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Negative synergistic impacts of ocean warming and acidification on the survival and proteome of the commercial sea bream, *Sparus aurata*

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Abstract

Global change is impacting aquatic ecosystems, with high risks for food production. However, the molecular underpinnings of organismal tolerance to both ocean warming and acidification are largely unknown. Here we tested the effect of warming and acidification in a 42-day experiment on a commercial temperate fish, the gilt-head seabream *Sparus aurata*. Juvenile fish were exposed to *control* (C 18ºC pH 8), *ocean warming* (OW 22ºC pH 8), *ocean acidification* (OA 18ºC pH 7.5) and *ocean warming and acidification* (OWA 22ºC pH 7.5). Proxies of fitness (mortality; condition index) and muscle proteome changes were assessed; bioinformatics tools (Cytoscape, STRAP, STRING) were used for functional analyses. While there was no mortality in fish under OW, fish exposed to OA and both OWA showed 17% and 50% mortality, respectively. Condition index remained constant in all treatments. OW alone induced small proteome adjustments (up-regulation of 2 proteins) related to epigenetic gene regulation and cytoskeletal remodeling. OA and both OWA induced greater proteome changes (12 and 8 regulated proteins, respectively) when compared to OW alone, suggesting that pH is central to proteome modulation. OA exposure led to increased glycogen degradation, glycolysis, lipid metabolism, anion homeostasis, cytoskeletal remodeling, immune processes and redox based signaling while decreasing ADP metabolic process. OWA led to increased lipid metabolism, glycogen degradation, glycolysis and cytoskeleton remodeling and decreased muscle filament sliding and intermediate filament organization. Moreover, as rates of change in temperature and acidification depend on region we tested as proof of concept an (i) acidification effect in a hot ocean (22ºC pH 8 *vs* 22ºC pH 7.5) which led to the regulation of 7 proteins, the novelty being in a boost of anaerobic metabolism and impairment of proteasomal degradation; and (ii) warming effect in an acidified ocean (18ºC pH 7.5 *vs* 22ºC pH 7.5) which led to the regulation of

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5 proteins, with an emphasis on anaerobic metabolism and transcriptional regulation. The negative synergistic effects of ocean warming and acidification on fish survival coupled to the mobilization of storage compounds, enhancement in anaerobic pathways and impaired proteasomal degradation could pose a serious threat to the viability of sea bream populations.

Key-words: global change, proteome, phenotypic plasticity, fish, temperature, pCO_2

Abbreviation list

ACT, actin isoform
ACT2, actin, muscle-type/alpha cardiac muscle 2
ACTS, actin alpha skeletal muscle
ACTSB, actin alpha skeletal muscle B
ADP, adenosine diphosphate
Ambic, ammonium bicarbonate
APOA1, apolipoprotein A-I
ATP, adenosine triphosphate
CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
Da, dalton
DOC/TCA, Na-deoxycholate/trichloroacetic acid
DTT, dithiothreitol
G3P, glyceraldehyde-3-phosphate dehydrogenase
GDE, glycogen debranching enzyme
GO, gene ontology
Hsp, heat shock proteins
IF2A, eukaryotic translation initiation factor 2 subunit 1
IPG, immobilized pH gradient
KAD1, adenylate kinase isoenzyme 1
KDM3A, lysine-specific demethylase 3A
LDHBA, L-lactate dehydrogenase B-A chain
MALDI TOF-TOF, Matrix-assisted laser desorption/ionization time-of-flight
MAPK, mitogen activated protein kinase
NADH, nicotinamide adenine dinucleotide reduced form
NADPH, nicotinamide adenine dinucleotide phosphate reduced form
NEBU, nebulin
OA, ocean acidification
OW, ocean warming
OWA, ocean warming and acidification
PERI, peripherin

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PMF, peptide mass fingerprints
PSA4, proteasome subunit alpha type-4
PSA6, proteasome subunit alpha type-6
SDS, sodium dodecyl sulphate
TPISB, triose phosphate isomerase B
ZFP69, zinc finger protein ZFP69

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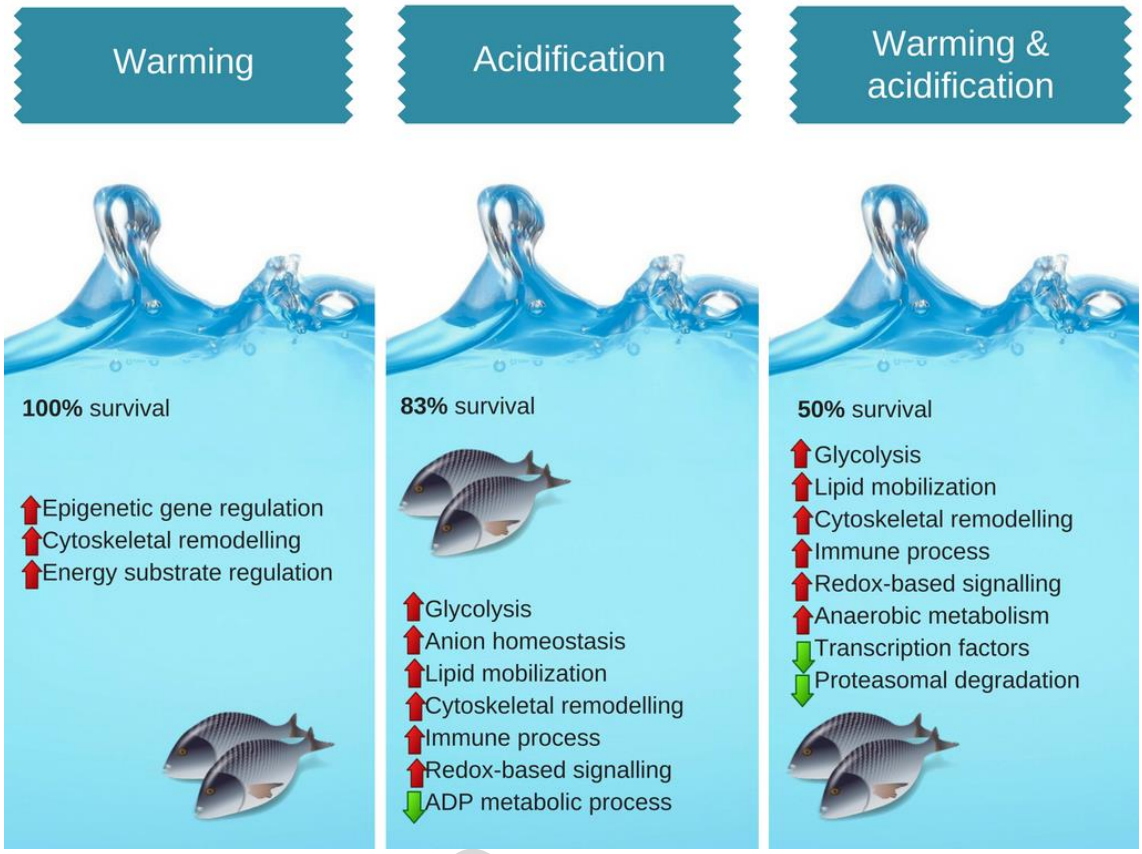
Graphical abstract



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Introduction

Global change forcing owing to greenhouse gas emissions (e.g. global warming and ocean acidification) is imposing biodiversity changes across terrestrial, coastal and oceanic habitats, with high risks for food production (IPCC, 2014; Walther et al., 2002). Sea surface temperature has risen 0.8ºC over the past century concomitantly with a decrease of 0.1 in ocean pH, which corresponds to a 26% increase in water acidity (IPCC, 2014). Model projections further indicate that oceans will warm up by 3 to 4ºC and will undergo additional acidification (ΔpH -0.3 to -0.5) until 2100, depending on region, habitat and emission scenario (Mora et al., 2013). Such changes are expected to decrease the fitness of marine biota (Kroeker et al., 2010; Mora et al., 2013) even though the sensitivity to environmental change may depend on taxonomic group. Organisms in high trophic levels such as carnivore fish will be highly impacted via elevated metabolic costs due to a rise in temperature coupled to a decrease in secondary production due to acidification (Nagelkerken and Connell, 2015). Some species (e.g. temperate killifish and tropical damselfish) are expected to show phenotypic plasticity over short (one generation) and long (transgenerational) time-scales allowing them to acclimate to new environmental conditions (Donelson et al., 2014, 2011; Fangue et al., 2006). However, negative effects may be exacerbated in exploited fish populations as their genetic diversity is highly reduced, further decreasing their potential for adaptation facing the changing world (Ottersen et al., 2006).

Physiological mechanisms of acclimation may not be specific for each stressor and overall they can be related to changes in gene expression (and protein levels), metabolism, behavior, life history traits, growth and reproductive tactics. For instance, organisms adjust metabolic rates (oxygen consumption) and modulate cellular pathways related to cytoskeleton dynamics, protein quality control system, antioxidant response,

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metabolic reprogramming, immune system, transcriptional regulation and signal transduction in response to elevated water temperature and/or acidification, in order to promote survival (Bresolin de Souza et al., 2014; Carter et al., 2013; Garland et al., 2015; Gunnarsson, 2010; Jayasundara et al., 2015; Madeira et al., 2016; Pörtner, 2010; Stillman and Tagmount, 2009; Timmins-Schiffman et al., 2014; Tomanek, 2014). However, elevated mortality rates and changes in the distributional range of species associated with global change and extreme climatic events have already been observed in the marine environment (Pearce and Feng, 2013; Walther et al., 2002; Wernberg et al., 2013, 2011), suggesting that physiological limits can be exceeded. Demersal sea breams may be particularly vulnerable to global change as their Critical Thermal Maxima values are not far from mean coastal and estuarine water temperatures and these could be surpassed by maximum temperatures reached during heat waves (Madeira et al., 2014, 2012). Additionally, thermal stress has been shown to induce tissue damage and mortality in the commercial gilthead seabream, *Sparus aurata* (*Linnaeus* 1758), paralleled to an increase in mitogen activated protein kinase (MAPK) signaling, glycolytic potential and markers of protein denaturation and oxidative stress (Feidantsis et al., 2009; Madeira et al., 2016b; Madeira et al., 2014). Warming is expected to have greater effects in physiology than acidification, as marine biota seem to be quite tolerant to a pH decrease (Byrne et al., 2009; Fabry et al., 2008; Findlay et al., 2010; Perry et al., 2015), although acidification may especially affect early-life stages (metabolic suppression, lower condition and impaired olfactory discrimination), particularly when combined with elevated temperature (Byrne, 2011; Fabry et al., 2008; Flynn et al., 2015; Philip L Munday et al., 2009; Rosa et al., 2014). Nevertheless, there seems to be no agreement on the combined effects of temperature and acidification. Some authors report antagonistic effects (Davis et al., 2013; Ferrari et al., 2015;

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Pistevos et al., 2016) while others report additive (Anlauf et al., 2011; Talmage and Gobler, 2011) and synergistic effects (Ferrari et al., 2015; Flynn et al., 2015), depending on species, developmental stage and parameters analyzed (reviewed by Byrne & Przeslawski 2013). Thus, knowledge on the interactive effects of global change drivers in marine biota is still limited (Byrne, 2011; Ferrari et al., 2015),

Sea breams are ecologically and economically relevant species in Southern Europe and North Africa. As predators, they exert top-down control of coastal ecosystem functioning and are a highly relevant group for the fishing (6,703 tonnes in 2014) and aquaculture industries (158,389 tonnes in 2014) (EUMOFA, 2015; FAO, 2015). Thus, the aim of this study was to investigate the long-term combined effects of ocean warming and acidification on a relevant sea bream, *Sparus aurata*. We hypothesized that (i) temperature and the combination of temperature and acidification induce greater physiological effects on fish than acidification alone; (ii) fish alter their proteome in response to single and both stressors, inducing proteins with cytoprotective functions and enhancing glycolytic potential to try to sustain the cellular stress response. To test these hypotheses we subjected fish to a 42-day experiment simulating global change conditions for 2100 (+4ºC) and calculated mortality and Fulton's K condition index concomitantly with the use of proteomics tools to assess protein changes in the muscle of fish. Such tools allow the establishment of direct links between molecular responses and phenotypes/fitness and the unravelling of pathways that characterize acclimation and adaptation processes (Dalziel and Schulte, 2012; Diz et al., 2012; Dupont et al., 2007; Karr, 2008), providing a mechanistic insight into the impacts of global change drivers on exploited high trophic level fish.

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2. Material and methods

2.1 Ethical statement

This study was approved by *Direcção Geral de Alimentação e Veterinária* and followed EU legislation for animal experimentation (Directive2010/63/EU). Two authors have a level C (persons responsible for directing animal experiments) certification by the Federation of European Laboratory Animal Science Associations.

2.2 *Sparus aurata* housing and husbandry

Fish (n = 48, mean ± sd; total lenght of 11 ± 3 cm and 38 ± 8 g of weight) were obtained from a fish farm (MARESA, Spain) and transported to the laboratory in 100 L opaque plastic boxes with constant aeration and stable temperature conditions (100% survival during transport). Sample sizes were calculated following previous omics studies (Jayasundara et al., 2015; Logan and Somero, 2011). Fish were placed in a re-circulating system consisting of two 400 l glass tanks (57 × 100 × 70 cm) with 24 individuals *per* tank. The tanks were filled with clean natural aerated sea water (95 - 100% air saturation), with a constant temperature of 18°C, salinity 35‰ and pH 8.0 (conditions of the fish farm). The fish were acclimated for 2 weeks and their welfare was assessed (e.g. presence/absence of wounds, external parasites, spots, ragged fins, lack of appetite). During the acclimation and experimental trials, a regime of period feeding was carried out (twice a day) with commercial food pellets (Gemma Diamond 1.8, Skettring).

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2.3 Experimental design

Fish were randomly divided into four 227 l tanks (37 × 98 × 62.5 cm) (1) *control* (C) 18ºC, pH 8 similar to natural water conditions to wild fish; (2) simulating conditions of *ocean warming* (OW, +4ºC) 22ºC, pH 8; (3) *ocean acidification* (OA) 18ºC, pH 7.5; (4) *ocean warming and acidification* (OWA) 22ºC, pH 7.5 (according to IPCC 2013) and maintained at these conditions for 42 days (n=12 fish per tank). Temperature was maintained using thermostats and pH levels were adjusted with CO_2 gas mixture injection. Water parameters (ammonia, nitrites, nitrates, O_2, temperature and pH) were monitored daily. Salinity was kept at 35 ± 1, the photoperiod was 12L:12D and normoxia levels were kept at pO_2>150 mmHg. All experiments were carried out following the guidelines described in (Riebesell *et al.* 2010), including carbonate chemistry manipulation. At the end of the experiment, fish (n=3 per treatment) were euthanized via cervical transection and white muscle was collected and stored at -80ºC for analyses. Muscle was chosen as target tissue because (i) of its commercial value; (ii) its proteome has already been characterized in *S. aurata* (Addis et al., 2010; Piovesana et al., 2016), and (iii) muscle activity requires an elevated energy expenditure and has been linked to fish well-being (Lembo et al., 2007).

2.4 Mortality rates and Fulton's K condition index

Cumulative mortality and Fulton's K condition index were calculated at the end of the experiment in each tank. The comparison of Fulton's K between treatments was carried out via a one-way ANOVA, given that data met the assumptions of normality (Shapiro-Wilk's test) and homoscedasticity (Levene's test).

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2.5 Sample preparation

Approximately 150 - 200 mg of muscle tissue per individual was homogenized in phosphate buffer saline (PBS, pH 7.4). After centrifugation (10 min at 16,000 ×*g*), the supernatants were precipitated by DOC/TCA method adapted from (Peterson, 1977) with minor changes (Madeira et al., 2016). The protein pellets were solubilized in rehydration buffer and protein concentration was determined by Bradford's method (Bradford, 1976).

2.6 Two dimensional gel electrophoresis

Samples containing 200 µg of protein were loaded onto IPG strips (pH 3-10, 7 cm, Bio-Rad) and isoelectric focusing was carried out in a Protean® IEF Cell (Bio-Rad), according to the manufacturer's instructions for 7 cm strips: 250 V for 20 min (linear mode), 4000 V for 2 h (linear mode) and 4000 V for 10,000 V-h (rapid mode). Following, strips were incubated in equilibration buffer I (15 min) followed by equilibration buffer II (15 min). Then, IPG strips were placed on top of SDS-PAGE 12.5% polyacrylamide gels and overlay with agarose sealing solution. Gels were run in Mini-Protean® 3 Cell (Bio-Rad) at 200 V, 400 mA, for 60 min. Two replicate gels were carried out for each sample.

2.7 Gel staining and image analysis

The gels were then stained overnight with colloidal coomassie brilliant blue G-250 and gel imaging was carried out with the PropicII-robot (Genomic Solutions™, Cambridgeshire, UK). Digitalized images of the gels were analysed with Progenesis SameSpots software (version 4.0, NonLinear Dynamics, Totallab, UK) and

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differentially expressed spots were detected using the incorporated statistical package (ANOVA, alpha=0.05) followed by Tukey's post-hoc tests (alpha=0.05).

2.8 In-gel protein digestion

The spots of interest were manually excised from gels and trypsin digested (0.02 $\mu g.\mu L^{-1}$ in Ambic 12.5 mM / 2% acetonitrile). The supernatants were collected to new tubes and dried-down in SpeedVac (Thermo Fisher Scientific Waltham, MA, USA). The dried peptides were stored at -60ºC until mass spectrometry analysis.

2.9 Mass spectrometry (MS) analysis and database search

The peptides were re-suspended in formic acid 0.3% and mixed (1:1) with a saturated solution of a-cyano-4-hydroxycinnamic acid and spotted onto the MALDI sample target plate (3 replicates per sample). 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 analyzed by the Global Protein Server Workstation (Applied Biosystems), which uses internal MASCOT software (v2.1.0 Matrix Science, London, UK) for searching the peptide mass fingerprints and MS/MS data. Either NCBI *Sparus aurata* database or Swiss-Prot nonredundant protein sequence database (October 2014) under the taxonomy Chordata were used for searches following parameters: (i) fixed modifications: carbamidomethylation and propionamide of cysteine; (ii) variable modification: oxidation of methionine; (iii) Missed Cleavages: two; (iv) peptide mass tolerance: 40 ppm; (v) fragment tolerance: 0.3 Da and. The significance threshold was set to a minimum of 95% ($p \leq 0.05$).

2.10 Bioinformatics

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Protein GO annotation was carried out in STRAP v1.5 (Bhatia et al., 2009). A two-way hierarchical cluster analysis plus heat map was carried out in Cluster 3.0+Java TreeView using normalized average spot volumes. A protein-protein interaction network was constructed in STRING v10.0 (Szklarczyk et al., 2015) using *Homo sapiens* as model organism. Functional association protein networks were constructed in ClueGo 2.2.6 + CluePedia 1.2.6 plugins from Cytoscape v3.4.0 platform. A Venn diagram was constructed in Venny 2.1.0 (Oliveros 2007) to detect shared and exclusively regulated proteins between different treatments (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

For methodological details see supplementary material (Table S1).

3. Results

3.1 Mortality rate and Fulton's K condition index

Mortality was 0% in both control (18ºC, pH 8) and warming (22ºC, pH 8) treatments but reached 17% in the ocean acidification treatment (18ºC, pH 7.5) and 50% in the ocean warming and acidification treatment (22ºC, pH 7.5). Fulton's K condition index did not differ between any of the treatments ($F=1.76$, $p=0.19$).

3.2 Proteomic analysis

All gels from *S. aurata* were matched and compared to the reference gel to detect differences in the protein spots (total of 407 detected spots). The analysis of variance ($p<0.05$) showed that 43 protein spots were differentially expressed between the four temperature/pH groups. Of the 43 spots, 24 were successfully identified (55.8%) (Fig. 1). For the successfully identified spots, different

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normalized volume levels were obtained between the control group (18ºC and pH 8) and the different global change groups (22ºC and pH 8; 18ºC and pH 7.5; 22ºC and pH 7.5). Expression patterns were more similar between temperatures and more distant between different pH (Fig. 2). In the *ocean warming* treatment, only two proteins were significantly up-regulated when compared to control conditions (PERI, KDM3A); in the *ocean acidification* treatment, 10 proteins were up-regulated (5 isoforms of ACT; 3 isoforms of G3P; APOA1; GDE) and 2 were down-regulated (TPISB; KAD1) when compared to control conditions; in the *ocean warming and acidification* treatment 6 proteins were up-regulated (3 isoforms of G3P; APOA1; ACT2; GDE) and 2 were down-regulated (PERI; NEBU) when compared to the control (Fig. 2 and supplementary Table S2). Moreover, as rates of change in temperature and acidification depend on region and local conditions we tested an (i) acidification effect in a hot ocean (regions with faster rate of warming than acidification) by comparing 22ºC pH 8 *vs* 22ºC pH 7.5 (Tukey's post-hoc comparisons). In this case, four proteins were up-regulated (APOA1; G3P; ACT2; LDHBA) while 3 were down-regulated (PERI; PSA4; PSA6); (ii) warming effect in an acidified ocean (regions with faster rate of acidification than warming) by comparing 18ºC pH 7.5 *vs* 22ºC pH 7.5 (Tukey's post-hocs). This led to an up-regulation of LDHBA and a down-regulation of four proteins (PERI; NEBU; ZFP69; G3P).

The proteins were classified into eight categories (Fig. 3a) according to their biological function namely cellular process (14.30%), regulation (12.26%), metabolic process (8.17%), developmental process (3.70%), immune system process (2.40%), localization (1.20%), response to stimulus (1.20%) and other (5.11%). Furthermore, the molecular function of these proteins was classified into

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five categories namely catalytic activity (11.35%), binding (15.48%), structural molecule activity (3.10%), molecular transducer activity (1.30%) and other (1.30%) (Fig. 3c). These proteins exist within several cellular components including cytoplasm, ribosome, nucleus, cytoskeleton, plasma membrane, macromolecular complex, other intracellular organelles, extracellular and other components (Fig. 3b). Functional analysis in Cytoscape showed that the proteins were involved in pathways such as glycolysis/gluconeogenesis, aldehyde biosynthetic process, muscle filament sliding, polyamine metabolic process and mesenchyme migration (Fig. 3d). The up-regulation/down-regulation of such processes varied according to the global change treatment (Fig. 4). Ocean acidification (18ºC pH 8 *vs* 18ºC pH 7.5) led to the up-regulation of mesenchyme migration, skeletal muscle fiber adaptation, anion homeostasis, negative regulation of cell adhesion molecule production while down-regulating ADP metabolic process and glyceraldehyde-3-phosphate biosynthetic process (glycolytic process and NADH regeneration were up- and down-regulated) (Fig. 4a). Ocean warming (18ºC pH 8 *vs* 22ºC pH 8) led to the up-regulation of histone demethylation, formaldehyde biosynthetic process and intermediate filament cytoskeleton organization (Fig. 4b). Acidification in a hot ocean (22ºC pH 8 *vs* 22ºC pH 7.5) led to the up-regulation of glucose catabolic process, glycolysis/gluconeogenesis, fat digestion and absorption, negative regulation of cell adhesion molecule production, mesenchyme migration and glomerular mesangial cell development, while down-regulating intermediate filament cytoskeleton organization and the proteasome (Fig. 4c). Warming in an acidified ocean (18ºC pH 7.5 *vs* 22ºC pH 7.5) led to an up-regulation of glucose catabolic process to lactate via pyruvate (anaerobic metabolism) and a down-regulation of

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peptidyl cysteine S-trans-nitrosylation, intermediate filament cytoskeleton organization and muscle filament sliding (glucose catabolic process was up- and down-regulated) (Fig. 4d). When compared to control conditions, ocean warming and acidification (18ºC pH 8 vs 22ºC pH 7.5) led to an up-regulation of glomerular mesangial cell development, negative regulation of cell adhesion molecule production, peptidyl cysteine S-trans-nitrosylation, fat digestion and absorption, negative regulation of cytokine secretion involved in the immune response, positive regulation of triglyceride catabolic process, cholesterol import and a down-regulation of intermediate filament cytoskeleton organization and muscle filament sliding (Fig. 4e). The Venn diagram (Fig. 5) showed that proteins exclusively regulated in OA were ACT2, TPISB, KAD1, ACTSB, and two isoforms of ACTS; the protein exclusively regulated in OW was KDM3A; the protein exclusively regulated in OWA was NEBU. Proteins shared between OA and OWA were APOA1, GDE, ACT2 and three isoforms of G3P. The protein shared between OW and OWA was PERI. The STRING protein-protein interaction (PPI) enrichment analysis showed that this protein network has significantly more interactions than expected for a random set of proteins ($p=0.0005$), thus being biologically connected (supplementary Fig S1).

Discussion

Phenotypic plasticity ("environmentally induced changes that occur within individual organisms during their lifetimes or physiological adaptation" as defined by Kelly, Panhuis and Stoehr 2012) can partly arise from differences in gene expression and is a key process that will determine the ability of organisms to survive and thrive in future oceans. In this long-term acclimation study, we

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showed that the effect of ocean acidification and the combined effect of ocean warming and acidification on *S. aurata* were greater than the warming effect alone. Physiological limits were surpassed in the OA and OWA treatment, as opposed to the OW. This finding is supported by the elevated mortality rates in these treatments, which reached 50% when both global change stressors were combined, indicating that ocean warming and acidification have negative synergistic effects on the survival of fish. Such elevated mortality rates under these conditions have also been observed in other organisms such as corals (Cumbo et al., 2013), foraminifera (Schmidt et al., 2014), gastropods (Noisette et al., 2014; Zhang et al., 2014) and sharks (Rosa et al., 2014). Nevertheless, a literature review shows that this is highly dependent on species, developmental stage and sex (Dupont et al., 2010; Ellis et al., 2017; Melatunan, 2012). Despite the increase in mortality, no changes were detected in condition index, suggesting that fish maintained foraging activity and did not lose weight throughout the experiment in any condition. This result is unexpected because stressful conditions (i.e. warm and acidified waters) are energetically demanding for marine organisms, as they have to invest their energy reserves in cellular defense, potentially leading to decreased condition and growth, as observed in other fish (Ishimatsu et al., 2005; Rosa et al., 2014). However, as there is a lag time between molecular and organism-level responses, a 42 day experiment may not be long enough to reflect changes on condition. Nevertheless, trends may vary. For instance, Pope et al. (2014) detected increased survival and growth of early life stages of sea bass *Dicentrarchus labrax* under global change conditions, highlighting that some species may be particularly resistant to change, even

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though developmental domino effects should be investigated to take further conclusions.

Ocean warming

S. aurata is a widely distributed species, associated with shallow coastal and estuarine waters in sub-tropical latitudes in the Atlantic Ocean and Mediterranean and Black Seas. Therefore, it is frequently exposed to temperatures in the range of 18ºC to 25ºC (Madeira, 2016), even though high temperatures only occur for a limited period of time (a couple of weeks at the most). However, this may explain why the ocean warming treatment alone (22ºC pH 8) did not induce many molecular adjustments nor increased mortality when compared to control conditions. Moreover, there was no induction of heat shock proteins (hsp) at 42 days of exposure. Alternatively, hsp could have been up-regulated earlier in white muscle (and other organs, especially oxidative tissues). However, if this is the case, hsp returned to control levels at day 42 in muscle, suggesting that these proteins were able to repair any potential cellular damage in this organ. Still, a +4ºC chronic rise in temperature may not be enough to elicit a heat shock response (HSR), since previous studies have shown that even a +6ºC chronic rise in temperature does not elicit a HSR in most tissues of *S. aurata* (Madeira, 2016). Hsp are commonly up-regulated upon exposure to heat stress in organisms such as fish, shellfish, crustaceans, polychaetes and gastropods in order to stabilize unfolded proteins (Dilly et al., 2012; Feder and Hofmann, 1999; Hofmann and Somero, 1995; Logan and Somero, 2010; Madeira et al., 2014; Pöhlmann et al., 2011; Stillman and Tagmount, 2009; Tomanek, 2002), although their induction may be stronger during acute stress and in cold-adapted species in comparison to

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chronic stress and warm-adapted species (Dilly et al., 2012; Logan and Somero, 2010). Nevertheless, the lack of a heat shock response at day 42 and the lack of elevated mortality supports the hypothesis that *S. aurata* was not under stress at 22ºC, following the rationale that hsp hardly change with temperature within the species' native thermal range (Tomanek, 2002).

The major processes associated with warming included the up-regulation of histone demethylation, formaldehyde biosynthetic process and intermediate filament cytoskeleton organization. The remodeling of cytoskeletal components upon warming is well documented (see Tomanek 2011, 2014). Cytoskeleton stabilization is crucial to homeostasis and has also been detected in transcriptome and proteome studies using *S. aurata* (Madeira et al., 2016), other fish species (Buckley et al., 2006; Jayasundara et al., 2015; Logan and Buckley, 2015; Podrabsky and Somero, 2004) and crustaceans as models (Harms et al., 2014). Histone methylation state has been linked to mechanisms of DNA repair and changes in gene transcriptional activation/repression (Kouzarides, 2007), with demethylation inducing transcriptional activation. Methylation/demethylation states of chromatin and histones are associated with an epigenetic regulation of cellular processes (Bernstein et al., 2007; Kouzarides, 2007) and several authors have suggested that acclimation and adaptation to global change may involve epigenetic mechanisms that can be transmitted to future generations (Donelson et al., 2011). Moreover, the up-regulation of the demethylation of histones is known to generate formaldehyde and succinate, which may enter the pentose phosphate pathway and the tricarboxylic acid cycle (TCA) for NADPH production, nucleic acid biosynthesis and energy production. As metabolic rates usually increase with temperature in marine biota (Clarke and Fraser, 2004; Rosa et al., 2014; Rosa et

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al., 2014), these changes should sustain the metabolism of fish in a warmer ocean. Adjustments in these pathways suggest that warmer temperatures induce a switch from pro-oxidant NADH to anti-oxidant NADPH metabolic pathways as also observed by Tomanek and Zuzow (2010) and Tomanek (2014). Moreover, changes in succinate in response to variable abiotic conditions have been associated with anaerobic metabolism (in bivalves, Grieshaber et al., 1994) and the maintenance of membrane potential, ATP synthesis and anaplerosis in *Mycobacterium* (Eoh and Rhee, 2013). Therefore, succinate may play a similar role in other organisms. Even though we did not measure succinate levels, an increased production of this metabolite under stress has indeed been reported for oysters (e.g. Lannig et al., 2010).

Ocean acidification

Several studies have shown that acidification has deleterious impacts on marine species, including lowered abundance, reduced predator-escape response, metabolic suppression, oxidative stress, decreased digestive capacity, reduced oxygen transport capacity, impaired olfactory discrimination and calcification (Fabry et al., 2008; Manríquez et al., 2016; Munday et al., 2010; Munday et al., 2009; Pimentel et al., 2015; Ross et al., 2011). Such effects may be dependent on the molecular plasticity of organisms and capacity for efficient cellular reprogramming and protection.

Metabolic reprogramming in the ocean acidification treatment involved the down-regulation of triosephosphate isomerase B (TPISB) and up-regulation of glyceraldehyde-3-phosphate dehydrogenase (G3P) and glycogen debranching enzyme (GDE). Glycolytic enzymes can undergo antagonistic changes upon

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stressful conditions with enzymes at the preparatory phase decreasing and enzymes involved in the pay-off phase increasing (Garland et al., 2015). The down-regulation of TPISB indicates a decrease in the conversion of dihydroxyacetone phosphate into gyceraldehyde-3-phosphate. However, the increase in GDE indicates the mobilization of glucose from glycogen in the muscle to be used as an energy source, which coupled to the up-regulation of G3P suggests an increase in glycolytic potential. This may indicate higher energy demand in fish subjected to acidification. Hypercapnia leads to a reduction in intracellular pH (Michaelidis et al. 2007) and therefore ion regulatory and transport mechanisms modulate the energy budget of marine organisms exposed to acidification (Kreiss et al., 2015; Pan et al., 2015). Our results indicate an up-regulation of anion homeostasis *via* APOA1, corroborating potential changes in ion concentrations and acid-base status. Interestingly, the disruption of ion homeostasis under ocean acidification seems to promote an altered neural function and consequent maladaptive behavioral changes (Nilsson and Lefevre, 2016). Moreover, APOA1 is also involved in fat digestion and absorption, suggesting indeed a higher energetic demand under acidification scenarios. Similarly to warming, skeletal muscle fiber adaptation was induced via the up-regulation of actin isoforms, thus cytoskeleton remodeling is also important under acidification conditions. Other up-regulated processes include mesenchyme migration and negative regulation of cell adhesion molecule production, which are relevant mechanisms in immune system processes. Both ocean warming and acidification have been shown to alter the immune response of shellfish and fish with induction or suppression depending on the target organ and stress levels (mild or moderate levels lead to induction while extreme levels lead to

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suppression) (Matozzo and Marin 2011; Mackenzie et al. 2014; Wang et al. 2016; Machado et al., 2016). However, in this study, warming alone did not induce immune responses as opposed to acidification. Protein modifications (i.e. peptidyl-cysteine S-trans-nitrosylation) were also induced as opposed to the ocean warming treatment, suggesting a boost in redox-based signaling pathways (Bolotina et al., 1994; Stamler et al., 2001) under acidification scenarios.

Combined effect of ocean warming and acidification

Ocean warming and acidification had the greatest physiological effect of all tested treatments. Several studies have put forward that the combination of global change drivers leads to physiological, functional and behavioral impairments with potential consequences for reproduction, growth and survival (Faleiro et al., 2015; Ferrari et al., 2015; Rosa et al., 2014; Rosa and Seibel, 2008).

The molecular adjustments of *S. aurata* exposed to ocean warming and acidification were similar to the acidification effect alone, suggesting that pH may be the main factor modulating gene expression in sea breams under global change scenarios. Up-regulated mechanisms were related to glycolysis, cellular signaling, immune processes and lipid mobilization suggesting not only an increase in energy demand but also the activation of immune responses, probably in response to inflammation, which has been shown to occur in *S. aurata* exposed to acute stress (Madeira et al., 2014).

As rates of warming and acidification may differ between regions, organisms may live in warm or acidified waters and then face a subsequent stressor. As proof-of-concept, we compared the proteome of fish (via Tukey's post-hocs

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results following ANOVA) from group 18ºC pH 7.5 *vs* 22ºC pH 7.5 (simulating regions in which the rate of acidification would be faster than warming – acidified waters get warmer) and 22ºC pH 8 *vs* 22ºC pH 7.5 (simulating regions in which the rate of warming would be faster than acidification – warm waters get acidified). Our results suggest that regulated molecular processes may differ depending on local conditions (temperature variations in an acidified area or pH variations in a warmed area). When warming takes place in an acidified area, anaerobic metabolism is up-regulated (glucose catabolic process to lactate via pyruvate) while cell signaling and muscle contraction decrease, suggesting possible alterations in swimming activity and deleterious lactate accumulation as predicted by the OCLTT (oxygen and capacity limited thermal tolerance) (Pörtner, 2012). In fact, global change drivers are reported to induce lethargy and reduce swimming speed and foraging behavior in fish, potentially affecting the ability to capture prey and escape predators (Faleiro et al., 2015; Johansen et al., 2014). Transcriptional activity and cellular structural properties also underwent changes as indicated by the down-regulation of intermediate filament cytoskeleton organization, muscle filament sliding, peptidyl-cysteine S-trans-nitrosylation and zinc finger protein ZFP69 (transcriptional regulator). Regulation of transcription factors upon exposure to warming has also been detected in *S. aurata* larvae (D. Madeira et al., 2016), intertidal gobies (Logan and Somero, 2010) and chinook salmon (Tomalty et al., 2015) and may be crucial to maintain homeostasis.

When acidification occurs in warm waters then glycolytic potential, anaerobic metabolism, cell migration, cell signaling and fat metabolism show an enhancement while proteasomal degradation and intermediate filament

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cytoskeleton organization undergo down-regulation at 42 days of exposure. Thus higher energetic demands but lowered aerobic scope, impaired protein degradation and decreased cellular mechanical strength seem to occur in this case. To confirm the assumption of higher energetic demands, future studies should combine the assessment of oxygen consumption (metabolic rates) with sub-cellular markers. Proteasomal degradation of proteins is a crucial part of the cellular stress response and is usually up-regulated under stressful conditions in order to prevent cytotoxicity due to the accumulation of denatured proteins (Hofmann and Somero, 1995; Madeira et al., 2014). In fact, ocean warming and acidification should lead to gene expression changes that coordinate acid-base balance, metabolic adjustments and cellular stress response mechanisms (Harms et al., 2014). However, such stress response mechanisms may be attenuated if organisms are not capable of compensating for ion and acid-base changes (Harms et al., 2014). Previous transcriptomic studies conducted in sea urchin showed that molecular chaperones, ubiquitin-proteasome pathway and anti-oxidant defense were impaired by ocean acidification (Todgham and Hofmann, 2009). Accordingly, either no changes or a decrease in cellular stress response proteins (e.g. ubiquitin, heat shock proteins, anti-oxidants) were detected suggesting impaired cellular stress response at 42 days of exposure. This finding is corroborated by the 50% mortality found in OWA treatment. Thus, *S. aurata* may be unable to compensate for protein damage paralleled to lactate accumulation from anaerobic metabolism. Moreover, previous studies have shown that global change drivers may decrease the metabolic rate and ATP turnover in fish, thus reducing the available energy for cellular reparative mechanisms (Munday et al., 2009). Such effects could lower the fitness of fish *via* inflammation, reduced

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growth and reproduction and decrease their tolerance to additional stressors. This is crucial considering that *S. aurata* inhabits estuarine and coastal waters, which are highly subjected to further anthropogenic forcing (e.g. pollutants, exotic species invasions *via* ballast water, fishing).

Conclusions

Here we show that *S. aurata* are able to face a 4ºC increase in ocean temperature without undergoing cellular damage and mortality, solely requiring cytoskeletal adjustments coupled to metabolic regulation of substrates of the pentose phosphate pathway and the tricarboxylic acid cycle to meet the higher energetic demands of a warmer ocean. Moreover, temperature induced changes in histone methylation patterns suggesting that epigenetic gene regulation is important in acclimation mechanisms. Nonetheless, acidification and the combined effect of warming and acidification induced mortality and immune processes in *S. aurata*. Moreover, the transition to anaerobic metabolism due to acidification is more prone to occur in the presence of elevated temperature. However, long-term hypercapnia alone has been shown to induce anaerobic metabolism in the muscle of *S. aurata* (Michaelidis et al., 2007). Yet, such conclusions were reached for an exposure period of 10 days, while in this study the exposure time was 42 days. Similarly, it seems that warming only induces anaerobic metabolism in waters that are already acidified. This is in accordance with the idea that acidification will narrow thermal tolerance breadths (Pörtner and Farrell, 2008) possibly through the oxygen and capacity limitation of thermal tolerance, in which a mismatch between oxygen demand and supply leads to a decrease in aerobic

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metabolism and an increase in anaerobic metabolism that can only be sustained for short periods (Pörtner, 2010; Pörtner and Knust, 2007).

Prolonged warming and acidification may also influence ontogenetic processes. However, in this experiment with juvenile fish, distinguishing ontogenetic impacts from the stressful impacts of temperature *per se* was not possible, warranting further research on this issue.

The negative synergistic effects of warming and acidification on survival coupled to elevated energy demands, anaerobic metabolism and impaired proteasomal degradation could lead to cytotoxic effects and pose a serious threat to sea bream populations. Sea breams inhabit shallow waters that have little inertia and are therefore prone to environmental change, including heat waves, which may have the potential to deplete sea bream populations (see Madeira et al. 2012, 2014, 2016b; c). This paralleled to other environmental issues such as pollution and overfishing may exert strong selective pressures that can either result in lowered abundance/local extinction, distributional changes or adaptation. Nonetheless, our results highlight the need for the integration of physiological and molecular information in conservation strategies and management plans, contributing to the sustainability of fish stocks in future oceans.

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Author contributions

M.S.D. and R.R. designed the study; R.R. and T.R. performed the experiments; J.E.A. carried out sample preparation, electrophoresis, image analysis and protein digestion; R.V. performed mass spectrometry analysis and protein identification; D.M. carried out bioinformatics and statistical analysis; D.M. and J.E.A. wrote the manuscript with relevant inputs from the other co-authors; D.M. and J.E.A contributed equally to this work.

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Table 1. Spots differentially expressed in the muscle of juvenile *Sparus aurata* exposed to *control* (18ºC, pH 8), *ocean warming* (22ºC, pH 8), *ocean acidification* (18ºC, pH 7.5), and *ocean warming and acidification* (22ºC, pH 7.5).

Spot	Acession number	Protein (Species)	Protein pI	Peptide Count	Protein Score	Protein Score C.I. %	Ion Score	Ion Score C.I. %	Peptide Sequence
585	PSA6_HUMAN	Proteasome subunit alpha type-6	6,34	6	127	100	45	98,676	HITIFSPEGR
							36	89,36	AINQGGLTSVAVR
861	KDM3A_HUMAN	Lysine-specific demethylase 3A	8,4	22	67,69	99,58			
466	G3P_MELGA	Glyceraldehyde-3-phosphate dehydrogenase (Fragment)	7,22	2	93,40	99,99	81	100	LVSWYDNEFGYSNR
420	ACTS_CARAU	Actin, alpha skeletal muscle	5,23	7	152	100	40	100	AVFPSIVGRPR
							85	100	SYELPDGQVITIGNER
886	G3P_DANRE	Glyceraldehyde-3-phosphate dehydrogenase	8,2	2	91,30	99,99	84	100	LVTWYDNEFGYSNR
185	ZFP69_HUMAN	Zinc finger protein ZFP69	8,78	10	62,40	95,19			
749	ACTSB_TAKRU	Actin, alpha skeletal muscle B	5,22	16	472	100	76	100	GYSFVTTAER
							44	99,64	AVFPSIVGRPR
							107	100	QEYDEAGPSIVHR
							144	100	SYELPDGQVITIGNER

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136	NEBU_HUMAN	Nebulin	9,11	37	71,40	99,39	32	98	GCKLSVTDDKNTVLALR
426	ACT2_XENTR	Actin, alpha cardiac muscle 2	5,23	7	205	100	51	99,756	IWHHTFYNELR
							89	1000	SYELPDGQVITIGNER
868	ACTS_CARAU	Actin, alpha skeletal muscle	5,23	3	114	100	80	1000	SYELPDGQVITIGNER
							37	1000	TTGIVLDAGDGVTHNVPVYEGYALPHAIMR
328	IF2A_CHICK	Eukaryotic translation initiation factor 2 subunit 1	5,07	12	66,90	98,29			
671	KAD1_CYPCA	Adenylate kinase isoenzyme 1	6,64	5	126	100	106	100	YGYTHLSSGDLLR
753	LDHBA_DANRE	L-lactate dehydrogenase B-A chain	6,4	4	66,30	98,04	11	0	VIGSGTNLDSAR
							25	0	IVADKDYSVTANSR
767	GDE_CANFA	Glycogen debranching enzyme	6,3	24	126	100	37	98,594	NIILAFAGTLR
							62	99,995	LEQGFELQFR
553	PSA4_HUMAN	Proteasome subunit alpha type-4	7,57	5	169	100	71	99,998	LLDEVFFSEK
							65	99,994	LSAEKVEIATLTR
867	ACT2_MOLOC	Actin, muscle-type	5,12	10	316	100	60	99,987	GYSFVTTAER
							32	90,796	AVFPSIVGRPR
							59	99,983	IWHHTFYNELR
							10	10	SYELPDGQVITIGNER

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							4	0	
608	APOA1_SPAAU	Apolipoprotein A-I	5,21	4	66,40	98,08	32	93,331	AVNQLDDPQYAEFK
840	G3P_PIG	Glyceraldehyde-3-phosphate dehydrogenase	8,51	2	99,09	99,99	87	100	LISWYDNEFGYSNR
841	PERI_BOVIN	Peripherin	5,28	13	64	96,67			
590	KCRM_HUMAN	Creatine kinase M-type	6,77	7	150	100	64	99,992	SFLVWVNEEDHLR
							49	99,754	GTGGVDTAAVGSVFDVSNADR
624	TPISB_DANRE	Triosephosphate isomerase B	6,45	11	248	100	80	100	WVILGHSER
							91	100	HVFGESDELIGQK
885	G3P_DANRE	Glyceraldehyde-3-phosphate dehydrogenase	8,2	5	235	100	110	100	VPTPNVSVVDLTVR
							96	100	LVTWYDNEFGYSNR
831	ACTS_CARAU	Actin, alpha skeletal muscle	5,23	5	183	100	60	99,958	IWHHTFYNELR
							74	99,998	SYELPDGQVITIGNER
791	G3P_MELGA	Glyceraldehyde-3-phosphate dehydrogenase (Fragment)	7,22	3	105	100	90	100	LVSWYDNEFGYSNR

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Figure legends

Fig. 1 Reference gel depicting the protein spots detected (n=407) in the muscle of *Sparus aurata* juveniles. Annotated spots were those that were differentially expressed between treatments and identified through mass spectrometry (24 spots) (ANOVA $p<0.05$).

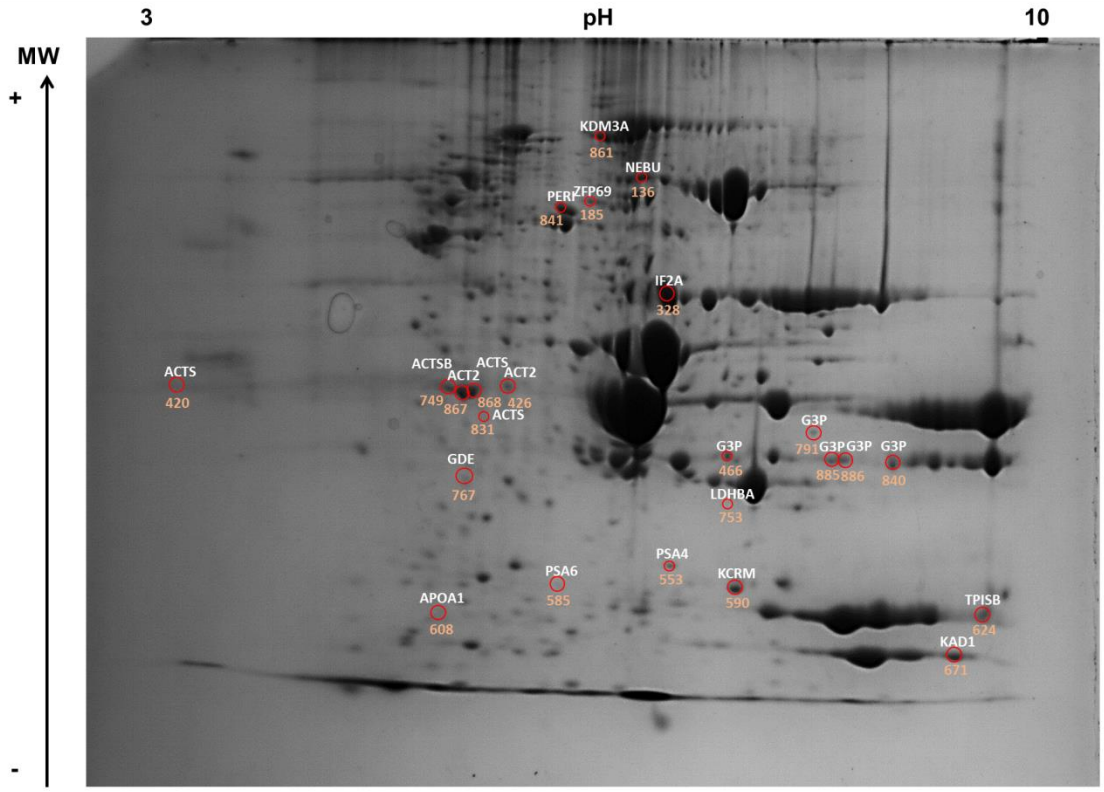

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Fig. 2 Two-way hierarchical clustering analysis of proteome data from *Sparus aurata* juveniles subjected to *control* (C) 18ºC, pH 8; *ocean acidification* (OA) 18ºC, pH 7.5; *ocean warming* (OW) 22ºC, pH 8; *ocean warming and acidification* (OWA) 22ºC, pH 7.5. Heat map of the clustered data matrix in which cells represent the $\log_2$ values of protein normalized volumes. The color scale ranges from green (lower than mean normalized volume) to red (higher than mean normalized volume). Columns represent different treatments while rows represent different proteins. C – cluster.

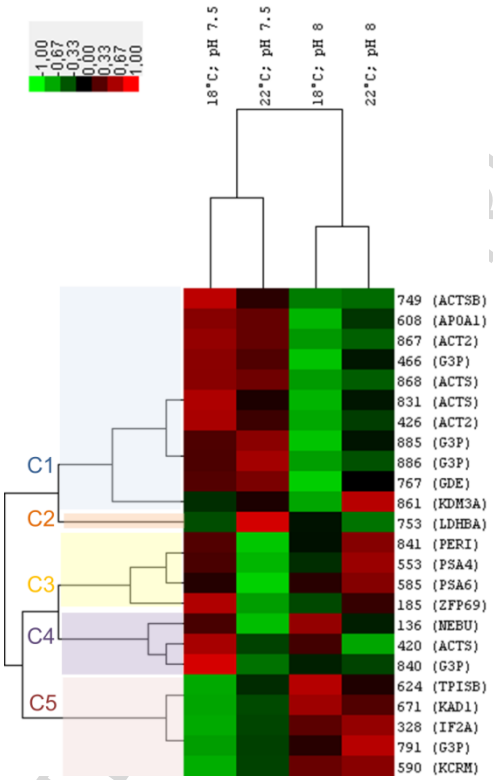

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Fig. 3 General and detailed functional categorization of identified proteins (a) biological process, (b) cellular component, and (c) molecular function obtained in STRAP v1.5 and (d) functional association protein network constructed in ClueGo 2.2.6 + CluePedia 1.2.6 plugin from Cytoscape v3.4.0 platform. Node size relates to statistical significance and number of genes associated with that biological process.

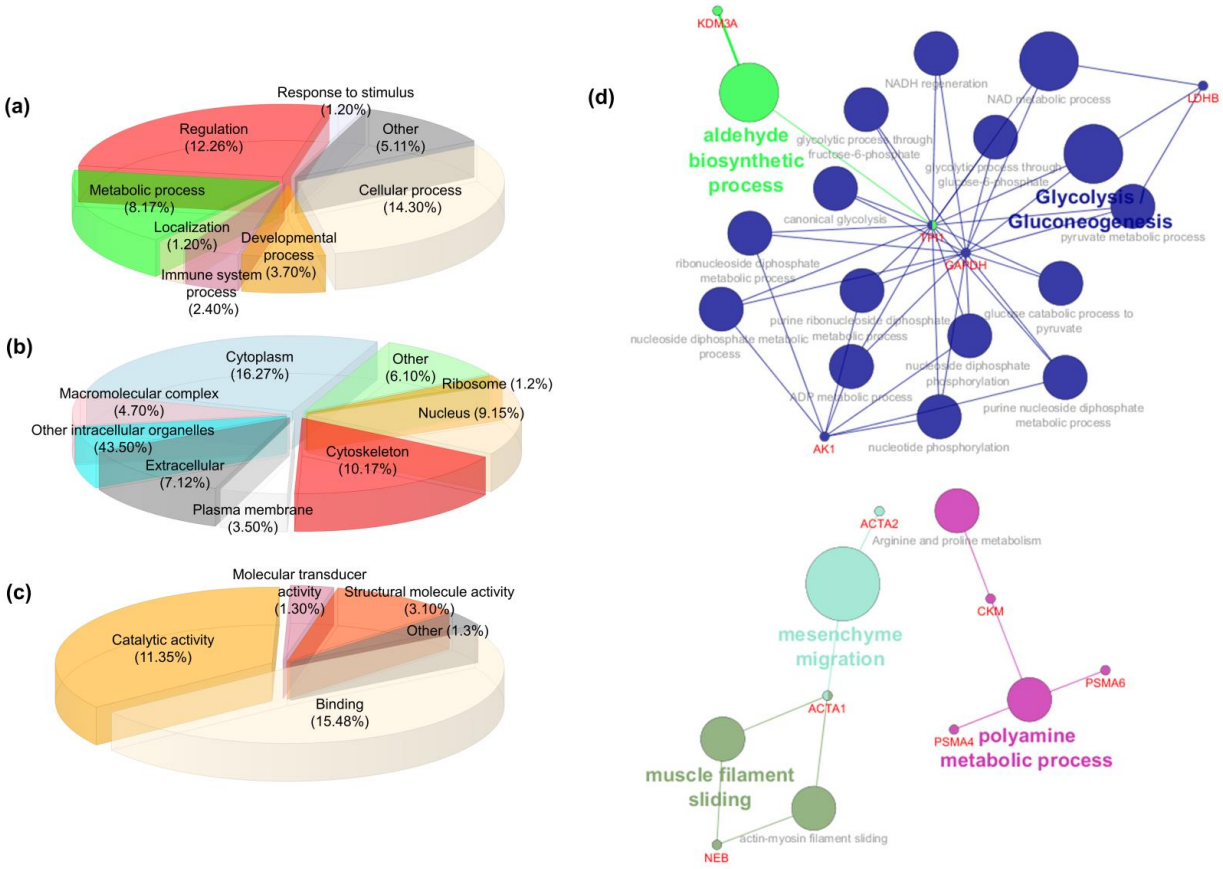

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Fig. 4 Functional association protein networks constructed in ClueGo 2.2.6 + CluePedia 1.2.6 plugins from Cytoscape v3.4.0 platform (a) ocean acidification effect (18ºC pH 8 *vs* 18ºC pH 7.5), (b) ocean warming effect (18ºC pH 8 *vs* 22ºC pH 8), (c) acidification effect in a hot ocean (22ºC pH 8 *vs* 22ºC pH 7.5), (d) warming effect in an acidified ocean (18ºC pH 7.5 *vs* 22ºC pH 7.5), (e) warming and acidification effect (18ºC pH 8 *vs* 22ºC pH 7.5). Red nodes – up-regulated; green nodes – down-regulated; grey nodes – both up- and down-regulated. Node size relates to statistical significance and number of genes associated with that biological process except in (b) in which significance is related to the red tones (darker red = greater significance).

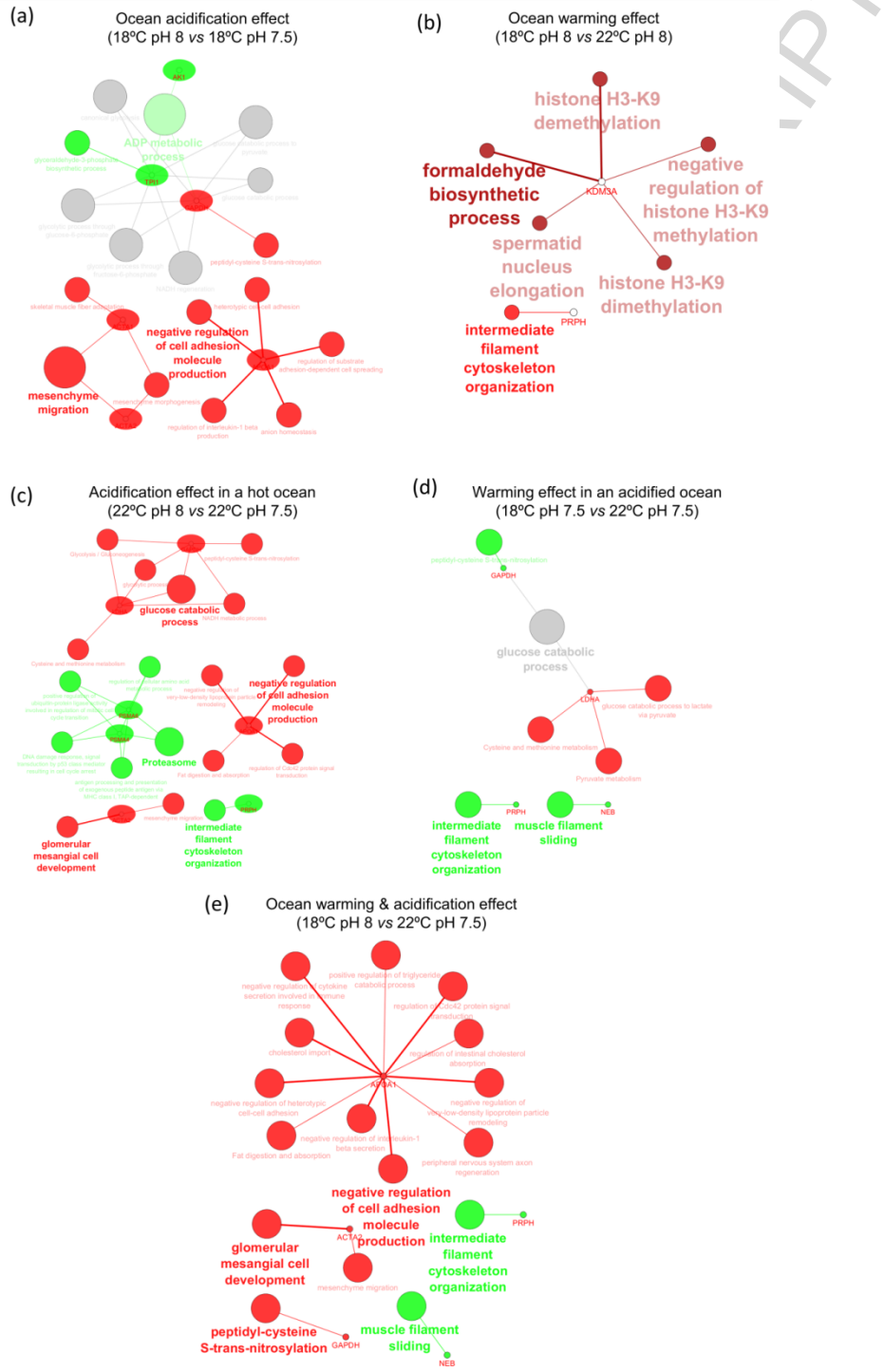

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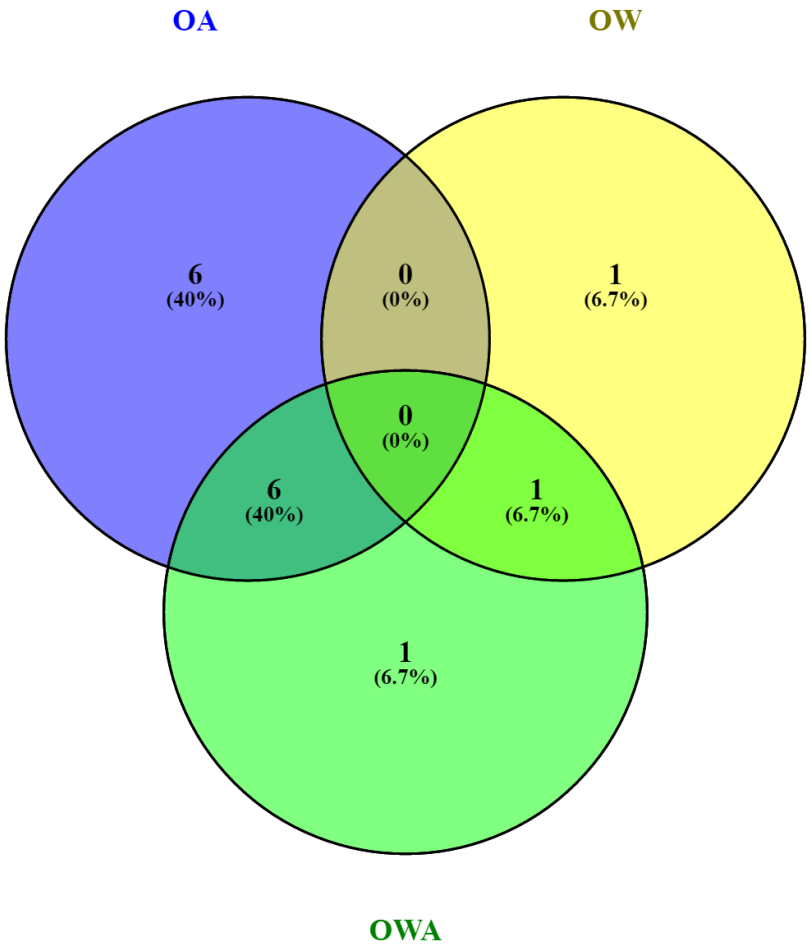
Fig. 5 Venn diagram showing shared and exclusively regulated proteins in the muscle of *Sparus aurata* exposed for 42 days to *ocean warming* (OW 22ºC, pH 8), *ocean acidification* (OA 18ºC, pH 7.5) and *ocean warming and acidification* (OWA 22ºC, pH 7.5). Proteins exclusively regulated in OA were ACT2, TPISB, KAD1, ACTSB, and two isoforms of ACTS; the protein exclusively regulated in OW was KDM3A; the protein exclusively regulated in OWA was NEBU. Proteins shared between OA and OWA were APOA1, GDE, ACT2 and three isoforms of G3P. The protein shared between OW and OWA was PERI.



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Highlights

- Fitness and proteome changes were assessed in *Sparus aurata* exposed to global change
- *S. aurata* are resilient to warming requiring solely cytoskeletal and metabolic adjustments
- Acidification decreased survival, boosted energy demands and immune processes
- OWA decreased survival synergistically, boosted energy demands and impaired stress responses
- Global change could pose a serious threat to sea bream populations

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