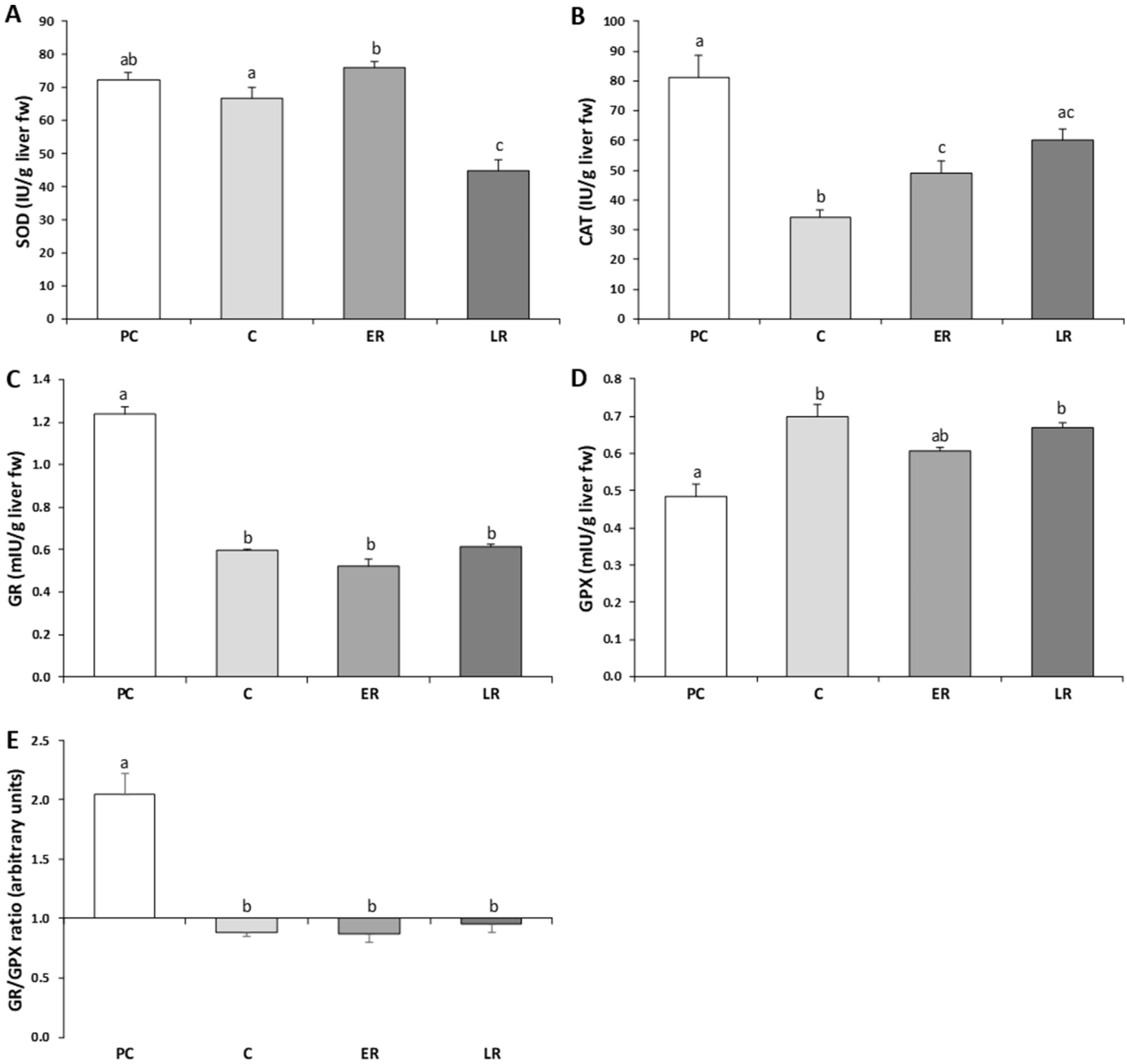

A
SOD (IU/g liver fw)
90
80
70
60
50
40
30
20
10
0
ab
a
b
c
PC
C
ER
LR
B
CAT (IU/g liver fw)
100
90
80
70
60
50
40
30
20
10
0
a
b
c
ac
PC
C
ER
LR
C
GR (mIU/g liver fw)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
b
b
PC
C
ER
LR
D
GPX (mIU/g liver fw)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
b
ab
b
PC
C
ER
LR
E
GR/GPX ratio (arbitrary units)
2.5
2.0
1.5
1.0
0.5
0.0
a
b
b
b
PC
C
ER
LR

Figure 2

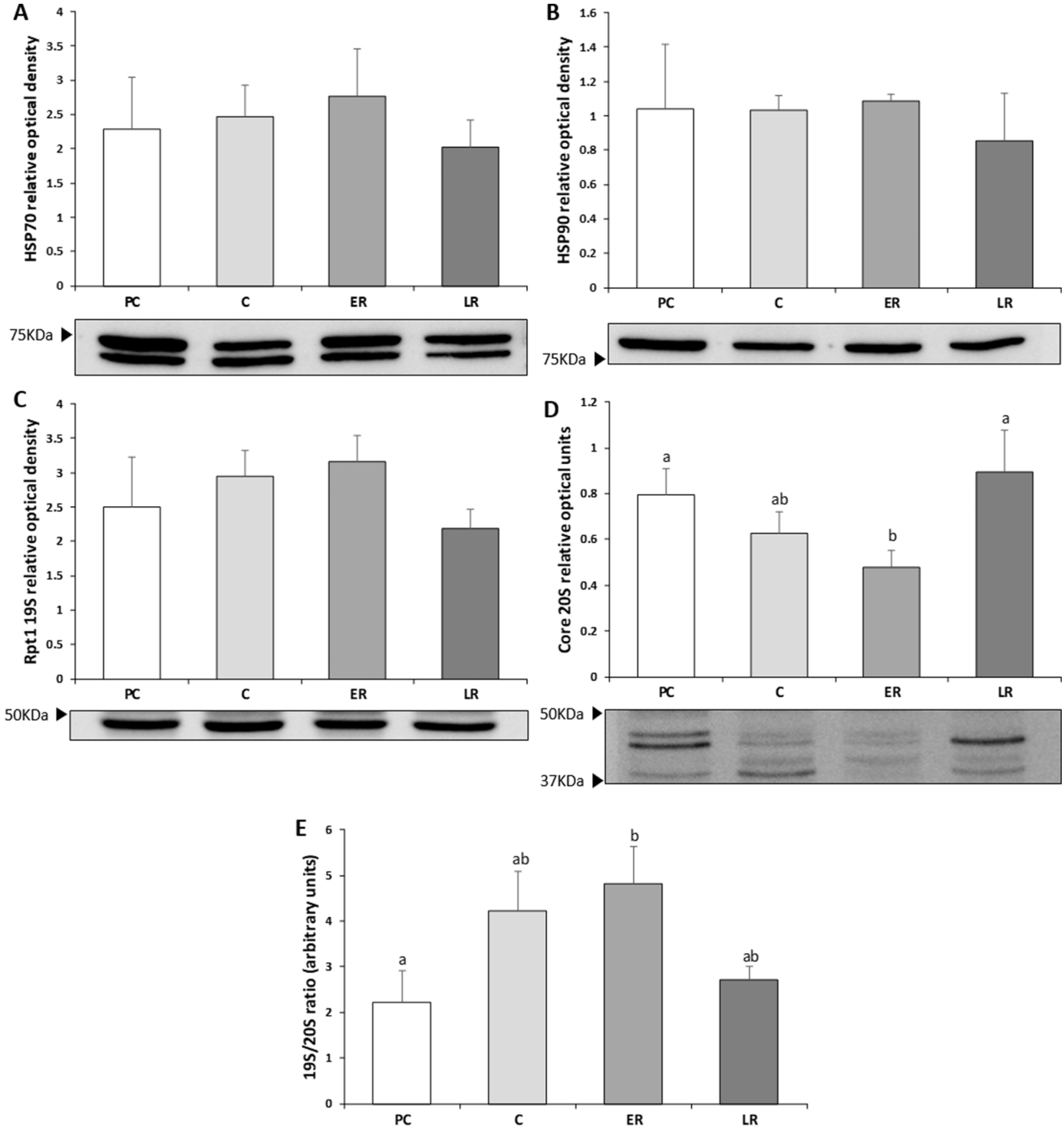
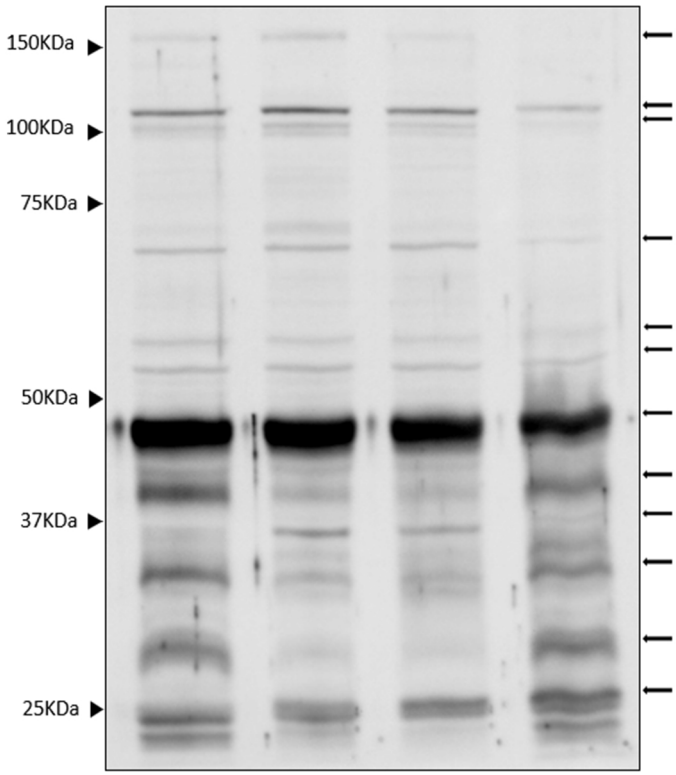
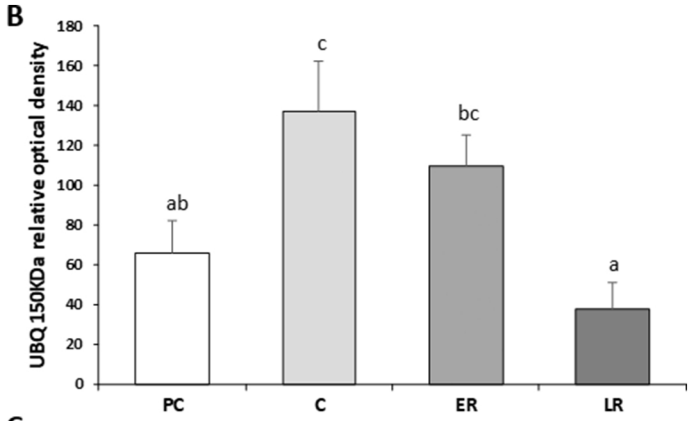
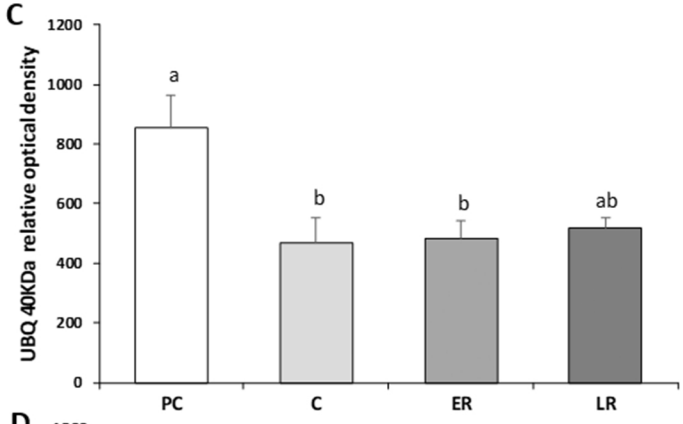
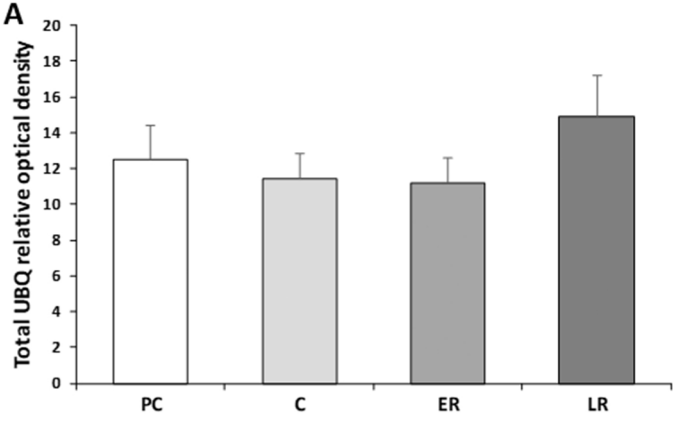

A
HSP70 relative optical density
4
3.5
3
2.5
2
1.5
1
0.5
0
PC
C
ER
LR
75KDa
B
HSP90 relative optical density
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
PC
C
ER
LR
75KDa
C
Rpt1 19S relative optical density
4
3.5
3
2.5
2
1.5
1
0.5
0
PC
C
ER
LR
50KDa
D
Core 20S relative optical units
1.2
1
0.8
0.6
0.4
0.2
0
a
ab
b
a
PC
C
ER
LR
50KDa
37KDa
E
19S/20S ratio (arbitrary units)
6
5
4
3
2
1
0
a
ab
b
ab
PC
C
ER
LR

Figure 3


150KDa
100KDa
75KDa
50KDa
37KDa
25KDa


B
UBQ 150KDa relative optical density
180
160
140
120
100
80
60
40
20
0
ab
c
bc
a
PC
C
ER
LR


C
UBQ 40KDa relative optical density
1200
1000
800
600
400
200
0
a
b
b
ab
PC
C
ER
LR


A
Total UBQ relative optical density
20
18
16
14
12
10
8
6
4
2
0
PC
C
ER
LR

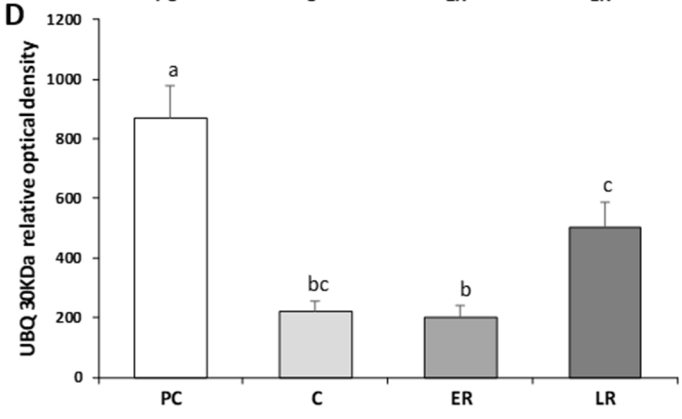

D
UBQ 30KDa relative optical density
1200
1000
800
600
400
200
0
a
bc
b
c
PC
C
ER
LR

Figure 4

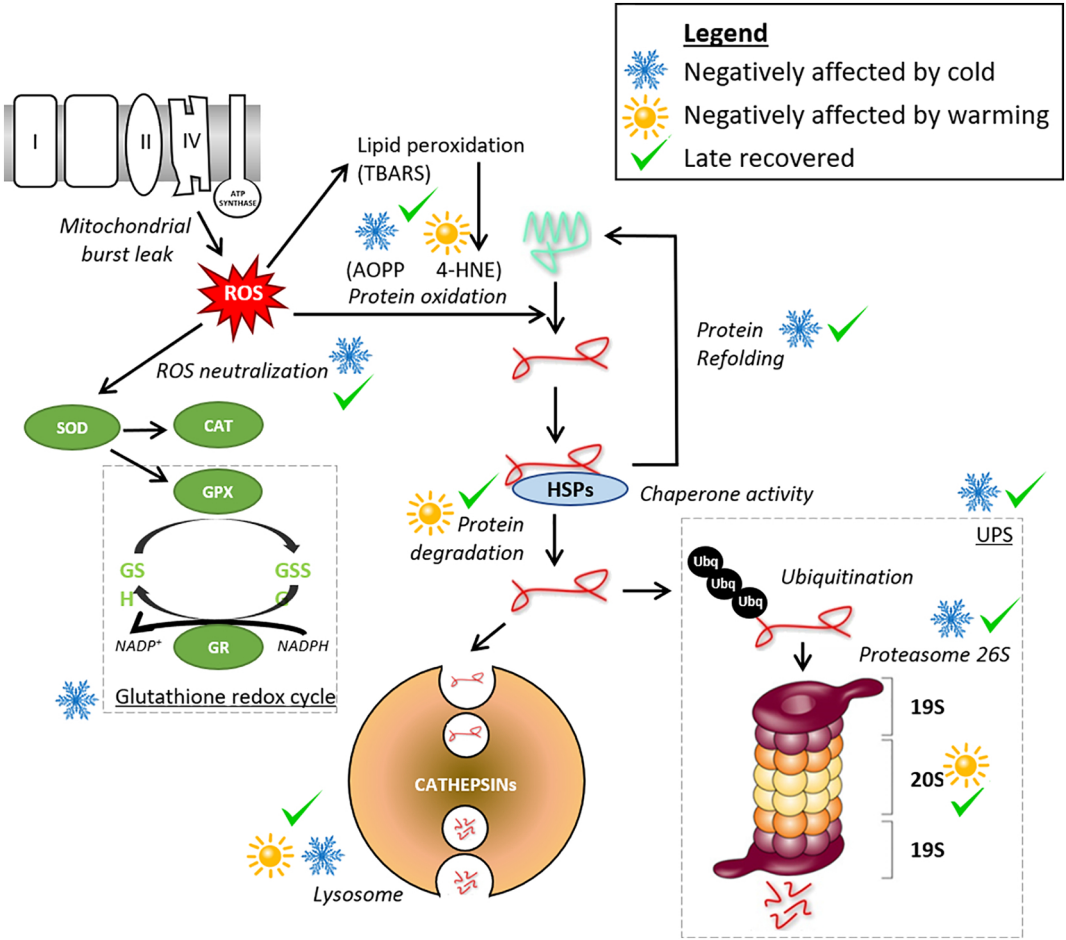

Legend
Negatively affected by cold
Negatively affected by warming
Late recovered
I
II
IV
ATP SYNTHASE
Mitochondrial burst leak
ROS
Lipid peroxidation (TBARS)
(AOPP 4-HNE)
Protein oxidation
ROS neutralization
SOD
CAT
GPX
GS H
GSS
NADP+
GR
NADPH
Glutathione redox cycle
Protein Refolding
HSPs
Chaperone activity
Protein degradation
UPS
Ubq
Ubiquitination
Proteasome 26S
19S
20S
19S
CATHEPSINs
Lysosome

Figure 5