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Ocean warming alters cellular metabolism and induces mortality in fish early life stages: A proteomic approach

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ABSTRACT

Climate change has pervasive effects on marine ecosystems, altering biodiversity patterns, abundance and distribution of species, biological interactions, phenology, and organisms' physiology, performance and fitness. Fish early life stages have narrow thermal windows and are thus more vulnerable to further changes in water temperature. The aim of this study was to address the sensitivity and underlying molecular changes of larvae of a key fisheries species, the sea bream *Sparus aurata*, towards ocean warming. Larvae were exposed to three temperatures: 18 °C (control), 24 °C (warm) and 30 °C (heat wave) for seven days. At the end of the assay, i) survival curves were plotted for each temperature treatment and ii) entire larvae were collected for proteomic analysis via 2D gel electrophoresis, image analysis and mass spectrometry. Survival decreased with increasing temperature, with no larvae surviving at 30 °C. Therefore, proteomic analysis was only carried out for 18 °C and 24 °C. Larvae up-regulated protein folding and degradation, cytoskeletal re-organization, transcriptional regulation and the growth hormone while mostly down-regulating cargo transporting and porphyrin metabolism upon exposure to heat stress. No changes were detected in proteins related to energetic metabolism suggesting that larval fish may not have the energetic plasticity needed to sustain cellular protection in the long-term. These results indicate that despite proteome modulation, *S. aurata* larvae do not seem able to fully acclimate to higher temperatures as shown by the low survival rates. Consequently, elevated temperatures seem to have bottleneck effects during fish early life stages, and future ocean warming can potentially compromise recruitment's success of key fisheries species.

1. Introduction

The Earth's climate is dynamic; it has changed many times according to historical records. However, while past changes can be attributed to natural causes, more recent changes (and rate of change) have been associated with an increase in greenhouse gas emissions by direct anthropogenic activities (Brierley and Kingsford, 2009; National Research Council USA, 2010; Doney et al., 2012; Godbold and Calosi, 2013). This phenomenon has led to an increase in global temperature (ranging from +1.8 to +4.0 °C), a change in weather patterns and hydrodynamics, sea level rise, stratification of ocean waters and ocean acidification (Solomon et al., 2007; Brierley and Kingsford, 2009; Okey et al., 2012; Storch et al., 2014; Bradley et al., 2015). Growing evidence is showing that climate change has pervasive impacts on terrestrial and marine ecosystems, altering biodiversity patterns, abundance and distribution of species, biological interactions, phenology, and organisms' physiology, performance and fitness (Walther et al., 2002; Perry et al., 2005; Doney et al. 2012, Okey et al., 2012). However, the extent of these effects may differ regionally and is dependent upon several factors such as regional rate of warming, as well as organisms' tolerance limits, phenotypic plasticity, adaptive capacity, generation time, dispersal, and reproductive output (e.g. Pörtner and Farrell, 2008). Moreover, tolerance limits and performance are dependent upon ontogenetic stage, because throughout development organisms have different physiological requirements. Several studies have approached this issue by studying thermal window widths and physiological performance across life stages (i.e. Pörtner and Farrell, 2008; Pörtner and Peck, 2010). According to these studies, thermal windows widths are narrower for egg and larval stages, as well as for spawners. Broader thermal windows are characteristic of juveniles and growing adults (Pörtner and Farrell, 2008). As such, early life

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stages are highly vulnerable to ocean warming (e.g. Storch et al., 2001; Pörtner and Farrell, 2008). Therefore, exposure of early life stages to stress can induce harmful downstream effects, in a process called 'developmental domino effect' (Pechenik , 2006; Byrne 2012; Byrne et al., 2013).

In the context of climate change, an overview of the current scientific literature shows that warming has greater physiological effects than ocean acidification in many species (see Byrne et al., 2009; Findlay et al., 2010; Rodolfo-Metalpa et al., 2010; Pansch et al., 2012; Moya et al., 2015). However, Byrne (2012) states that research concerning the vulnerability of early developmental stages to climate change has mainly focused on ocean acidification. Nevertheless, some studies have shown that temperature induces both sublethal and lethal effects on larvae. Among sublethal effects, changes in metabolism, disease resistance, growth and development, and increased incidence of malformations were reported by several authors (e.g. Polo et al., 1991; Werner et al., 2007; Georgakopoulou et al., 2010; Byrne, 2011; Vinagre et al., 2013). Moreover, increasing temperatures can induce low performance and increased mortality, creating a bottleneck effect at early life stages, impairing recruitment and population sustainability (Houde, 1989, 2008; Faria et al., 2011; Landsman et al., 2011; Bartolini et al., 2013). This may have severe effects in several species including commercial ones, compromising fish stocks and ecosystem services.

A major limitation in climate change research and associated effects on marine larvae is the lack of information concerning the molecular basis of responses to stress. Even though this has been addressed by several authors, studies are usually focused on a few specific biomarkers. Some improvement was possible with the expansion of genomic and transcriptomic tools; however, these are focused on analyzing DNA sequences or transcripts and not the final product of gene expression (i.e. proteins). Therefore, direct links with phenotype and fitness are hard to establish especially due to the poor correlation between transcript and protein levels (Vogel and Marcotte, 2012; Diz et al., 2012). Proteomics overcame this issue by enabling the study of a vast set of proteins within the cell, which can be linked to the cell's phenotype and can thus be related to fitness and adaptation (Dupont et al., 2007; Feder and Walser, 2005; Diz et al., 2012). Surprisingly, proteomics has not been widely applied in marine ecology, possibly due to the lack of sequencing data for marine organisms. Indeed, Tomanek (2014) already stated that proteomic studies are still scarce and restricted to few species and habitat types. Therefore, proteomic research applied to ecology is still in its infancy and may unravel new pathways that play a role in adaptation processes (Karr, 2008; Dalziel and Schulte, 2012). The few proteomic studies performed on marine organisms have shown that several pathways are affected by temperature including cytoskeletal dynamics, energetic metabolism, oxidative stress metabolism, chaperoning activity, immune response, transcriptional regulation, protein turnover and signal transduction (e.g. López et al., 2002; Gardeström et al., 2007; Tomanek and Zuzow, 2010; Tomanek, 2011; Fields et al., 2012; Garland et al., 2015).

The aims of this study were to assess the sensitivity of larval fish to ocean warming and extreme events in a highly commercial species, the sea bream *Sparus aurata* (*Linnaeus,* 1758) (IUCN Red List – Least Concern). *S. aurata* has a distributional area ranging from the Mediterranean and Black Seas to Eastern Atlantic and from the British Isles to Cape Verde (Froese and Pauly 2006, Sola et al. 2007). Spawning takes place between October and April in the open sea (Kissil et al., 2001; Dimitriou et al., 2007; Mylonas et al., 2011; Ibarra-Zatarain and Duncan, 2015). Early life stages are planktonic and the larval phase lasts about 50–60 days at 18 °C (Andrades et al., 1996; Sola et al., 2007). Larvae migrate across several environments during spring and summer to settle in shallow water habitats such as estuaries and coastal lagoons (Suau and Lopez, 1976; Arabaci, 2010; Verdiell-Cubedo et al., 2013), where they will grow until the adult stage. We used an integrative approach connecting proteome changes with organism-level indicators to unravel both molecular and fitness alterations providing a mechanistic insight into stress tolerance pathways and consequent fitness outcomes. We hypothesize that for larvae to survive heat stress, they must regulate at least two crucial processes, i) pro-survival pathways by up-regulating proteins with cytoprotective functions and ii) adjusting the energetic metabolism to cope with higher energetic demands of warm water, promoting homeostasis and sustaining performance. Proteomic approaches allow us to explore stress response networks and their regulatory steps, identify new candidate proteins (see Diz et al., 2012) and predict the vulnerability of fish larvae to climate change and environmental extremes, improving our understanding of ecological processes.

2. Methods

2.1. Assessment of S. aurata thermal environments

Both Portuguese coastal water temperatures and estuarine temperatures were assessed. Coastal water temperatures were retrieved from the sea temperature database (satellite data available from http://seatemperature.info/portugal-water-temperature.html), which has monthly sea surface temperatures for the main coastal cities of Portugal (data from the past five years – 2011–2015 for January until October and data from 2010 to 2014 for November and December). Maximum ± sd and minimum ± sd temperatures were calculated from this time-series considering all locations. Water temperatures in the Tagus estuary were obtained from the Marine and Environmental Sciences Center (MARE) database (data obtained from measurements carried out with YSI loggers) considering a time-series from 1978 to 2006. Moreover, future thermal environments were projected taking into account that Portuguese waters will undergo 2–3 °C increase by 2100 (Miranda et al., 2002).

2.2. Housing and husbandry of larvae

Larvae (n = 75; 35 days post-hatch – dph – larvae from a brood stock of 50 males and 25 females – breeding scheme in Fig. SM1a; total length range of 10–15 mm) were obtained from a fish farm (MARESA, Ayamonte, Huelva, Spain) and transported to the laboratory in 10 L opaque plastic containers with constant aeration and stable temperature conditions (18 ± 0.5 °C). The sample size was calculated considering that natural daily mortalities expected for larvae at 18 °C are around 20% at 30 dph, 9.9% at 40 dph and 4.9% at 50 dph (Andrades et al., 1996).

Distant and recent thermal history of the larvae were assessed considering i) the origin of parental fish, and ii) culture conditions at the fish farm. The first parental fish of the hatchery (collected in the late '90s) were wild fish caught in the nearby coastal lagoon mixed with adults obtained from an aquaculture in Almería region (Spain). In the fish farm, larvae are reared under tightly controlled conditions in indoor tanks (20 °C, high water quality) until they reach 0.1 g (approximately at 60 days post-hatch). Afterwards, they are placed in other less controlled indoor tanks but keep being reared at 20 °C. When they reach 1 g (approximately 90 days post-hatch), they are moved into land-based outdoor ponds (with water from the nearby coastal lagoon) and subjected to a natural temperature regime (temperate climate with seasonal variation: colder during winter and warmer during summer). According to data (from 1984 to 2010) obtained from the Spanish Agencia

Estatal de Meteorologia, mean air temperatures in the area range from 11.0 °C in January to 26 °C in July/August. Maximum air temperatures can reach 33.0 °C and minimum air temperatures can reach approximately 6 °C.

The experimental system consisted of a re-circulating structure (total volume of 2000 L) with six 70 L opaque polyvinyl tanks (35 × 35 × 55 cm). The flow rate of clean water in each tank was 300 mL min^{-1}. Larvae were randomly placed in smaller transparent polyvinyl containers (17.5 × 17.5 × 15 cm^3, approximately 4.5 L; n=12–13 larvae.$tank^{-1}$) positioned inside the 70 L tanks with water flowing through small punctures (this allowed for a gentle water flow, preventing physical stress). All the tanks were filled with clean and aerated sea water (95–100% O_2), with a constant temperature of 18 ± 0.5 °C, salinity 35‰ and pH 8 ± 0.01 (same conditions of the fish farm). Larvae were allowed to acclimate for 24 h before the experiment (a longer acclimation period was not feasible because it would interfere with the objective of studying larval stages). During the acclimation and experiment larvae were fed under a regime of period feeding (every 6 h) with *Artemia salina* metanauplii and 2 different grain-sized feeds (0.3–0.6 mm and 0.6–1.0 mm).

2.3. Experimental assays

Following the acclimation period in the experimental system, temperature was gradually increased during 2 days (6 °C.day^{-1}) until the experimental temperatures were reached (controls were kept at 18 ± 0.5 °C; experimental temperatures 24 ± 0.5 °C and 30 ± 0.5 °C; n=2 tanks for each temperature, Fig. SM1b in supplemental material). These temperature treatments were chosen to simulate control conditions (18 °C), present-day warm waters (summer in shallow nursery grounds –24 °C) and future heat wave situations in nursery areas (30 °C). At the beginning of the experimental trial, the total sample size was 60 larvae, 10 individuals per tank). Temperatures were maintained using thermostats (TetraTec® HT 100, 100-150L, Tetra Werke, GmbH, Melle, Germany). Larvae (whole body; n=4 larvae per temperature group, 2 per tank) were sampled for proteomic analysis after seven days of exposure (greater sample sizes were not possible due to mortality rates at higher temperatures). The experiments were carried out in shaded day light (15L; 09D). The number of dead larvae was counted every day. To keep environmental parameters constant throughout the experiment, a monitoring scheme was employed. Temperature was monitored in all tanks everyday using a digital thermometer and other parameters i.e. salinity (kept at 35%), pH (kept at 8 ± 0.01), ammonia (kept at 0 mg L^{-1}) and nitrites (kept under 0.3 mg L^{-1}), were monitored twice a week using a handheld refractometer (Atago, Japan), a digital pH probe (model HI9025, Hanna Instruments, USA), and Tetra Test Kits (Tetra Ammonia Test Kit and Tetra Nitrites Test Kit, USA), respectively.

2.4. Statistical analysis on survival

Kaplan-Meier survival curves were plotted using GraphPad Prism v5. The curves were compared using Log-rank (Mantel-Cox) test and the log-rank test for trend, using an α level of 0.05.

2.5. Protein extraction

Each larvae was homogenized in 500 μL of cold buffer saline solution (PBS, pH 7.4: 0.14M NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4) by using Tissue Master 125 homogenizer (Omni International, Kennesaw, USA). Afterwards, homogenates were centrifuged (10 min. at 16,000*g*) and the supernatant fraction was transferred to microcentrifuge tubes (1.5 mL) and stored at −80 °C until further analysis.

2.6. Proteomic analysis

2.6.1. Sample preparation

The homogenized and centrifuged samples (as previously described in Section 2.5) were precipitated through the DOC/TCA (Na-deoxycholate/trichloroacetic acid) method. Briefly, for each 100 μL of sample, 1 μL of 2% DOC was added and samples were incubated 30 min on ice. Then, 18 μL of 100% TCA was added to the mixture and microtubes were incubated overnight on ice. Afterwards, samples were centrifuged at 14,000*g* for 20 min at 4 °C. Supernatant was removed and pellets were washed with 200 μL of ice cold acetone, followed by another centrifugation (14,000*g* for 20 min at 4 °C). This washing step was performed twice. Subsequently, pellets were resuspended in rehydration buffer (7 M urea, 2 M thiourea, 2% w/v CHAPS - cholamidopropyl-dimethylammonio-propanesulfonic acid, 0.2% v/v IPG buffer, 0.002% bromophenol blue, 50 mM DTT – dithioerythritol). Protein content was determined through the Bradford method (Bradford, 1976). The analysis was carried out in 96-well microplates (Nunc) by adding 150 μL of Bradford reagent (Bio-Rad, USA) and 150 μL of sample or standards to each well (previously diluted to fit the calibration curve). This calibration curve was constructed using BSA standards (0–25 μg.mL^{-1}). After 10 min of reaction, the absorbance was read at 595 in a microplate reader (model LT-4000, Labtech, United Kingdom).

2.6.2. Two dimensional gel electrophoresis (2-DE)

Samples containing 100 μg of protein were loaded onto IPG strips (pH 3–10, 7 cm, Bio-Rad) for separation according to their isoelectric point (pI). IPG strips had been previously rehydrated overnight with 7M urea, 2M thiourea, 0.5% w/v CHAPS, 0.2% v/v IPG buffer, 0.002% bromophenol blue, 10 mM DTT. Isoelectric focusing was carried out in a Protean® IEF Cell (Bio-Rad), according to the manufacturer's instructions for 7 cm strips: 250V for 20 min (linear mode), 4000 V for 2 h (linear mode) and 4000 V for 10,000 V-h (rapid mode). Strips were immediately incubated in equilibration buffer I (6 M urea, 75 mM Tris–HCl, 20% v/v glycerol, 2% w/v SDS – sodium dodecyl sulfate, 0.002% bromophenol blue, 2% w/v DTT) for 15 min with continuous shaking, and then equilibration buffer II (6 M urea, 75 mM Tris–HCl, 20% v/v glycerol, 2% w/v SDS, 0.002% bromophenol blue, 2,5% w/v IAA – iodoacetamide) for 15 min with continuous shaking. Afterwards, IPG strips were placed on top of 12,5% polyacrylamide gels and were covered with an agarose sealing solution (0.5% w/v agarose and 0.002% bromophenol blue in running buffer – 25 mM Tris base, 192 mM glycine, 0.1% SDS). Gels were run in Mini-Protean® 3 Cell (Bio-Rad) at 200 V for 45 min and were then stained for 48 h with a solution of colloidal Coomassie Blue G-250 (0.12% w/v Coomassie G-250, 10% w/v ammonium sulfate, 10% w/v orthophosphoric acid, 20% methanol). Following, gels were de-stained with milli-Q water in several washes. Four individuals were used in each temperature group (2 from each tank) and duplicate gels were carried out for each individual to ensure gel reproducibility.

2.6.3. Gel image analysis

Gel imaging was carried out with the PropicII-robot (Genomic Solutions™, Cambridgeshire, UK) and digitalized images of the gels were analyzed with Progenesis SameSpots software (version 4.0, NonLinear Dynamics, Totallab, UK). A master gel was automatically defined by the software and match vectors were also automatically created to align the gels (match the spots within all the gels). Protein spot volumes were normalized against total spot volume of all proteins in a gel image. Then, the software calculated spot intensities and automatically compared temperature treatments (via a one-way analysis of variance – ANOVA) to detect differentially expressed proteins. A final gel image of the master

gel was created, indicating (with specific numbers) the differentially expressed spots between temperature groups.

2.6.4. Protein digestion

The spots of interest were manually excised from gels and transferred to 0.5 mL low bind tubes (protein Lobind tubes, Eppendorf, Germany). Gel spots were washed with water and destained twice with 50% acetonitrile/25 mM Ambic (ammonium bicarbonate) until the blue color disappeared and then, dehydrated with 100% acetonitrile. Posteriorly, 15 μL of trypsin (Sigma-Aldrich, USA) (0.02 μg/μL in Ambic 12.5 mM /2% acetonitrile) was added to the gel spots and incubated for 60 min on ice. Afterwards, gel spots were inspected and all the trypsin solution not absorbed into the gel was removed. Then, the gels were covered with 25-50 μL of 12.5 mM Ambic depending on the spot volume. The samples were incubated for 18 h, overnight at 37 °C. Tubes were chilled to room temperature, the gel pieces spin down and the supernatants collected to a new tube. Then 25 μL formic acid 5% (v/v) were added to the remaining gel pieces, vortexed and incubated for 15 min at 37 °C. The supernatants were collected once again to the tubes and about 25 μL 50% ACN/0.1% TFA was added. Once more the supernatant was collected to the first tube, solution dried-down in SpeedVac (Thermo Fisher Scientific Waltham, MA, USA) and the dried peptides stored at −20 °C until MS and MS/MS analyses.

2.6.5. Mass spectrometry

Tryptic peptides were re-suspended in 10 μL of a 50% acetonitrile/0.1% formic acid solution. The samples were mixed (1:1) with a matrix consisting of a saturated solution of a-cyano-4-hydroxycinnamic acid prepared in 50% acetonitrile/0.1% trifluoracetic acid. Three aliquots of each sample (0.5 μL) were spotted onto the MALDI sample target plate. Peptide mass spectra were obtained on a MALDI-TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, Europe) in the positive ion reflector mode. Spectra were obtained in the mass range between 900 and 4500 Da with ca. 1500 laser shots. For each sample spot, a data dependent acquisition method was created to select the six most intense peaks, excluding those from the matrix, or acrylamide peaks, for subsequent MS/MS data acquisition.

2.6.6. Database Search

Spectra were processed and analyzed by the Global Protein Server Workstation (Applied Biosystems), which uses internal MASCOT software (v2.1.0 Matrix Science, London, UK) on searching the peptide mass fingerprints and MS/MS data. Swiss-Prot nonredundant protein sequence database (October 2014) was used for all searches under taxonomy Chordata (an additional search was run against a list of common contaminant proteins). Database search parameters were as follows: carbamidomethylation and propionamide of cysteine as a variable modification as well as oxidation of methionine, and the allowance for up to two missed tryptic cleavages. The peptide mass tolerance was 40 ppm and fragment ion mass tolerance was 0.3 Da. Protein identifications were considered as reliable when the MASCOT score was >60, calculated as $-10\times\log P$ (where P is the probability that the observed match is a random event). This is the lowest score indicated by the program as significant ($P<0.05$) and indicated by the probability of incorrect protein identification. In a few spots (seven of them), there were several significant matches but these represented the same protein (in different species). In these cases, the species with the highest score was chosen.

2.6.7. Expression analysis

A cluster and heat map analysis was carried out to visualize level changes of proteins of interest using Cluster 3.0 plus Java TreeView. The aim was to gain insight on data structure and group proteins by similarity in their expression profiles. Normalized average spot volumes (mean normalized volume of four individuals in each temperature group) were used for this analysis, which followed the criteria (chosen following Gibbons and Roth 2002, D'haeseleer 2005 and Kreil and Russel 2005):

i) hierarchical cluster analysis: model based on distance connectivity appropriate for small datasets and no *a priori* knowledge of the number of clusters
ii) before carrying out the analysis, adjustments were made to data: log transformation (in order to represent data on the same scale, as fold changes, and to equalize variances); center rows in order to subtract the row-wise mean from the values in each row of data, so that the mean value of each row is 0. By centering the rows (proteins), the values represent the relative $\log_2$ fold change compared to the mean of the protein.
iii) similarity metric: Spearman rank correlation (correlation metrics are appropriate to group proteins in terms of shape of expression i.e. profiles)
iv) clustering method: average linkage (not so sensitive to outliers as complete linkage and counterbalances the tendency of single linkage to form long chains instead of compact clusters)

2.6.8. Categorization of identified proteins into functional classes

The identified proteins were categorized using STRAP v1.5 (Bhatia et al., 2009) according to biological process, cellular component and molecular function. As STRAP only provides general categories, an independent search was carried out using gene ontology tools (i.e. UniProt, GeneCards, neXtprot beta, InterPro and Qiagen) in order to assess the proteins' specific biological functions.

Moreover, a functional protein association network was constructed in String (v10) using *Homo sapiens* homolog proteins (entry proteins: heme binding protein 1, proteasome subunit beta type 3, heat shock 70 kDa protein 8, tubulin alpha 4a, tropomyosin 1, keratin 8, actin related protein 2/3 complex subunit 2, myosin light chain 1, growth hormone 1, heat shock protein 90 kDa alpha, POTE ankyrin domain family member F, actin alpha 1 skeletal muscle, kinesin heavy chain member 2A, IQ motif containing with AAA domain 1).

3. Results

3.1. Temperature data

Coastal water temperatures were lowest in February (average of 13.9 °C, minimum of 12.2 °C and maximum of 15.2 °C) and highest in September (average of 19.3 °C, minimum of 17.2 °C and maximum of 21.8 °C) (Fig. 1). Considering the Tagus estuary, average temperatures were lowest in January (11.7 °C) and highest in July (23.6 °C) (Fig. 1). Current heat waves make estuarine waters reach 28 °C. Following the scenario of a 2–3 °C increase in Portuguese coastal waters by 2100 (Miranda et al., 2002), future heat waves will lead to coastal water temperatures in the range of 19–23 °C and estuarine waters in the range of 26–30 °C.

3.2. Survival curves

Survival curves were significantly different (Log-rank test for trend; Chi square=18.64; df=1; $p<0.001$). At the end of seven days, survival decreased from 55% at 18 °C to 20% at 24 °C (Log-rank Mantel-Cox test; Chi square=4.129; df=1; $p=0.04$). At 30 °C, all larvae had died after 5 days of exposure (Log-rank Mantel-Cox test: 18 °C vs 30 °C - Chi square=20.92; df=1; $p<0.0001$; 24 vs

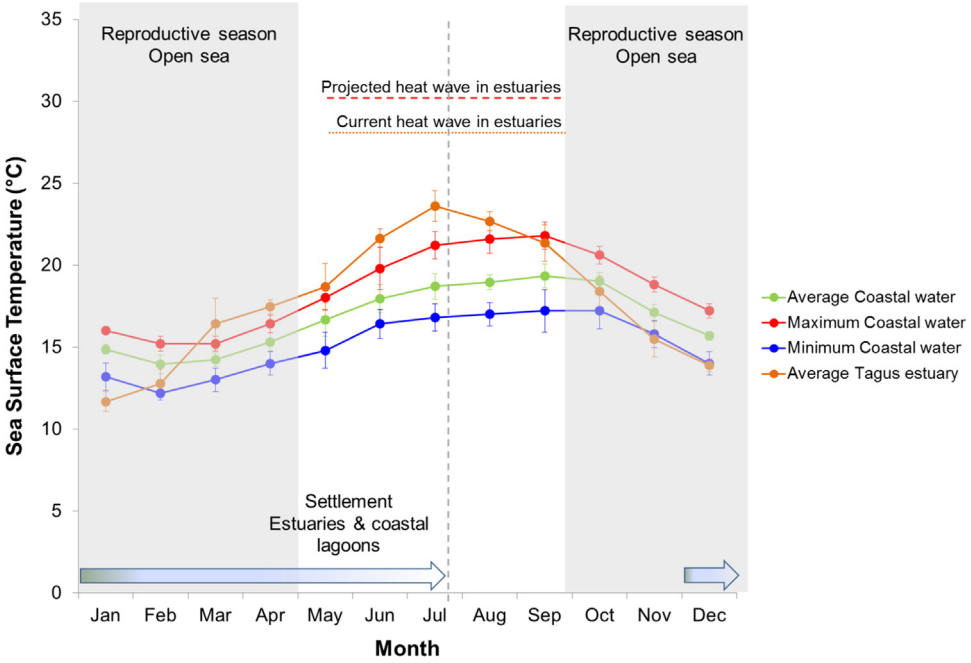

Fig. 1. Current and predicted water temperatures in Portuguese coastal areas and Tagus estuary. Temperatures are in the range of 16–20 °C in coastal waters and 12–24 °C in the Tagus estuary. Portuguese waters will undergo a 2–3 °C increase by 2100 (see Miranda et al., 2002) leading to temperatures in the range of 19–23 °C in the coastal area and 26–30 °C in estuaries and coastal lagoons during summer.

30 °C - Chi square = 12.09, df = 1, $p < 0.0005$) (Fig. 2). As such, further analyses were only possible between 18 °C and 24 °C temperature groups.

3.3. Proteomic analysis

Concerning proteomic analysis, all gels were automatically matched and compared to the master gel (Fig. 3) to detect differences in protein spots. The analysis of variance ($p < 0.05$) showed that 23 protein spots were differentially expressed between 18 °C and 24 °C. Of the 23 spots, 15 (65%) were successfully identified (Table 1, Fig. 3), seven of which were up-regulated (tropomyosin alpha-1 chain, Hsp90 alpha, proteasome subunit beta 3, somatotropin, myosin light chain, actin related protein 2/3 complex subunit 2, IQ and IAA domain containing protein) and 8 were down-regulated at 24 °C (heme binding protein 1, actin isoforms, heat shock cognate 71 kDa, kinesin heavy chain, POTE ankyrin domain family member F, tubulin alpha-4 chain, keratin II cytoskeletal 8) (Fig. 4).

Functional categorization analysis carried out in STRAP v1.5 (Fig. 5) showed that identified proteins were involved in biological processes such as regulation (4.22%), response to stimulus (1.6%), cellular processes (5.28%), immune system processes (1.6%), interaction with cells and organisms (1.6%), localization (1.6%), metabolic process (1.6%) and other (4.22%) (Fig. 5a). Concerning cellular components, proteins were localized in cytoplasm (11.30%), macromolecular complex (1.3%), extracellular (5.14%), plasma membrane (3.8%), cytoskeleton (9.24%), nucleus (3.8%), mitochondria (1.3%), endosome (1.3%), other intracellular organelles (2.5%) and other (1.3%) (Fig. 5b). The molecular functions of the identified proteins include binding (11.58%), catalytic activity (4.21%), enzyme regulatory activity (1.5%) and structural molecule activity (3.16%) (Fig. 5c). In detail, gene ontology search tools showed that identified proteins were mainly involved in cytoskeleton dynamics (actin, actin related proteins, myosin, tropomyosin, tubulin, keratin), chaperoning (heat shock cognate 71 kDa, heat shock protein 90 kDa, IQ motif and AAA domain containing protein), intracellular transport (kinesin, IQ motif and AAA domain containing protein), growth (somatotropin), porphyrin metabolism

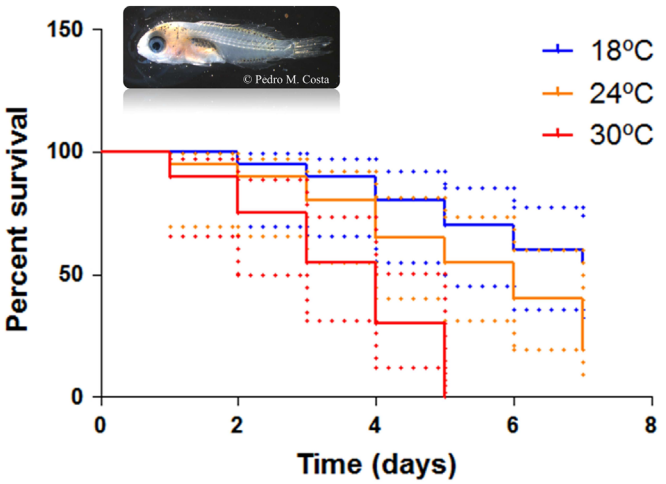

Fig. 2. Survival curves (solid lines) and 95% confidence intervals (dotted lines) of *Sparus aurata* larvae exposed to 18 °C, 24 °C and 30 °C for a period of seven days. These curves were compared through the Log-rank test for trend (Chi square = 18.64; df = 1; $p < 0.001$). Specific comparisons were carried out using Log-rank Mantel-Cox test (18 °C vs 24 °C - Chi square = 4.129, df = 1, p = 0.04; 18 °C vs 30 °C - Chi square = 20.92, df = 1, $p < 0.0001$; 24 vs 30 °C - Chi square = 12.09, df = 1, $p < 0.0005$).

(heme-binding protein), proteolysis (proteasome subunit, IQ motif and AAA domain containing protein), regulation (POTE ankyrin F), cell-cycle regulation and transcription (IQ motif and AAA domain containing protein) (Table SM2, supplemental material).

The functional protein association network constructed in String (v10) was significantly enriched in interactions (p-value = 3.86e-4), mainly between heat shock proteins, cytoskeletal components and cargo transporting (see supplementary Fig. SM2).

4. Discussion

Molecular plasticity is one of the most important mechanisms by which organisms can respond to and survive environmental

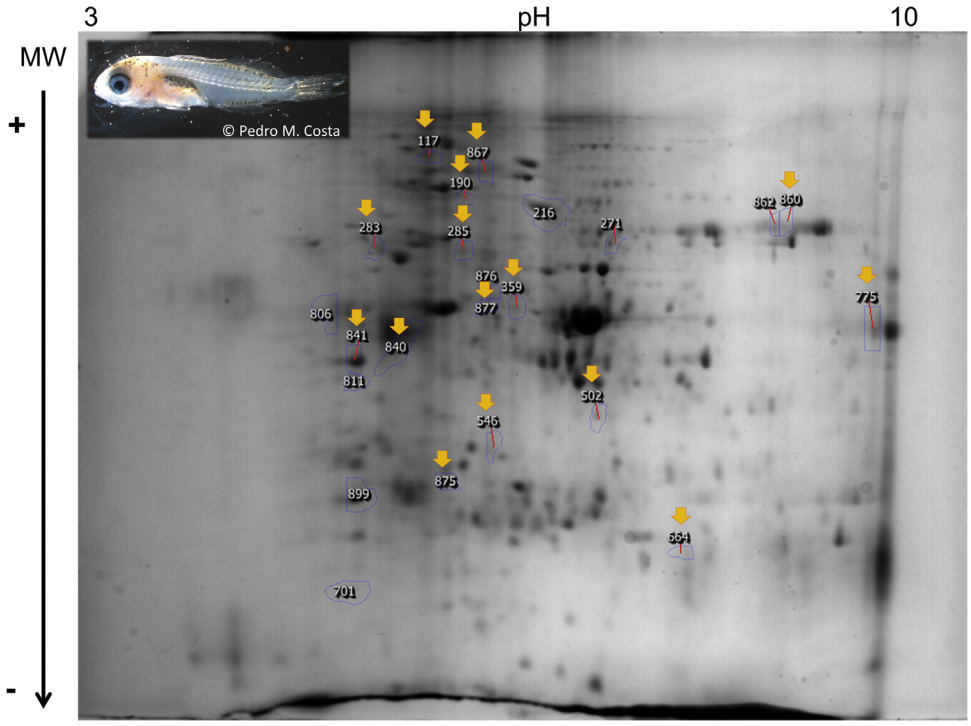

Fig. 3. Image of the master gel depicting the protein spots detected in *Sparus aurata larvae*. Numbered spots were those that were differentially expressed between temperature groups (n=4 larvae in each group, 2 technical replicates, ANOVA, $p < 0.05$). Arrows mark the spots that were successfully identified by mass spectrometry. Only 18 °C and 24 °C were compared because there was 100% mortality in larvae exposed to 30 °C for 7 days.

changes (e.g. acclimation). In marine organisms, protein regulation in response to a change in the environment is elicited some degrees above the acclimation temperature. Usually, the onset temperature for protein regulation is close to the upper pejus temperature (loss of performance and increased mortality), reaching a peak and decreasing close to the upper thermal limit (protein denaturation, compromised survival). However, such temperatures are dependent on acclimation temperature and frequency and duration of exposure (and therefore habitat and season dependent) (see Hofmann, 1999; Tomanek and Somero, 1999; Anestis et al., 2007; Pörtner, 2010; Schiffer et al., 2014). According to Aubin-Horth and Renn (2009), identifying up- and down-regulated genes allows us to detect a unique gene expression pattern that reflects the biological phenotype. This is especially important in early life stages, for which molecular assessments are lacking. In this study, we showed that larvae exposed to three different thermal environments (reflecting current temperatures and projected warming) for a period of seven days change their proteome, promoting protein folding and turnover, cytoskeletal re-organization, cell cycle regulation and transcription regulation while inhibiting cargo transporting and oxygen transport. Interestingly, no changes were detected in proteins related to energetic

Table 1
Proteins differentially expressed between temperature groups (18 °C and 24 °C) in *Sparus aurata* larvae. Only 18 °C and 24 °C were compared because there was 100% mortality in larvae exposed to 30 °C. These proteins were identified using MASCOT under the taxonomy Chordata.

Spot no.	Protein Name	Species	Accession number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C. I. %
546	Actin, muscle	*Styela plicata*	ACTM_STYPL	42,327.06	5.23	6	138	100
860	Kinesin heavy chain isoform	*Homo sapiens*	KIF5C_HUMAN	109,427.15	5.86	18	65.9	97.85
502	Actin-related protein 2/3 complex subunit 2	*Homo sapiens*	ARPC2_HUMAN	34,311.48	6.84	8	203	100
283	IQ and AAA domain-containing protein 1-like	*Rattus norvegicus*	IQCAL_RAT	95,564.65	9.55	10	66.8	98.25
664	Myosin light chain 1, skeletal muscle isoform	*Liza ramada*	MLE1_LIZRA	20,054.03	4.54	3	63.7	96.44
775	Somatotropin	*Prionace glauca*	SOMA_PRIGL	21,057.59	6.66	9	64.6	97.10
867	Heme-binding protein 1	*Gallus gallus*	HEBP1_CHICK	21,091.45	5.76	8	64.3	96.89
875	Proteasome subunit beta type-3	*Oncorhynchus mykiss*	PSB3_ONCMY	23,005.41	5.05	7	177	100
359	POTE ankyrin domain family member F	*Homo sapiens*	POTEF_HUMAN	121,366.69	5.83	18	134	100
877	Actin, muscle-type OS	*Molgula oculata*	ACT2_MOLOC	42,235.95	5.12	11	189	100
190	Heat shock cognate 71 kDa protein	*Ictalurus punctatus*	HSP7C_ICTPU	71,296.14	5.19	16	369	100
841	Tropomyosin alpha-1 chain	*Liza aurata*	TPM1_LIZAU	32,709.66	4.69	26	483	100
117	Heat shock protein HSP 90-alpha	*Oryctolagus cuniculus*	HS90A_RABIT	79,683.10	4.88	6	116	100
285	Tubulin alpha-4A chain	*Homo sapiens*	TBA4A_HUMAN	49,892.37	4.95	14	76.9	99.83
840	Keratin, type II cytoskeletal 8	*Danio rerio*	K2C8_DANRE	57,723.45	5.15	20	105	100

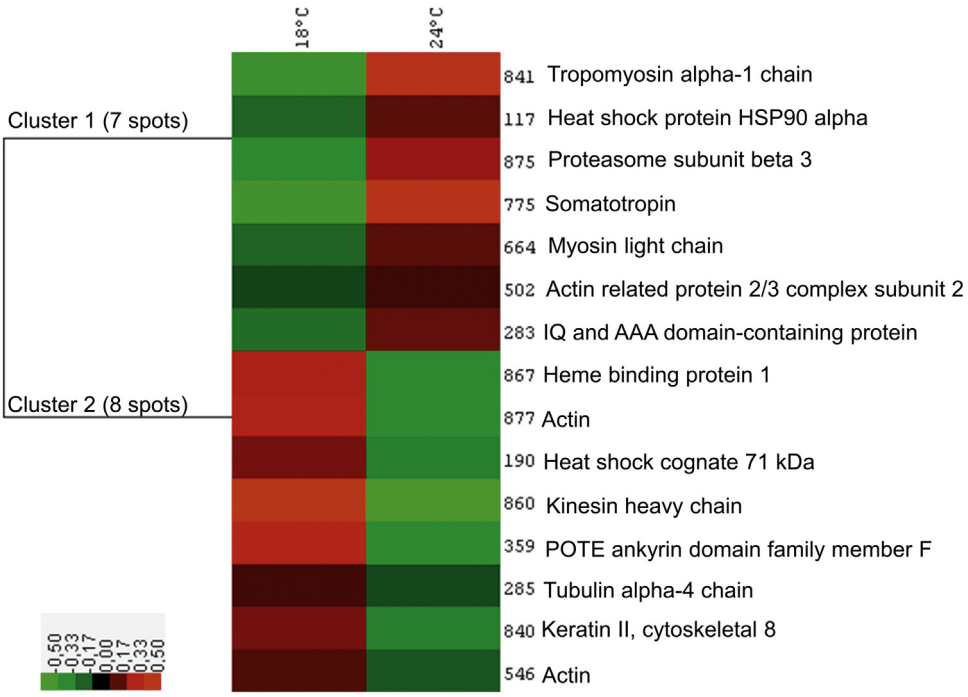

Fig. 4. Protein expression in *Sparus aurata* larvae exposed to 18 °C and 24 °C for seven days. Only 18 °C and 24 °C were compared because there was 100% mortality in larvae exposed to 30 °C for seven days. Data is presented as a heat map with clusters to visualize protein Log2 expression values ranging from green (lower than the mean expression level) to red (higher than the mean expression level). Columns represent different temperatures while rows represent different proteins (identified by spot number and the correspondent identification by mass spectrometry). Full details concerning protein identification are given in Table 1 and Table SM1.

metabolism as opposed to many other organisms subjected to elevated temperature including *S. aurata* juveniles, other fish and invertebrates, e.g. Madeira et al., in preparation; Tomanek and Zuzow, 2010; Jayasundara et al., 2015; Garland et al., 2015). One could argue that this could be due to 2D gel limitations such as imperfect resolution (highly abundant and soluble proteins are easier to resolve than low abundant and lipid-soluble proteins – see Rabilloud and Lelong, 2011), but changes in energetic metabolism of juvenile *S. aurata* exposed to warming were detected using this same method (Madeira et al., in preparation). Thus, this suggests that larvae may not have the energetic plasticity (enhanced glycolytic potential) needed to sustain cellular protection in the long-term. Accordingly, despite proteome changes related to cytoprotection, survival rates of larvae decreased with temperature suggesting that they were pushed beyond their physiological limits and cannot acclimate to these temperatures 24 °C (summer average for estuaries) and 30 °C (projected heat wave in estuaries).

In the following subsections of discussion, we will focus on the 15 identified proteins and their roles in cellular and thermal compensation processes.

4.1. Chaperoning and protein degradation

Overall, changes were detected in molecular chaperones (Hsp90 and Hsc70) and the proteasome system. Molecular chaperones (both inducible and constitutive isoforms) are involved in cellular processes and regulation, playing crucial roles in protein folding and preventing the formation of cytotoxic aggregations upon exposure to stressful conditions (Moseley, 1997; Fink, 1999; Vabulas et al., 2010), improving survival upon heat shock (Sørensen et al., 2003). Several studies have shown that these proteins (and transcripts) are up-regulated when aquatic organisms are exposed to heat stress, including fish and invertebrates such as corals, bivalves, dog whelks, sea cucumbers and crustaceans (e.g. Feder and Hofmann, 1999, 2005; Portune et al., 2010; Dong et al., 2011; Madeira et al., 2012a; Smith et al., 2013; Tomalty et al., 2015). Thus, chaperones contribute to the success of species across environmental gradients (Hofmann 2005). Accordingly, Hsp90 was up-regulated in *S. aurata* larvae exposed to warming, potentially contributing to the stabilization of proteins and other processes such as signal transduction and intracellular transport (Li and Buchner 2013). Similarly, Silvestre et al. (2010) also detected increases in Hsp90 in sturgeon larvae exposed to heat. Thus, the Hsp90 chaperone may play a crucial role in larval fish thermotolerance. However, Hsc70 (constitutive isoform) was unexpectedly down-regulated. Constitutive chaperones also have other functions other than protein folding regulation, and their down-regulation can therefore influence cell cycle regulation, transcriptional activation and scaffolding of the spliceosome (see UniProt database). Nevertheless, chaperone changes may not be straightforward; as several authors have reported, there is a complex interplay between several chaperones and their threshold for induction/repression may be dependent on several factors including stress levels, hormone levels, type of tissue and species (e.g. Dietz and Somero, 1993; Wegele et al., 2004; Celi et al., 2012; Madeira et al., 2012b, 2013; Alvira et al., 2014).

Despite the increase in Hsp90, there was an increase in proteolysis (as shown by the up-regulation of the proteasome subunit beta type-3) suggesting that chaperones could not rescue all proteins and thus protein damage can occur when larvae are exposed to warming. Exposure to warming is known to induce changes in the rate of protein degradation, up-regulating proteins related to ubiquitin tagging and proteasome degradation in *S. aurata* and other aquatic species (e.g. Parsell and Lindquist, 1993; Hoffman and Somero, 1996; Buckley et al., 2006; Madeira et al., 2014), including larval zebrafish (Long et al., 2012).

4.2. Cytoskeleton dynamics

Cytoskeletal filaments are essential in several cellular and regulation processes including cell motility, muscle contraction, cell division, cell signalling, and organelle movement. Changes in

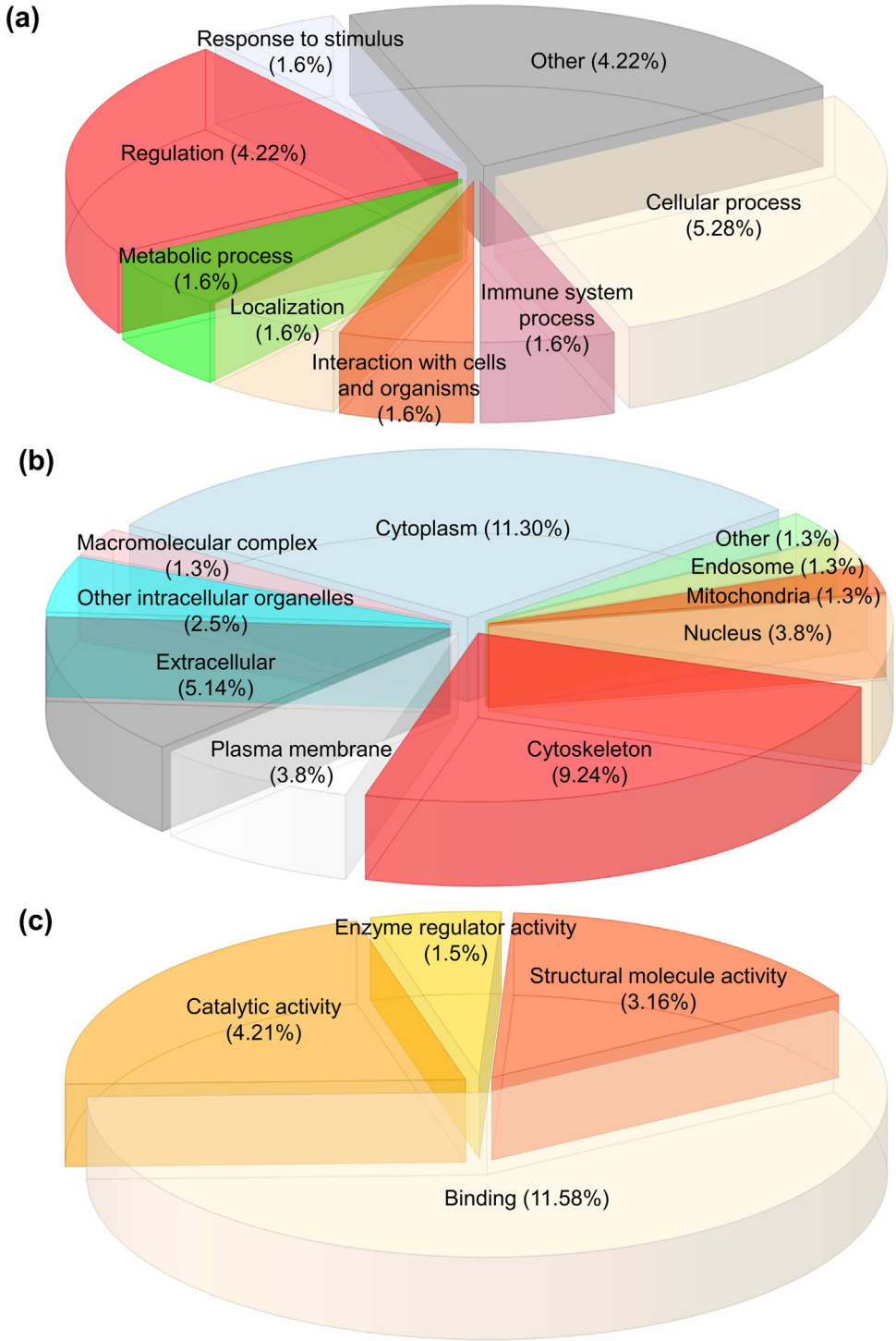

Fig. 5. Distribution of identified proteins by functional classes according to STRAP v1.5, (a) biological process, (b) cellular component and (c) molecular function. Detailed functions are described in Table SM2.

cytoskeletal proteins point to a restructuring of cytoskeleton when larvae are exposed to warming conditions, as observed in other studies (both acute and chronic exposures) carried out in marine organisms, including fish, crustaceans and bivalves (e.g. Podrabsky and Somero, 2004; Buckley et al., 2006; Tomanek and Zuzow, 2010; Tomanek, 2014; Jayasundara et al., 2015; Garland et al., 2015; Artigaud et al., 2015; Tomalty et al., 2015). Additional studies in different organisms and cell lines have also concluded that cytoskeleton elements are very sensitive to heat shock (Holubářová et al. 2000; Mounier and Arrigo 2002; Gavriolova et al. 2013) and that stabilization of cytoskeleton is crucial to maintain homeostasis (Garlick and Robertson, 2007). In fact, Buckley et al. (2006) described two scenarios for changes in cytoskeletal proteins upon heat stress i) either cytoskeletal restructuring is an important mechanism to counterbalance the effects of heat or ii) as cytoskeletal components are highly dynamic and can rapidly polymerize and depolymerize, they are more susceptible to environmental perturbations and synthesis of new filaments is required. Interestingly, actin-related protein 2/3 complex subunit 2 was up-regulated, indicating increased nucleation of actin filaments and

the formation of branched actin networks (see Table SM2). Moreover, this protein is also involved in immune processes, which suggests that warming injures cells and tissues. Tomanek and Zuzow (2010) have suggested that changes in cytoskeletal proteins are a trigger for the expression of molecular chaperones. In fact, several authors have reported that an interaction exists between cytoskeletal components and heat shock proteins. Small heat shock proteins can modulate actin properties and protect cytoskeleton upon stressful conditions (Mounier and Arrigo 2002). Moreover, large Hsp such as Hsp90 and Hsp70 can bind to the microtubule and centrosome network (Liang and MacRae, 1997), modulating and protecting cytoskeletal components.

4.3. Intracellular transport

The down-regulation of kinesin heavy chain indicates a slow-down of cargo transporting. Although the adaptive significance of this finding is not clear, it is possible that some down-regulation of intracellular transport is a strategy to partially reduce energy expenditure, in favour of survival functions. Other studies have detected changes in transport proteins in fish exposed to heat stress but the trend was opposite as fish mostly up-regulated transport proteins in response to temperature increases (Logan and Somero, 2011; Tomalty et al., 2015). Interestingly, in this study, IQ and AAA domain-containing protein 1-like was up-regulated This protein has diverse functions including intracellular transport, suggesting that some cargo still needs to be transported to other cellular locations to maintain homeostasis.

4.4. Cell-cycle regulation and transcription regulation

IQ and AAA domain-containing protein 1-like also has other functions including cell-cycle and transcription regulation, proteolysis and chaperoning. This corroborates the up-regulation of other proteins involved in proteolysis and chaperoning and suggests that *S. aurata* larvae modulate gene expression when exposed to warming, which is supported by the observed changes in protein levels in this study. In addition, POTE ankyrin domain family member F was down-regulated from 18 to 24 °C, suggesting changes in PAK (p21 protein activated kinase) and Ras pathways. These pathways are usually activated in response to extracellular signals. While PAK pathway is involved in gene expression regulation and cytoskeletal actin assembly, the Ras pathway is involved in signal transduction and gene expression regulation. Several authors have also detected changes in transcriptional regulators in marine and freshwater fish exposed to heat (Buckley et al., 2006; Long et al., 2012; Tomalty et al., 2015), including sturgeon larvae (Silvestre et al., 2010). According to these authors, this is crucial to respond to extracellular signals and trigger or repress gene expression. Even though transcriptional regulation is surely important in molecular adaptation, it is not entirely possible to determine which specific genes and pathways are being mediated. Nevertheless, besides regulating cytoskeletal dynamics, POTE ankyrin domain family member F is also involved in cell adhesion and migration so these mechanisms might be down-regulated in larvae exposed to warming. Cell adhesion and migration play important roles in development and immune and inflammatory responses (see Casazza et al., 2007; Schwab et al., 2012), pinpointing that larvae could be subjected to tissue injury, which has been posteriorly confirmed via microscopy analysis (Madeira et al., in preparation).

4.5. Growth metabolism

Interestingly, larvae exposed to warming up-regulated somatotropin, a known growth hormone, indicating that warming may increase growth and affect developmental processes. Literature concerning temperature effects on growth is extensive in marine species and overall it is acknowledged that warmer temperatures increase growth until a threshold is reached, in which extreme temperatures lead to a decrease in growth (e.g. Houde, 1989; Kucharczyk et al. 1997; Vinagre et al. 2012; 2013). One could argue that if growth is stimulated at 24 °C, then growth related proteome changes could also occur, including cytoskeleton and protein turnover changes (e.g. Papakostas et al., 2010). However, we do not know if there was a significant difference in growth rate between temperature groups. Moreover, cytoskeleton and protein turnover are highly associated to stress responses (e.g. Tomanek, 2011), which is supported by the observed fitness changes (high mortality levels) and concomitant changes in other stress-related proteins such as chaperones. Thus, it is more likely that these protein changes are directly associated with thermal stress, especially considering that the optimum temperature for *S. aurata* larvae is in the range of 16–22 °C and temperatures outside this range are deleterious to these fish (e.g. Polo et al., 1991). Interestingly, growth hormones and growth factors have also been shown to increase under abiotic stressful conditions and could be important for the mobilization of energy reserves to meet the higher energetic demands of living under stress (Barrett, 1988; Reinecke et al., 2005; Deane and Woo, 2009). Thus, growth hormones could also function as metabolic hormones (Reinecke et al., 2005). Despite no changes being detected in energetic pathways, this role should be investigated for somatotropin in larval fish.

4.6. Porphyrin metabolism

Oxygen transport seemed to be compromised in larvae exposed to warming. This finding is supported by the down-regulation of heme-binding protein 1 protein, which binds porphyrins i.e. organic aromatic compounds that act as co-factors of several proteins (e.g. cytochrome c, haemoglobin and myoglobin). In fact, several studies have reported that thermal tolerance is related to the capacity to transport oxygen to organs (Pörtner and Knust, 2007; Pörtner and Farrell, 2008; Pörtner and Peck, 2010). When organisms reach a certain temperature threshold, onset of hypoxaemia takes place and organisms may have to rely on anaerobic metabolism to survive heat shock. Therefore, a down-regulation of heme-binding proteins suggests a drop in aerobic scope, which characterizes the onset of thermal limitation, as reported in Pörtner and Knust (2007).

4.7. Conclusions

In this study, we used an integrative approach connecting proteome changes with organism-level indicators (see Fig. 6, summary figure). We showed that *S. aurata* exposed to warming rely on protein turnover mechanisms, gene expression regulation, cytoskeletal re-arrangements and a fined-tuned regulation of cargo transporting to promote homeostasis, muscle integrity and organ functioning. Oxygen transport seemed compromised suggesting the onset of hypoxaemia. As larvae showed no changes in proteins related to energetic pathways, we put forward the hypothesis that this lack of energetic adjustments could have limited the coping abilities of larval fish. This may be partially corroborated by the fact that detected changes in proteome did not provide acclimation, as shown by low survival rates at the highest temperatures. Thus, temperature may have bottleneck effects at early life stages of *S. aurata*, compromising recruitment's success, and suggesting the need for improved stock management. Nevertheless, as this study was performed on farmed fish, such assumption should be the scope of future studies in wild animals. It should be noted that farmed animals may be genetically related

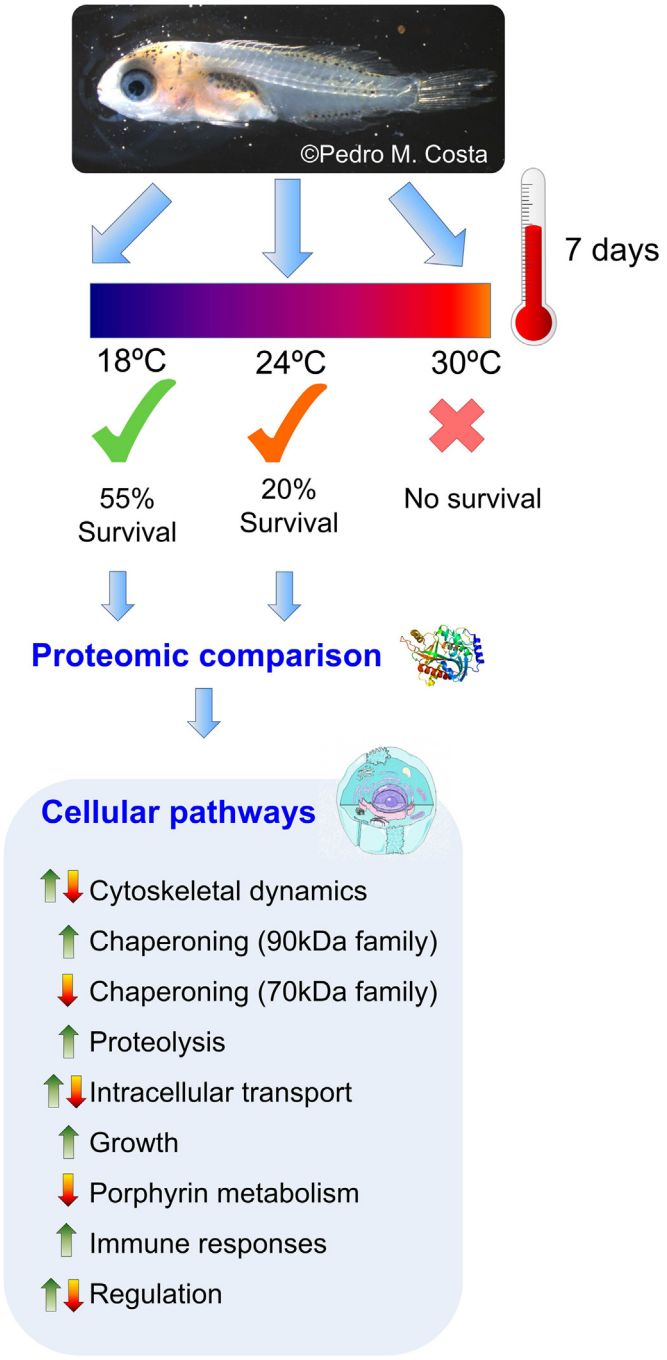

Fig. 6. Summary of the study. *Sparus aurata* larvae exposed to warm temperatures (control 18 °C vs 24 °C and 30 °C) showed decreased survival, reaching 100% mortality at 30 °C. Proteomic comparison of whole larvae at 18 °C vs 24 °C showed that larvae modulated proteins involved in cytoskeleton dynamics (actin, actin related proteins, myosin, tropomyosin, tubulin, keratin), chaperoning (heat shock cognate 71 kDa, heat shock protein 90 kDa, IQ motif and AAA domain containing protein), intracellular transport (kinesin, IQ motif and AAA domain containing protein), growth (somatotropin), porphyrin metabolism (heme-binding protein), proteolysis (proteasome subunit, IQ motif and AAA domain containing protein), regulation (POTE ankyrin F), cell-cycle regulation and transcription (IQ motif and AAA domain containing protein). These processes may be important in cell-functioning and survival upon challenging environmental conditions but high levels of mortality suggest that full acclimation may not be achieved. Green arrows (↑) indicate up-regulation and red arrows (↓) indicate down-regulation.

and could therefore be less resistant than wild ones due to possible inbreeding. However, the genetic structure of farmed fish is mostly unknown due to the lack of an established methodology regarding breeding programmes (see Arabaci, 2010). Thus, it is crucial to uncover stress thresholds in wild fish in order to fully understand acclimation potential. The possible influence of re-stocking programmes, spawning in off-shore cages and farm escapees on wild populations should also be evaluated in the context of resilience and vulnerability to climate change, since these events are quite frequent and could lead to an altered genetic structure of *S. aurata* populations (Sola et al., 2007; Dimitriou et al., 2007; Chavanne et al., 2008; Arabaci et al., 2010; Somarakis et al., 2013; Šegvić-Bubić et al., 2014).

Harsh extreme events are predicted to be more frequent, intense and extended in time (Miranda et al., 2002; IPCC, 2007, 2013). Such events have been previously associated with high mortality in marine species and can alter biodiversity patterns (see Garrabou et al., 2009; Dudgeon et al., 2010; Rose et al., 2012; Wernberg et al., 2013; Pearce and Feng, 2013). Therefore, the sustainability of key fisheries species may be in jeopardy, especially considering that these species are already under other anthropogenic pressures such as overfishing. Cheung et al. (2013) and Pecl et al., (2014) have reported the urgent need to develop adaptation plans to minimize the effects of warming on marine fisheries and, in this context, knowing the sensitivity of fish early life stages to warming is crucial, improving ecological and economic forecasting. This study provides insights into proteome regulation of larval fish and highlights the need for developing improved stock management plans for sea breams because larval stages seem to lack the ability to acclimate to chronic warming.

Author contributions

MSD and CV designed the study; MSD and DM collected larvae and performed the experiment; DM and JEA carried out sample treatment, 2D electrophoresis and image analysis; RV performed mass spectrometry analysis and MASCOT search; DM performed expression analysis and functional categorization of proteins; JLC contributed new reagents and analytical advice; DM wrote the manuscript with relevant inputs from all authors.

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Ethical statement

This study was approved by national authorities (Direcção Geral de Alimentação e Veterinária, DGAV) and followed EU legislation for animal experimentation (Directive 2010/63/EU). Three authors have a level C certification by FELASA (Federation of European Laboratory Animal Science Associations).

Data accessibility

Accession numbers ACTM_STYPL, KIF5C_HUMAN, ARPC2_HUMAN, IQCAL_RAT, MLE1_LIZRA, SOMA_PRIGL, HEBP1_CHICK, PSB3_ONCMY, POTEF_HUMAN, ACT2_MOLOC, HSP7C_ICTPU, TPM1_LIZAU, HSP90A_RABIT, TBA4A_HUMAN, K2C8_DANRE.

Sequences were uploaded as online supporting information (Table SM1).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.envres.2016.03.030.

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