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Mestre em Ecologia Marinha

Effects of ocean warming throughout the life cycle of *Sparus aurata*: a physiological and proteomic approach

Dissertação para obtenção do Grau de Doutor em
Química Sustentável

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RESUMO

As alterações climáticas são um problema ambiental com efeitos persistentes nos ecossistemas marinhos, afectando os serviços ambientais fornecidos por estes à sociedade, provocando alterações na biodiversidade, abundância e distribuição das espécies, interacções biológicas, fenologia e fisiologia dos organismos. No entanto, a vulnerabilidade dos recursos pesqueiros ao aquecimento do oceano é pouco clara devido à falta de abordagens integradas que avaliem processos fisiológicos e moleculares, e que elucidem a forma como estes se alteram ao longo do ciclo de vida dos organismos. Esta tese tem como objectivo estudar a vulnerabilidade duma espécie de peixe comercial, a dourada (*Sparus aurata*), ao aquecimento do oceano ao longo do seu ciclo de vida, ao nível molecular, celular e do indivíduo. Os bioensaios realizados revelaram que a fase larvar é especialmente sensível a um aumento agudo ou crónico de temperatura (>22ºC). O CTmax (máximo térmico crítico) das larvas foi de 30ºC e estas mostraram uma capacidade reduzida de induzir a resposta celular ao *stress* (levando à desnaturação de proteínas), assim como de alterar o seu metabolismo energético de forma a lidar com o aumento de temperatura. Como tal, foram registadas taxas de mortalidade elevadas, associadas a lesões nos tecidos (ex: músculo e rins). A fase juvenil foi a mais resistente ao aquecimento da água, apresentando um CTmax de 35.5ºC, embora temperaturas iguais ou superiores a 28ºC no ensaio de *stress* agudo tenham provocado lesões severas nos tecidos, possivelmente associadas a uma desnaturação proteica e a stress oxidativo. A exposição a *stress* térmico crónico levou à indução da resposta celular ao *stress* (CSR) associada a um aumento do potencial glicolítico (os órgãos vitais foram especialmente reativos), no entanto, após 21 dias de exposição foram detectados sinais de inflamação nos tecidos analisados e observou-se um aumento significativo da mortalidade. A fase adulta mostrou-se menos resistente do que a juvenil uma vez que os órgãos vitais não apresentaram uma grande capacidade de resposta, e foram os que sofreram maiores alterações nos tecidos (ex: danos nos lípidos e proteínas, inflamação, atrofia), causando uma mortalidade elevada. Isto sugere que os adultos não possuem muita plasticidade para lidar com o aumento da temperatura da água. Por conseguinte, o esforço e mecanismos induzidos como defesa ao *stress* térmico são diferencialmente alocados consoante o órgão e o estadio de desenvolvimento. Deste modo, conclui-se que a fase larvar é o estadio de desenvolvimento chave, que irá determinar o fecho do ciclo de vida da dourada num cenário de aquecimento global.

Palavras-chave: temperatura, alterações climáticas, peixes, fisiologia, proteómica, ciclo de vida

ABSTRACT

Climate change is a major environmental problem, known to cause pervasive effects on marine ecosystems, impacting goods and services provided to society. It alters biodiversity patterns, abundance and distribution of species, biological interactions, phenology, and organisms' physiology. However, the vulnerability of fish stocks towards ocean warming is still far from clear due to the lack of integrative approaches that address physiological and molecular compensation mechanisms throughout all the stages of the life-cycle. This thesis aimed at uncovering the vulnerability of a highly commercial sea bream *Sparus aurata* to ocean warming throughout the various stages of its life cycle, considering organism-, cellular- and molecular-level approaches. The first two assays revealed that larvae are very sensitive to both acute and chronic thermal stress (>22ºC), showing a CTmax (Critical Thermal Maximum) of 30ºC and a reduced capacity to employ the cellular stress response (leading to protein denaturation) and perform the energetic adjustments that would have been necessary to sustain a temperature increase. This probably led to the observed tissue injury (in muscle and kidneys) and elevated mortality rates. Juveniles were the most resistant and plastic to warming with a CTmax of 35.5ºC although temperatures of ≥ 28ºC in the acute assay induced significant tissue damage related to protein denaturation and oxidative stress. Exposure to chronic warming elicited the cellular stress response coupled with an enhanced glycolytic potential (vital organs were highly responsive). However, after 21 days fish showed signs of inflammation and mortality was significantly increased. Adult fish were less resistant than juveniles because vital organs were less responsive and showed the highest tissue injury level (damage to lipids and proteins, inflammation, atrophy) leading to elevated mortality. This suggests a response to damage rather than a plastic response. Thus, the effort and mechanisms of protection against thermal challenge are differentially allocated by distinct organs, depending on developmental stage. Moreover, the larval phase is the key developmental stage that will determine life cycle closure of *S. aurata* under the projected ocean warming scenarios.

Keywords: temperature, climate change, fish, physiology, proteomics, life-cycle

LIST OF SYMBOLS AND ABBREVIATIONS

A

AAA ATPases, ATPases Associated with diverse cellular Activities

Abs, absorbance

ac, acinar cells

ACN, acetonitrile

ACT2, Actin, muscle-type 2
ACTM, actin muscle
ADP, adenosine diphosphate
Ambic, ammonium bicarbonate
AMP, adenosine monophosphate
ANOVA, analysis of variance
Anti-DNP, anti-dinitrophenol
AOX, anti-oxidant
Arg, arginine
ARPC2, actin-related protein 2/3 complex subunit 2
at, adipose tissue
ATP, adenosine triphosphate

B

b, brain
BSA, bovine serum albumin
bv, blood vessels

C

CAH1, carbonic anhydrase 1
CaM, calmodulin
CANFA, *Canis familiaris*
CAT, catalase
CDNB, chloro-2,4-dinitrobenzene
CHAAC, *Chaenocephalus aceratus*
CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHIHA, *Chionodraco hamatus*
CI, confidence interval
CO_2, carbon dioxide
CRIGR, *Cricetulus griséus*
CSR, cellular stress response
CTmax, Critical Thermal Maximum
CYP1A, cytochrome P450 1A
CYPCA, *Cyprinus carpio*

D

Da, Dalton
DANRE, *Danio rerio*
DGAV, Direcção Geral de Alimentação e Veterinária
DHE3, Glutamate dehydrogenase, mitochondrial
DNA, deoxyribonucleic acid
DNPH, 2,4-dinitrophenylhydrazine
DOC, Na-deoxycholate
dph, days post-hatch

DPX, Distrene, Plasticiser, Xylene
DTT, dithioerythritol

E
E1, ubiquitin-activating enzyme
E2, ubiquitin-conjugating enzyme
E3, ubiquitin-ligase
EC, Enzyme Nomenclature
EDTA, ethylenediaminetetraacetic acid
EGR1, early growth response protein 1
ELISA, Enzyme-Linked Immunosorbent Assay
ENOA, alpha-enolase
EPON 821, epoxy resin 821
Eq, equation
ER, endoplasmic reticulum
er, erythrocyte
ERSST v4, Extended Reconstructed Sea Surface Temperature version 4
EST, Expressed Sequence Tag
ey, eye

F
f, filament
FAO, Food and Agriculture Organization of the United Nations
fc, fascicles
FELASA, Federation of European Laboratory Animal Science Associations
FishStat, fishery statistical time series
fv, fat vacuolation

G
G, gills
G3P, glyceraldehyde-3-phosphate dehydrogenase
G6PI, glucose-6-phosphate isomerase
gc, goblet cell
GDIB, Rab GDP dissociation inhibitor beta
GDP, guanosine diphosphate
GISS, Goddard Institute for Space Studies
Glu, glutamate
GO, gene ontology
GPx, glutathione peroxidase
GST, glutathione-S-transferase
GTP, guanosine triphosphate

H
H&E, Haematoxylin and eosin histological stain
ha, hepatic arterioles
HCl, hydrochloric acid
HEBP1, Heme-binding protein 1
hp, hepatocytes
HRP, horseradish peroxidase
HS90A or HSP90AA1, Heat shock protein HSP 90-alpha
Hsc70, heat shock cognate 70 kDa
HSD, honest significant difference
HSF1, heat shock factor 1

Hsp70, heat shock protein 70 kDa
HSP71, Heat shock 70 kDa protein 1
HSP7C, Heat shock cognate 71 kDa protein
Hsp90, heat shock protein 90 kDa
HSPA8, heat shock 70kDa protein 8
Hsps, heat shock proteins

I
I, intestine
IAA, iodoacetamide
IBR, Integrated Biomarker Response
ICTPU, *Ictalurus punctatus*
IEF, isoelectric focusing
IF4A2, eukaryotic initiation factor 4A-II
IgG, immunoglobulin G
IPCC, Intergovernmental Panel on Climate Change
IPG, immobilized pH gradient
IQCA1, IQ motif containing with AAA domain 1
IQCAL, IQ and AAA domain-containing protein 1-like
IUCN, International Union for Conservation of Nature and Natural Resources

K
K2C8, keratin, type II cytoskeletal 8
KAD1, adenylate kinase isoenzyme 1
KCl, potassium chloride
KCRM, creatine kinase M-type
KCRT, creatine kinase testis isozyme
kDa, kilodalton
KH_2PO_4, monopotassium phosphate
KIF2A, kinesin heavy chain member 2A
KIF5C, kinesin heavy chain isoform
KRT, keratin 8
KRT19, keratin 19

L
L, liver
L:D, light:dark
Leu, leucine
LIZAU, *Liza aurata*
LIZRA, *Liza ramada*
lm, lamella
lp, lamina propria
LPO, lipid peroxidation

M
m, muscle
MACFA, *Macaca fascicularis*
MALDI, matrix-assisted laser desorption/ionization
MAPK, mitogen-activated protein kinase
MARE, Marine and Environmental Sciences Centre
MDA, malondialdehyde bis(dimethylacetal)
MELGA, *Meleagris gallopavo*
MESAU, *Mesocricetus auratus*

ml, melanin
MLE1, myosin light chain 1, skeletal muscle isoform
MOLOC, *Molgula oculata*
mRNA, messenger ribonucleic acid
MS, mass spectrometry
MS/MS, tandem mass spectrometry
mv, microvilli
MW, molecular weight

N
Na_2HPO_4, disodium phosphate
NaCl, sodium chloride
NAD, nicotinamide adenine dinucleotide
NaOH, sodium hydroxide
NASA, National Aeronautics and Space Administration
NBT, nitroblue tetrazolium
NCBI, National Center for Biotechnology Information
NEC1, Neuroendocrine convertase 1
NOAA, National Oceanic and Atmospheric Administration of USA
NPF, nucleation-promoting factor
NSIDC, National Snow and Ice Data Center
Nt, notochord

O
OCLTT, oxygen and capacity limited thermal tolerance
Ol, olfactory lobe
ONCMY, *Oncorhynchus mykiss*
ONCTS, *Oncorhynchus tshawytscha*
ORYLA, *Oryzias latipes*

P
p, p-value
PAK, p21 protein activated kinase
PARK2, parkin RBR E3 ubiquitin protein ligase
PAS, Periodic Acid-Shiff's
PBS, phosphate-buffered saline
PC, protein carbonylation
PCA, Principal Component Analysis
Phe, phelylalanine
PI, isoelectric point
PKC, protein kinase C
Poly(A), polyadenylation
PONAB, *Pongo abelii*
POTEF, POTE ankyrin domain family member F
ppm, parts per million
pr, pancreatic tissue
PRIGL, *Prionace glauca*
PSB3 or PSMB3, proteasome subunit beta type-3
PTM, post-translational modification
PYGB, Glycogen phosphorylase brain form

R
RAMSES, Reconciling Adaptation, Mitigation and Sustainable Development for Cities

RGL2, ral guanine nucleotide dissociation stimulator-like 2
RNA, ribonucleic acid
ROS, Reactive Oxygen Species

S
SAM9L, Sterile alpha motif domain-containing protein 9-like
sc, spinal chord
SD, standard deviation
SDS, sodium dodecyl sulphate
SIAM, Scenarios, Impacts and Adaptation Measures (Climate Change in Portugal)
sk, skeletal muscle
Sm, striated myocytes
SOD, superoxide dismutase
SOMA, somatotropin
SRES, Special Report on Emission Scenarios
SST, sea surface temperature
STYPL, *Styela plicata*

T
TCA, trichloroacetic acid
Tub, total ubiquitin
Tcritical, critical temperature
TBARS, thiobarbituric acid reactive substances
Tl, telencephalon
TPIS, Triosephosphate isomerase fragments
TPISB, Triosephosphate isomerase B
TFA, trifluoroacetic acid
TOF, time-of-flight
TUBA4A, tubulin alpha 4a
TPM1, tropomyosin 1
Tyr, tyrosine
$T_{end\text{-}point}$, temperature at which the end-point is reached
T, tegument
TNF, tumor necrosis factor
TMB, 3, 3', 5, 5' – Tetramethylbenzidine
T7, 7 days of exposure
T14, 14 days of exposure
T21, 21 days of exposure
T28, 28 days of exposure

U
UV, ultraviolet radiation

V
vc, vacuolation

X
XENLA, *Xenopus laevis*
XENTR, *Xenopus tropicalis*
XOD, xanthine oxidase

W
WHO, World Health Organization

Z
Zm, zymogen granules

Other
2D, two-dimensional
5'-UTR, leader sequence
↑, up-regulation
↓, down-regulation

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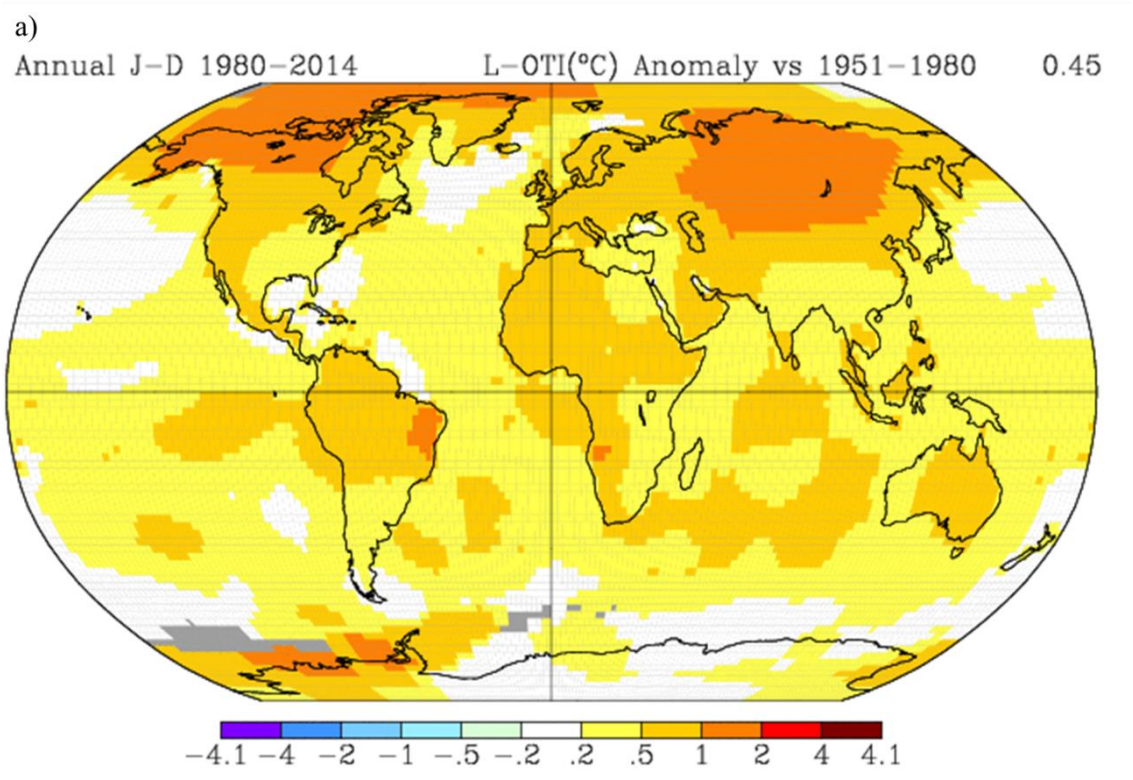
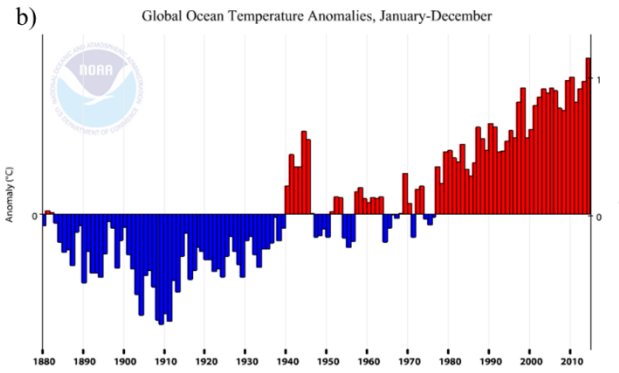
CHAPTER 1. GENERAL INTRODUCTION

1. Climate change: global and regional trends

Global average temperatures have been rising over the past century and a half. The Intergovernmental Panel on Climate Change (IPCC, 2014) reports an average increase of 0.85ºC in global temperature since 1880 and refers that the last three decades have been successively warmer (approximately +0.2ºC per decade, Hansen et al., 2006) (**Fig. 1.1**). This additional energy is mostly absorbed by the world's oceans which caused on average a 0.8ºC rise in sea surface temperature over the past 100 years. The scientific community has attributed this warming to human activities, mainly the increase of CO_2 and other greenhouse gases in the atmosphere largely due to industrialization processes and consequent burning of fossil fuels (e.g. Barnett et al., 2005; Hansen et al., 2006; Solomon et al. 2009; Godbold and Calosi, 2013; IPCC, 2014).





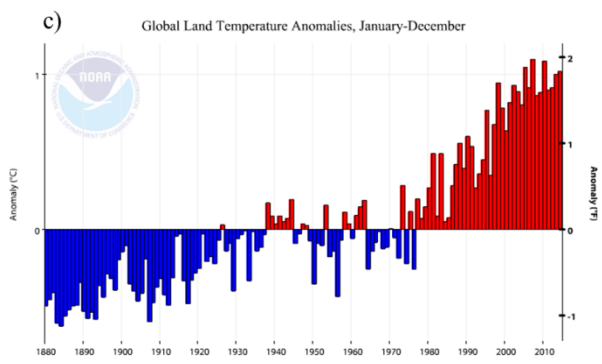


Figure 1.1 Global temperature anomalies **a)** Temperature anomaly for the period of 1980-2014 when compared to the reference period of 1951-1980. The analysis was carried out in Goddard Institute for Space Studies website (NASA, http://data.giss.nasa.gov/gistemp/maps/) following the parameters: data sources land (GISS analysis) and ocean (ERSST_v4); mean period annual; time interval 1980-2014; base period 1951-1980; smoothing radius 1200 km; projection type Robinson. Notes: Gray areas are missing data; ocean data are not used over land nor within 100km of a reporting land station**; b)** and **c)** Global ocean and land (respectively) temperature anomalies by year, starting at 1880 until 2014. The right Y axis gives temperature anomaly in degrees Fahrenheit while the right axis is in degrees Celsius (an anomaly of 1ºF is equivalent to an anomaly of 0.556ºC). Source: graphs were obtained using data and tools available in NOAA (National Oceanic and Atmospheric Administration of USA, Department of Commerce, http://www.ncdc.noaa.gov/sotc/global/201509, accessed 16-11-2015).

Global circulation models predict a rise in temperature and changes in ocean chemistry, mostly a decrease in ocean pH (IPCC, 2001, 2007, 2014; Santos et al., 2002). Additionally, changes in climate patterns will escalate extreme events considering their frequency and intensity (heat waves, cold spells, La Niña and El Niño, droughts or heavy rain) (e.g. Fischer and Schär, 2010; IPCC, 2013; Cai et al., 2015). Altogether, such events lead to an altered dynamic of physical and chemical processes, causing a shift in ocean biogeochemistry, hydrodynamics and climate interactions, changes in ocean currents and stratification of ocean waters, a sea level rise (**Fig. 1.2**), shifts in precipitation patterns, a decrease in ice cover and consequent expansion of the open water season (e.g. Roessig et al., 2004; Gruber, 2011; Hordoir and Meier, 2012; Golshani et al., 2012; Barnhart et al., 2015). However, such impacts are not uniform across the globe and are thus dependent on geographic location.

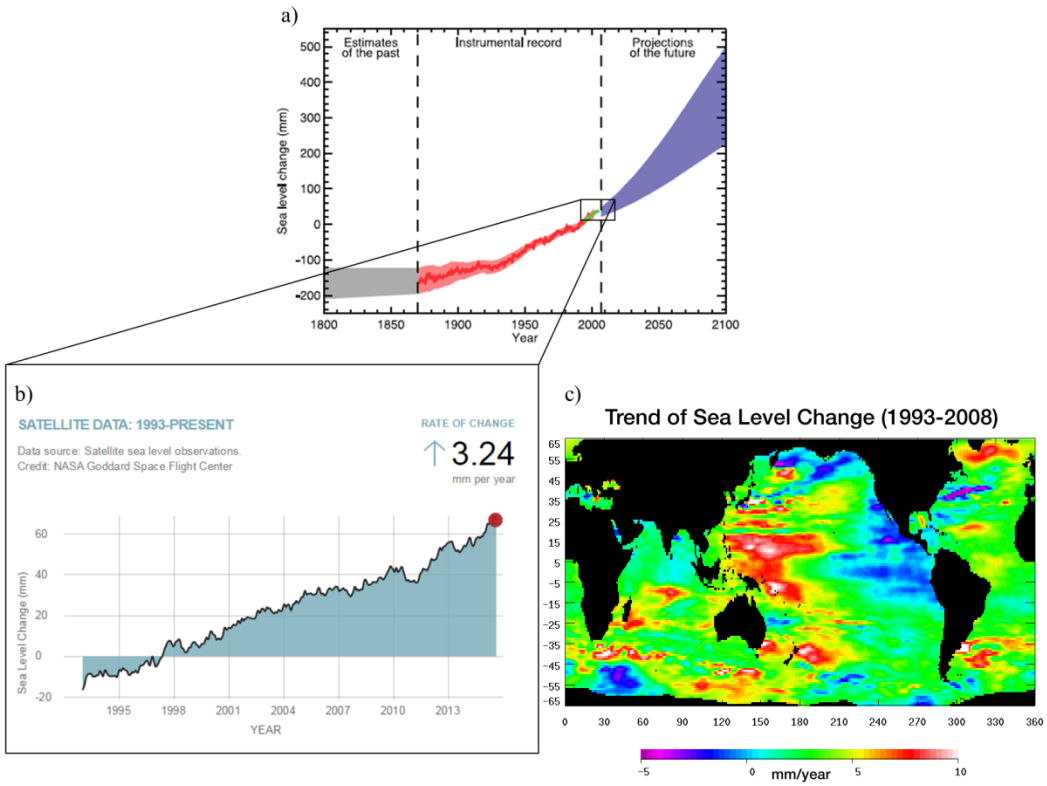


Figure 1.2 Trend of sea level change**. a)** Global mean sea level change compared to the mean of the baseline period 1980-1999. The gray area shows uncertainty due to the lack of data before 1870. The instrumental record provided in red is based on tidal gauges (mean and variations) and the green line is based on satellite data on altimetry. The blue area represents projections for the future considering scenario SRES A1B. Source: IPCC 2007, with permission. **b)** Inset of the period 1993-present considering global sea level change based on satellite data. Source: NASA Goddard Space Flight Center (http://climate.nasa.gov/vital-signs/sea-level/, accessed 16-11-2015), with permission. **c)** Trend of sea level change between 1993-2008 based on satellite data (sea surface height data from the Topex/Poseidon and Jason-1 satellites). Trends of sea level change vary across regions. Source: NASA Jet Propulsion Laboratory, California Institute of Technology (http://photojournal.jpl.nasa.gov/catalog/PIA11002, accessed 20-11-2015), with permission.

Several studies have shown that the Northern and Southern hemispheres are thermally asymmetric and so are correspondent warming trends, which have been greater in the Northern hemisphere (**Fig. 1.3**). This is possibly due to different spatial impacts of greenhouse emissions and forcing, the dynamics of warm/cold anomalies and nonlinearities in radiative balance, ice cover and evapotranspiration (Friedman et al., 2013; Robeson et al., 2014; Good et al., 2015).

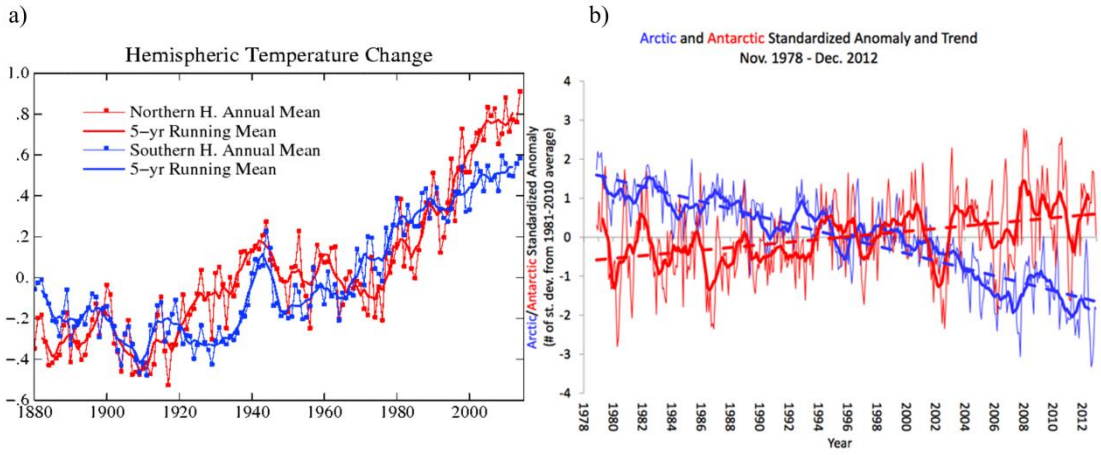


Figure 1.3 Differences in warming trends and ice extent between the Northern and Southern hemispheres. **a)** Annual and five-year running mean temperature change for northern and southern hemispheres considering the baseline period from 1951 to 1980. Source: NASA, Goddard Institute for Space Studies (http://data.giss.nasa.gov/gistemp/graphs_v3/, accessed 16-11-2015), with permission; **b)** Arctic and Antarctic sea ice extent anomalies and trends concerning the period from 1979 to 2012. Thick lines: twelve-year running means; thin lines: monthly anomalies. Source: National Snow and Ice Data Center (authors: Stroeve J, Meier W), University of Colorado, Boulder, USA (https://nsidc.org/cryosphere/sotc/sea_ice.html, accessed 16-11-2015), with permission.

According to IPCC (2001), southern Europe is one of the regions where warming is expected to be most severe, with predicted air temperature increases up to 4 to 7ºC by 2100 in the Iberian Peninsula (IPCC, 2001; Santos and Miranda, 2006). More specifically, climate change models predict a 2ºC increase in Portuguese waters (Miranda et al., 2002) and 3-4ºC in the Mediterranean (Fischer and Schär, 2010). Considering that water temperatures in the Portuguese coastal area range approximately from 15 to 20ºC during the summer and 10-16ºC during the winter, temperatures for 2100 are in the range of 18-23ºC and 13-19ºC during the summer and winter respectively. Furthermore, the mean temperature in estuaries during summer ranges approximately from 20 to 24ºC, depending on geographic region (north or south of Portugal), reaching maximum temperatures between 25 and 28ºC during heat waves, and persisting for over 2 weeks (e.g. see Costa, 1990; Azevedo et al., 2006; Cabral et al., 2007; Madeira et al., 2012a). Therefore, by 2100, Portuguese estuaries will have mean water temperatures approximately from 23-27ºC, reaching 30-31ºC during heat waves. Predicted shifts in extreme events can occur due to a shift of temperature distributions towards a warmer climate, an increase in temperature variability and/or an asymmetric distribution of temperatures towards hot weather (**Fig. 1.4**). Heat waves may be intense and prolongued since a significant increase in the number of days surpassing 35ºC (air temperature) and number of tropical nights (>20ºC air temperature) is expected as well as an increase in the maximum temperature attained during these events (Miranda et al., 2002; Fischer and Schär, 2010; Tapia et al., 2015). Moreover, a reduction in rainfall is also expected, mostly during summer (Miranda et al., 2002; EURO-CORDEX initiative). Concerning sea-level, it has already risen 10 to 20 cm along the

Portuguese coast during the last century (Santos and Miranda, 2006). Altogether, such changes will affect marine and terrestrial communities and associated goods and services provided to society.

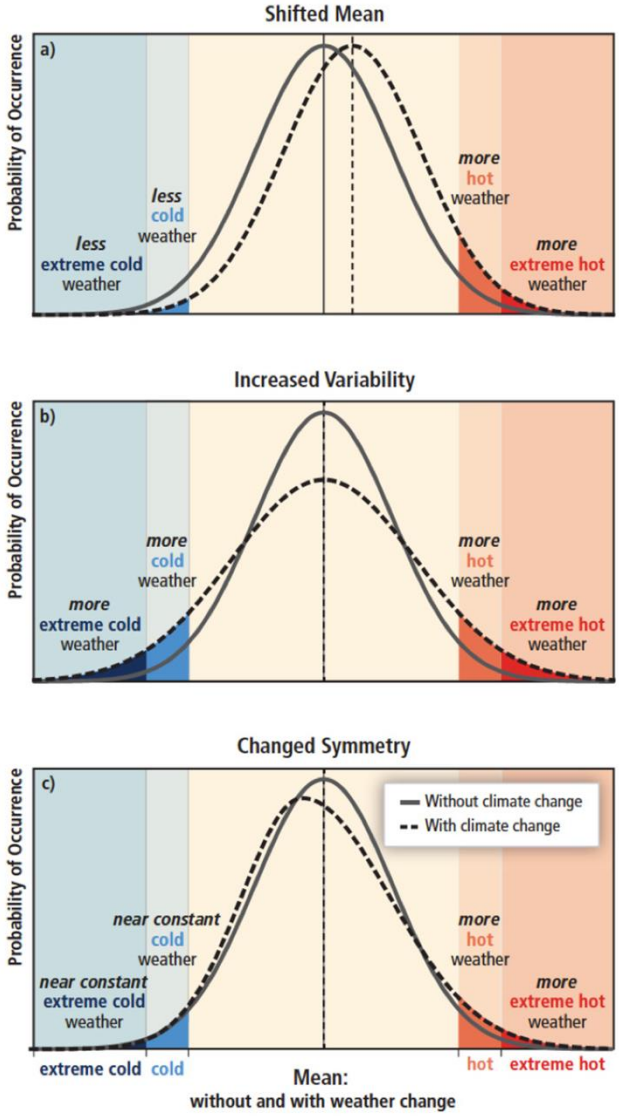


Figure 1.4 Predicted shifts in extreme events. Shifts in extreme events due to changes in temperature distribution can occur in three ways **a)** shift of the entire distribution towards a warmer climate; **b)** increase in temperature variability with no shift in the mean and **c)** asymmetric distribution towards hot weather. Source: IPCC 2012, with permission.

2. Marine organisms under climate warming scenarios: impacts of rising temperature and the study of thermal tolerance mechanisms

Climate change has been associated with alterations in biodiversity, phenology, abundance and distribution of species, shifts in biological interactions, and changes in organisms' physiology, performance and fitness (Walther et al., 2002; Perry et al., 2005; Peck et

al., 2012; Poloczanska et al., 2013). Temperature is one of the most relevant factors affecting marine organisms, mainly because it has great impacts at lower levels of biological organization, setting the kinetic energy of molecules and reaction rates. As most marine organisms are ectotherms and thus do not regulate body temperature, they are highly impacted by fluctuations in environmental temperature, with downstream effects on their activities such as foraging, reproduction, locomotion, behavior and physiology (e.g. Fry, 1971; Mora and Ospina, 2001). Therefore, population dynamics, competition, and mortality are also set by temperature in the marine environment (Glynn, 1988; Southward et al., 1995; Perry et al., 2005; Munday et al., 2008; Pörtner et al., 2008; Boyd et al., 2015) (**Fig. 1.5**).

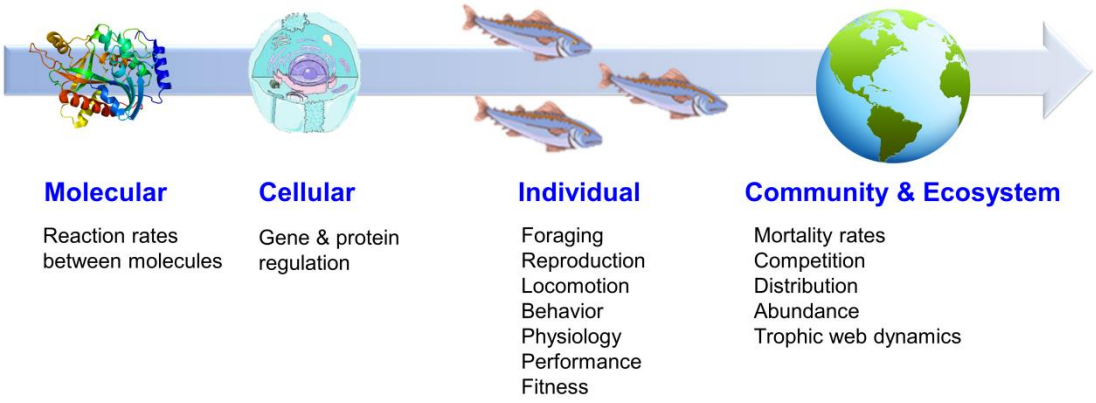


Figure 1.5 Up-scaling of temperature effects from the molecular level to communities and ecosystems (constructed using SmartDraw and PowerPoint tools).

The impacts of such temperature changes, coupled with the effects of other anthropogenic activities (e.g. pollution and overfishing) may exert strong influences on ecosystem services, affecting some economic activities such as fisheries and aquaculture. Climate change is predicted to affect such activities not only through effects of temperature (and ocean acidification) on species physiology but also through changes on aquaculture practices (see Brander, 2007; Cochrane et al., 2009; Doubleday et al., 2013). In this context, it is important to determine species' vulnerability towards environmental forcing. Species' vulnerability is dependent upon several factors, including thermal tolerance limits, phenotypic plasticity, generation time, population connectivity and recruitment success.

Thermal tolerance limits are related to the concept of thermal tolerance window, which is defined as the favorable range of temperatures for performance. This range includes an optimum temperature interval, at which performance is maximum and suboptimum intervals, for which performance starts to decrease. Outside this range, performance is severely decreased and the organism can only survive for a limited period of time. Pörtner and Knust (2007) further developed this concept and proposed the theory of oxygen and capacity limited thermal tolerance (OCLTT). According to this theory, the thermal window for performance in a species matches the window of aerobic scope of that species. In other words, oxygen supply to tissues

is the primary mechanism limiting thermal tolerance. When environmental temperature falls out of the optimum zone, loss of performance starts to take place (at *pejus* temperatures) due to hypoxaemia caused by a mismatch of oxygen supply to tissues and the demand for oxygen (**Fig. 1.6**). This is dependent on the capacity of the circulatory and ventilatory systems. In fish, venous oxygen tensions have a thermal optimum, limiting the amount of oxygen that reaches the heart thus limiting cardio-circulatory function (Pörtner et al., 2004).

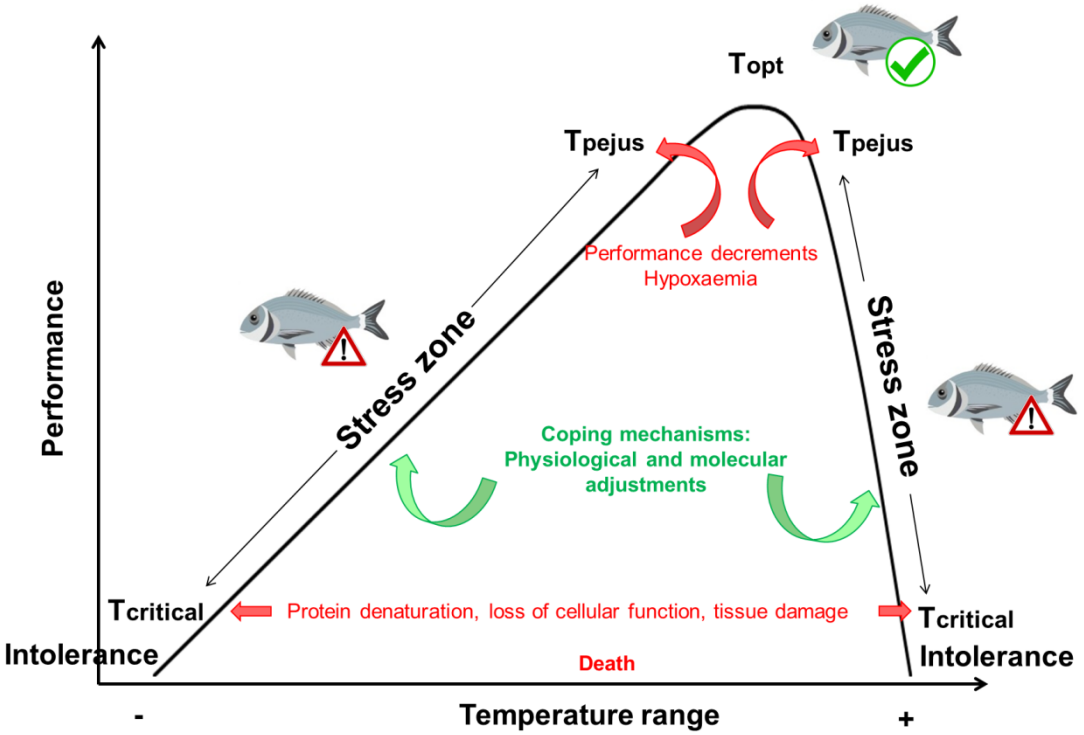


Figure 1.6 Thermal window and the concept of oxygen and capacity limited thermal tolerance (OCLTT) (adapted from Pörtner and Farrell, 2008 and Pörtner, 2010 using PowerPoint tools). When environmental temperature falls out of the optimum zone, loss of performance starts to take place (at *pejus* temperatures - Tpejus) due to hypoxaemia caused by a mismatch of oxygen supply to tissues and the demand for oxygen. At that point, organisms enter the stress zone and several coping mechanisms can be activated, including physiological and molecular adjustments. When the thermal limits are reached (Tcritical), performance is severely affected due to protein denaturation, tissue damage and loss of cellular function. Beyond that temperature point, organisms are intolerant and will not be able to survive.

Thus, understanding the molecular underpinnings of thermal tolerance and of organismal responses to ocean warming is of major importance to improve biogeographic forecasting. There is a need to obtain molecular information and connect it with physiological and ecological patterns (Hofmann, 2005; Basu et al., 2002) in order to establish an integrative approach to address climate change impacts on biodiversity.

Such data can be obtained using biomarker approaches or targeted/untargeted 'omics' techniques. According to the World Health Organization (WHO, 1993) a biomarker is defined as "any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and

physiological, biochemical, at the cellular level, or a molecular interaction". To consider such measurements as biomarkers, one must first establish the relationship and shape of response of the potential biomarker considering the environmental variable being studied (integrating several levels of exposure). As suggested by Bucheli and Fent (1995), biomarkers are early-warning signals that reflect the injurious biological responses towards some environmental factor, providing information on ecosystem's health. Biomarkers can be divided into three sub-classes, following the World Health Organization (1993): biomarkers of exposure, biomarkers of effect and biomarkers of susceptibility. Even though such classification was mainly based on chemical exposure, these categories may apply to other circumstances. In more detail, biomarkers of exposure include exogenous substances, their metabolites or the interaction between a substance and a target molecule within an organism; biomarkers of effect are measurable alterations within an organism, including biochemical, physiological and behavioral changes that indicate health impairment; biomarkers of susceptibility are indicators of the ability of the organism to respond to environmental challenges (World Health Organization, 1993). Furthermore, a good biomarker should reflect a qualitative or quantitative trait, that changes when the organism is exposed to the factor of interest; should be reproducible in the short and long-term; should be common to individuals within the population of interest with known variability under control conditions; and should be ubiquitous (or at least common between many species) (World Health Organization, 1993). Therefore, biomarkers can be useful in environmental monitoring to assess the level of exposure of an organism to a specific factor, given that the dose-effect relationship has been previously established.

Considering high throughput 'omics' techniques, these comprise four main areas: genomics, transcriptomics, proteomics and metabolomics (see Ge et al., 2013 for a review). These technologies contribute greatly to unravel pathways involved in the response to stress, elucidating how organisms cope with environmental challenges. Such 'omics' tools have helped scientists to face the substantial challenge of improving the predictive power concerning ecological consequences of climate change by integrating molecular, organismal, community-ecosystem and cascade-effect approaches – systems biology (Kordas et al. 2011, Sarà et al. 2014). 'Omics' approaches have enabled the simultaneous study of many cellular components and how these change in response to environmental fluctuations (Hollywood et al. 2006, Joyce and Palsson 2006). Specifically, proteomics tools are extremely relevant in the study of environmental adaptation (see Karr, 2008). Proteins are the final products of gene expression (i.e. functional units) regulating virtually all cellular processes (including the transcriptional competency of the genome - Baer and Millar, 2015) and thus biological function. In this context, the proteome can be directly linked to fitness (Feder and Walser 2005) as it encompasses the entire set of proteins within a cell, revealing the cell's phenotype under certain conditions (Dupont et al. 2007). Ultimately, complex protein networks and proteome plasticity is the basis of phenotypic plasticity (Karr, 2008; Valcu and Kempenaers, 2015; Baer and Millar, 2015) and can thus reveal ecological function (Gotelli et al, 2012). Proteomics research can fall within 3 categories: expression proteomics, functional proteomics and structural proteomics (Valcu and

Kempenaers, 2015). The first two categories are especially relevant in thermal biology research as they include the identification of differentially expressed proteins between different treatments and the investigation of protein functions and interaction networks (Valcu and Kempenaers, 2015), elucidating molecular adaptation mechanisms and their influence on the organism's physiology. Despite all of these advantages, the application of such technology in the marine environment remains scarce and the number of proteomic studies related to environmental stress is still limited, restricted to very few species and few habitat types (Tomanek, 2014). This is related to the fact that many marine organisms do not have sequenced genomes. However, with expanding genome sequencing techniques, the interest in omics technology is escalating and environmental proteomics is now seen as promising field to ecologists (Gotelli et al. 2012; Ge et al., 2013), advancing our knowledge of the adaptations of organisms to stress. Moreover, omics technologies are very important tools that help scientists to generate new hypotheses (see Baer and Millar, 2015 for an example) and promote further biomarker development for environmental monitoring. According to Veldhoen et al. (2012) omics techniques allow the assessment and characterization of molecular profiles (i.e. signatures) that represent the ability of an organism to respond or adapt to changing conditions, helping scientists to determine and monitor population and environmental status.

Proteomic approaches can follow different work-flows, but the most standard approach includes sample preparation (homogenization and clean up), separation of proteins from a complex mixture (2-dimensional – 2D – gel electrophoresis, liquid chromatography), enzymatic digestion followed by protein identification by mass spectrometry (MS) techniques or the study of post-translational modifications (PTMs) and data analysis (analysis of biological functional using gene ontology tools, clustering analysis and protein networks) (**Fig. 1.7**).

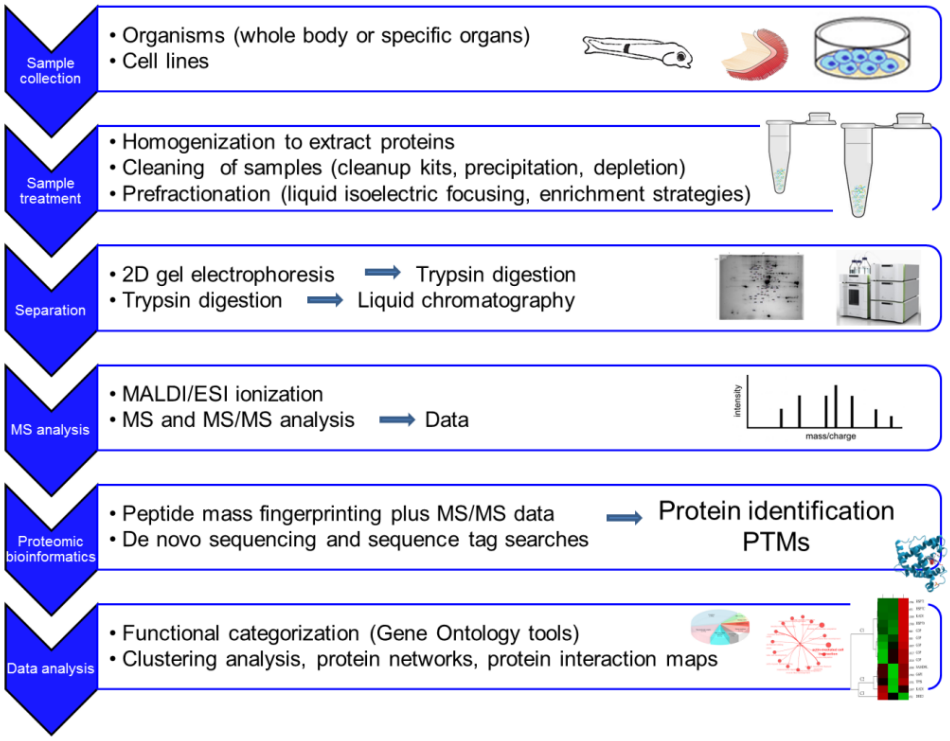


Figure 1.7 Typical workflow for proteomic studies (constructed using PowerPoint tools).

As organisms respond to changing environmental conditions by modulating gene expression and correspondent protein levels (Logan and Somero, 2011), both the biomarker and omics approaches are useful to understand organismal responses to ocean warming. In marine organisms, protein regulation in response to a change in the environment is usually elicited some degrees above the acclimation temperature. This response is elicited by the activation of the neuroendocrine/endocrine system, with the production of hormones that activate molecular cascades (Iwama, 1999; Barton, 2002) (**Fig. 1.8**). Specific metabolic pathways are activated leading to physiological and biochemical adjustments including changes in respiratory, cardiovascular and immune functions, as well as changes in the energetic metabolism, including the activation of gluconeogenesis and glycogenolysis to obtain more energy for cellular defense (Barton and Iwama, 1991; Iwama, 1999; Barton, 2002). Moreover, several studies have shown that the main molecular pathways modulated by temperature include heat shock proteins (e.g. Feder and Hofmann, 1999), the ubiquitin-proteasome pathway (Hofmann and Somero, 1995; Madeira et al., 2014), anti-oxidant defenses (Abele and Puntarulo, 2004; Madeira et al., 2013), energy related pathways (Kassahn et al., 2007; Tomanek, 2011), cytoskeleton dynamics and cell signaling (Tomanek, 2011; Jayasundara et al., 2015). The modulation of such pathways under challenging environmental conditions is likely to contribute to enhanced fitness and increased survival of individuals. In the next paragraphs, an overview of the main biomarkers of thermal stress is provided.

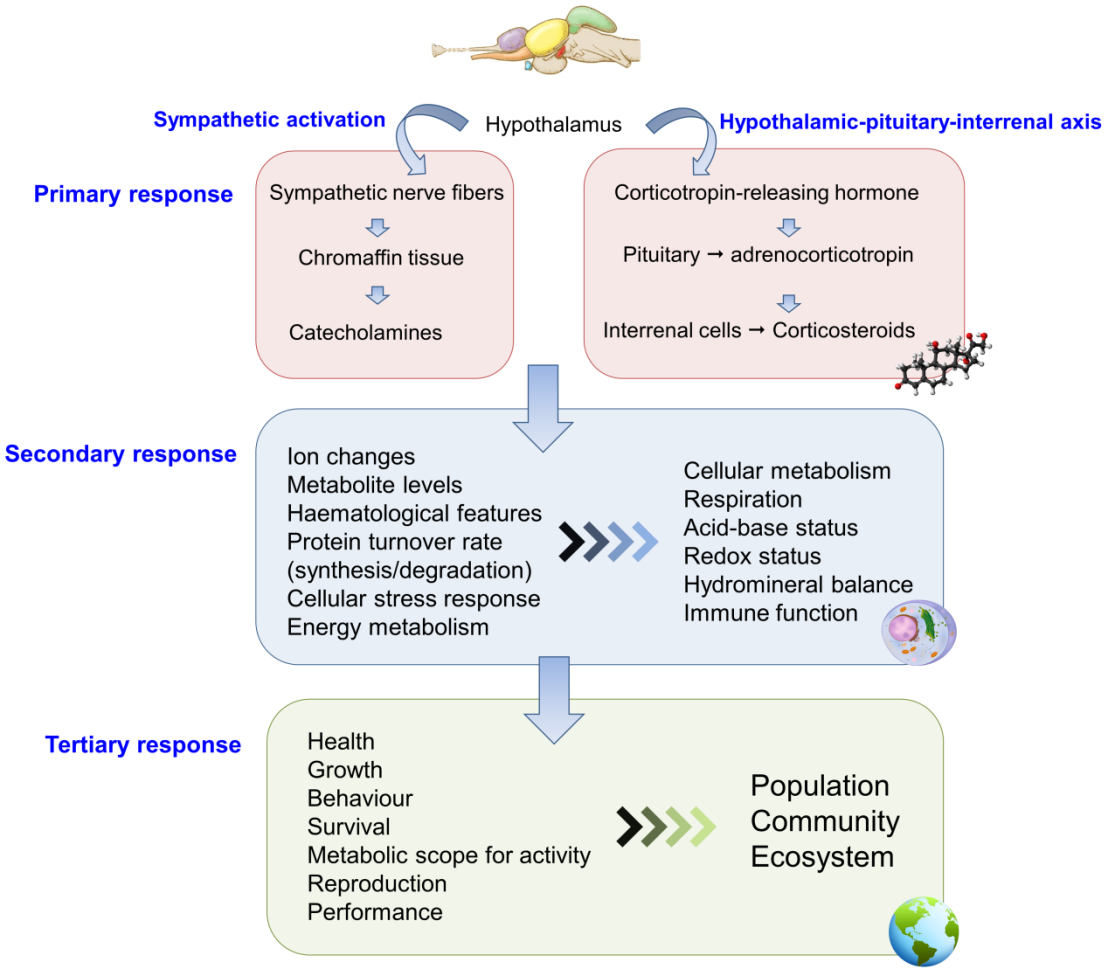


Figure 1.8 Stress responses in fish (constructed using SmartDraw and PowerPoint tools).

Heat shock proteins (Hsp) have been considered relevant biomarkers in thermal stress studies. These proteins are chaperones (ATPase activity) that play a primary role in intra-cellular defense (Csermely and Yahara, 2003). They stabilize denatured and nascent proteins, mediating their folding and/or preventing them from forming cytotoxic aggregations (Moseley, 1997; Fink, 1999). Thus, these chaperones are crucial to maintain the integrity of the protein pool contributing to cellular functioning under regular (constitutive Hsp isoforms) and stressful conditions (inducible Hsp isoforms). Several stress factors can induce Hsp, including temperature, hypoxia, Reactive Oxygen Species (ROS), pollution, UV radiation, viral and bacterial infections, and osmotic stress, among others (Köhler et al., 2001; Tine et al., 2010; Madeira et al., 2012a; Tiedke et al., 2014; Rola et al., 2014; Das et al., 2015). Therefore, these proteins are not specific towards a certain type of stress, but respond to multiple physical, chemical and biological factors providing tolerance and cross-tolerance ("the ability of one stressor to transiently increase tolerance to a second heterologous stressor" – Todgham et al., 2005) (Todgham et al., 2005; Ely et al., 2014). In fact, Hsp have a selective value and contribute to the success of organisms across environmental gradients as they increase the ability of an organism to temporarily survive environmental extremes (Feder and Hofmann, 1999; Hofmann

et al., 2002; Hofmann, 2005; Sørensen et al., 2003). Moreover, several studies have shown that the induction of Hsp has effects on life history traits (e.g. development, stress resistance, longevity and reproductive capacity), affecting individual fitness (Rutherford, 2003, Sørensen and Loeschcke, 2001), albeit not free of developmental and reproductive trade-offs (Hofmann and Somero, 1995; Sørensen et al., 2003). The most studied Hsp in thermal stress studies have been the Hsp70 kDa and Hsp90 kDa. Both of these families occur in several cellular organelles including the nucleus, cytosol, peroxisome, lysosome, mitochondria and endoplasmic reticulum. While both types of Hsp are important in protein folding, Hsp90 is also related to the regulation of hormone receptors (glucocorticoid receptor – Iwama et al., 2006) and cytoskeletal dynamics (Liang and McRae, 1997). Furthermore, Hsp70 also has anti-apoptosis activity and it is important in the targeting of proteins for the ubiquitin-proteasome pathway (Alberti et al., 2002; Patterson and Höhfeld 2006; Kriegenburg et al., 2012).

The ubiquitin-proteasome pathway is crucial for the elimination of irreversible misfolded proteins which have lost their native function and tend to form cytotoxic aggregations (Amm et al., 2014). This is carried out via the selective tagging of proteins by ubiquitin and their translocation to the proteasome for degradation (Hershko and Ciechanover, 1992) (**Fig. 1.9**). This pathway maintains cellular homeostasis and plays important roles in several cellular processes including cell cycle regulation, apoptosis, trafficking, transcription, immune and inflammatory responses (Wang and Maldonado, 2006; Hanna et al., 2007). The main functions of this system include 1) continuous turnover to rapidly modulate protein levels; 2) elimination of proteins in response to specific signals; 3) elimination of defective proteins that arise through physical or chemical factors and transcriptional/translational errors or gene mutations; and 4) the turnover of excess free forms of proteins (from multi subunit complexes) (Finley 1991). Moreover, Lecker et al. (2006) state that the rapid degradation of certain proteins is important in the adaptation to new physiological conditions.

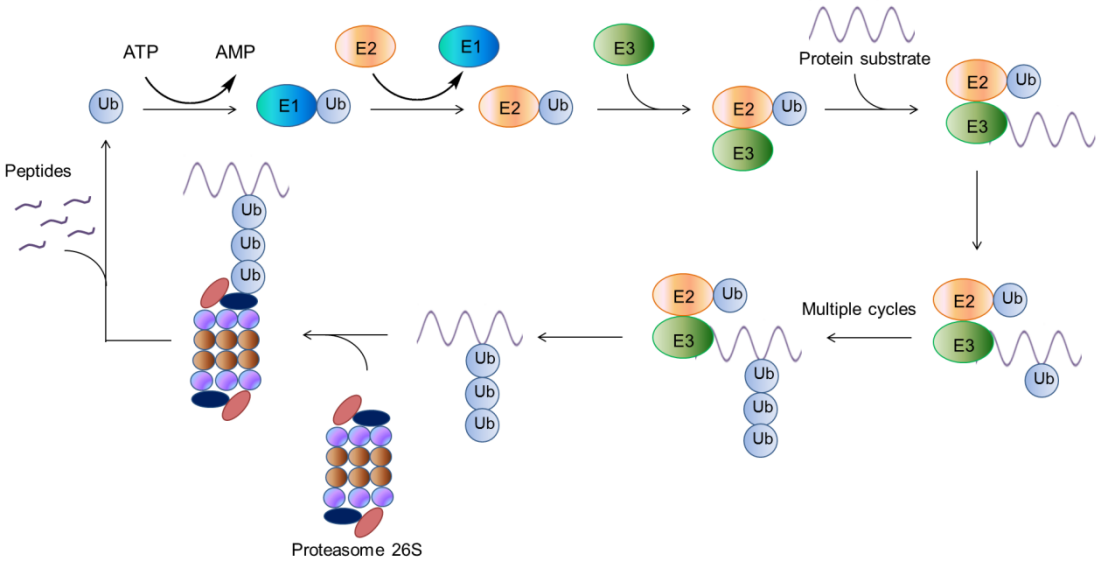


Figure 1.9 Schematic of the ubiquitin-proteasome pathway. Ubiquitin tagging is ATP dependent and is based on the action of 3 enzymes, E1, E2 and E3. Ubiquitin-activating enzyme (E1) binds to ubiquitin via a thio-ester bond. This allows for the subsequent step to take place and thus ubiquitin is transferred to the ubiquitin-conjugating enzyme (E2). Following, ubiquitin-ligase (E3) recognizes the protein substrate and catalyzes the transfer of ubiquitin to that substrate. After multiple cycles, the protein substrate is polyubiquitinated and can be linked to the proteasome subunit for degradation of the misfolded protein (peptide bond hydrolysis) with subsequent release of reusable free ubiquitin (via deubiquitinating enzyme) (adapted from Glickman and Ciechanover, 2002; Rahimi, 2012; Amm et al., 2014; Cell Signaling Technology Pathways). Note: mono-ubiquitination has also been recognized as a proteasome degradation signal. Constructed using PowerPoint tools.

Another major pathway in the cellular stress response is the elimination of free radicals (e.g. Reactive Oxygen Species - ROS) which can be induced by several factors, including temperature changes, pollution and UV radiation (Lesser, 2006). ROS have a single unpaired electron in the outer orbit which leads to an unstable energy state (Riley, 1994). This configuration makes free radicals extremely reactive with adjacent molecules (i.e. cellular components) (Rahman, 2007). It should be noticed that ROS are synthesized by cells under normal conditions (via the electron transport chain, peroxisomes, cytochrome P450 metabolism and inflammatory cell activation – Cadenas, 1989; Inoue et al., 2003) and they play significant roles in cellular signaling (Halliwell and Gutteridge, 1999). Nevertheless, the increased production induced by stressful conditions can lead to an unbalanced state that causes oxidative stress and consequent damage to proteins (carbonylation), DNA (deletions and mutations), carbohydrates, free amino acids and lipids (lipid peroxidation) (Toyokuni, 1999; Abele and Puntarulo, 2004). Lipid peroxidation and protein carbonylation are amongst the most relevant mechanisms of cellular injury. Lipid peroxidation occurs when ROS attack polyunsaturated fatty acids leading to a chain reaction that destroys cellular membranes (Ahmed, 2005; Lesser, 2006). Moreover, protein carbonylation leads to inactivation of enzymes, receptors and transport proteins, affecting major cellular functions (Przekwas et al., 2003; Lesser, 2006). The increase in ROS due to a temperature increase can arise by 1) higher respiration rates and 2) as a consequence of tissue re-oxygenation during the recovery phase (Halliwell and Gutteridge, 1999; Abele et al., 2002; Freire et al., 2011). In order to prevent or reduce the aforementioned damages by maintaining a steady-state of ROS, organisms possess a set of anti-oxidant agents (synthesized or dietary origin), including enzymes, vitamins, glutathione, ubiquinone, uric acid and lipoic acid (Martínez-Álvarez et al., 2005). The major enzymes that act as ROS scavengers are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx, selenium dependent) and glutathione-S-transferase (GST, selenium independent). Interestingly, these enzymes can be activated by Hsp chaperones and this interplay seems to be crucial in the survival of organisms exposed to critical temperatures (Mocanu et al., 1993; Currie et al., 1998; Heise et al., 2006; Kalmar and Greensmith, 2009). Such enzymes quench ROS by catalyzing the reactions that transform toxic ROS into non-toxic products (**Fig. 1.10**). Superoxide dismutase integrates the first line of defense by catalyzing the dismutation of the superoxide radical into oxygen and hydrogen peroxide ($2\ O_2^{\bullet-} + 2H^+ \rightarrow O_2 + H_2O_2$). Following, both catalase and glutathione peroxidase detoxify hydrogen peroxide into water and oxygen (CAT: $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$) or solely water (GPx: $H_2O_2 \rightarrow 2\ H_2O$).

Additionally, glutathione related enzymes (peroxidase and S-transferase) detoxify lipid peroxides (lipid-OOH) by transforming them into alcohols (lipid-OH). Several studies have shown that anti-oxidant enzymes increase when marine organisms are exposed to thermal stress, suggesting that ocean warming can have impacts in the oxidative status of marine biota (e.g. Madeira et al., 2013; Vinagre et al., 2014).

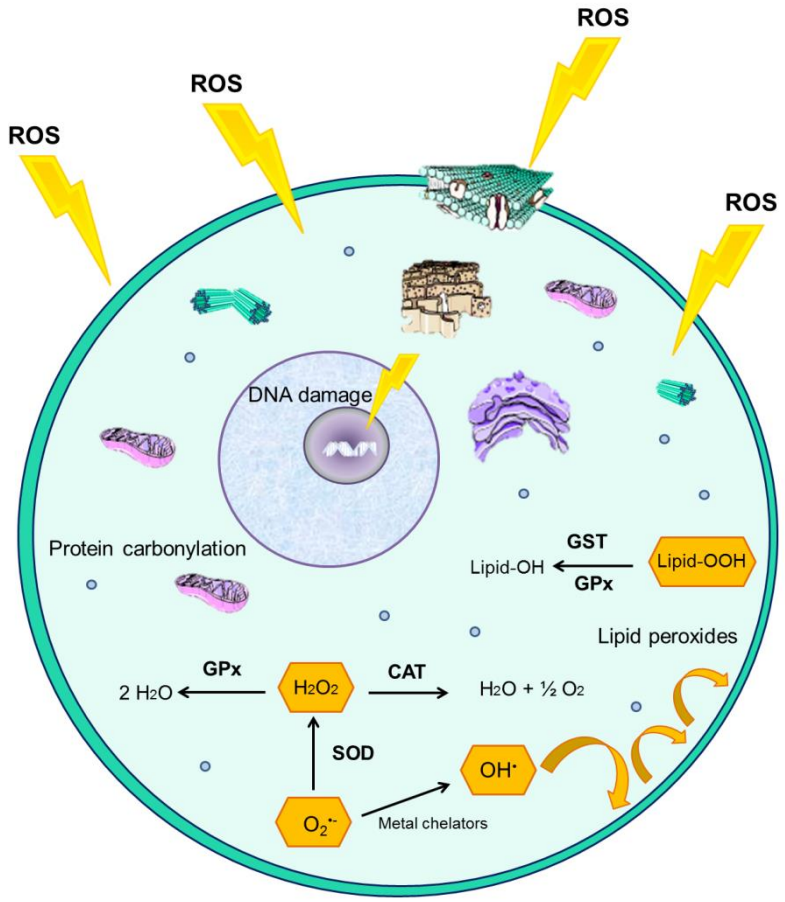


Figure 1.10 Schematic of cellular oxidative damage and anti-oxidant enzyme pathways. Reactive Oxygen Species (ROS) are enhanced under stressful conditions leading to potential oxidative damage, including DNA deletions and mutations, protein carbonylation and lipid peroxidation. Several enzymes (superoxide dismutase – SOD; catalase – CAT; glutathione peroxidase – GPx; glutathione-S-transferase – GST) counterbalance these effects by catalyzing reactions that transform free radicals into less toxic or non-toxic products (constructed using SmartDraw and PowerPoint tools).

3. Model organism: *Sparus aurata*

The teleost fish *Sparus aurata* (Linnaeus, 1758) (**Fig. 1.11** for taxonomy) is a commercially important sea-bream, especially in Southern Europe. This species is highly valuable both in fisheries and aquaculture. According to the Food and Agriculture Organization of the United Nations (FAO), the global aquaculture production of *S. aurata* was 173,062 tonnes in 2013, the main producers being Greece, Turkey, Spain and Italy. Other producer countries include Portugal, Croatia, Cyprus, Egypt, France, Tunisia, Morocco and Israel. Additionally, the mean global capture production of *S. aurata* since 1990 is around 8,000 tonnes per year. Thus,

this fish species can be viewed as proxy of commercially important sea breams, whose vulnerability towards climate change will have a direct impact on society.

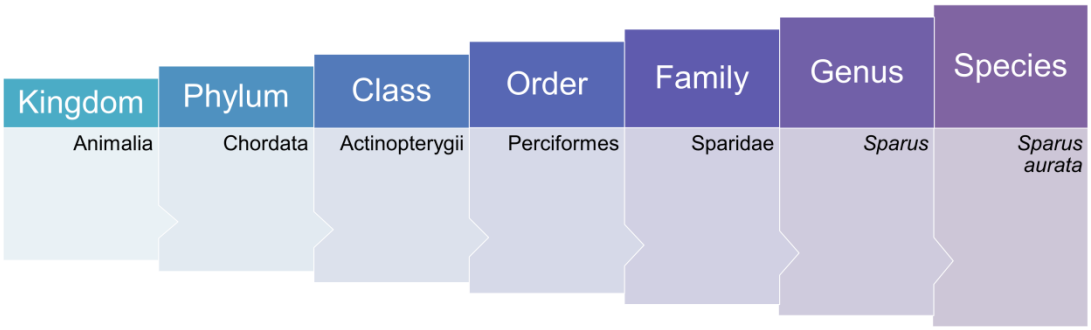


Figure 1.11 Taxonomy of *Sparus aurata* (constructed using PowerPoint tools).

S. aurata is a warm-temperate eurythermal fish that occurs in coastal seagrass beds, on sandy bottoms, and in the surf zone, as well as in estuaries and coastal lagoons (Altimiras et al. 1994). Its native distribution is subtropical and extends from the Mediterranean to the east Atlantic coast (including Cape Verde and the Canaries to the English Channel) (Froese and Pauly, 2006; Sola et al., 2007) (**Fig. 1.12**). It is an euryhaline species and it usually inhabits shallow waters, from 1 to 150 m deep (commonly 1 to 30 m) (Froese and Pauly, 2006).

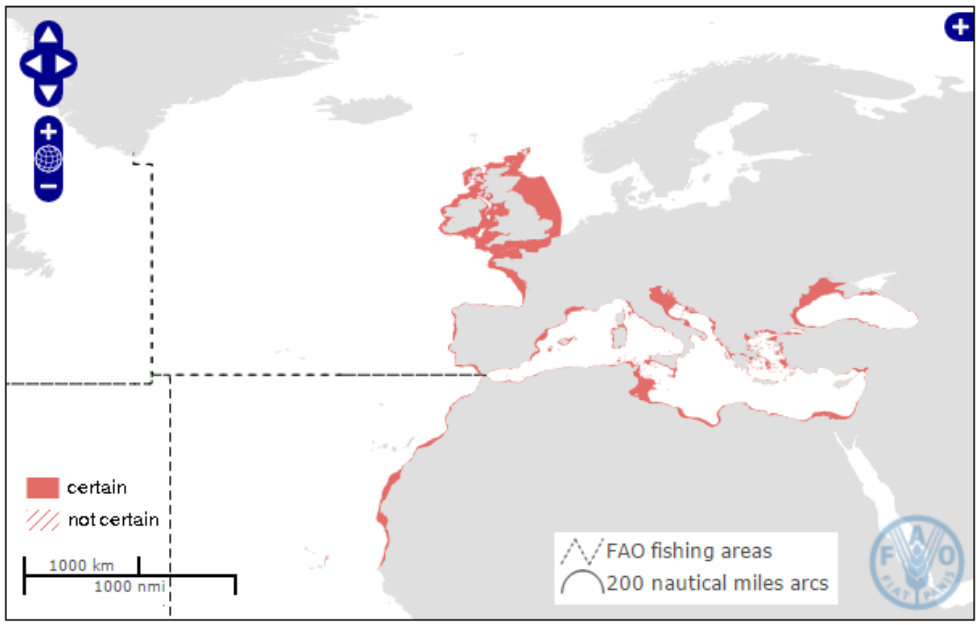


Figure 1.12 Native distribution of the sea bream *Sparus aurata* (source: Food and Agriculture Organization of the United Nations).

Considering its life cycle, adults reproduce in the open sea during autumn, winter and early spring. Early life stages are planktonic and the larval phase lasts about 50 to 60 days at

18ºC (Andrades et al, 1996; Sola et al. 2007). Then, late 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 school as juveniles (Craig et al., 2008). As *S. aurata* is a protandric hermaphrodite, it first matures as a male at about 1-2 years of age and as a female at 2-3 years of age (Froese and Pauly, 2006). Adult fish usually inhabit coastal waters but return to open sea to breed. This type of life cycle is common to many species of coastal fish, thus rendering this model species of general interest for the understanding of fish eco-physiology and vulnerability in the context of climate change. The next table (**Table 1.1**) summarizes all relevant information about *S. aurata*.

Table 1.1 Life history traits of *Sparus aurata* and commercial importance.

Sparus aurata

Domains	Specific traits	Eggs	Larvae	Juveniles	Adults	References
Geographic location and environment	Habitat	Open ocean	Open ocean; migrate to estuaries and coastal lagoons	Estuarine; coastal lagoons; seagrass beds; sandy and rocky bottoms	Estuarine; coastal; open ocean; seagrass beds; sandy and rocky bottoms	Froese & Pauly, 2006; Mariani, 2006
	Domain	Pelagic	Pelagic	Demersal	Demersal; pelagic	Froese & Pauly, 2006
	Distribution	Subtropical; Eastern Atlantic (British Isles to Cape Verde); Mediterranean Sea; Black Sea				Bauchot & Hureau, 1986; Froese & Pauly, 2006
	Depth	Surface layers (presumed)	Surface layers (presumed)	1-30	1-150	Suau & Lopez, 1976; Froese & Pauly, 2006
	Home range	Travel in ocean currents; larvae/juveniles reach nursery grounds by spring/summer		Small; sedentary fish	Sedentary until reproductive migration towards open sea takes place	Suau & Lopez, 1976; Froese & Pauly, 2006; Sola et al., 2007; Abecasis & Erzini, 2008; Arabaci et al., 2010; Franchini et al., 2012; Verdiell-Cubedo et al., 2013
Reproduction	Reproductive biology	Multiple batch spawner; sequenced spawning; protandric hermaphrodites maturing as males at 2 years and as females at 3 years of age				Froese & Pauly, 2006; Jerez et al., 2012
	Daily mean relative fecundity	Approximately 5,000-40,000 eggs.kg^{-1} of female, depending on age and time of the breeding season (in captivity); one female can lay between 20,000-80,000 eggs per day during the spawning period				Sola et al., 2007; Scabini et al., 2011; Jerez et al., 2012
	Spawning period	October to April; peak spawning in December				Froese & Pauly, 2006; Dimitriou et al., 2007; Basurco et al., 2011; Kissil et al., 2011; Mylonas et al., 2011; Ibarra-Zatarain & Duncan, 2015

	Egg duration (days)			2		Moretti et al., 1999;
	Maturation age (years)			2		Froese & Pauly, 2006
	Maturation size (cm)			20-40		Froese & Pauly, 2006; Ahmed, 2011
	Egg size (mm)			0.9-1.1		Froese & Pauly, 2006; Basurco et al., 2011
	Egg buoyancy			Positive at 35-37ppt (salinity)		Moretti et al., 1999
	Yolk conversion efficiency index			Maximal at 16ºC		Polo et al., 1991
	Larval duration (days)			50-60 at 18ºC		Andrades et al., 1996; Sola et al., 2007
	Larval length at hatching (mm)			2.5-3.0 (maximal size at 16-19ºC)		Polo et al, 1991; Parra & Yúfera, 2000; Froese & Pauly, 2006
	Distance from spawning areas to nursery grounds (km)			Unknown		See Franchini et al., 2012
Life span	**Maximum length reported (cm)**			70		Froese & Pauly, 2006
	Life span (maximum age reported in years)			11		Froese & Pauly, 2006
	Duplication time (years)			1.4-4.4		Froese & Pauly, 2006
Temperature range	**Optimal temperature (ºC)**	15-17	16-22	18-?	15-?	Polo et al., 1991; Moretti et al., 1999; Chaoui et al., 2006; scope of this thesis

	Critical Thermal Maximum (ºC)	?	?	?	?	Scope of this thesis
	Critical Thermal Minimum (ºC)	Unknown; lower lethal limit is reported to be around 2ºC; threshold temperature for metabolic activity 12-14ºC				Ibarz et al., 2003; Sola et al, 2007; Kyprianou et al., 2010
	Temperature for spawning activity (ºC)	Optimum 15-17 (min 13 to max 20); Gametogenesis blocked at 24				Moretti et al., 1999
	Temperature for maximum hatching rate (91-95%)	14-26ºC				Polo et al., 1991
	Temperature (ºC) for which % survival from fertilization is greater	16-22				Polo et al., 1991
Trophic characterization	**Feeding**	Yolk sac	Zooplankton	Polychaeta, nematodes, molluscs, crustaceans, small fish	Molluscs, crustaceans, small fish	Tancioni et al., 2003; Froese & Pauly, 2006; Abecasis & Erzini, 2008; European Comission, 2015
	Trophic level	3.7; Carnivorous; accessorily herbivorous				Froese and Pauly, 2006
Genetic traits	**Dispersal capacity and genetic differentiation between populations**	Passive dispersal due to planktonic eggs and larvae; degree of gene flow between populations is uncertain; both panmitic and subdivided populations have been reported; significant genetic differentiation has been reported between Mediterranean and Atlantic populations; mixed evidence within the Mediterranean but some connectivity is expected; studies concerning other Mediterranean sea breams report dispersal distances of at least 200 km				Ben Slimen et al., 2004; Rossi & Sola, 2006; Sola et al., 2007; Chaoui et al., 2009; Arabaci et al., 2010; Di Franco et al., 2012; Coscia et al., 2012; Franchini et al., 2012; Pujolar et al., 2013
Commercial importance	**Mean global capture production per year**	8,000 tonnes				FAO, 2015
	Aquaculture production	173 062 tons in 2013				FAO, 2015
	Price	Very high				Froese & Pauly, 2006

		Farmed fish 13€.kg^{-1}; Wild fish 25€.kg^{-1} (Portugal, 2015)	
Conservation	**IUCN Red List**	Least Concern	The IUCN Red List of Threatened Species, accessed 7 Dec 2015

Due to its commercial importance, *S. aurata* has been fairly studied and its genome has been partially sequenced (NCBI EST library → 114,155 entries; and NCBI protein database → 17,507 entries), making this species an appropriate model for molecular studies. Several proteomic studies have been carried out on *S. aurata*, including profiling of the skin mucous under control conditions (Jurado et al., 2015) and under overcrowding stress (Cordero et al., 2016); plasma changes induced by handling and overcrowding stress (Alves et al., 2010); proteome changes due to copper exposure (Isani et al., 2011); proteomic changes induced by dietary additives (Rufino-Palomares et al., 2011), probiotic intake (Cordero et al., 2016) and different feed formulations (Ghisaura et al., 2014); systematic characterization of muscle proteome (Addis et al., 2010; Piovesana et al., 2016); salinity-induced proteome changes (Varó et al., 2009); and hepatic changes following potentiated sulfa drugs (Varó et al., 2013) and invermectin administration (Varó et al., 2010). Most of these studies relate to aquaculture optimization practices. Considering thermal stress, very few proteomic studies have been carried out using *S. aurata* as model species. A literature search retrieved only studies related to low temperature stress and the consequent winter syndrome disease (e.g. Ibarz et al., 2010; Silva et al., 2014). As such, knowledge on *S. aurata*'s molecular thermal biology has been mainly produced from biomarker studies, measuring parameters such as protein kinases, heat shock proteins, and metabolic enzymes (e.g. Feidantsis et al., 2009, 2012, 2013, 2015). Thus, further studies using other biomarkers and a more holistic approach such as proteomics are crucial. Also, there are no studies concerning the thermal tolerance across several stages of the life cycle of *S. aurata*. This information is crucial to know if life cycle closure is hampered in a warming ocean, allowing for the improvement of conservation practices of exploited fish stocks.

4. Relevance to the scientific community and society

Climate change is one of the main environmental concerns of modern society. To understand and mitigate its impacts, a multidisciplinary approach is needed, as well as coordinated efforts between researchers, stakeholders, politicians and citizens. Basic research is needed to gather sufficient amount of information and enlighten the most appropriate trajectories for environmental management (i.e. sustainable, mitigation-based) in the face of observed and predicted changes. Moreover, in a recent Nature Climate Change editorial (2014), it is stated that researchers are critical players in the policy decision process concerning climate change and should produce policy-relevant research. This thesis fits such concepts by addressing one important question in ecology and conservation: how will ocean warming affect one of the main commercial fish species in Europe and North Africa? The multidisciplinary approach taken to carry out this study allowed us to assess effects at several levels of biological organization, from the molecular, cellular, tissue and organismal level, providing information on the health and plasticity of *S. aurata* throughout its lifecycle. Moreover, this allowed the identification of which life stages are subjected to strong selection pressures and thus should be protected to ensure that the life cycle is complete under climate change scenarios. Determining

the thermal tolerance and underpinning molecular mechanisms of commercial fish species is essential to ecological risk assessments. It allows us to understand potential acclimation mechanisms and how climate phenomena affect mortality, distribution and abundance of exploited fish stocks, with potential impacts on socio-economic activities such as fisheries. In fact, Munday et al., (2009) suggest that information concerning the physiology of species should be taken into account in fisheries management plans to mitigate adverse impacts of climate change. Furthermore, an additional demand for animal products (including fish) is expected in the future (e.g. FAO, 2014 - based on macroeconomic, demographic and environmental scenarios) and high-value species such as sea breams are highly traded (not only in prosperous markets but also in emerging economies through import) (FAO, 2014). Under these circumstances, it is relevant to maintain sustainable practices and contribute to the equilibrium between demand and supply by defining the vulnerability of exploited species.

In addition, thermal tolerance studies may also be useful when designing marine protected areas, which should consider for instance which life stage is more vulnerable and where it inhabits, recruitment's success in the face of climate change and adaptive capacity of species, in order to provide effective conservation. Ultimately, research concerning the vulnerability of commercial species towards ocean warming will help the improvement of conservation practices and contribute to the sustainability of fish stocks. In addition, aquaculture industries may have to adapt their practices to the reality of climate change, mitigating potential negative economic impacts.

5. Aims, scopes and thesis layout

The current thesis focuses on temperature effects on the gilthead sea bream at the organismal, tissue and cellular level, uncovering thermal limits and molecular mechanisms that might be responsible for resistance/vulnerability of *S. aurata* to climate changes, in particular ocean warming. The main aims are to determine the thermal tolerance and underpinning proteome plasticity in *S. aurata* throughout its life cycle using an integrative approach, which combines specific biomarker analyses with proteomics tools, histopathological analysis of several organs and fitness measures. Studying protein changes during ontogeny should provide insights into how the environment affects cellular pathways that establish the adult form. Moreover, the aim is to enlighten which life stage(s) is(are) more vulnerable and which will suffer more severe effects, considering that ocean temperatures are rising. Determining if high temperatures differently affect eco-physiological traits of fish throughout their life cycles is crucial to provide accurate forecasts of ecological changes arising from ocean warming. An additional aim is to infer on which proteins (up- or down-regulated) could be further used as biomarkers of thermal stress.

Such studies are crucial to evaluate current population status of exploited fish stocks, since determining the full scope of proteins produced in stress conditions allows us to assess the consequences both on individual health (and thus performance and fitness) and

consequently predict population changes (e.g. mortality, recruitment's success). This potentially gives us a chance to predict impacts at higher levels of organization, as has already been shown in other biomarker/proteomic studies (e.g. Costa et al., 2009). Once temperature affects physiological, behavioral and ecological processes, there is a need to understand what mechanisms are behind the organisms' response, enhancing our predictive and environmental management capacities considering climate change scenarios. This is of great importance, in particular to countries with a sea-based economy, like Portugal.

In detail, the objectives were to:

1. Unravel upper thermal limits of *S. aurata* throughout its life cycle

2. Determine the vulnerability of life stages of *S. aurata* to acute and chronic warming

3. Unravel molecular mechanisms that are employed by *S. aurata* under acute and chronic thermal stress and how these may affect its health and acclimation capacity by

i) Studying the capacity of *S. aurata* to employ specific cellular defense mechanisms throughout its life cycle,

ii) Analyzing histopathological changes (i.e. phenotypical anchoring) caused by warming,

iii) Determining the proteome plasticity of *S. aurata* under chronic warming conditions to unravel its potential for acclimation (considering several life stages),

iv) Analyzing if this plasticity is enough to maintain the health and survival of *S. aurata* (measuring mortality rates and condition index)

v) Determining protein stress response networks and metabolic pathways (based on biomarker and proteomic results) affected by ocean warming,

4. Based on the previous information, infer on the possible consequences of ocean warming on *S. aurata* populations, improving our understanding of ecological processes and conservation practices needed to maintain the sustainability of fish stocks.

The next table (**Table 1.2**) summarizes all parameters used in the thesis, their biological significance and expected results under ocean warming scenarios.

Table 1.2 Parameters used in this thesis, their biological significance and expected results under warming scenarios.

Parameter	Details	Biological significance	Expected response under warming scenarios	Based on references (e.g.)
Biomarker	Heat shock proteins (70kDa, 90 kDa)	Chaperones, important in folding and stabilizing unfolded proteins, preventing cytotoxic aggregations	Increase at moderate stress levels and decrease at extreme stress levels, but depends on the targeted organ	Feder and Hofmann, 1999; Madeira et al., 2012a
	Ubiquitin and the proteasome	Ubiquitin targets damaged proteins to be degraded in the proteasome, preventing cytotoxic aggregations	Increase at moderate stress levels and decrease at extreme stress levels, but depends on the targeted organ	Hofmann and Somero, 1995; Madeira et al., 2014
	Catalase	Anti-oxidant enzyme that catalyzes the reaction that transforms hydrogen peroxide into water and oxygen (2 $H_2O_2 \rightarrow 2\ H_2O + O_2$)	Increase at moderate stress levels and decrease at extreme stress levels, but depends on the targeted organ	Madeira et al., 2013; Vinagre et al., 2014 Almroth et al., 2015
	Superoxide dismutase	Anti-oxidant enzyme that catalyzes the dismutation of the superoxide radical into oxygen and hydrogen peroxide (2 $O_2^{\bullet-} + 2H^+ \rightarrow O_2 + H_2O_2$)	Increase at moderate stress levels and decrease at extreme stress levels, but depends on the targeted organ	Vinagre et al., 2014; Almroth et al., 2015
	Glutathione-S-transferase	Phase II metabolic isozyme that detoxifies xenobiotic substrates and lipid peroxides	Increase at moderate stress levels and decrease at extreme stress levels, but depends on the targeted organ	Madeira et al., 2013; Vinagre et al., 2014; Almroth et al., 2015
	Lipid peroxidation	These are oxidation products that indicate cellular damage due to oxidative stress	Increase, but depends on the targeted organ	Madeira et al., 2013; Vinagre et al., 2014; Almroth et al., 2015
	Protein carbonylation		Increase, but depends on the targeted organ	Almroth et al., 2015
Histopathological	Signs of inflammation,	These signs indicate tissue damage	Increased tissue damage but also potential	Dash et al., 2011; Rojas

evaluation	atrophy, immune responses, apoptosis or necrosis	(reversible or irreversible)	adaptive alterations	et al., 2013; Raina et al., 2015
Proteomics	Analysis of the full muscle proteome and how it varies with temperature	Measure of the cell's phenotype via the analysis of the full proteome	Variations in proteins related to cellular defense, integrity and energy utilization	Tomanek, 2011; 2014
CTmax	Critical Thermal Maximum	Estimation of the upper thermal limit based on loss of equilibrium	Between 25-35ºC, depending on life stage; might vary if fish are subjected to different acclimation temperatures	Madeira et al., 2012b; Vinagre et al., 2016
K Fulton	Condition index	Measure of health and weight changes	Potential increase at moderate temperature and decrease at high temperature	Walther et al., 2010
Mortality/survival	Measured as % mortality or survival curves	Ability to survive stress	High mortality/Low survival, depending on the level of thermal stress	Houde, 1989; Clapp et al., 1997; Landsman et al., 2011

This thesis comprises of 8 chapters, the first being a general view of the main themes related to climate change and thermal biology and the structure of the present thesis; chapters 2 and 3 concern the upper thermal limits of *S. aurata* larvae and their vulnerability to acute and chronic warming based on an integrative approach that comprises measurements at the individual (mortality), tissue (histopathology) and molecular level (biomarker analysis and proteomics); chapter 4 and 5 analyze upper thermal limits of *S. aurata* juveniles and their capacity to employ specific cellular defense mechanisms upon acute exposure to warming and how that capacity reflects on tissue histopathological traits; chapter 6 evaluates the survival, condition and proteome plasticity of juvenile *S. aurata* exposed to chronic warming; chapter 7 includes a thorough analysis of *S. aurata*'s thermal tolerance throughout larvae, juvenile and adult stages based on molecular and individual level analyses (biomarkers, mortality and condition index) upon exposure to chronic warming; and chapter 8 presents the concluding remarks and future perspectives (**Fig. 1.13**).

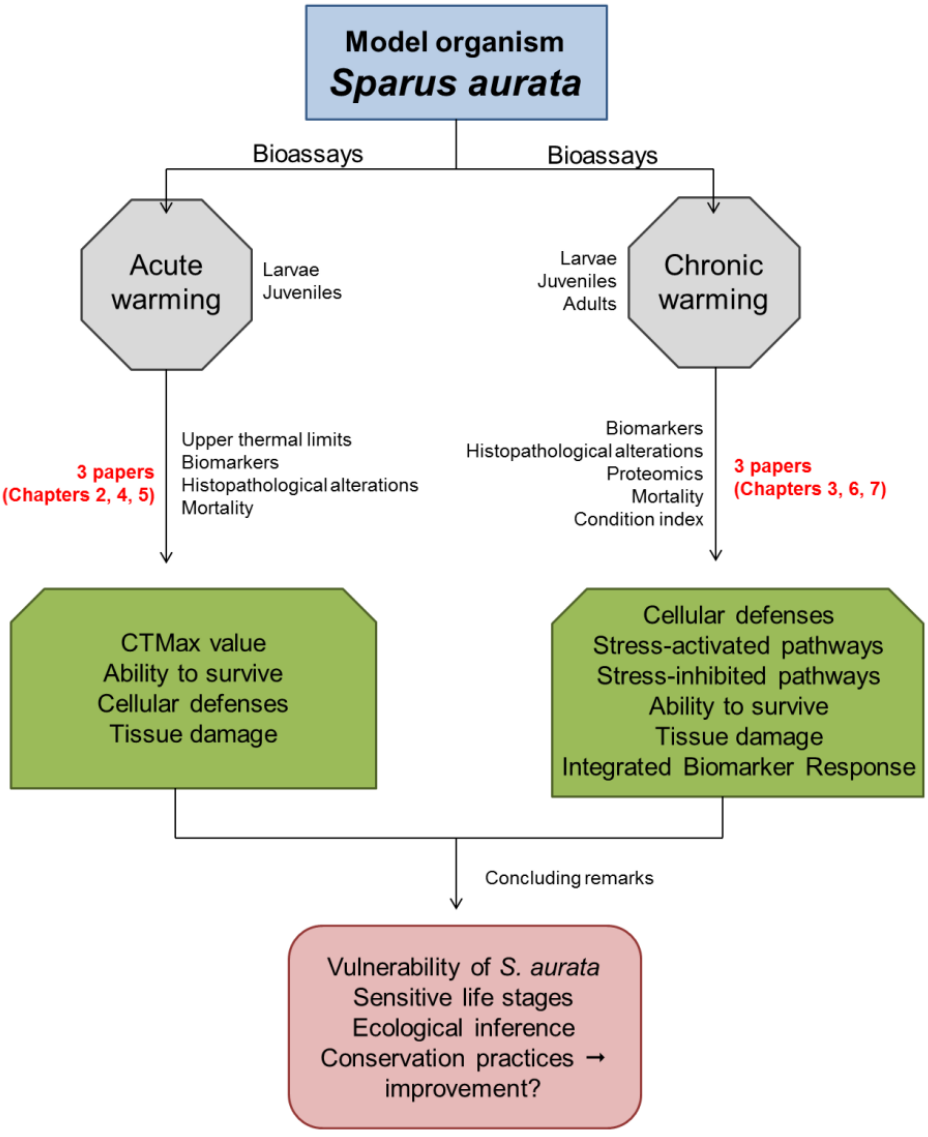


Figure 1.13 Scheme of the thesis workflow (constructed using Power Point tools).

6. Acknowledgements

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CHAPTER 2. WHEN WARMING HITS HARDER: SURVIVAL, CELLULAR STRESS AND THERMAL LIMITS OF *Sparus aurata* LARVAE UNDER GLOBAL CHANGE[1]

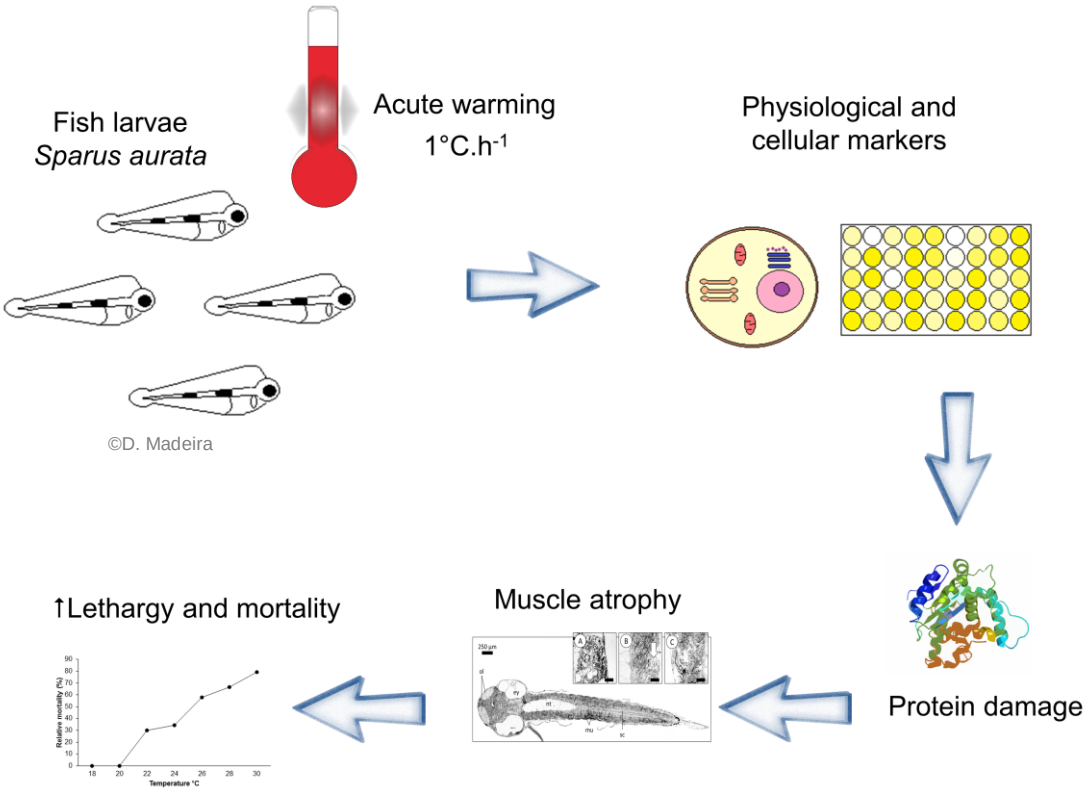
[1]Madeira D, Costa PM, Vinagre C, Diniz MS (2016) Marine Biology 163, 91-104. DOI: 10.1007/s00227-016-2856-4

Abstract

Understanding physiological and molecular compensation mechanisms that shape thermotolerance is crucial for estimating the effects of ocean warming on fish stocks, especially during early life stages, whose tolerance determines recruitment success and population viability. The aims of this study were to assess the sensitivity of fish larvae towards ocean warming and heat wave events in the commercial species, *Sparus aurata*, whose habitat is likely to be affected by rising water temperatures. We 1) estimated its critical thermal maximum (CTmax) and mortality rates upon warming, 2) quantified stress biomarkers: heat shock protein 70kDa, total ubiquitin, antioxidant enzymes (superoxide dismutase, catalase, glutathione-S-transferase), lipid peroxidation and protein carbonylation, and 3) analyzed histopathological changes as a result of thermal stress. Larvae showed increasing levels of lethargy with increasing temperature, attaining a cumulative CTmax value of 30ºC. Relative mortality increased upon warming, reaching 80% at 30ºC. Oxidative damage was higher at moderate temperatures and decreased at 24ºC probably due to a significant increase in superoxide dismutase's activity. Hsp70 chaperone levels also increased at 26ºC, but unfolding persisted at higher temperatures as shown by the increase in total ubiquitin at 26 and 28ºC, indicating protein damage. Skeletal muscle showed disorganization of muscle fibers from 24ºC onwards. Overall, protein denaturation seems to be the major cause of larval mortality, potentially compromising recruitment's success from 22ºC onwards, considering that larvae migrate into nursery grounds by spring and summer (i.e. high temperatures), thus hindering the viability of local fish stocks. These data demonstrate that the biochemical homeostasis of fish can be disturbed within an ecologically realistic thermal range and emphasize the risks of rising global temperatures for larval fishes.

Key words: biomarkers, histology, larvae, thermal stress, climate change

Graphical abstract



1. Introduction

Global average temperatures have risen 0.85ºC in the past century, at an average rate of 0.2ºC per decade over the past thirty years – likely caused by increased concentration of CO_2 and other greenhouse gases in the atmosphere (Hansen et al. 2006; Godbold and Calosi 2013; IPCC 2014a, b). According to several studies, climate-driven changes, especially ocean warming (+0.8ºC in sea surface temperature over the past century, Godbold and Calosi 2013), are already leading to changes in species' abundance and distribution, with consequences at the community and ecosystem level (Cabral et al. 2001; Walther et al. 2002; Perry et al. 2005; Vinagre et al. 2009; Peck et al. 2012). Species' resilience and therefore community structure in the marine environment is dependent upon several factors, mainly organism's physiology, phenotypic plasticity, population connectivity and recruitment success. Thus, understanding physiological and molecular mechanisms deployed by organisms to cope with stress is important to improve biogeographic forecasting (Woodin et al. 2013).

Early life stages are reported to be the most vulnerable life-stages to environmental changes (e.g. Storch et al. 2001; Pörtner and Farrell 2008; Pimentel et al. 2014). Moreover, several climate-driven processes have been associated with the probability of larval survival (Peck et al. 2012). Considering that most marine species have planktonic eggs and larvae (that

are subjected to high mortality rates) further changes in water temperature may create a bottleneck effect through low performance and increased mortality, compromising recruitment and sustainability of fish stocks (Houde 1989; Houde 2008; Faria et al. 2011; Landsman et al. 2011; Bartolini et al. 2013). Furthermore, temperature has been shown to cause sublethal effects on larvae, influencing metabolism, disease resistance, motor function, growth and development and increasing the incidence of developmental malformations (e.g. Polo et al. 1991; Werner et al. 2007; Georgakopoulou et al. 2010; Dionísio et al. 2012; Vinagre et al. 2013; Linares-Casenave et al. 2013; Rosa et al. 2014). As a result, determining thermal tolerance and physiological performance of early life stages upon exposure to thermal stress is critical to understand the impacts of global change on biodiversity and maintenance of commercial stocks. In this context, the model species selected for the present study was the gilt-head seabream *Sparus aurata* (*Linnaeus* 1758) (see **Table 2.1** for details), which is highly important in fisheries and aquaculture. The species' habitats in Eastern Atlantic (especially in areas of Southern Europe) and Mediterranean are judged to be one of the most endangered of the European region. Climate models predict an increase in frequency and severity of heat waves and rising water temperature (e.g. Fischer and Schär 2010), hence the importance of understanding the risks posed to local fish populations. This is especially relevant considering that developing or early metamorphosing larvae (<20mm total length) move to shallow waters in spring and summer to settle in nursery grounds such as estuaries and coastal lagoons (Suau and Lopez 1976; Chícharo and Teodósio 1991; Arabaci 2010; Verdiell-Cubedo et al. 2013). As such, migrating larvae travel across several habitats, being exposed to different environmental regimes (e.g. Bodinier 2010) that can be physiologically challenging, especially under climate change scenarios. This coast-estuary larval migration process is common to numerous other coastal fish species, which means that in general coastal fish go through this physiological challenge during this life-stage with a potential effect on their later recruitment.

Table 2.1 *Sparus aurata* life history traits.

Traits	***Sparus aurata***	References
Distribution	Subtropical; Mediterranean Sea; Black Sea; Eastern Atlantic (from the British Isles to Cape Verde)	Froese & Pauly 2006, Sola et al. 2007
Habitat	Seagrass beads; sandy bottoms usually down to 30m (adults can occur down to 150m deep). Early life stages are planktonic and juveniles and adults are demersal	Froese & Pauly 2006
Spawning	In the open sea from October to April	Kissil et al., 2001; Dimitriou et al., 2007; Mylonas et al., 2011; Ibarra-Zatarain and Duncan, 2015
Duration of egg phase	48h	Moretti et al., 1999
Duration of larval phase	50 days at 17-18ºC	Sola et al. 2007

Importance	Commercial species (capture production 8,000-10,000 tons.year^{-1}; aquaculture production 150,000 tons in 2012)	FAO, FishStat 2015
	Predators in estuarine systems (top down effects on communities);	Froese & Pauly 2006
	Life cycle is similar to many other coastal species	

During summer, Portuguese coastal water temperatures are usually in the range of 17-20ºC while in estuaries and coastal lagoons water temperature is in the range of 24ºC-26ºC (databases from Instituto Português do Mar e da Atmosfera and Centro de Ciências do Mar e do Ambiente). Rates of temperature increase have been calculated for different geographic locations, showing that southern Europe is one of the regions more prone to warming (IPCC 2001). The main impacts predicted for Portugal over the 21st century are i) a significant increase (+2-3ºC) in sea-surface temperature (SST) and ii) a significant increase in the number of days surpassing 35ºC (HadRM2 model, Miranda et al. 2002). Climate models also predict an increase in the frequency, intensity and length of extreme events (IPCC 2007, 2013), which have already been associated with fish, invertebrate and macroalgae mortalities in other parts of the world (see Garrabou et al. 2009; Rose et al. 2012; Wernberg et al. 2013; Pearce and Feng 2013). During heat waves (at least 5 days in a row with a maximum air temperature of ≥ 35ºC – Santos and Miranda, 2006), Portuguese coastal waters can currently attain 23ºC (Madeira et al. 2014) while estuaries and coastal lagoons can reach 28ºC (due to lower thermal inertia). Moreover, mean SST (sea surface temperatures) in the Mediterranean area can also range from 26 to 28ºC in summer (Damianides and Chintiroglou 2000). Under such scenarios, by 2100, the expected estuarine water temperature during heat waves is ≥ 30ºC.

These temperatures may be harmful once they are close to the thermal limits of several species, including seabreams (Madeira et al. 2012a; Vinagre et al. 2014). Although still a matter of debate, the limits of thermal tolerance seem to be firstly determined by the capacity to transport oxygen to organs (Pörtner and Farrell 2008). At critical temperatures, organisms experience severe performance decrements. At these stages, organisms modulate gene expression to maintain homeostatic balance and promote functional capacity and cell survival (e.g. Logan and Somero 2011). Nevertheless, the onset of such mechanisms depends on thermal history, acclimation temperature and duration of the stress. The cellular defense mechanisms generally include i) the activation of the ubiquitin-proteasome pathway, ii) the induction of chaperones (e.g. heat shock proteins - hsp) and iii) an increase in antioxidant enzymes. The induction of these mechanisms is crucial to counterbalance deleterious effects of temperature such as protein denaturation, cytotoxic aggregations and thermal induced-oxidative stress (e.g. Lesser 2006; Fulda et al. 2010; Logan and Somero 2011; Madeira et al. 2013). Heat shock proteins function as chaperones to stabilize unfolded proteins, while ubiquitin targets irreversibly damaged proteins to be degraded in the proteasome. Additionally, anti-oxidant enzymes are crucial elements to maintain the cellular redox state. They neutralize reactive oxygen species such as hydrogen peroxide and superoxide anion and detoxify endogenous

compounds such as peroxidized lipids, which usually increase with temperature. Some of these proteins have been shown to increase in larvae under different types of stress (Werner et al. 2007; Sung et al. 2008; Rhee et al. 2013; Linares-Casenave et al. 2013). Overall, the ability to deploy these compensating mechanisms and their associated phenotypic plasticity are two crucial factors in thermotolerance research, helping scientists determine how species respond to ocean warming (Somero 2010). However, these mechanisms, and consequently, thermal tolerance are dependent upon ontogenetic state (Pörtner and Farrell 2008) albeit the full molecular and physiological effects of temperature in larvae remain largely unknown.

Based on this background, the aim of this work was to determine the sensitivity of larval fish towards ocean warming and heat wave events and its relation to the molecular pathways of protein degradation and antioxidant defense. This issue was addressed in *S. aurata* larvae by: i) estimating its Critical Thermal Maximum (CTmax) at a rate of temperature increase of $1ºC.hr^{-1}$, simulating heat wave events on low depth nursery grounds and ii) calculating the mortality rate upon warming. Moreover, we also assessed iii) if larvae are capable of increasing cytoprotective proteins upon warming via the quantitation of heat shock protein 70 kDa (Hsp70) and total ubiquitin (Tub); iv) the role of thermal-induced oxidative stress in health and survival impairment of larvae by quantifying activity levels of anti-oxidant enzymes (catalase, superoxide dismutase and glutathione-S-transferase) and levels of oxidation products (lipid peroxidation - and protein carbonylation), and v) relate biomarkers with histopathological endpoints (i.e. phenotypic anchoring) to uncover pathological effects of high temperature.

2. Materials & Methods

2.1 Ethical statement

This study was approved by *Direcção Geral de Alimentação e Veterinária* and followed the recommendations of the Portuguese legislation for animal experimentation. All of the authors have a level C certification by FELASA (Federation of European Laboratory Animal Science Associations).

2.2 Larvae collection and acclimation

S. aurata larvae were obtained from a fish farm on the 3rd of October 2013 (MARESA, Ayamonte, Spain) and transported to the laboratory in 5 L containers with gentle aeration (11 day post-hatch larvae, 4.4±0.5 mm total length; n~400 based on the hatchery's rounded down estimate of number of larvae per liter; produced from a broodstock of 50 males and 25 females). Larvae were then transferred to a re-circulating system with filtration holding 30L opaque polyvinyl tanks at a constant temperature of 18°C, salinity of 25‰ and O_2 levels between 95-100% (same conditions of the fish farm). Larvae were maintained at above conditions for 24h prior to experimentation (a longer period was not feasible as developmental changes occur very fast, interfering with the objective of studying early larval stage). Larvae were fed under a strict periodic feeding regime (every 6 hours) with rotifers and *Artemia salina* nauplii. Larvae were 12

days post-hatch when the experiment was carried out. At this age, larvae have pectoral fins, eyes pigmentation, primary swim bladder inflation, around 100% of the yolk sac reabsorbed and 70% of oil droplet reabsorbed (see Moretti et al. 1999). Exotrophy started between day 3 and 4 post-hatch. At the age of 11 days post hatch, larvae show active feeding with high movement (José 2012).

2.3 Experimental setup

The rearing tanks (two 30L tanks with ~200 individuals each) were placed in a temperature-controlled bath connected to a MultiTemp III system (Pharmacia Biotech) heated/refrigerator circulator (**Fig. 2.1**). During the experiment, larvae (now 12 days post-hatch) were exposed to a constant rate of water-temperature increase of $1°C.h^{-1}$ and observed continuously, until they reached the Critical Thermal Maximum (CTmax, given in ºC). This rate of temperature increase was based on the study by Mora and Maya (2006), which analyzed several heating rates and concluded that $1°C.h^{-1}$ maximized thermal tolerance estimates and decreased lag time between reached environmental temperature and reached body temperature. In addition, this rate is suitable for simulating heat wave events in low depth nursery grounds. The estimation of CTmax was adapted from the dynamic method described in Mora and Ospina (2001). The CTmax is usually estimated following a behavioral end-point e.g. loss of equilibrium and/or onset of muscular spasms. As these end-points are extremely difficult to observe in ≈5mm larvae, a cumulative CTmax was used and defined as the temperature at which 100% of larvae had reached lethargic behavior (when they stopped swimming and were quiet in the bottom of the tank). Larvae were collected for biochemical and histological analysis every two degrees (18ºC, 20ºC, 22ºC, 24ºC, 26ºC, 28ºC, 30ºC). Larvae had to be pooled for biochemical analysis, in order to give a sufficient amount of protein. Therefore, two pooled samples (one from each tank) were taken at each temperature point, comprising of 20 live individuals each. Only two pooled samples were taken to ensure enough larvae numbers to sample until the end of the experiment (due to the expected rates of mortality). For optical microscopy analysis, three live individuals were taken at each temperature. Relative mortality was measured when random samples of larvae were being collected for analyses; briefly 30 individuals were monitored to detect if they reacted or not upon stimulation (by creating gentle water movement with a Pasteur pipette). If no reaction was detected, then larvae were considered dead and were removed, not being used in any biochemical or histological analysis. In summary, these 30 individuals were taken as 100% and then percent mortality was calculated at each sampling time (coefficient mortality).

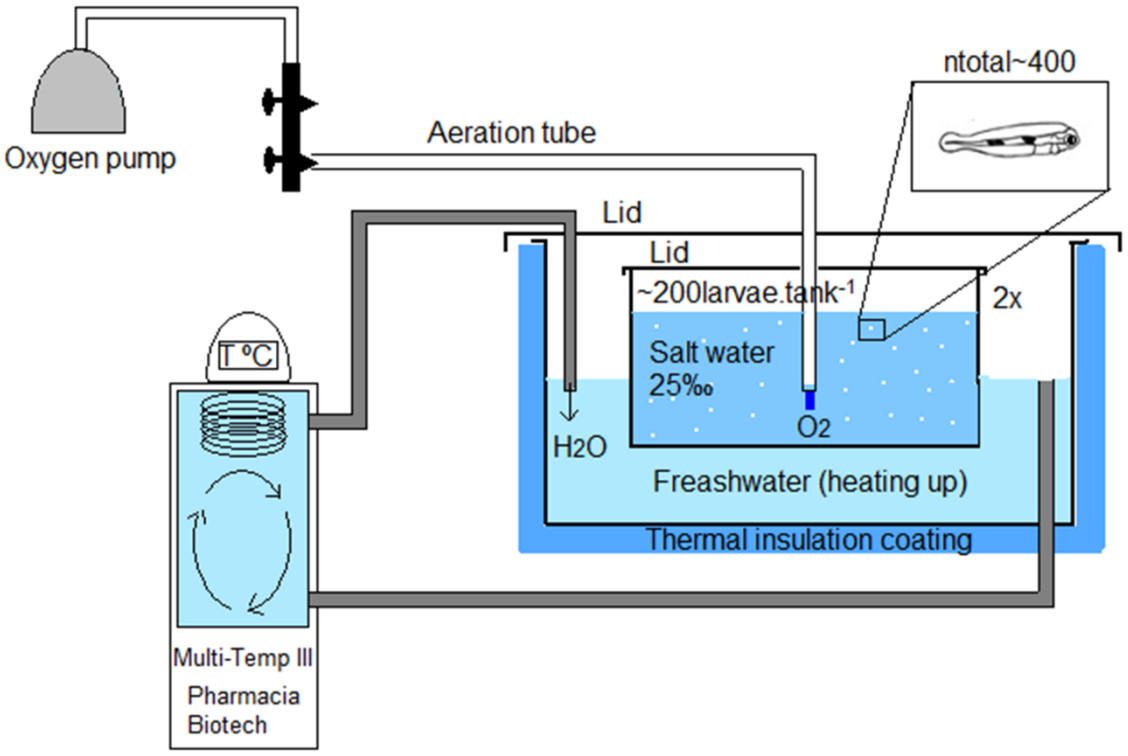


Figure 2.1 Experimental system: two 30L tanks were placed in a thermostatized bath connected to a heated/refrigerator circulator (MultiTemp III, Pharmacia Biotech). The aquarium was equipped with an aeration system. Both containers (aquarium and thermostatized bath) had a lid to prevent evaporation. The total number of larvae was approximately 400 based on the hatchery's rounded down estimate of number of larvae per liter. Half of the individuals were transferred to tank 1 and the other half to tank 2 (~200 larvae.tank^{-1}). Larvae were obtained from breeding 50 males with 25 females. Components are not to scale. Constructed in Paint.

2.4 Protein extraction

Samples of pooled larvae were homogenized in 200 µL of phosphate buffered saline (PBS) solution (0.14 M NaCl, 0.003 M KCl, 0.01 M Na_2HPO_4, 0.002 M KH_2PO_4, pH 7.4) to extract proteins, using disposable micro tissue grinders, in ice-cold conditions. The crude homogenates were centrifuged for 15 min at 10,000 × g at 4ºC (refrigerated centrifuge model Himac CT15RE, Hitachi Koki Co. Ltd., Japan) and frozen immediately (-80ºC) until subsequent biochemical analyses. Two pooled samples collected at each sampling point were used for all biochemical analyses described below.

2.5 Hsp70 and total ubiquitin (Tub) quantification

Heat Shock Protein 70 kDa was quantified using an indirect Enzyme Linked Immunosorbent Assay (ELISA) whereas total ubiquitin was quantified via a direct ELISA (both in 96-well microplates - Greiner, Germany) as detailed in Madeira et al. (2014a). In brief: samples for either analysis were diluted to 1:10 in 0.05 M carbonate-bicarbonate buffer (Sigma-Aldrich, USA). Then, two replicates of 50 µL were taken from each diluted sample and transferred to the

microplate wells for incubation overnight at 4ºC. The microplates were washed (3×) in PBS containing 0.05% Tween-20 (Sigma-Aldrich, USA) and then blocked by adding 200 µL of 1% (m/v) BSA) (Bovine Serum Albumin, Sigma-Aldrich, USA) in PBS. The microplate was afterwards incubated at 37°C for 90 min. After microplate washing as previously described, the primary antibody (for Hsp70: anti-Hsp70/Hsc70, Acris, USA; for total ubiquitin: Ub P4D1, sc-8017, HRP conjugate, Santa Cruz, USA), was diluted to 0.5 $\mu g.mL^{-1}$ in 1% BSA in PBS and added to the microplate wells (50 µL each). The microplates were then incubated for 90 min at 37°C and washed again (3×). After this step, the protocols diverge as described:

1) indirect ELISA for Hsp70: the secondary antibody (anti-mouse IgG, fab specific, alkaline phosphatase conjugate, Sigma-Aldrich, USA) was diluted (1 $\mu g.mL^{-1}$ in 1% BSA in PBS) and added (50 µL) to each well followed by incubation at 37°C for 90 min. After the washing stage (as previously described), 100 µL of substrate (SIGMA *FAST*™ p-Nitrophenyl Phosphate Tablets, Sigma-Aldrich, USA) was added to each well and incubated for 30 min at room temperature. Fifty µL of stop solution (3 N NaOH) was added to each well and the absorbance was measured at 405 nm using a BenchMark model microplate reader (BIO-RAD, USA). For quantification purposes, a calibration curve was constructed using serial dilutions of purified Hsp70 active protein (Acris, USA) to give a range from 0 $\mu g.mL^{-1}$ to 2 $\mu g.mL^{-1}$.

2) direct ELISA for total ubiquitin: 100 µL of substrate (TMB/E, Temecula California, Merck Millipore) was added to each well and incubated for 30 min at room temperature. One hundred µL of stop solution (1 N HCl) was added to each well and the absorbance was read in the aforementioned 96-well microplate reader at 415 nm. A calibration curve was constructed using serial dilutions of purified ubiquitin (UbpBio, E-1100, USA) to give a range from 0 $\mu g.mL^{-1}$ to 2 $\mu g.mL^{-1}$.

2.6 Antioxidant enzymes

The enzymatic activity assay for Catalase (CAT) (EC 1.11.1.6) was carried out according to the procedures of Johansson and Borg (1988). The assay was performed in 96-well microplates. Activity from a standard bovine catalase (Sigma-Aldrich, USA) solution of 1523.6 $U.mL^{-1}$ was used as a positive control. Formaldehyde standards (0-75 µM) were used to construct a calibration curve. Catalase activity was calculated considering that one enzyme unit (U) catalyzes the formation of 1.0 nmol of formaldehyde per minute at 25ºC.

The enzymatic assay of glutathione S-transferase (GST) activity (EC 2.5.1.18), using the substrate CDNB (1-Chloro-2,4-dinitrobenzene), was carried out based on the original method of Habig et al. (1974) and optimized for 96-well microplates. After reading the absorbance at 340 nm (at 25ºC) GST activity was calculated using a molar extinction coefficient for CDNB of $0.0053\varepsilon^{mM}$.

The enzymatic assay of superoxide dismutase (SOD) activity, using nitroblue tetrazolium (NBT) and xanthine oxidase (XOD), was carried out according to method adapted

from Sun et al (1988). After reading the absorbance at 560 nm (at 25ºC), SOD activity was calculated using the equation for the % inhibition:

$$\frac{(\frac{Abs}{min}\,negative\ control)-(\frac{Abs}{min}\,sample)}{\frac{Abs}{min}\,negative\ control}\,x\,100 \qquad (1)$$

2.7 Lipid peroxidation (LPO)

The lipid peroxides assay was adapted from the thiobarbituric acid reactive substances (TBARS) protocol (Uchiyama and Mihara 1978) adapted by Madeira et al. (2013). Five µL of each sample were added to 45 µL of 50 mM monobasic sodium phosphate buffer. Then 12.5 µL of SDS 8.1%, 93.5 µL of trichloroacetic acid (20%, pH=3.5) and 93.5 µL of thiobarbituric acid (1%) were added to each microtube. To this mixture, 50.5 µL of Milli-Q grade ultrapure water were added and the microtubes were vortexed for 10 seconds. Subsequently, they were incubated in boiling water for 10 minutes. The reaction was stopped on ice 62.5 µL of Milli-Q grade ultrapure water and 312.5 µL of n-butanol pyridine (15:1, v/v) were then added to extract the red pigment. The microtubes were then vortexed and centrifuged at 10 000 × g for 5 minutes. Duplicates of 150 µL of the supernatant per reaction were put into a 96-well microplate and absorbance was read at 530 nm. A six-point calibration curve (0-0.005 µM TBARS) was obtained using malondialdehyde bis(dimethylacetal) (from Merck) as standard.

2.8 Protein carbonylation (PC)

Protein carbonylation was quantified using an indirect Enzyme Linked Immunosorbent Assay (ELISA) adapted from Alamdari et al. (2005) for 96-well microplates. Samples were diluted to 1:2 in PBS (described in 2.3). Then, two replicates of 50 µL were taken from each diluted sample plus PBS (blanks) and transferred to the microplate wells for incubation overnight at 4ºC. The microplates were washed (3×) in PBS. Following, the proteins were derivatized for 45 min at room temperature in the dark with 0.05 mM DNPH (2,4-dinitrophenylhydrazine, Sigma-Aldrich, USA) solution (pH 6.2). Afterwards, the microplate was washed (5×) with PBS:ethanol (1:1 in v/v) and 2× with PBS. Two hundred microliters of blocking solution (1% m/v BSA in PBS) were added and the microplate was then incubated at 37°C for 90 min. After microplate washing with PBS containing 0.1% Tween-20, the primary antibody (anti-DNP, Acris, USA), was diluted to 1 µg.mL^{-1} in 1% (m/v) BSA in PBS and added to the microplate wells (50 µL each). The microplates were then incubated for 90 min at 37°C and washed again (3×). The secondary antibody (anti-mouse IgG, fab specific, alkaline phosphatase conjugate, Sigma-Aldrich, USA) was diluted (1 µg.mL^{-1} in 1% BSA in PBS) and added (50 µL) to each well followed by incubation at 37°C for 90 min. After the washing stage, 100 µL of substrate (SIGMA *FAST*™ p-Nitrophenyl Phosphate Tablets, Sigma-Aldrich, USA) was added to each well and incubated for 30 min at room temperature. Fifty µL of stop solution (3 N NaOH) was added to each well and the absorbance was read in a 96-well microplate reader at 405 nm.

The absorbance of the blanks was subtracted from the samples and all absorbance values were normalized to total protein. In absence of available commercial standards, the results are expressed as relative units (taking the control temperature 18ºC as reference).

2.9 Statistical analysis

An ANOVA followed by Dunnett's post-hoc tests were applied to test differences between control and other temperatures. A Spearman's rank order correlation was also applied to test if relative mortality was correlated with the temperature increase. Cluster analysis was carried out with biomarker data (using single linkage as amalgamation method and 1-Pearson r correlation statistic as distance – correlation metrics emphasize shapes of expression/activity i.e. profiles) in order to verify potential relations between the analyzed end-points. A significance level of α=0.05 was set for all statistics. Statistics were carried out using the software Statistica 10 (StatSoft Inc, USA) and GraphPad Prism v5 (GraphPad Software Inc, USA).

2.10 Histological analysis

Larvae (3 per temperature point) were fixed in Bouin's solution (10% v/v formaldehyde and 7% v/v acetic with picric acid to saturation) for 48 h at room temperature. The specimens were dehydrated in a progressive series of acetone and embedded in EPON 821 (Luft's mixture). Propylene oxide was employed for intermediate infiltration. Semi-thin sections (≈ 0.5 µm thick) were obtained with an LKB Bromma III 8800 ultramicrotome and stained with Toluidine Blue O for the histopathological appraisal. A qualitative assessment (mainly based on presence/absence, type and extent of lesions) was carried out and was based on both transversal and longitudinal sections of larvae. The analysis was conducted with a DMLB model microscope equipped with a DFC480 digital camera, all from Leica Microsystems.

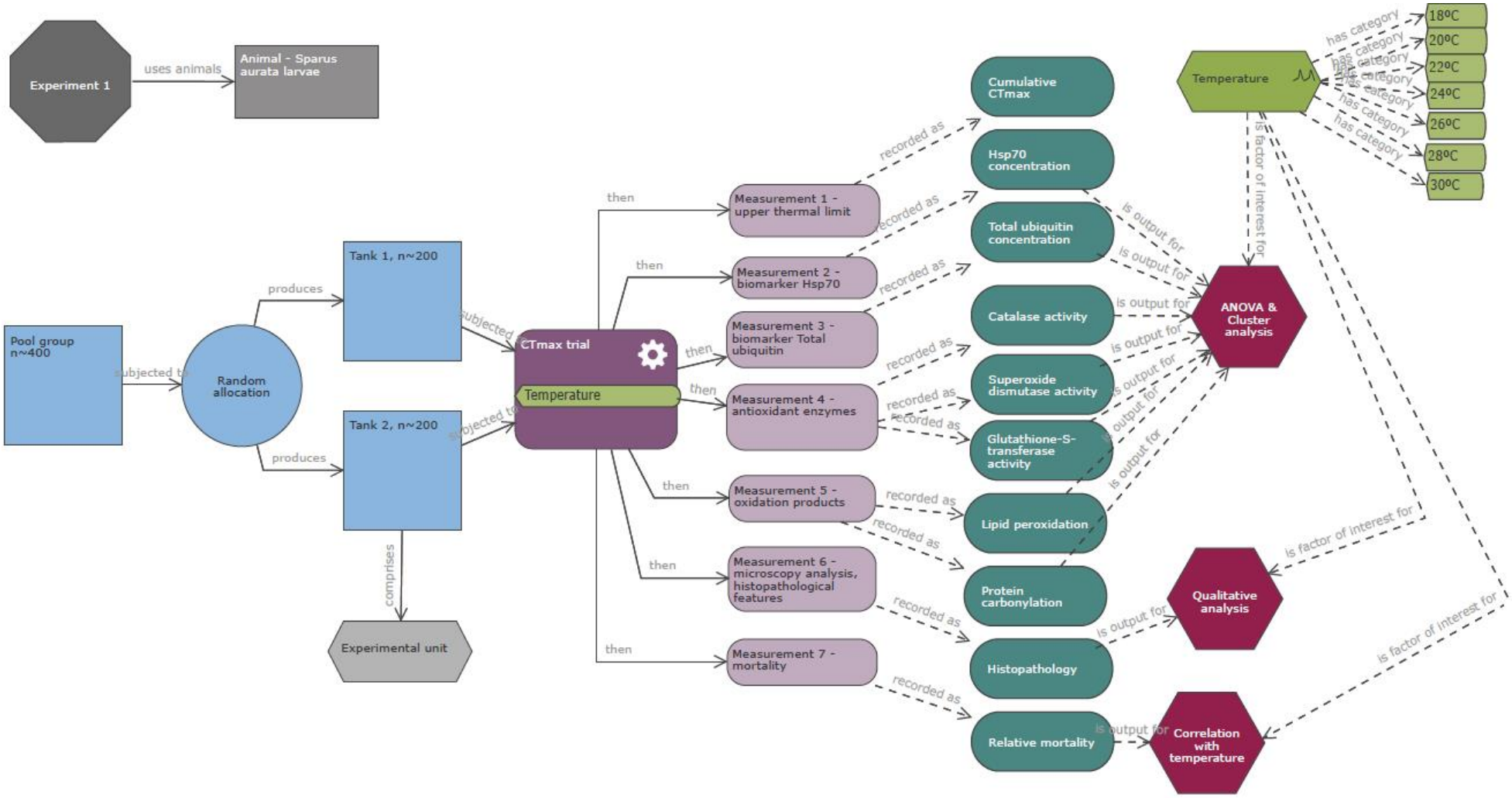


Figure 2.2 Experimental approach (diagram constructed using Experimental Design Assistant, https://eda.nc3rs.org.uk/).

3. Results

3.1 Critical Thermal Maximum

The cumulative CTmax value for 12d post-hatch larvae of *S. aurata* (4.4±0.5 mm total length) was 30ºC (temperature at which 100% of larvae showed lethargic behavior), with sublethal and lethal effects arising at lower temperatures (starting at 22ºC).

3.2 Biomarkers and percent mortality

Larvae showed no mortality at 18ºC and 20ºC, but at 22ºC there was 30% mortality, which continued to increase up to 80% at 30ºC. Moreover, Spearman rank-order R statistic test confirmed that relative mortality (**Fig. 2.3**) was strongly correlated with temperature ($R=0.99$, $p<0.05$).

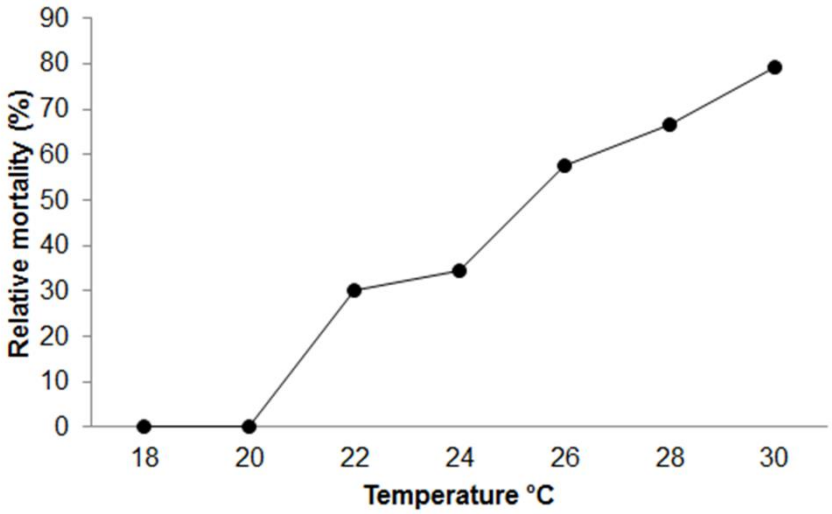


Figure 2.3 Relative mortality of *Sparus aurata* larvae (12d post-hatch, ≈5mm) along the temperature gradient (rate of temperature increase of 1ºC.h^{-1}).

Statistical analyses showed that 4 out of the 7 biomarkers tested were affected by temperature (**Table 2.2**). While levels of total ubiquitin (protein damage biomarker) increased at 26ºC (2.8-fold) and 28ºC (2.9-fold) when compared to control conditions (18ºC), Hsp70 only increased at 26ºC (2.6-fold) (**Fig. 2.4a**). Focusing on AOX (antioxidant enzymes), SOD increased its activity at 20ºC (2.7-fold) (**Fig. 2.4b**) while catalase and glutathione-S-transferase showed no changes (**Fig 2.4b**). Focusing on oxidation products, lipid peroxidation showed no changes along the temperature trial (**Fig 2.4c**) even though there was a trend to increase at the beginning of the trial. Concerning protein carbonylation, there was a trend to increase at 20ºC followed by a significant decrease in relation to control levels at 24ºC (2.2-fold).

Table 2.2 ANOVA results. Significant results are marked with an asterisk. Hsp70 – heat shock protein 70kDa; Tub – total ubiquitin; LPO – lipid peroxidation; GST – glutathione-S-transferase; SOD – superoxide dismutase; CAT – catalase; PC – protein carbonylation.

Biomarker	F	df	p-value
Hsp70	4.155	6	0.0421*
Tub	8.584	6	0.0060*
LPO	1.403	6	0.3315
GST	1.946	6	0.2020
SOD	4.191	6	0.0412*
CAT	2.501	6	0.1279
PC	4.122	6	0.0429*

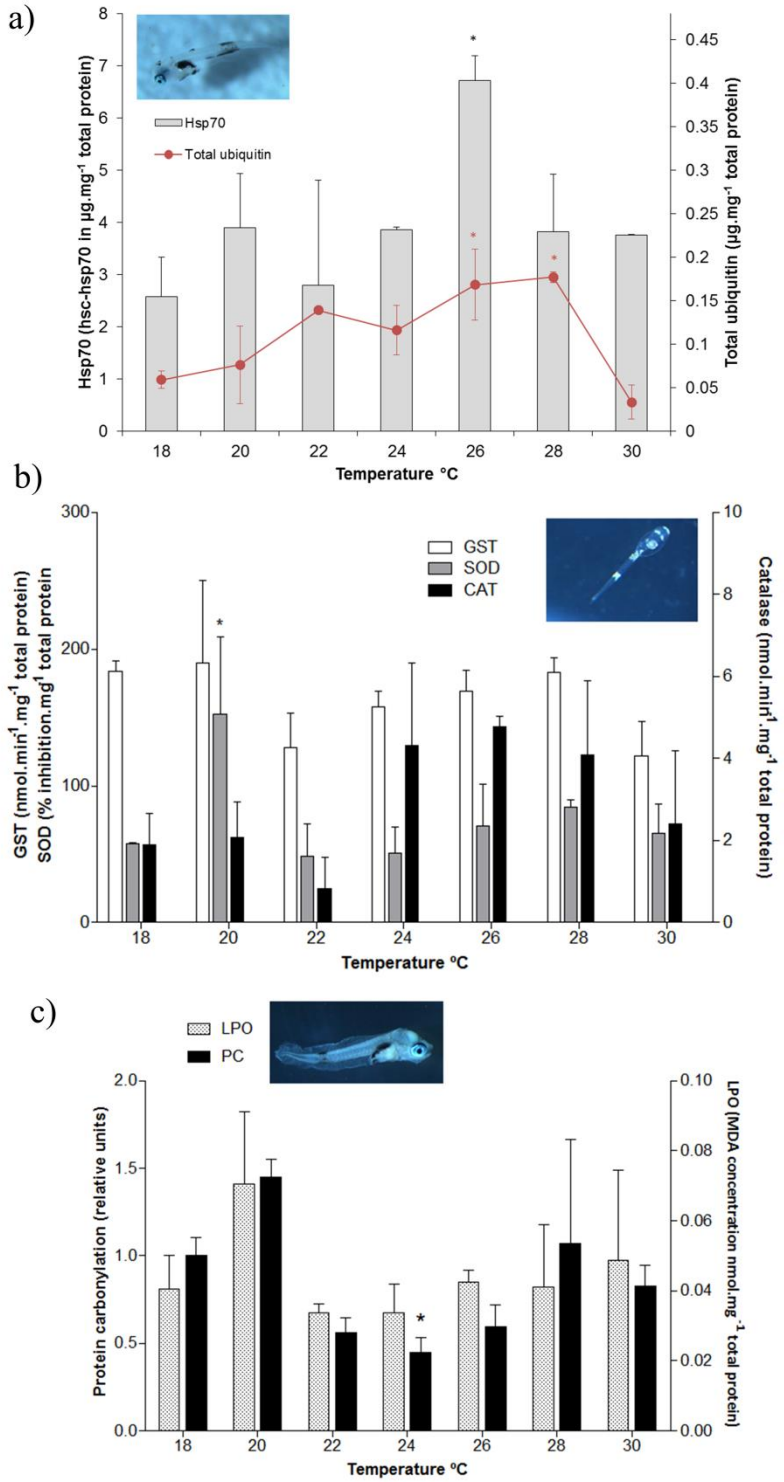


Figure 2.4 Thermal and oxidative stress biomarkers in larvae (12d post-hatch, ≈5mm) of *S. aurata* exposed to a temperature gradient (1ºC.h^{-1}) ranging from the control temperature (18ºC) until the cumulative Critical Thermal Maximum (temperature at which 100% of larvae showed lethargic behavior – 30ºC). **a)** Hsp70 (Hsc+Hsp70) and total ubiquitin, **b)** antioxidant enzymes (glutathione-S-transferase - GST, superoxide dismutase - SOD, catalase - CAT) and **c)** lipid peroxidation - LPO and protein carbonylation – PC. Two pooled samples (comprising of 20 larvae each) were taken at each temperature point. Results are shown as mean±SD. Groups significantly different from control are marked with an asterisk ($p<0.05$, ANOVA, Dunnett's post-hocs). Larvae photographs by M.S. Diniz.

Cluster analysis yielded two major groups of responses, one grouping oxidative stress-related responses (with the exception of CAT activity), the second comprising Tub, Hsp70, CAT and mortality (**Fig. 2.5**). Inside the first group, SOD and LPO were the most correlated whereas in the second group CAT and Hsp70 yielded the best correlation within pairs of variables.

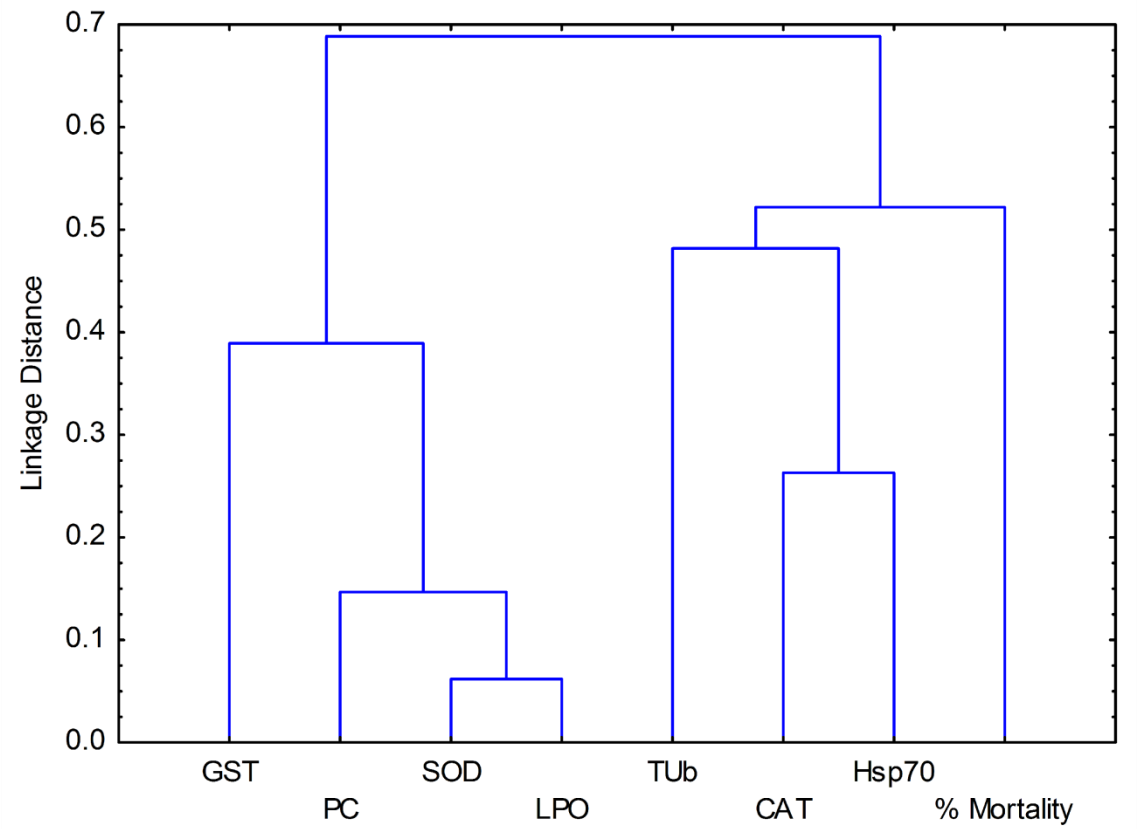


Figure 2.5 Cluster analysis dendrogram (amalgamation rule: single linkage; distance metrics: 1-Pearson r) of all variables measured in *S. aurata* larvae (12d post-hatch, ≈5mm) along a temperature gradient ($1^{o}C.h^{-1}$) ranging from the control temperature (18ºC) until the Critical Thermal Maximum (temperature at which 100% of larvae showed lethargic behavior – 30ºC). GST – glutathione-S-transferase; SOD – superoxide dismutase; LPO – lipid peroxidation; TUb – total ubiquitin; CAT – catalase; Hsp70 – Heat shock protein 70kDa; PC – protein carbonylation.

3.3 Histological observations

Alterations in visceral organs in animals subjected to temperatures higher than 18 ºC were either scarce or undetermined, in face of the early differentiation stage of tissues and organs (**Fig. 2.6**). Still, larvae subjected to temperatures from 24 ºC and above yielded foci of damaged pancreatic acinar cells (**Fig. 2.6A**). Also, small, restricted foci, of unspecific alterations such as hepatic vacuolation, abdominal hemorrhaging and even vacuolation of the retina were also found in few animals subjected to 24 ºC or higher, albeit without a clear temperature-related trend (not shown). The digestive tract, gills, kidneys, notochord and nervous system

were also screened for histopathological alterations but no lesions were found, comparative to larvae subjected to 18 ºC (controls). The most relevant and consistent lesions were found in the muscle, especially skeletal musculature, of animals enduring temperatures from 24 ºC upwards (**Figs. 2.6B** and **C**). These lesions consisted of foci of dystrophic muscle bundles, characterized by pronounced disorganization of muscle fibers with obvious loss of mass, creating lacunae within the muscle. These alterations were observed in animals subjected to temperatures of 24 ºC and higher. The frequency and size of foci depicted a trend to increase with test temperature.

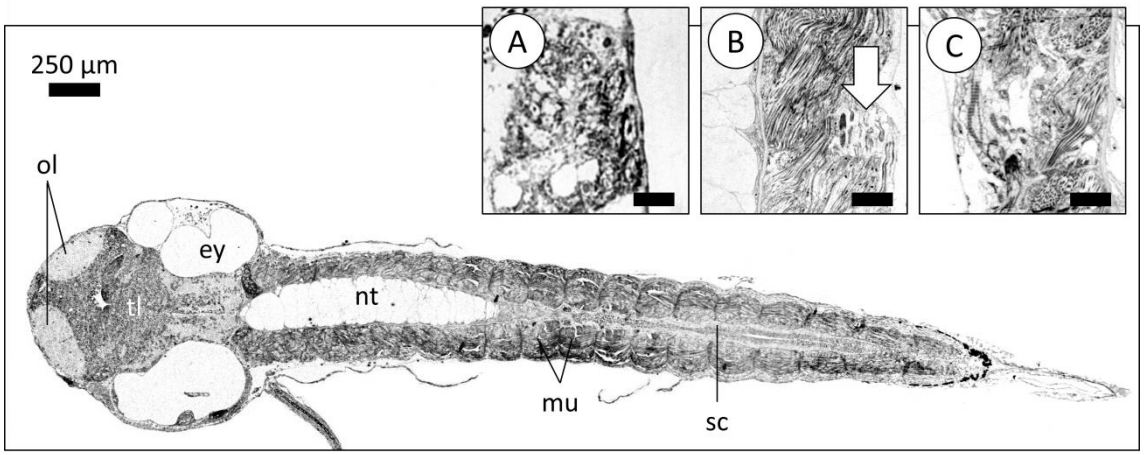


Figure 2.6 Dorsal overview of *S. aurata* larva 12d post-hatch, ≈5mm (longitudinal section). ey) eye; ol) olfactory lobe; nt) notochord; tl) telencephalon; sc) spinal cord; mu) muscle. A) damaged pancreatic tissue in a larva subjected to 24 ºC. Scale bar: 5 µm. B) focus of dystrophic skeletal muscle (arrow) in a larva subjected to 30 ºC. Compare with adjacent normal muscle bundles. Scale bar: 25 µm. C) Larger focus of dystrophic skeletal muscle of another larva subjected to 30 ºC. Note fiber loss and disorganization, with the creation of obvious lacunae within fiber. Epon-embedded samples stained with Toluidine Blue. Scale bar: 25 µm

4. Discussion

This study showed that *S. aurata* larvae, are very sensitive to rising temperature showing signs of impaired health and elevated mortality at temperatures beyond 22ºC (displaying lethargic behavior, protein damage and muscle atrophy upon exposure to warming). This information holds particular ecological relevance because larvae migrate to estuaries and coastal lagoons during spring and summer, in which waters can easily reach temperatures of ≥22ºC (see Madeira et al. 2012b). Altogether, the strong correlation between larvae relative mortality and temperature supports the hypothesis that temperature can have bottleneck effects on larval stages, determining population viability. This is especially evident considering that regular daily mortalities for *S. aurata* larvae at this age (≈10 days post-hatch) are in the order of 5% under standard rearing conditions – 18ºC (Andrades et al. 1996). These values are much lower than those herewith obtained in larvae subjected to short-term thermal stress. Also, several other studies have reported increased mortalities in larvae or juvenile stages of marine species exposed to high temperatures (Houde 1989; Pepin 1991; Vinagre et al. 2013; Dunphy et al. 2013; Rosa et al 2014). Such increased mortalities may severely affect wild populations,

particularly considering that in most marine fish species 99.9% of all offspring will die during the first year of life and thus will not reproduce, affecting recruitment success and maintenance of fish stocks (Houde 2008; Peck et al. 2012). Nevertheless, to assess the viability of fish stocks under ocean warming scenarios, mortality should be calculated for all life stages.

Larvae showed signs of thermal-induced oxidative damage at moderate temperatures. Both lipid peroxidation and protein carbonylation showed a trend to increase at 20ºC (although not significant) and superoxide dismutase significantly increased its activity at 20ºC, suggesting an increased need for anti-oxidant defenses. Following, protein carbonylation significantly decreased at higher temperatures (24ºC), which possibly indicates that oxidative damage is being counterbalanced by SOD. Altogether, larvae are seemingly able to cope with oxidative stress that may arise during heat challenge. In fact, other authors have suggested that embryos and larvae need a strongly regulated redox environment to coordinate between growth and differentiation (Skjærven et al. 2013), suggesting that antioxidant mechanisms are already well developed at those stages. Therefore, oxidative stress may not be a driver of larval mortality, which is also shown by the cluster dendrogram, since mortality is best correlated with Hsp70 and total ubiquitin. It should be noticed however that even though the rate of warming was chosen to decrease the lag time between environmental and body temperature, a delay in response time is still possible. Thus, temperatures thresholds for the onset of protection mechanisms could be lower.

Larvae showed signs of reversible and irreversible protein damage at temperatures ≥ 26ºC, which is in agreement with other studies on marine larvae (e.g. Werner et al. 2007; Meyer et al. 2011; Linares-Casenave et al. 2013). The increase in chaperones suggests an increased demand for protein stabilization potentially to prevent cytotoxic aggregations of denatured proteins (Basu et al. 2002). However, irreversible protein unfolding was not prevented as total ubiquitin increased at 26ºC and 28ºC, suggesting that larvae are prone to protein damage. Therefore, protein damage seems to correlate best with larval mortality in comparison to other end-points tested. This result is corroborated by other studies that state that larval mortality following acute heat stress may arise due to massive degradation of proteins with direct impacts on cell functioning (Somero and Hofmann 1997; Landsmann et al. 2011). Nevertheless, it should be noticed that other untested factors may be at play such as metabolic dysfunction and low ATP turnover. Valente et al. (2013), state that muscle atrophy occurs when rates of protein degradation exceed the rates of protein synthesis. Interestingly, microscopy analyses showed that the most relevant lesions occurred in muscle, from 24ºC upwards, which is in agreement with other studies (e.g. Alami-Durante et al. 2006). There was an evident disorganization of muscle fibers with loss of mass (and potential disruption of myogenesis), which probably contributed to the lethargic behavior of larvae and consequent lack of swimming, leading ultimately to death. Altogether, these results indicate that there is a loss of performance in larvae exposed to high temperature which may be associated primarily with proteolytic processes rather than general metabolic failure caused by oxidative stress. Further studies on metabolism should confirm this assertion. These proteolytic processes usually have high

metabolic costs and this energy deviation towards repair mechanisms will ultimately accelerate the consumption of energy stores (Blaxter 1992; Landsmann et al. 2011; Kristiansen et al. 2014), with potential impacts on growth.

Temperature has direct physiological effects that impact the behavior, physiological status and swimming of larvae (Blaxter 1992; Green and Fisher 2004; Koumoundouros et al. 2009; McLeod et al. 2013). Therefore, high temperature can affect population structure and recruitment success by affecting crucial components of the survival. These components include predator avoidance success, ability to capture prey and larval dispersal (Blaxter 1992; Plaut 2001; Leis and McCormick 2002; Fisher and Wilson 2004), which depend on physiological status and swimming ability. Still, temperature effects on recruitment and population dynamics are dependent on the ecology and habitat of the species (O'Connor et al. 2007). In the case of *S. aurata*, larvae migrate to shallow waters during spring and early summer, which is when temperature starts to increase and heat waves occur, potentially affecting their survival. Nevertheless, as the spawning period of this species is quite prolonged in time (from October to April), the first larvae might be able to reach nursery grounds before heat waves strike, whereas larvae from later spawning events may be more affected. Events such as this have already been described in other species of the Portuguese coast (Vinagre et al. 2013). Therefore, at least in the Atlantic Ocean, spring temperatures seem to be the most appropriate for larval migration into nursery grounds. Exposure to temperature extremes is known to inflict a selection pressure on wild populations (e.g. Williams et al. 2012). Therefore ocean warming could impose a selection pressure on spawning behavior and migration phenology (to occur earlier in the year), or on thermal tolerance of larvae (broader or shifted thermal tolerance windows). In this context, *S. aurata* populations in the Mediterranean Sea could be subjected to stronger selection pressures than Atlantic populations, considering that sea surface temperature is higher in the Mediterranean. Thus, in Southern Europe, larvae can be frequently exposed to temperatures ≥25ºC and unless they possess high phenotypic plasticity (acclimation potential) or some kind of specific adaptation (involving selection on genetic variation) to the local milieu, heat waves could affect larval survival. Concerning plasticity, *S. aurata* is an eurythermal species so it must have some potential for acclimatization. Moreover, transgenerational acclimatization (as described in Munday 2014) could also ameliorate climate change effects on fish, although this has been seldom studied. This type of plasticity is based on non-genetic inheritance, which may include a number of factors such as epigenetic variation, and parental effects which are dependent on nutritional, somatic or cytoplastic factors (Munday 2014). Such transgenerational plasticity implies that the effects of a stressor can be ameliorated when several generations experience that stressor, albeit not entirely free from developmental trade-offs. Transgenerational plasticity has been shown to occur in some fish species exposed to warming, including damselfishes (Donelson et al. 2011), sheepshead minnow (Salinas and Munch 2012) and three-spine sticklebacks (Shama et al. 2014) (see Munday 2014 for a review). Other factors should also be considered when assessing the viability of fish stocks in the face of climate change including the potential for local adaptation through selection on genetic variation

across generations (dependent on gene flow/larval dispersal, de-novo mutations, ancestral variation, and which genes/alleles involved in response mechanisms possess a selective value).

Notwithstanding, the final impact of ocean warming on *S. aurata* populations also depends on the thermotolerance of other life stages, such as juveniles and adults. The thermotolerance of *S. aurata* juveniles has already been investigated. While the CTmax value (measured through loss of equilibrium attaining 35.5 ± 0.5°C) was much higher than for larvae, signs of stress (measured through biochemical and histopathological markers) also arose at lower temperatures, approximately at 26-28ºC (Madeira et al. 2014). This has also been confirmed by other studies with the same species (Feidantsis et al. 2009, 2013). These findings follow the general acknowledgement that thermal window widths are larger for juveniles than for eggs and larvae (Pörtner and Farrell 2008). Still, if juveniles confined in estuaries and coastal lagoons cannot escape to deeper waters, they can also be vulnerable to heat wave events, which can make water temperature attain 28-30ºC in southern Europe (and 30-35ºC by 2100 – Miranda et al. 2002 and Fischer and Schär 2010). Therefore, *S. aurata* may be threatened through reduced physiological performance, leading to impaired recruitment or, at least, bottleneck effects on genotypic diversity, with unpredictable effects on population maintenance. As this is a major commercial species, such data should be considered in management strategies in order to mitigate the impacts of climate change, which will most likely worsen problems already created by overfishing and habitat destruction by direct anthropogenic action.

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CHAPTER 3. OCEAN WARMING ALTERS CELLULAR METABOLISM AND INDUCES MORTALITY IN FISH EARLY LIFE STAGES: A PROTEOMIC APPROACH[1]

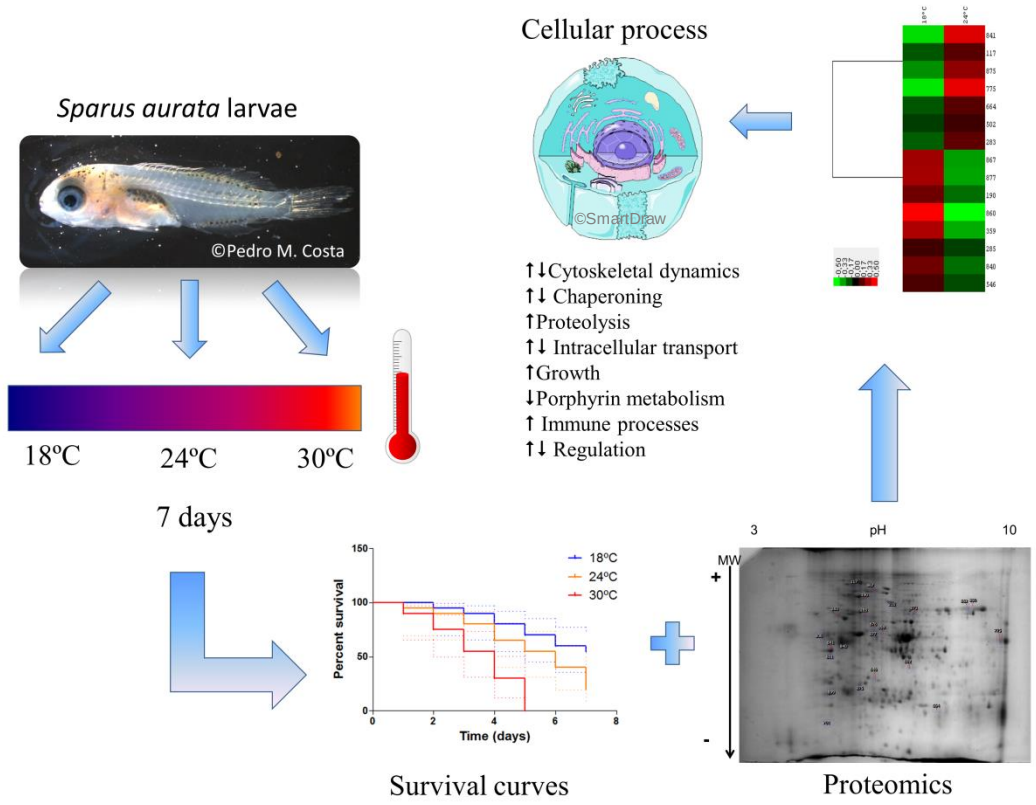
[1]Madeira D, Araújo JE, Vitorino R, Capelo JL, Vinagre C, Diniz MS (2016). Environmental Research 148, 164-176. DOI: 10.1016/j.envres.2016.03.030

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: 18ºC (control), 24ºC (warm) and 30ºC (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 30ºC. Therefore, proteomic analysis was only carried out for 18ºC and 24ºC. Proteomic analysis revealed that larvae up-regulated protein folding and degradation, cytoskeletal re-organization, transcriptional regulation and the growth hormone while 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 at the highest temperatures. 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.

Key words: temperature, larvae, *Sparus aurata*, proteome, climate change

Graphical abstract



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 will lead to an increase in global temperature (ranging from +1.8 to +4.0ºC), 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, 2015). 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 and adaptive capacity, generation time, dispersal, and reproductive output (e.g. Pörtner & 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 & Farrell, 2008; Pörtner & 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 & Farrell, 2008). As such, early life stages are highly vulnerable to ocean warming and potentially to a decrease in pH as well (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' (Pechenick, 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; Houde, 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 cells' 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 synthesis and signal transduction (e.g. López et al., 2002; Gardeström et al., 2007; Stillman et al., 2009; Tomanek and Zuzow, 2010; Tomanek, 2011; Fields et al., 2012).

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). We used an integrative approach connecting proteome changes with organism-level indicators to unravel both fitness and proteome changes 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 Ethical statement

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

2.2 Assessment of *S. aurata* thermal environments

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 to 60 days at 18ºC (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. Therefore, both 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 to 2015 for January until October and data from 2010-2014 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 Centre (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 2-3ºC increase by 2100 (Miranda et al., 2002).

2.3 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. 3.1a**; total length range of 10 to 15 mm) were obtained from a fish farm (MARESA, Spain) and transported to the laboratory in 10 L opaque plastic containers with constant aeration and stable temperature conditions (18.0±0.5ºC). The sample size was calculated considering that natural daily mortalities expected for larvae at 18ºC are around 20% at 30dph, 9.9% at 40dph and 4.9% at 50dph (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 (20ºC, high water quality) until they reach 0.1g (approximately at 60 days post-hatch). Afterwards, they are placed in other less controlled indoor tanks but keep being reared at 20ºC. When they reach 1g (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 11.0ºC in January to 26ºC in July/August. Maximum air temperatures can reach 33.0ºC and minimum air temperatures can reach approximately 6ºC.

The experimental system consisted of a re-circulating structure (total volume of 2,000 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, 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±0.5°C, salinity 35‰ and pH 8.00±0.01 (same conditions of the fish farm). Larvae were allowed to acclimate for 24h before the experiment trial (a longer acclimation period was not feasible because it would interfere with the objective of studying larval stages). During the acclimation and experimental trial larvae were fed under a regime of period feeding (every 6h) with *Artemia salina* metanauplii and 2 different grain-sized feeds (0.3-0.6 mm and 0.6-1.0 mm).

2.4 Experimental assays

Following the acclimation period in the experimental system, temperature was gradually increased during 2 days (6ºC.day^{-1}) until the experimental temperatures were reached (controls were kept at 18.0±0.5ºC; experimental temperatures 24.0±0.5ºC and 30.0±0.5ºC; n=2 tanks for each temperature, **Fig. 3.1b, c**). These temperature treatments were chosen to simulate control conditions (18ºC), present-day warm waters (summer in shallow nursery grounds – 24ºC) and future heat wave situations in nursery areas (30ºC). 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 8.00±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 hand-held 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.

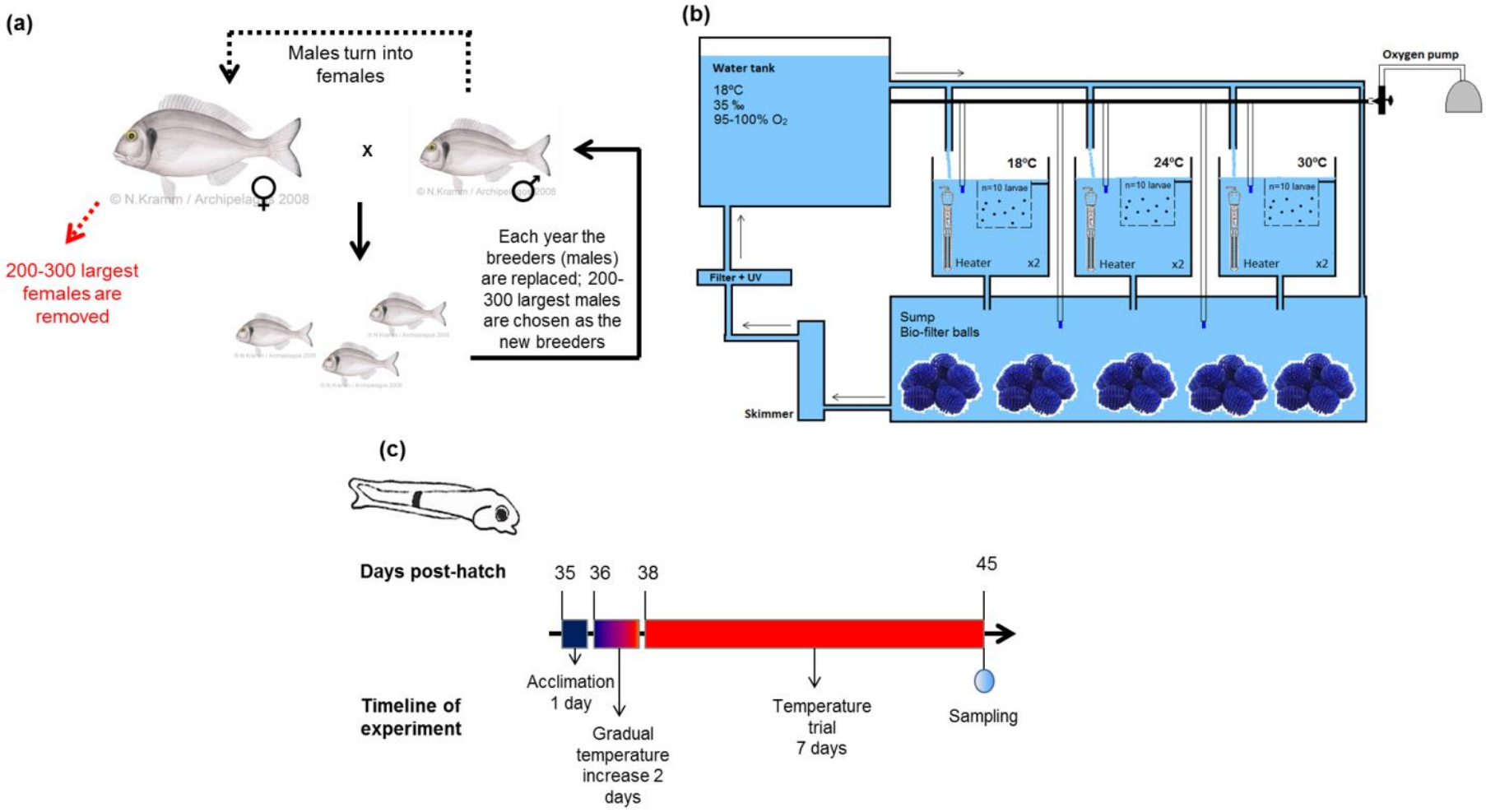

(a)
Males turn into females
x
200-300 largest females are removed
Each year the breeders (males) are replaced; 200-300 largest males are chosen as the new breeders
(b)
Water tank
18°C
35 ‰
95-100% O2
Oxygen pump
18°C
24°C
30°C
n=10 larvae
Heater
x2
Filter + UV
Sump
Bio-filter balls
Skimmer
(c)
Days post-hatch
35
36
38
45
Acclimation 1 day
Gradual temperature increase 2 days
Temperature trial 7 days
Sampling
Timeline of experiment

Figure 3.1 (a) Breeding scheme carried out in the hatchery (total breeding stock has about 400-600 animals). *Sparus aurata* is a protandric hermaphrodite species, maturing as male at about 2 years of age and turning into female at about 3 years of age. Therefore, the males that turn into females are replaced by new males (annual replacement; usually the 200-300 largest of one generation are chosen). Accordingly, the largest 200-300 females are removed. Drawings by N. Kramm retrieved from Archipelagos Wildlife Library; **(b)** Experimental setup (not to scale). Re-circulating system (total volume of 2,000L) consisting of six 70L white polyvinyl tanks (35 × 35 × 55 cm). The flow rate of clean water in each tank was 300 mL.min^{-1}. Larvae were randomly distributed in smaller plastic containers (17.5 × 17.5 × 15 cm, approximately 4.5 L) placed inside the 70L tanks with water flowing through small punctures. All the tanks were filled with clean and aerated sea water (95-100% O_2), with a constant temperature of 18.0±0.5°C (n=2 tanks), 24.0±0.5ºC (n=2 tanks) and 30.0±0.5ºC (n= 2 tanks). Salinity was kept at 35‰ and pH at 8.00±0.01. Experimental temperatures were maintained using thermostat heaters (TetraTec® HT 100, 100-150L, Tetra Werke, GmbH, Melle, Germany); **(c)** Timeline of experiment (larva drawing by D. Madeira).

2.5 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.

2.6 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 -80ºC until further analysis.

2.7 Proteomic analysis

2.7.1 Sample preparation

The homogenized and centrifuged samples (as previously described in section 2.3) 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 4ºC. 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 4ºC). 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).

2.7.2 Two Dimensional Gel electrophoresis (2-DE)

Samples containing 100 µg of protein were loaded onto IPG strips (pH 3-10, 7cm, 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 sulphate, 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.

2.7.3 Gel image analysis

Gel imaging was carried out with the PropicII-robot (Genomic Solutions™, Cambridgeshire, UK) and digitalized images of the gels were analysed 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.

2.7.4 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 de-stained twice with 50% acetonitrile/ 25 mM Ambic (ammonium bicarbonate) until the blue color disappeared and then, dehydrated with 100 % acetonitrile (ACN). 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 37ºC. 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 15min at 37ºC. 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 -20ºC until MS and MS/MS analyses.

2.7.5 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 (0.5µL) 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.

2.7.6 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.

2.7.7 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)

2.7.8 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 70kDa 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 90kDa alpha, POTE ankyrin domain family member F, actin alpha 1 skeletal muscle, kinesin heavy chain member 2A, IQ motif containing with AAA domain 1).

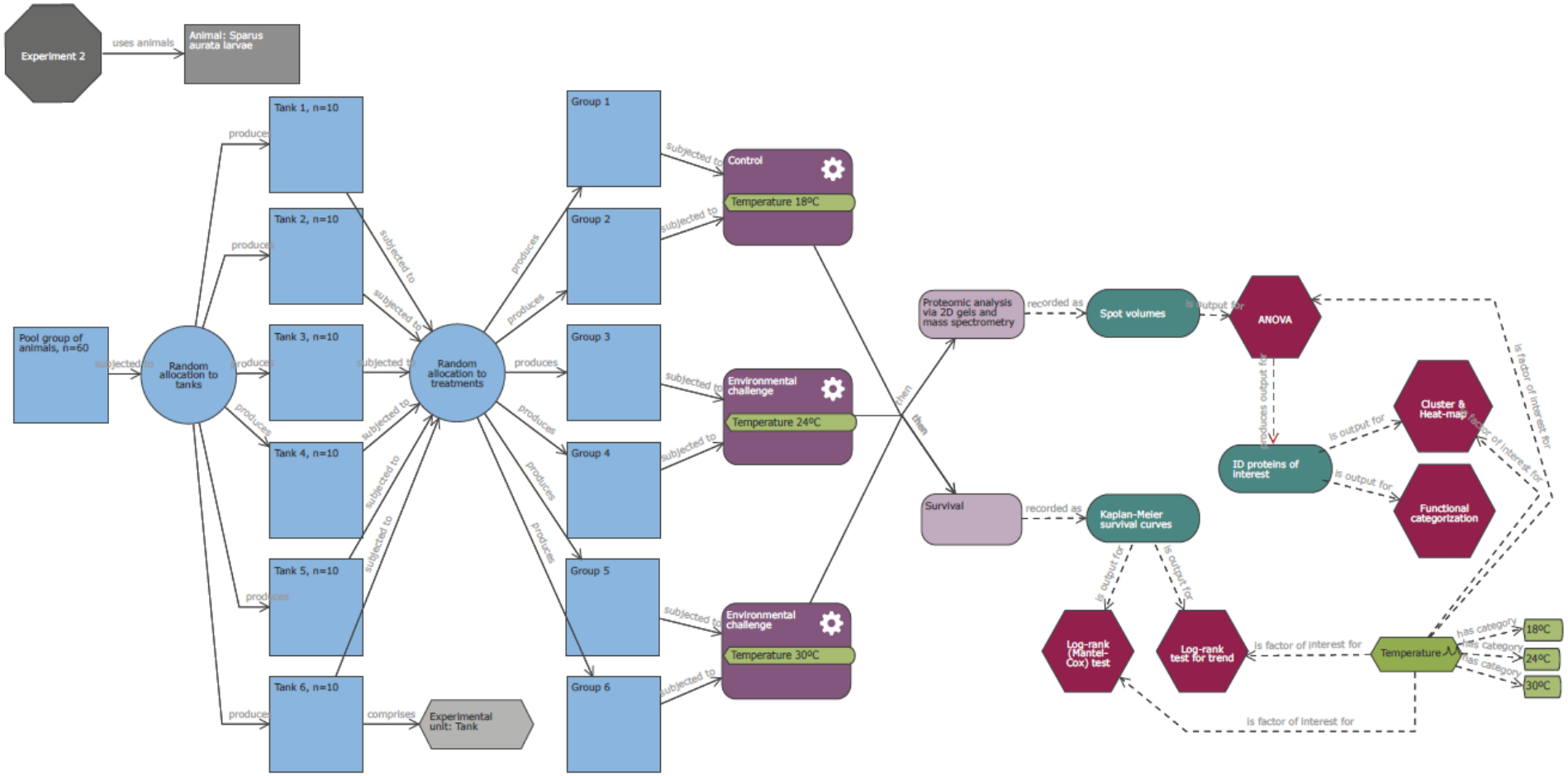


Figure 3.2 Experimental approach (diagram constructed using Experimental Design Assistant, https://eda.nc3rs.org.uk/).

3. Results

3.1 Temperature data

Coastal water temperatures were lowest in February (average of 13.9ºC, minimum of 12.2ºC and maximum of 15.2ºC) and highest in September (average of 19.3ºC, minimum of 17.2ºC and maximum of 21.8ºC) (**Fig. 3.3**). Considering the Tagus estuary, average temperatures were lowest in January (11.7ºC) and highest in July (23.6ºC) (**Fig. 3.3**). Current heat waves make estuarine waters reach 28ºC. Following the scenario of a 2-3ºC increase in Portuguese coastal waters by 2100 (Miranda et al., 2002), future heat waves will lead to coastal water temperatures in the range of 19-23ºC and estuarine waters in the range of 26-30ºC.

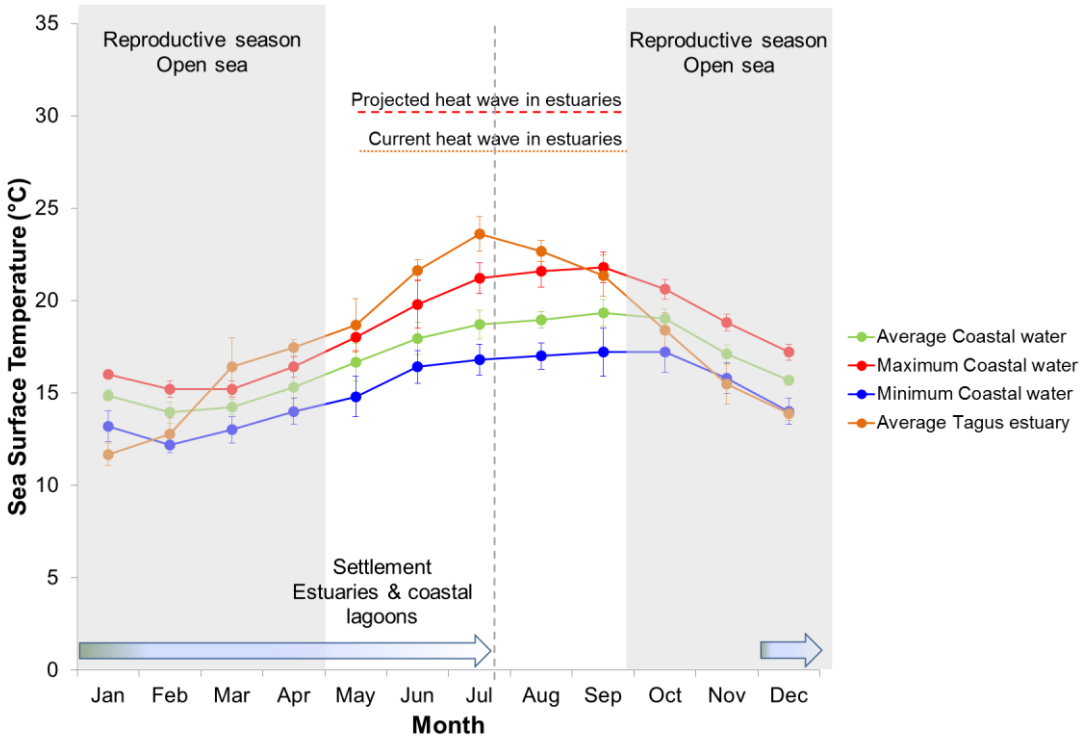


Figure 3.3 Current and predicted water temperatures in Portuguese coastal areas and Tagus estuary. Temperatures are in the range of 16-20ºC in coastal waters and 12-24ºC in the Tagus estuary. According to Miranda et al. (2002) Portuguese waters will undergo a 2-3ºC increase by 2100 leading to temperatures in the range of 19-23ºC in the coastal area and 26-30ºC in estuaries and coastal lagoons. Data are shown as mean±SD.

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 18ºC to 20% at 24ºC (Log-rank Mantel-Cox test; Chi square=4.129; df=1; p=0.04). At 30ºC, all larvae had died after 5 days of exposure (Log-rank Mantel-Cox test: 18ºC vs 30ºC - Chi square=20.92; df=1; p<0.0001; 24 vs 30ºC - Chi square=12.09, df=1, p<0.0005) (**Fig. 3.4**). As such, further analyses were only possible between 18ºC and 24ºC temperature groups.

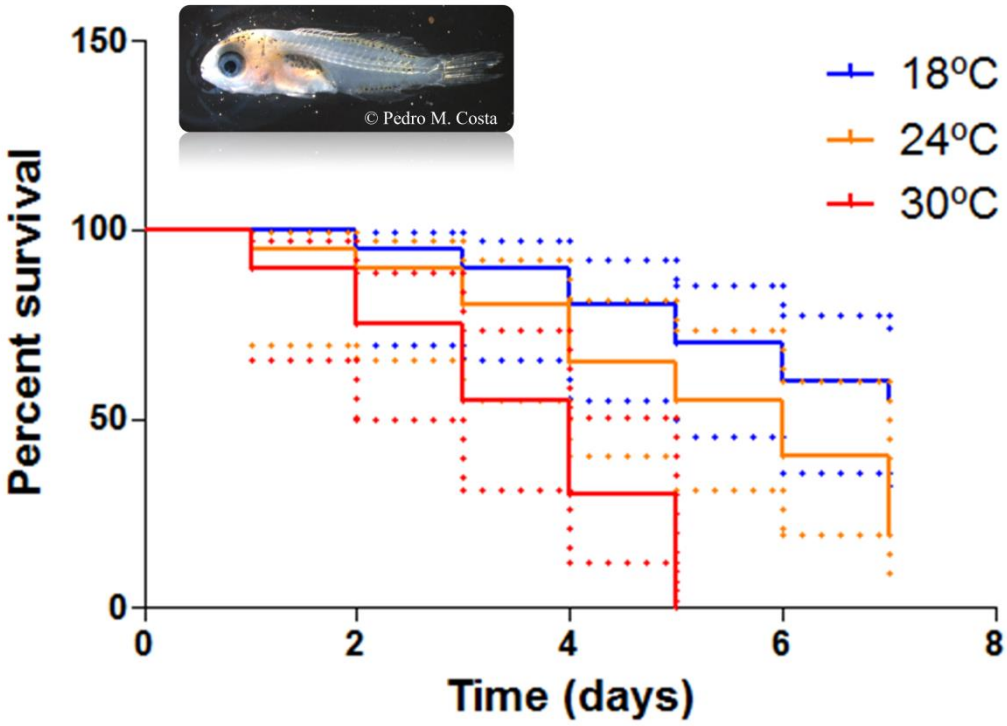


Figure 3.4 Survival curves (solid lines) and 95% confidence intervals (dotted lines) of *Sparus aurata* larvae exposed to 18ºC, 24ºC and 30ºC for a period of seven days. These curves were compared through the Log-rank test for trend (Chi square=18.64; df=1; p<0.001). Specific comparisons were carried out using Log-rank Mantel-Cox test (18ºC vs 24ºC - Chi square=4.129, df=1, p=0.04; 18ºC vs 30ºC - Chi square=20.92, df=1, p<0.0001; 24 vs 30ºC - Chi square=12.09, df=1, p<0.0005).

3.3 Proteomic analysis

Concerning proteomic analysis, all gels were automatically matched and compared to the master gel (**Fig. 3.5**) to detect differences in protein spots. The analysis of variance (p<0.05) showed that 23 protein spots were differentially expressed between 18ºC and 24ºC. Of the 23 spots, 15 (65%) were successfully identified (**Table 3.1, Fig. 3.5**), 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 24ºC (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. 3.6**).

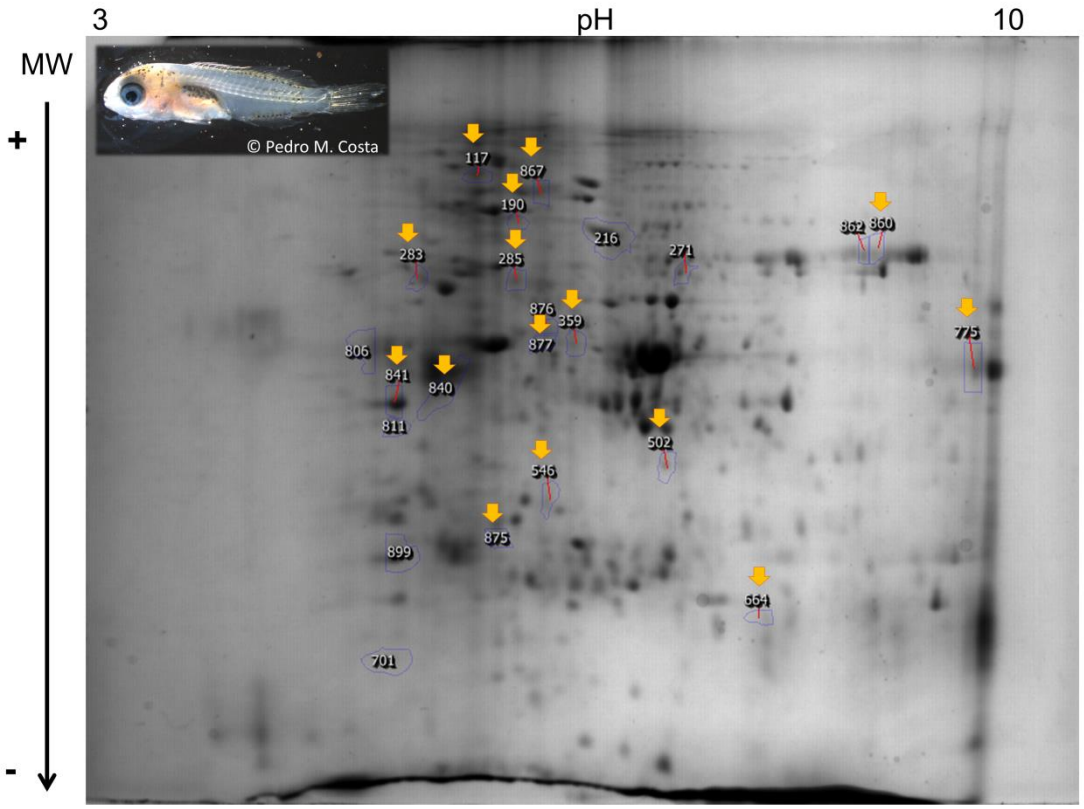


Figure 3.5 Image of the master gel depicting the 638 protein spots detected in *Sparus aurata larvae*. Annotated spots were those that were differentially expressed between temperature groups (n=4 larvae in each group, 2 technical replicates, ANOVA, $p<0.05$). Yellow arrows mark the spots that were successfully identified by mass spectrometry. Only 18ºC and 24ºC were compared because there was 100% mortality in larvae exposed to 30ºC for 7 days.

Table 3.1 Proteins differentially expressed between temperature groups (18ºC and 24ºC) in *Sparus aurata* larvae. Only 18ºC and 24ºC were compared because there was 100% mortality in larvae exposed to 30ºC. 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	42327.06	5.23	6	138	100
860	Kinesin heavy chain isoform	*Homo sapiens*	KIF5C_HUMAN	109427.15	5.86	18	65.9	97.85
502	Actin-related protein 2/3 complex subunit 2	*Homo sapiens*	ARPC2_HUMAN	34311.48	6.84	8	203	100
283	IQ and AAA domain-containing protein 1-like	*Rattus norvegicus*	IQCAL_RAT	95564.65	9.55	10	66.8	98.25
664	Myosin light chain 1, skeletal muscle isoform	*Liza ramada*	MLE1_LIZRA	20054.03	4.54	3	63.7	96.44
775	Somatotropin	*Prionace glauca*	SOMA_PRIGL	21057.59	6.66	9	64.6	97.10
867	Heme-binding protein 1	*Gallus gallus*	HEBP1_CHICK	21091.45	5.76	8	64.3	96.89
875	Proteasome subunit beta type-3	*Oncorhynchus mykiss*	PSB3_ONCMY	23005.41	5.05	7	177	100
359	POTE ankyrin domain family member F	*Homo sapiens*	POTEF_HUMAN	121366.69	5.83	18	134	100
877	Actin, muscle-type OS	*Molgula oculata*	ACT2_MOLOC	42235.95	5.12	11	189	100
190	Heat shock cognate 71	*Ictalurus punctatus*	HSP7C_ICTPU	71296.14	5.19	16	369	100

	kDa protein							
841	Tropomyosin alpha-1 chain	*Liza aurata*	TPM1_LIZAU	32709.66	4.69	26	483	100
117	Heat shock protein HSP 90-alpha	*Oryctolagus cuniculus*	HS90A_RABIT	79683.10	4.88	6	116	100
285	Tubulin alpha-4A chain	*Homo sapiens*	TBA4A_HUMAN	49892.37	4.95	14	76.9	99.83
840	Keratin, type II cytoskeletal 8	*Danio rerio*	K2C8_DANRE	57723.45	5.15	20	105	100

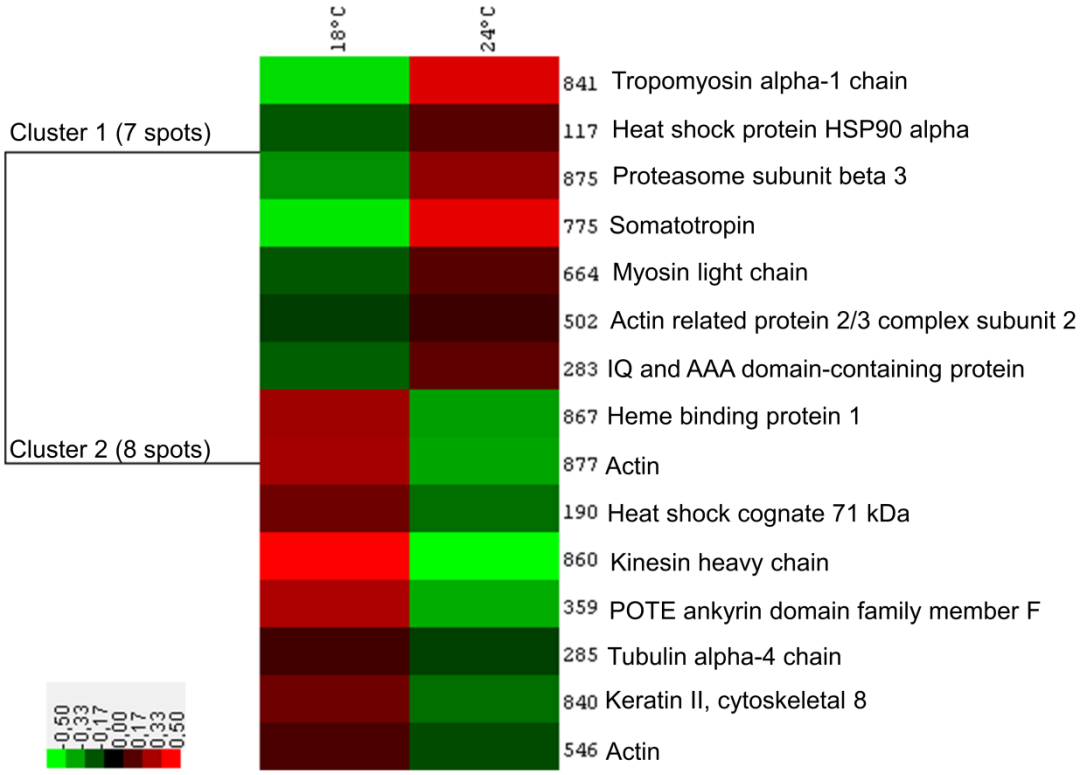


Figure 3.6 Protein expression in *Sparus aurata* larvae exposed to 18ºC and 24ºC for seven days. Only 18ºC and 24ºC were compared because there was 100% mortality in larvae exposed to 30ºC for seven days. Data is presented as a heat map with clusters to visualize protein Log2 expression values ranging from green (down-regulated) to red (up-regulated). 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 3.1 and supplementary Table S3.1 (annex 2).

Functional categorization analysis carried out in STRAP v1.5 (**Fig. 3.7**) 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. 3.7a**). 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. 3.7b**). 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. 3.7c**). 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 71kDa, heat shock protein 90kDa, 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) (**supplementary Table S3.2, annex 2**).

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 (**Fig. 3.8**).

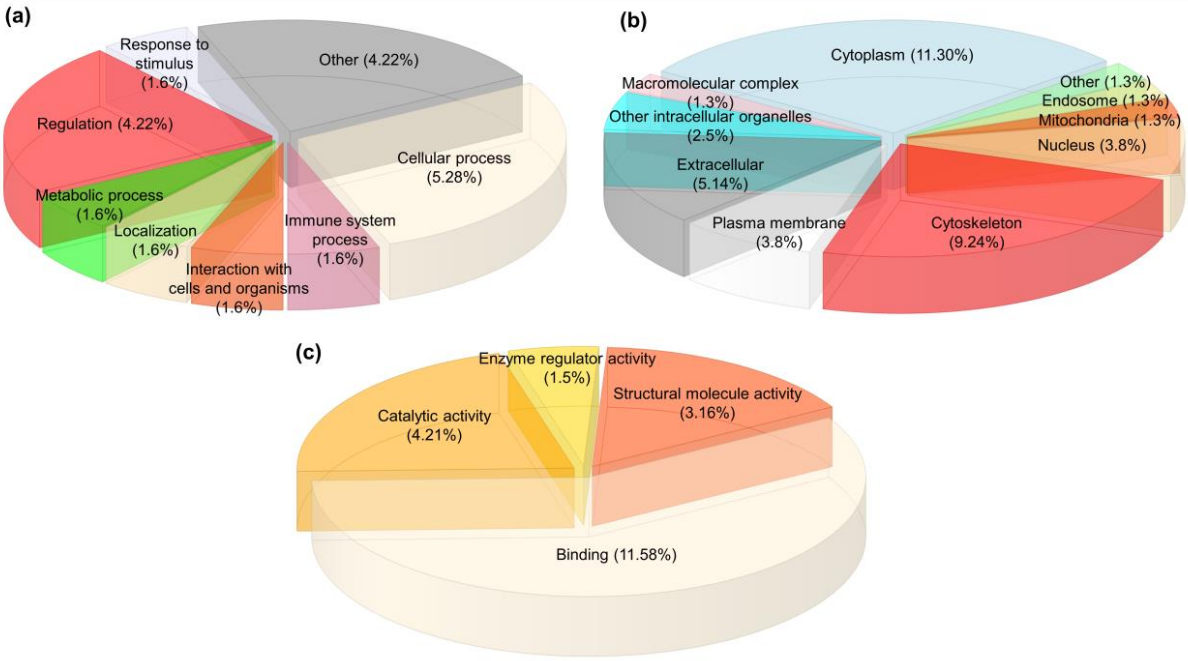


Figure 3.7 Distribution of identified proteins by functional classes according to STRAP v1.5, **(a)** biological process, **(b)** cellular component and **(c)** molecular function. Detailed functions are described in supplementary **Table S3.2** (annex 2).

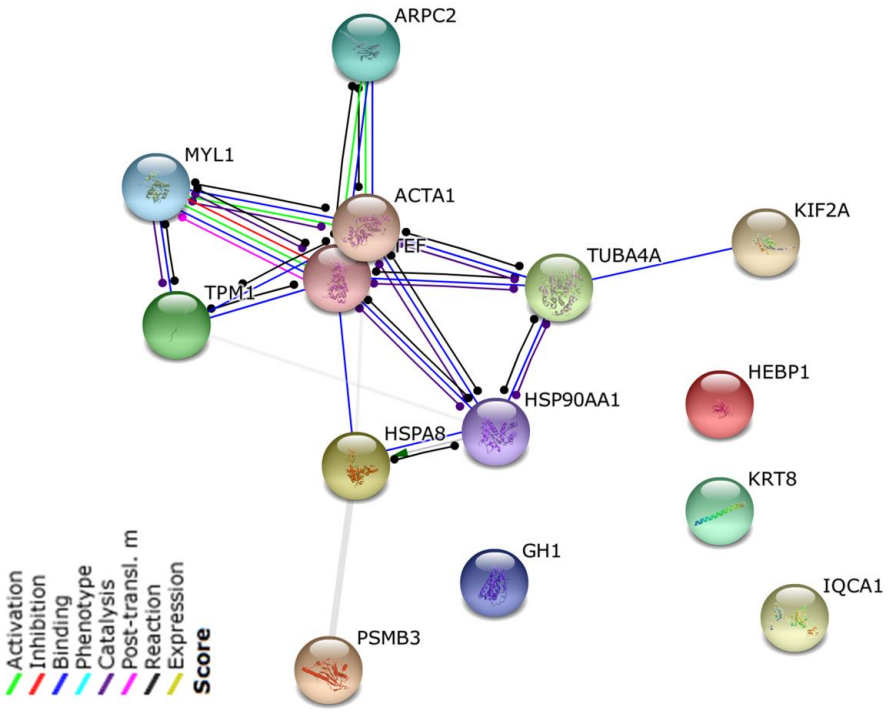


Figure 3.8 Functional protein association network retrieved from String (v10) using *Homo sapiens* as model species. This network is enriched in interactions (p-value=3.86e-4), mainly between heat shock proteins, cytoskeletal components and cargo transporting. HEBP1 – heme binding protein 1; PSMB3 – proteasome subunit beta type 3; HSPA8 – heat shock 70kDa protein 8; TUBA4A – tubulin alpha 4a; TPM1 – tropomyosin 1; KRT - keratin 8; ARPC2 – actin related protein 2/3 complex subunit 2; MYL1 – myosin light chain 1; GH1 – growth hormone 1; HSP90AA1 – heat shock protein 90kDa alpha; POTEF – POTE ankyrin domain family member F; ACTA1 – actin alpha 1 skeletal muscle; KIF2A – kinesin heavy chain member 2A; IQCA1 – IQ motif containing with AAA domain 1.

4. Discussion

Molecular plasticity is one of the most important mechanisms by which organisms can respond to and survive environmental 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 Dietz and Somero, 1992; Hofmann, 1999; Tomanek and Somero, 1999; Anestis et al., 2007; Pörtner, 2010; Schiffer et al., 2014). According to Aubin-Horth et al. (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 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 24ºC (summer average for estuaries) and 30ºC (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; Hofmann 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 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; Buckely 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; Gavrilova 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 S3.2**). 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 slowdown 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 in *S. aurata* larvae exposed to warming. 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 24ºC, 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 (Buckely 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 increases growth and affects 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 24ºC, 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 to 22ºC 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).

5. Conclusions

In this study, we used an integrative approach connecting proteome changes with organism-level indicators (see **Fig. 3.9**, summary figure). For the first time, 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 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 restocking 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 such 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.

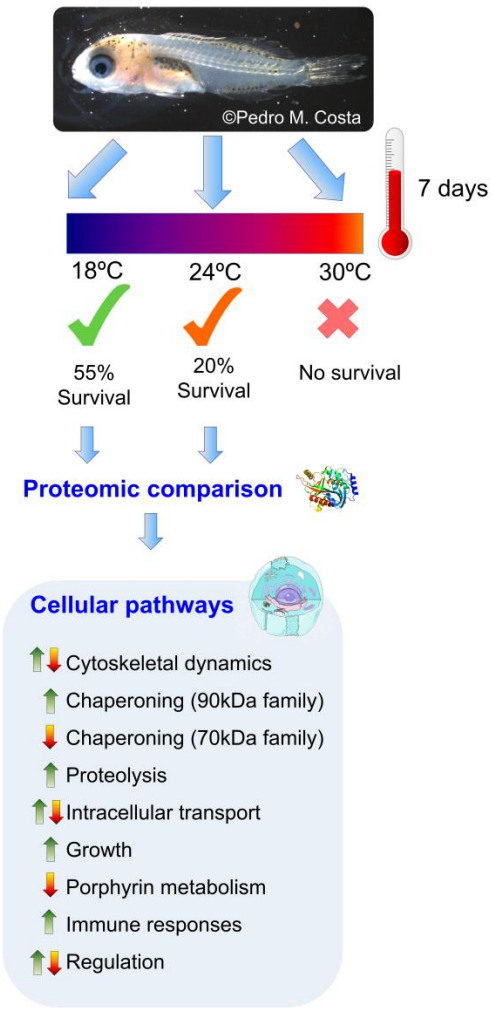


Figure 3.9 Summary of the study. *Sparus aurata* larvae exposed to warm temperatures (control 18ºC vs 24ºC and 30ºC) showed decreased survival, reaching 100% mortality at 30ºC. Proteomic comparison of whole larvae at 18ºC vs 24ºC showed that larvae modulated proteins involved in cytoskeleton dynamics (actin, actin related proteins, myosin, tropomyosin, tubulin, keratin), chaperoning (heat shock cognate 71kDa, heat shock protein 90kDa, 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.

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PTDC/MAR/119068/2010 and PTDC/AAG-REC/2139/2012; strategic project grants UID/Multi/04378/2013, UID/MAR/04292/2013 and UID/BIM/04501/2013]. The funding sources had no involvement in the study design, analysis, writing or publication of this manuscript. The authors have no conflicts of interest to declare.

7. 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.

Supplementary information (annex 2):

Table S3.1 Masses and sequences of peptides obtained for each spot.

Table S3.2 Detailed functional categorization of proteins. Information was retrieved from UniProt, GeneCards, neXtprot beta, InterPro and Qiagen.

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CHAPTER 4. HISTOPATHOLOGICAL ALTERATIONS, PHYSIOLOGICAL LIMITS AND MOLECULAR CHANGES OF JUVENILE *Sparus aurata* IN RESPONSE TO THERMAL STRESS[1]

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Abstract

Current concerns about climate change have led to a great body of literature on thermal tolerance. However, it is still difficult to understand and relate biological changes at different organizational levels. This is especially important in commercial species. We aimed to test the effects of temperature stress in juvenile gilthead seabream *Sparus aurata* in order to contribute to the understanding of the vulnerability of this species to temperature changes, heat waves, and potentially climate warming. Here we applied an integrative approach, considering indicators at the biochemical, cellular, and physiological levels. Firstly, the upper thermal limit was estimated via the critical thermal maximum (CTmax); secondly, levels of Hsp70 (reversible protein damage) and total ubiquitin (irreversible protein damage) were quantified in several tissues via ELISAs; and thirdly, histological analyses were performed to identify cellular structural damage due to temperature and how it correlates to biochemical alterations. Results showed that mean (±SD) CTmax was 35.5 ± 0.5°C. Absolute amounts of both Hsp70 and total ubiquitin varied significantly among organs, with gills having the highest amounts of both. Biomarker and histopathological results indicated that *S. aurata* might be particularly sensitive to water temperatures ≥28°C. At 30°C, *S. aurata* juveniles showed severe signs of stress, with increased biomarker levels in almost every organ tested and significant cellular damage (atrophy, inflammation, micro-hemorrhage, hyperemia, hyperplasia). Therefore, *S. aurata* may be vulnerable to heat wave events that currently make water temperature attain 28°C for several weeks (30°C by 2100 under a climate change scenario). Thus, this species might be vulnerable to a rise in sea temperature, and our research may be important from a management perspective, as *S. aurata* is a major commercial species.

Key words: Hsp70, ubiquitin, cellular alterations, CTmax, Sea bream, temperature

1. Introduction

Stressful conditions can act as evolutionary forces, leading to adaptive changes in populations (Sorensen et al. 2003). A variety of stressors (physical, chemical, biological) operate in aquatic ecosystems, and among them, temperature is one of the most important. All organisms have a thermal performance curve, meaning that when temperature reaches values close to the organism's upper thermal limits, performance decreases (see Huey & Stevenson 1979). Moreover, several studies defined the concept of oxygen-limited thermal tolerance, implying that the oxygen supply to organs is optimal between limits (lower and upper pejus temperatures; Portner 2001, 2010). Furthermore, organisms cannot survive (except for a limited period of time) at temperatures above or below the *pejus* temperatures (see Portner & Peck 2010 for a review).

Metabolic pathways and biochemical reactions are affected by temperature, determining an organism's physiological state and behavior. Therefore, activities such as foraging, reproduction, and growth will be altered and may change the fitness of the individuals (Mora & Ospina 2001, Hochachka & Somero 2002, Portner & Peck 2010) and community and ecosystem structure (e.g. Portner & Farrell 2008).

Within marine aquatic environments, estuaries and other confined coastal areas are amongst the most variable. These areas are economically important, providing food resources and serving as nursery areas for juveniles of many species (Haedrich 1983, Vinagre et al. 2010). Nevertheless, as estuaries are shallow water bodies, anthropogenic impacts (e.g. pollution, overfishing) and environmental change are greater than in deep sea or open-ocean sites. Additionally, significant variations in abiotic variables that follow seasonal or even tidal rhythms may be permanent sources of stress to residing biota. Hence, juvenile fish inhabiting these ecosystems may endure stressful conditions, including significant temperature variations (see Madeira et al. 2012a). Considering climate change scenarios for Portuguese waters (a 2°C increase in mean seawater temperature by 2100; Miranda et al. 2002), juveniles may undergo severe thermal stress which may compromise their growth (Vinagre et al. 2012a, 2013a,b). Madeira et al. (2012a) showed that sea bream juveniles inhabiting Portuguese estuaries may already be subjected to thermal stress under the present conditions. It is therefore important to study the molecular and biochemical mechanisms behind the thermal stress response in commercial species to determine whether they may be undergoing cellular and tissue damage. Biomarker quantification may be very useful to achieve this aim. A biomarker is defined as 'any measurement reflecting an inter action between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction' (World Health Organi zation 1993). Knowing and measuring these biomarkers may help us understand ecological patterns of response to increasing seawater temperature and, therefore, improve the management of commercial fish populations.

From this perspective, the model chosen for this study was the gilthead seabream *Sparus aurata*, which is a very important commercial species, both in fishing activities and

aquaculture. It is a euryhaline, warm-temperate species that occurs in coastal seagrass beds, on sandy bottoms, and in the surf zone, as well as in estuaries and coastal lagoons (Altimiras et al. 1994). It usually inhabits shallow waters, from 1 to 150 m deep (commonly 1 to 30 m). Juveniles tend to school in coastal lagoons and estuarine brackish waters (Craig et al. 2008), which are warmer than coastal waters. Its native distribution is subtropical and extends from the Mediterranean to the east Atlantic coast (including Cape Verde and the Canaries to the English Channel).

Amongst stress biomarkers, heat shock proteins (Hsps) have been the most widely studied, especially Hsp70 (Tomanek 2011). Temperature leads to proteotoxic stress and induces a heat shock response in several species (e.g. Somero 1995, Madeira et al. 2012b). Hsps have chaperoning activity and can be seen as molecular protection mechanisms with a selective value, contributing to the organism's and species' success across environmental gradients (Hofmann 2005). They confer stress tolerance (Li et al. 1995, Feder 1996, Basu et al. 2002, Sorte & Hofmann 2005) and are associated with an increase in the ability to temporarily survive temperature extremes (Feder & Hofmann 1999, Hofmann et al. 2002, Sorensen et al. 2003). Hsps are considered an indirect measure of protein damage and biochemical indicators of reversible protein unfolding in the cell (Hofmann 2005, Tomanek 2010, 2011). Another important biomarker, which complements the information provided by Hsps, is ubiquitin. Ubiquitination is critical to cell cycle regulation, transcription, apoptosis, protein sorting, and protein quality control, among other cell processes (Hanna et al. 2007). The ubiquitin metabolic pathway involves the selective tagging of proteins for proteasomal degradation (Hershko & Ciechanover 1992) and falls into 4 categories: (1) continuous turnover to rapidly modulate protein levels; (2) elimination of proteins in response to specific signals; (3) elimination of defective proteins that arise through physical or chemical factors and transcriptional/translational errors or gene mutations; and (4) the turnover of excess free forms of proteins (from multi subunit complexes; Finley 1991). Regarding these aspects, ubiquitin levels are considered a direct measure of protein loss (Ciechanover 1998), indicating irreversible protein damage in the cells (Hofmann & Somero 1995). Moreover, a relationship may exist between the heat shock and ubiquitin systems (Finley et al. 1984, Alberti et al. 2002). However, it is still unclear which features of damaged proteins mediate proteasome activity, whether they are eliminated through ubiquitination or recovered by Hsps (Finley 1991, Patterson & Hohfeld 2006). Nonetheless, substrate recognition and processing may require cooperation between these two systems (Patterson & Hohfeld 2006). During stress, accumulations of misfolded proteins (aggresomes) tend to form in the cells (Johnston et al. 1998, Kopito 2000). Interestingly, components of the ubiquitin system and chaperone system are present and seem to be actively recruited to these aggresomes (Garcia-Mata et al. 1999, Wyttenbach et al. 2000). Moreover, increased Hsp70 can reduce aggresome formation by increasing protein degradation (Dul et al. 2001). Thus, there seems to be a protein triage system within the cells, in which Hsps seem to play an important part. However, little information is available on these matters concerning marine species. All of these hypotheses were tested in

mammals or human cell lines, so further studies are needed using aquatic organisms as models.

Histopathological changes are also important indicators of health changes in species of aquatic and other ecosystems (e.g. Bignell et al. 2011, Costa et al. 2013a,b). Even though several studies have investigated histopathological changes induced by temperature, they mainly focused on freshwater fish (e.g. Day & Butler 2005, Sollid & Nilsson 2006). These changes included morphometric changes in the attributes of muscle tissue, changes in axon and fiber diameter and thickness, changes in the respiratory surface area, and changes in several tissues (gills, liver, kidney, and heart), such as hyperplasia, prominent vacuolization, increased melanin production, canaliculi formation, hypertrophy, and necrosis of cells.

The aims of this study were to test the effects of temperature stress in juvenile gilthead seabream *S. aurata* in order to contribute to the understanding of the vulnerability of this species to temperature changes, heat waves, and potentially climate warming. In detail, our aims were (1) to test the upper thermal limit, (2) to assess the biochemical thermal stress response, quantifying the levels of Hsp70 (reversible protein damage) and total ubiquitin (irreversible protein damage) in several tissues of this species (gills, intestine, muscle, brain, hepatopancreas−liver, and infiltrated pancreatic acini), (3) to identify any correlations between Hsp70 levels and total ubiquitin levels, and (4) to perform histological analysis of the samped tissues in order to identify cellular structural damage due to temperature and how it correlates to biochemical alterations.

2. Materials and methods

2.1 Ethical statement

This study was carried out in strict accordance with the recommendations of the Portuguese legislation for animal experimentation following the approval of Direcção Geral de Alimentação e Veterinária. Moreover, three of the authors have a level C certification by FELASA (Federation of European Laboratory Animal Science Associations). All efforts were made to minimize animal suffering.

2.2 Thermal tolerance method

Juvenile *Sparus aurata* (mean ± SD total length of 92.1±8.1 mm and weight of 12.2±2.9 g) were obtained from a fish farm (MARESA, Spain) and acclimated in a re-circulating system with 150 L aquaria containing clean, permanently aerated sea water, at the constant temperature of 18°C and a salinity of 35‰ (maintenance conditions in the fish farm). The dissolved O_2 level of the water varied between 95% and 100%. The fish were acclimated for one week prior to bioassays and were fed commercial pellets twice a day. They were starved for 24h before the experiments. The use of farmed organisms reduces potential confounding factors (i.e. thermal history). According to several authors, thermal history and parental effects

are determinant and induce irreversible changes in the thermal tolerance of the species (Cossins and Bowler 1987; Shaefer and Ryan 2006).

Thermal tolerance was determined using the dynamic method described in Mora and Ospina (2001). The parameter measured was the Critical Thermal Maximum (CTmax given in °C), which is defined as the "arithmetic mean of the collective thermal points at which the end-point is reached" (Mora and Ospina 2001). This end-point is defined as the loss of equilibrium. The CTmax method was chosen for this study because it has been the most used to determine upper thermal limits (Lutterschmidt and Hutchison 1997) and because dynamic trials are accurate predictors of the responses of organisms to natural conditions (Bennet and Judd 1992, Bennet and Beitinger 1997).

To determine the CTmax, the organisms were subjected to a thermostatized bath, using a heated/refrigerator circulator (MultiTemp III, Pharmacia Biotech). They were placed in five 40.5 L white plastic tanks (30 x 30 x 45cm) with lids to prevent evaporation (10-11 fish per tank). Each tank was equipped with an aeration system (**Fig. 4.1**). During the experiment, animals were exposed to a constant rate of water-temperature increase of 1°C.h^{-1}, and observed continuously, until they reached the end-point. Fish were collected every 2ºC, killed by cervical decapitation with the aid of a scalpel, and organs removed. Samples (brain, gills, intestine, hepatopancreas, muscle) from 6 individuals were taken and immediately frozen in liquid nitrogen or processed fresh for histological analyses (in this case, samples of 3 animals per sampling point, out of the total of 6). The total number of individuals was 54.

The temperature at which each animal reached its end-point was measured with a digital thermometer, registered and then CTmax (and its standard deviation) were calculated, through the following equation:

$$CTMax = \frac{\sum_1^n (Tend - point\ n)}{n}$$

Where $T_{end\text{-}point}$ is the temperature at which the end-point (loss of equilibrium) was reached for individual 1, individual 2, individual n, divided by the n individuals that were in the sample. The experiments were carried out in shaded day light (15 light; 09 dark). Total length and weight was measured at the end of each trial using an icthyometer and a scale, respectively.

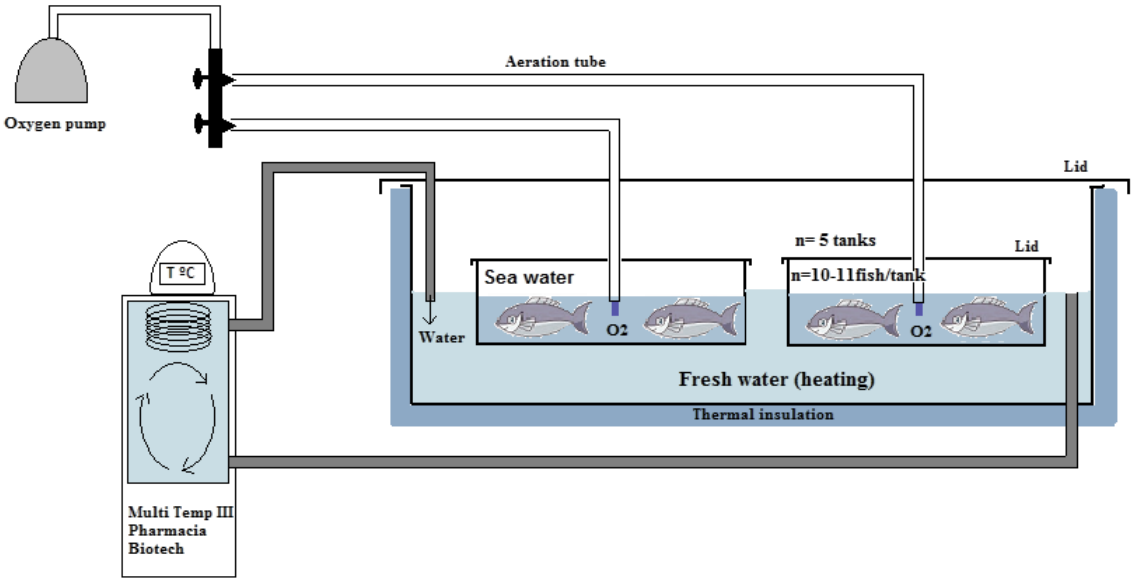

Figure 4.1 Experimental setup: the fish were randomly placed in 40.5 l white plastic tanks (n=5 tanks, 30 × 30 × 45cm with water from the home tank), which were transferred to a thermostatized bath, connected to a heated/refrigerator circulator (MultiTemp III, Pharmacia Biotech). Each tank had a lid to prevent evaporation and was equipped with an aeration system (10-11 fish.tank^{-1}). Note: not to scale. Constructed in Paint.

2.3 Protein extraction

Samples (approximately 200-250mg of gills, muscle, intestine, brain and hepatopancreas) were placed in 1.5 mL microtubes and homogenized in 1mL of phosphate buffered saline solution (0.14M NaCl, 0.003M KCl, 0.01M Na_2HPO_4, 0.002M KH_2PO_4, pH 7.4) to extract most soluble cytosolic proteins, using a glass/teflon Potter Elvejhem tissue grinder, in ice-cold conditions. The crude homogenates were then centrifuged for 15 min at 10000 × g. Afterwards, the supernatant was collected, transferred to new microtubes (1.5 mL) and frozen immediately (-80ºC) until further biochemical analyses.

2.4 Hsp70 quantification

Heat Shock Protein 70 was quantified using an indirect Enzyme Linked Immunosorbent Assay (ELISA) (Njemini et al. 2005) with 96-well microplates (Greiner, Germany). Either ELISA or western blot can be successfully employed in Hsp quantification (Brun et al. 2008). Samples were diluted to 1:100 in 0.05M carbonate-bicarbonate buffer (Sigma-Aldrich, USA). This dilution was chosen to give optimum signal and was achieved after previous optimization.

Three replicates of 50 µL were taken from each diluted sample, transferred to the microplate wells and incubated overnight at 4ºC. The microplate was washed 3 times in PBS 0.05% Tween-20 and then blocked by adding 200 µL of 1% BSA (%w/v) (Bovine Serum Albumin, Sigma-Aldrich, USA) in PBS. The microplate was incubated at 37°C for 90 min. After microplate washing, the primary antibody (anti-Hsp70/Hsc70, Acris, USA), diluted to 0.5µg.mL^{-1} in 1% BSA in PBS, was added to the microplate wells (50µL each). Then the microplates were

incubated for 90 min at 37°C. After another washing stage, the secondary antibody (anti-mouse IgG, fab specific, alkaline phosphatase conjugate, Sigma-Aldrich, USA) was diluted (1µg.mL^{-1} in 1% BSA in PBS) and added (50 µL) to each well followed by incubation at 37°C for 90 min. After the washing stage, 100µL of substrate (SIGMA *FAST*^{TM} p-Nitrophenyl Phosphate Tablets, Sigma-Aldrich, USA) was added to each well and incubated for 30 min at room temperature. Fifty µL of stop solution (3N NaOH) was added to each well and the absorbance was read in a 96-well microplate reader at 405nm (BIO-RAD, Benchmark, USA). For quantification purposes, a calibration curve was constructed using serial dilutions of purified Hsp70 active protein (Acris, USA) to give a range from 0 µg.mL^{-1} to 2 µg.mL^{-1}.

2.5 Ubiquitin and ubiquitinated/poly-ubiquitinated protein quantification

Quantification was achieved through a direct Enzyme Linked Immunosorbent Assay (ELISA) with 96-well microplates (Greiner, Germany). After previous dilution optimization, samples were diluted to 1:100 in 0.05M carbonate-bicarbonate buffer (Sigma-Aldrich, USA) to give optimum signal.

Three replicates of 50 µL were taken from each diluted sample, transferred to the microplate wells and incubated overnight at 4ºC. The microplate was washed 3 times in PBS 0.05% Tween-20 and then blocked by adding 200 µL of 1% BSA in PBS (Bovine Serum Albumin, Sigma-Aldrich, USA). The microplate was incubated at 37°C for 90min. After microplate washing, the primary antibody (Ub P4D1, sc-8017, HRP conjugate, Santa Cruz, USA), diluted to 0.5µg.mL^{-1} in 1% BSA in PBS, was added to the microplate wells (50µL each). Then the microplates were incubated for 90 min at 37°C. After another washing stage, a 100 µL of substrate (TMB/E, Temecula California, Merck Millipore) was added to each well and incubated for 30 min at room temperature. One hundred µL of stop solution (1 N HCl) was added to each well and the absorbance was read in a 96-well microplate reader at 415nm (BIO-RAD, Benchmark, USA). For quantification purposes, a calibration curve was constructed using serial dilutions of purified ubiquitin (UbpBio, E-1100, USA) to give a range from 0 µg.mL^{-1} to 2 µg.mL^{-1}.

For normalization purposes, the Bradford Assay was used to quantify the total amount of protein in each sample (Bradford, 1976). The analysis was carried out in 96-well microplates (Nunc-Roskilde, Denmark) by adding 200 µL of Bradford reagent in each well and 10 µL of each sample or standards. After 10 minutes of reaction, the absorbance was read at 595 nm in a microplate reader (BIO-RAD, Benchmark, USA). A calibration curve was constructed using BSA standards (0-10 µg.mL^{-1}). The results for Hsp70 and ubiquitin conjugates were divided by the total amount of protein in order to give the final data values.

2.6 Histological analysis

The procedure followed essentially Martoja and Martoja-Pierson (1967) for light microscopy. In brief, freshly-dissected samples from 3 individuals per temperature group (of

gills, muscle, intestine, hepatopancreas), were fixed in Bouin's solution (10% v/v formalin and 7% v/v acetic acid with Picric Acid added to saturation) for 48h at room temperature. The samples were dehydrated in a progressive series of ethanol and embedded in liquid paraffin (Panreac, Spain) overnight at 60ºC. Sectioning was done with a Jung RM2035 model rotary microtome. Sections of 5µm thick were stained with Harris' alum haematoxylin (Riedel de-Haën, Germany) and counterstained with alcoholic eosin Y (Panreac, Spain) for structural analysis. The slides were mounted with DPX resinous media (VWR International, USA). Microscopy analysis was performed with a DMLB model optical microscope equipped with a DFC 480 digital camera, all from Leica Microsystems (Germany).

2.7 Statistical analysis

Failure to comply with the assumptions of parametric ANOVA (normality of the data and homoscedasticity) led to the application of non-parametric statistics. The Kruskal-Wallis by ranks and multiple comparisons test was used to compare Hsp70 and ubiquitin levels between different temperature groups and different organs. Correlation analyses (Spearman R, since the data did not follow bivariate normality) were performed for Hsp70 and ubiquitin levels in each tissue. A significance level of $\alpha=0.05$ was set for all statistics. Statistics were carried out using the software Statistica (Version 10 StatSoft Inc, USA).

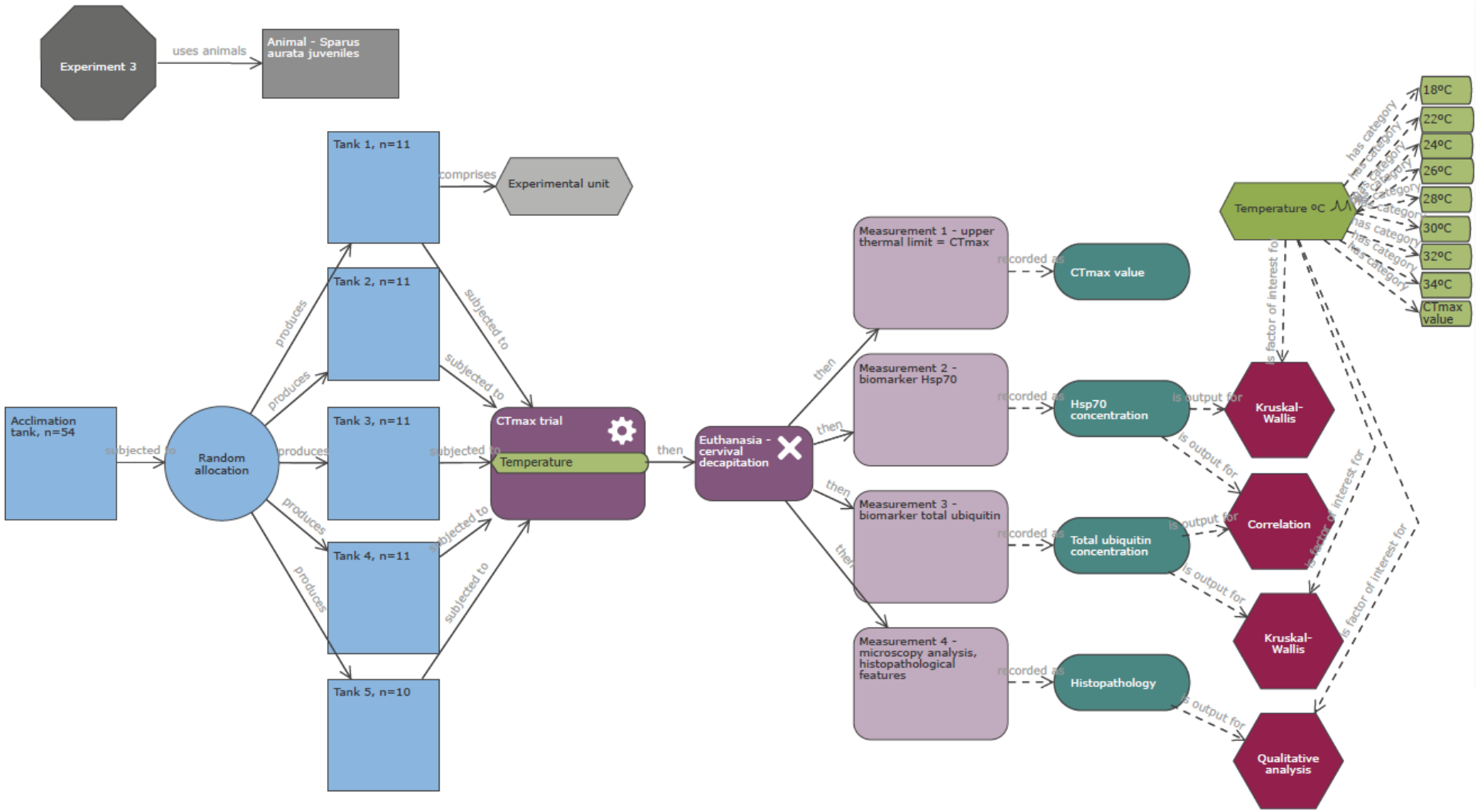

Figure 4.2 Experimental approach (diagram constructed using Experimental Design Assistant, https://eda.nc3rs.org.uk/).

3. Results

3.1 CTmax and Hsp70

Following **Eq. (1)**, *Sparus aurata* juveniles had a mean ± SD CTmax of 35.5 ± 0.5°C. The Kruskal-Wallis results showed significant differences in Hsp70 levels among temperature groups in every tissue tested. For easier interpretation, **Fig. 4.3a** only shows significant differences to the control group (18°C). The results showed significant increases from control at temperatures ≥30°C, namely in the brain (1.4-fold, peak at 30°C), gills (2.1-fold, peak at 34°C), muscle (2.2-fold, peak at 34°C), and intestine (3.1-fold, peak at 36°C). These results show that during increasing temperatures, changes in the brain are observed sooner than in the rest of the organs. Gills expressed the highest absolute amount of Hsp70 when compared to other tissues ($p < 0.001$). Values of Hsp70 in the gills were followed by those measured in the brain and muscle (these 2 showed equal amounts of Hsp70 but higher amounts in relation to other tissues; $p < 0.001$). Hepatopancreas and intestine showed equivalent amounts of Hsp70, but these levels were lower compared to other tissues ($p < 0.001$). Intraspecific variability in Hsp70 was elevated in all tissues.

3.2 Total ubiquitin

The Kruskal-Wallis results showed significant differences in total ubiquitin levels between temperature groups in all tissues tested except intestine and brain. For easier interpretation, **Fig. 4.3b** only shows significant differences to the control group (18°C). The results showed significant increases from controls in muscle (+2.9 fold, peak at 32°C) and gills (+4.1-fold, peak at 34°C), indicating that muscle responds faster than gills. When comparing total ubiquitin amounts in different tissues, gills, brain, and muscle had equivalent levels to each other, but had significantly higher levels than hepatopancreas and intestine ($p < 0.001$). Hepatopancreas and intestine showed significant differences, with intestine having higher amounts of total ubiquitin ($p < 0.01$). Intraspecific variability in total ubiquitin was also elevated in all tissues.

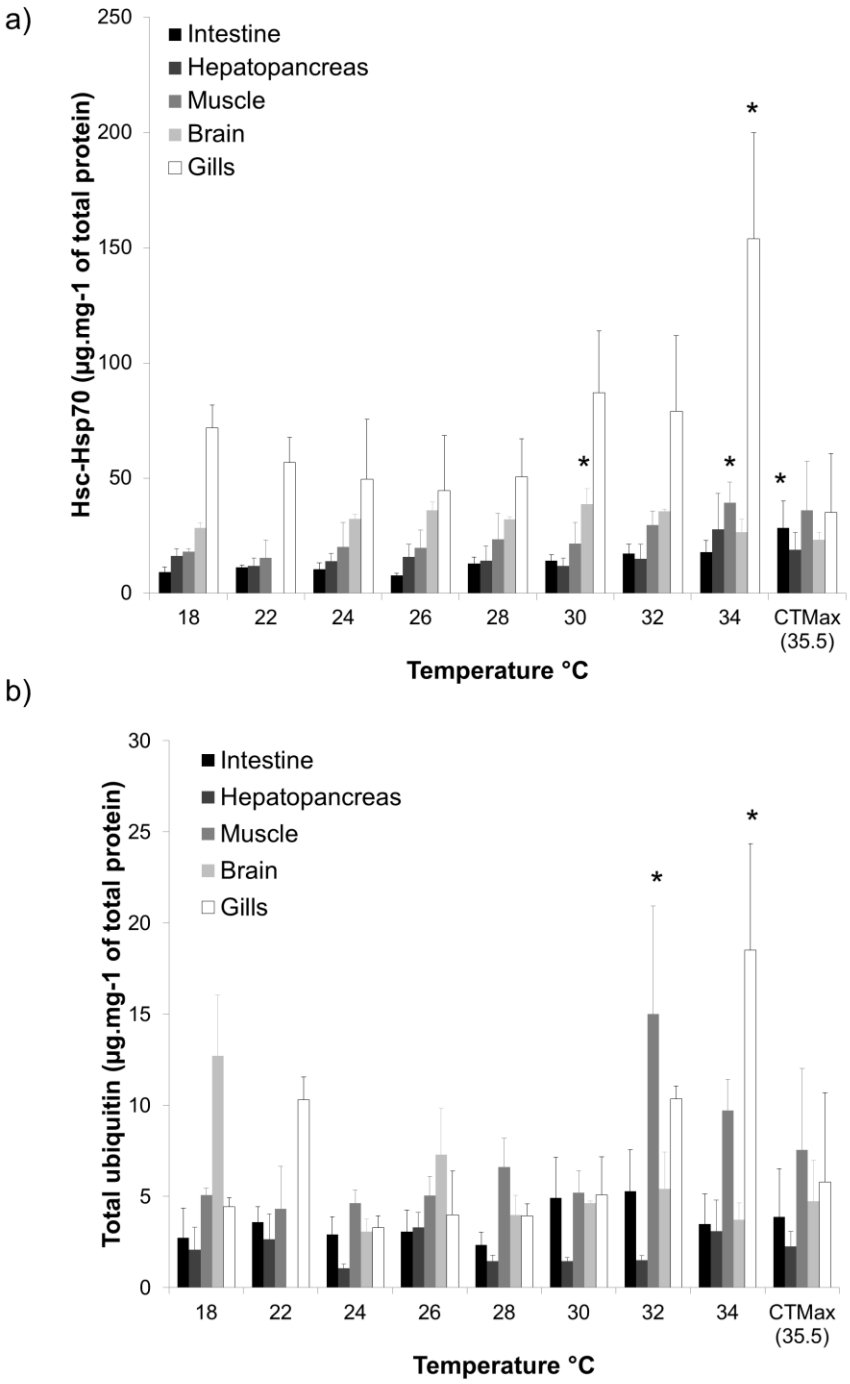

Figure 4.3 a) Levels of Hsp70 (Hsc+Hsp70) in juveniles of *Sparus aurata* along a temperature gradient ranging from the control temperature (18ºC) until the Critical Thermal Maximum (CTmax). Six individuals were sampled at each temperature point except 22ºC in brain). Results are shown as mean + SD. Groups with Hsp70 levels significantly different from control are marked with an asterisk ($p<0.05$). Hsp70 levels differ significantly between tissues. Gills expressed the highest levels ($p<0.001$), followed by levels in brain and muscle, which showed equivalent levels. Hepatopancreas (liver and infiltrating pancreatic acini) and intestine showed equivalent amounts of Hsp70 but these levels were lower when compared to other tissues ($p<0.001$). **b)** Levels of ubiquitin conjugates in juvenile *Sparus aurata* along a temperature gradient ranging from the control temperature (18ºC) until the Critical Thermal Maximum (CTmax). Six individuals were sampled at each temperature point (except 22ºC in brain). Results are shown as mean + SD. Groups with ubiquitin conjugates levels significantly different from control are marked with an asterisk ($p<0.05$). Ubiquitin conjugates levels differ significantly between some of the tissues. Gills, brain and muscle showed

equivalent levels of ubiquitinated conjugates, but showed significant differences from hepatopancreas and intestine ($p<0.001$). Hepatopancreas (liver and infiltrated pancreatic acini) and intestine showed significant differences, intestine having higher amounts of ubiquitin conjugates ($p<0.01$).

3.3 Correlation between Hsp70 and total ubiquitin

Spearman R correlations showed that Hsp70 and total ubiquitin were significantly correlated in gills (Spearman $R = 0.43$, $p < 0.005$), hepatopancreas (Spearman $R = 0.44$, $p < 0.002$), and muscle (Spearman $R = 0.67$, $p < 0.000$). Brain ($p > 0.61$) and intestine ($p > 0.18$) did not show a significant correlation between these 2 biomarkers.

3.4 Histological observations

Gills. Lesions in this organ started to occur at 28−30°C. The normal microanatomy of gills (i.e. in control individuals) consisted of lamellae attached to gill filament. Lamellae were covered by a single layer of squamous epithelial cells (1 cell thick) jointed by pillar cells between which were located the lamellar capillaries, with few erythrocytes (**Fig. 4.4a**). Goblet (mucus-secreting) and chloride cells (presenting a dense cytoplasm and a visible crypt) were mostly (but not exclusively) found in the interlamellar space (usually 1 or 2 cells per interlamellar space). At low temperatures (20−22°C), some occasional and localized alterations were visible, such as epithelial hyperplasia (especially in the interlamellar space), occasional hyperemia in the lamellar capillaries, and localized epithelial lifting from lamellae. From 28°C to CTmax, alterations to lamellar structure and epithelia became more frequent and diffuse. Between 28 and 30°C, the epithelial cells showed some degree of hypertrophy, and a few chloride cells became vacuolated (hypertrophied) in aspect due to fluid retention (see e.g. Costa et al. 2010; **Fig. 4.4b**). Compared to preceding conditions, epithelial lifting also became more disseminated (**Fig. 4.4d**). At 32−34°C, the aforementioned alterations were also conspicuous; however, at CTmax, deformation of lamellae (convoluted lamellae; **Fig. 4.4e**) was common, as was focal or partial lamellar fusion due to hyperplastic events (**Fig. 4.4c**) and some epithelial desquamation (shedding of squamous epithelial cells). Despite significant interlamellar epithelial hyperplasia, rod-shaped filaments did not occur.

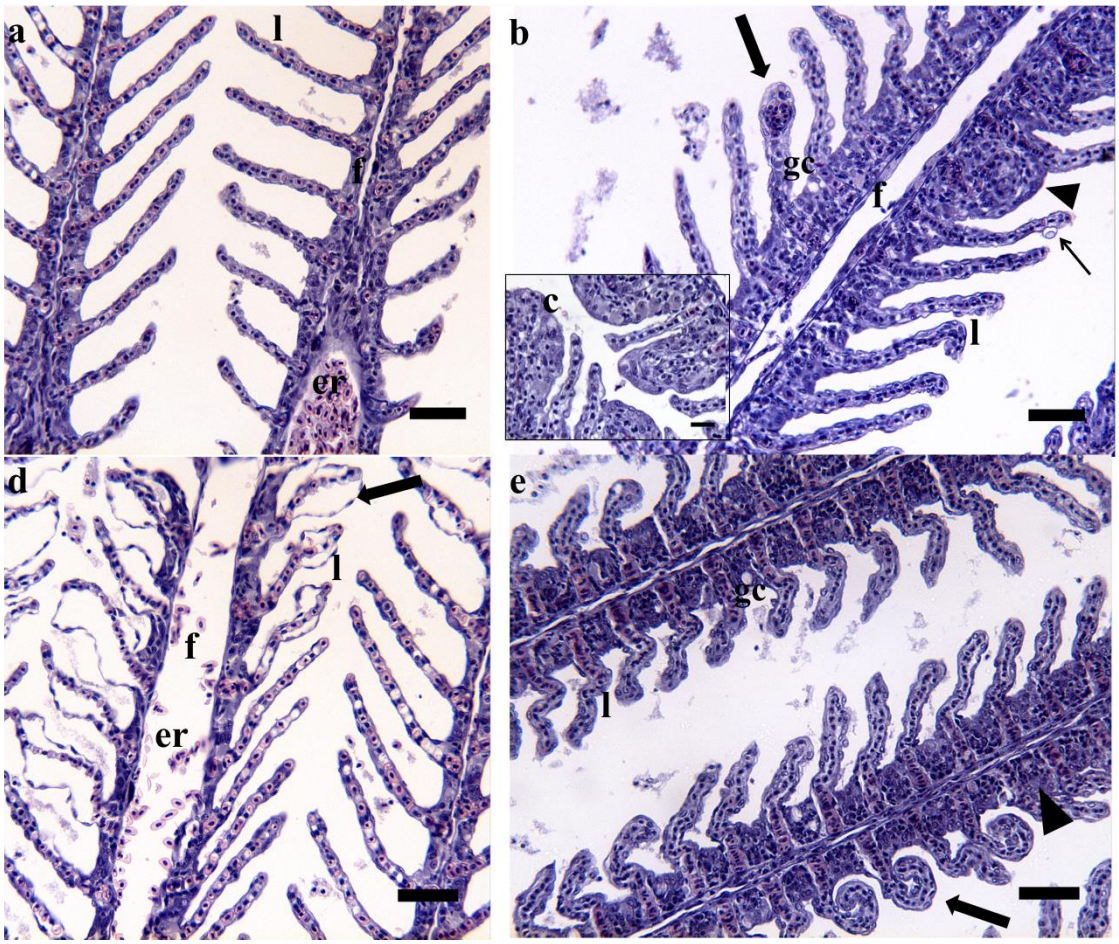

Figure 4.4 Representative micrographs of the gills of juvenile *Sparus aurata* exposed to control or high temperature. a) Normal structure of the gills at control temperature (18ºC)., b) Gill filament showing a lamellar edema (thick arrow), hyperplasia (arrow head) and hypertrophied chloride cell (thin arrow) (32ºC)., c) Detail of several hyperplasic foci (34ºC)., d) Epithelial lifting of lamellae (34ºC)., e) Convoluted lamellae (arrow) at the Critical Thermal Maximum temperature (35.5ºC) and moderate hyperplasia in the interlamellar space (arrow head). f – filament, l – lamellae, er – erythrocyte, gc – goblet cell. Scale bars: (a, b, d, e) 25µm; (c) 15µm.

Muscle. The normal microanatomy of muscular fibers (as observed in control animals) consisted of polynucleated striated myocytes (rhabdomyocytes) grouped into fascicles surrounded by a sheath of connective tissue, between which blood vessels were common (**Fig. 4.5a**). Overall, gross lesions were either reduced or absent in animals exposed to elevated temperatures; however, at 34°C and CTmax, a few muscle bundles showed atrophy of the fibers, probably caused by focal necrosis or autophagy (**Fig. 4.5b**).

Figure 4.5 Representative micrographs of the muscle of juvenile *Sparus aurata* exposed to control or high temperature. a) Normal structure of skeletal muscle at control temperature 18ºC with striated myocytes (Sm) grouped into fascicles (fc) and surrounded by a sheath of connective tissue. Blood vessels (bv) are evident between fascicles and adjacent to the tegument (T), in which melanin is visible (ml)., b) Muscle at the Critical Thermal Maximum temperature (35.5ºC) showing localized atrophy of muscle fibers probably caused by focal necrosis or autophagy. Scale bars: 25µm.

Liver. The normal structure of liver tissue, observed in control animals, consisted of parenchymal tissue composed of polygonal hepatocytes with 1 or 2 round nuclei holding conspicuous nucleoli (**Fig. 4.6a**). Sinusoids were visible in the parenchyma, as well as intrusion of pancreatic tissue (thus forming the hepatopancreas). Small fat vacuoles or microvesicles were common features in control organisms. At 24°C, a higher number of defense cells were visible inside blood vessels, albeit without significant intrusion into the tissue. At 26°C, extensive areas of vacuolization (**Fig. 4.6b**) were prominent, and at 28°C, both hyperemia and melanomacrophage aggregates were visible (**Fig. 4.6c**) inside sinusoids and infiltrating the adjacent tissue. When the temperature reached 30°C and 32°C, vacuolization became more diffuse, and small necrotic foci appeared in the parenchyma, typically involving hyperemia, micro-hemorrhage, and macrophage infiltration (**Fig. 4.6d**), thus indicating an inflammatory response to the lesions. At 34°C, the alterations were similar to those that occurred at 30−32°C, but hepatocyte degeneration became apparent (involving altered shape and differential cytoplasm staining, probably indicative of a change in function; **Fig. 4.6e**). When organisms reached their CTmax, there was a boost in hyperemia, and de generate hepatocytes were clearly more eosinophilic, likely indicating an early stage of hepatocellular alteration (**Fig. 4.6f**).

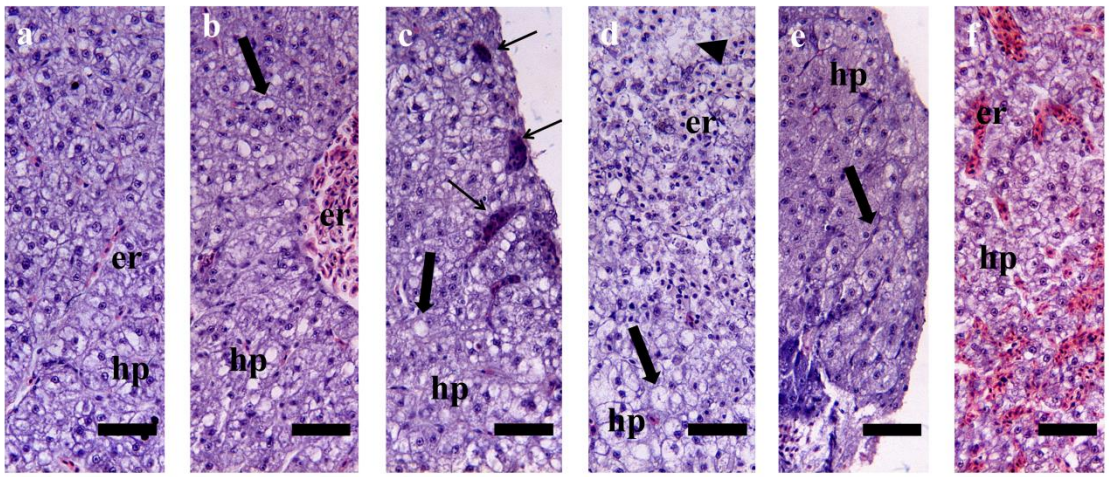

Figure 4.6 Representative micrographs of the liver of juvenile *Sparus aurata* exposed to control or high temperature. a) Normal structure of the liver at control temperature (18ºC) consisting of parenchymal tissue constituted by polygonal hepatocytes (hp) with round nuclei (one or two) and conspicuous nucleoli. Sinusoids with few erythrocytes (er) were visible in the parenchyma., b) Fat vacuoles (arrow) and slight hyperemia in hepatic vessels (26ºC), c) Fat vacuoles (thick arrow), hyperemia and melanomacrophage aggregates (thin arrows) (28ºC), d) Fat vacuoles (thick arrow), small necrotic foci in the parenchyma (arrow head) involving hyperemia, micro-hemorrhage and melanomacrophage infiltration (30ºC), e) Moderate hepatocellular alteration (arrow) and melanomacrophage infiltration (34ºC). Note the alteration to hepatocyte size and shape. f) Increased hyperemia and hemorrhage, with hepatocytes clearly more eosinophilic (at the Critical Thermal Maximum temperature of 35.5ºC) . Scale bars: 25µm.

Pancreatic acini. Control animals (18°C) revealed normal micro anatomy of pancreatic acini, consisting of highly basophilic (with zymogen granules) cells arranged concentrically in clusters (**Fig. 4.7a**). Pancreatic tissue infiltrated the hepatic parenchyma, forming the hepatopancreas typical of this species and other teleosts. Overall, this tissue yielded lesions that developed in a gradual mode as temperature in - creased, similar to the liver. Occasional alterations started to occur at 24°C, namely moderate hyperemia and macrophage infiltration, which may indicate the beginning of an inflammation process (**Fig. 4.7b**). At 28°C, vacuolization of acinar cells became evident, accompanied by hyperemia (**Fig. 4.7c**). At temperatures around 30−32°C, agglomerates of lipofuscin like pigments were visible inside pancreatocytes and nearby melanomacrophages (**Fig. 4.7d**). At 34°C and CTmax, the acini were atrophied, showing diffuse inflammation and necrosis (**Fig. 4.7e,f**).

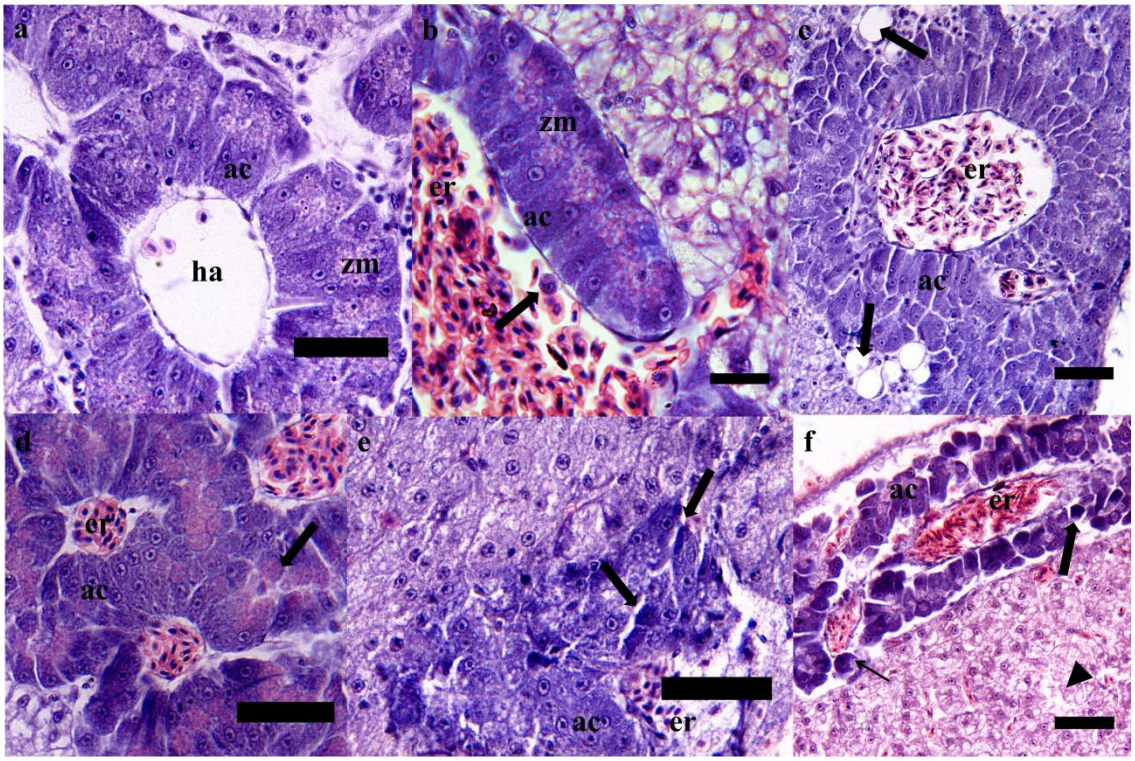

Figure 4.7 Representative micrographs of the hepatopancreas of juvenile *Sparus aurata* exposed to control or high temperature. a) Normal structure of the pancreas at control temperature (18ºC) consisting of acini composed of highly basophilic acinar cells (ac) (with zymogen granules, zm) arranged concentrically in clusters. Hepatic arterioles (ha) are visible holding few erythrocytes., b) Detail of pancreas at 24-26ºC showing some hyperemia (erythrocytes – er) and leucocytes (arrow)., c) Vacuolization of acinar cells accompanied by hyperemia (28ºC)., d) Detail of lipofuscin-like pigments (arrow) accumulated in acinar cells (30-32ºC) e) Pancreatic tissue at 34ºC with atrophied acini, showing diffuse inflammation and necrosis (arrows)., f) Macrophage infiltration (thick arrow) and hyperemia at the Critical Thermal Maximum temperature (35.5ºC). Lipofuscin-like pigments (thin arrow) and necrotic liver tissue (arrow head) are visible. Scale bars: (a, c, d, e, f) : 25µm; (b): 15µm.

Intestine. Control animals (18°C) exhibited the normal intestinal architecture, consisting of mucosal folds lined internally by microvilli-bearing absorptive cells and goblet cells that secrete mucin (which, in contact with water, forms mucus). Underneath the epithelial layer, a thin layer of connective tissue in the middle (the lamina propria) was visible, with scattered lymphocytes in side adjacent lymph vessels (**Fig. 4.8a,b**). The muscularis of the mucosa was also visible as well as the tunica submucosa, tunica muscularis externa, and tunica serosa at the edge. At low temperatures (20 and 22°C), no alterations were visible except for occasional epithelial vacuolization. Lesions started to become visible in fish subjected to 28 and 30°C, namely with atrophy of epithelial cells and defense cell infiltration within the lamina propria, accompanied by a slight hyperemia, which became more prominent at higher temperatures (**Fig. 4.8c,d**). At 34°C, hyperplasia of absorptive cells seemed to occur, as well as moderate focal necrosis in the muscle layer, which was more obvious in animals subjected to CTmax.

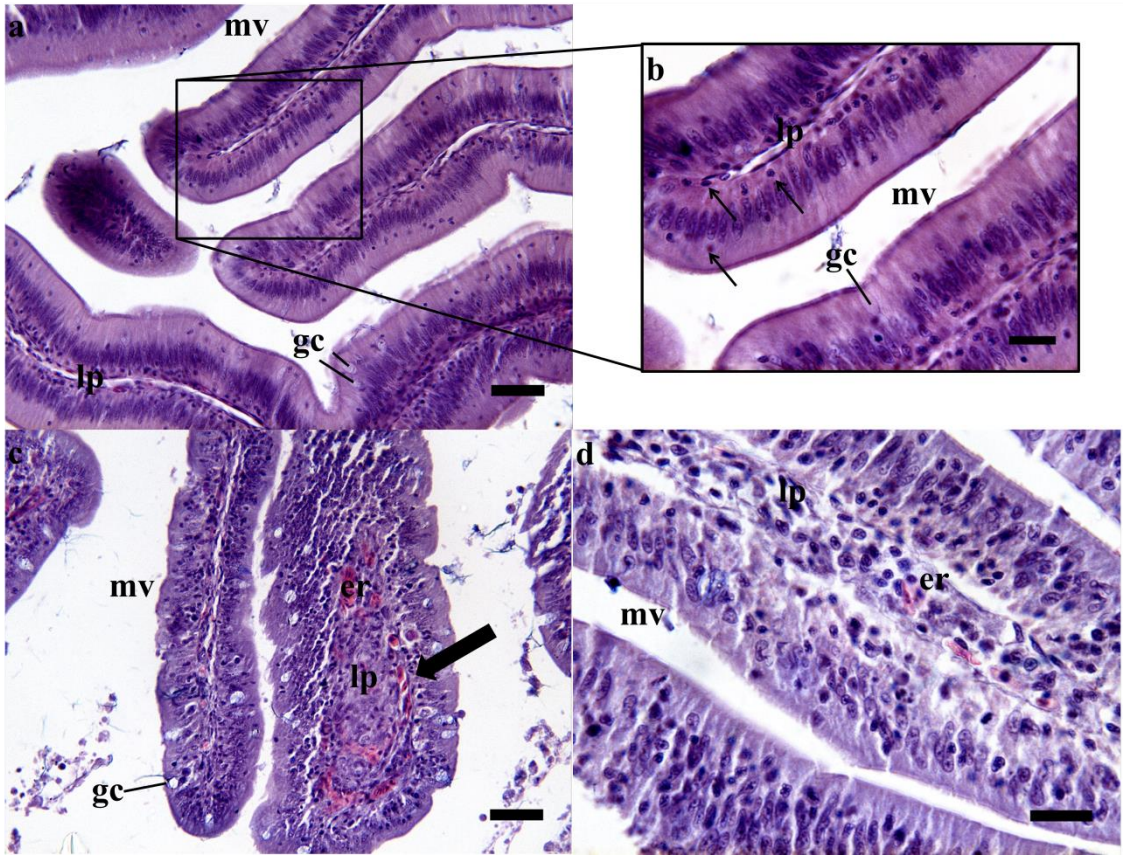

Figure 4.8 Representative micrographs of observed histopathological alterations in the intestine of juvenile *Sparus aurata* exposed to control or high temperatures. a and b) Normal intestinal architecture (18ºC) consisting of mucosal folds lined internally by microvilli (mv) bearing absorptive cells and goblet cells (gc). Underneath the epithelial layer lays the lamina propria (lp) with scattered lymphocytes (thin arrows in b) inside adjacent lymph vessels., c and d) Atrophy of epithelial cells, lymphocyte infiltration, dilated lamina propria (caused by fluid retention) and hyperemia (arrow) at 34ºC. Scale bars: (a, c): 25µm; (b, d): 15µm.

4. Discussion

Sparus aurata juveniles had a CTmax value of 35.5 ± 0.5°C. As gilthead seabream is a warm temperate marine species and juveniles inhabit warmer waters of estuaries and coastal lagoons, a relatively high CTmax value was expected. Living in these confined habitats, with a lower thermal inertia (in comparison to the sea), increases the probability of exposure to high temperature conditions. Thus, this species must be adapted to the thermal regime of estuaries and coastal lagoons. The intraspecific variability in CTmax was somewhat low, which is in accordance with other studies (Mora & Ospina 2001, Madeira et al. 2012a, Vinagre et al. 2013c).

Hsp70 levels significantly increased with rising temperature in several tissues (i.e. gills, muscle, brain, intestine), starting at warmer tem peratures of ≥30°C. Hsp70 levels generally undergo a gradual increase until they reach a maximum amount and start to decrease as the thermal stress becomes more extreme and exhaustion of the biological system starts to take place. This is a common pattern that has been identified in other marine species (Madeira et al.

2012b). There are some exceptions, considering that neither hepatopancreas nor intestine followed this trend. Total ubiquitin levels showed significant increases from control (18°C) in gills and muscle at temperatures ≥32°C. In other marine organisms, biochemical stress is indicated by an increase in Hsps and ubiquitin levels, and these proteins can be overexpressed in response to thermal stress (Hofmann & Somero 1995, Iwama et al. 1998, Feder & Hofmann 1999). During thermal stress, an increase in denaturation and misfolding of proteins occurs, creating a demand for chaperoning activity and/or protein degradation mechanisms. However, ubiquitin levels are not always increased during thermal stress, probably due to adaptation to stressful habitats (i.e. the concentration of Hsps is adequate to remediate protein denaturation, reducing irreversible damage to proteins; Berger & Emlet 2007).

In the present study, Hsp70 and ubiquitin levels varied according to the tissue examined. These results are in accordance with several other studies in marine species (i.e. Feidantsis et al. 2009, Yamashita et al. 2010). In the current study, gills showed the highest amount of Hsp70 and also high amounts of total ubiquitin which may be due to direct contact with water. As warm water passes directly through the gills, they are more exposed to heat. High levels of these proteins may help this tissue maintain its integrity and vital functions (i.e. gas exchange, osmoregulation, and acid-base regulation). This may be achieved by chaperoning activity of Hsp70 and by triage events, preventing irreversibly abnormal proteins to accumulate. Increased Hsp70 can also reduce abnormal protein accumulation by increasing protein degradation (Dul et al. 2001). In addition, maximum Hsp70 and ubiquitin level peaks in gills occurred only at 34°C. This may indicate that gills are able to deal with high temperatures. In fact, according to histopathological results, structural alterations only start taking place between 28 and 30°C, peaking at 34°C and CTmax, supporting the biochemical biomarker results. Other studies in freshwater species (e.g. Sollid et al. 2005, Sollid & Nilsson 2006) have shown that gill structure is altered under high temperatures; however, those were major restructuring events, different from the reported alterations in this study. In addition, our results showed that Hsp70 and total ubiquitin were significantly correlated in gills, indicating that there may be an important interaction of these 2 biomarkers in the protein quality control system.

Brain and muscle had the second highest Hsp70 levels but showed equivalent ubiquitin levels when compared to gills. Brain tissue showed signs of biochemical stress earlier than muscle, when water temperature reached 30°C (Hsp70 peak). However, in relation to total ubiquitin levels, brain and muscle showed different responses: brain did not show any increases with higher temperature, unlike muscle (peak at 32°C). Thus, muscle apparently processes higher amounts of irreversibly damaged proteins than brain. In addition, the tissue-specific expression of stress proteins might be due to different rates of tissue protein synthesis (Kawabe & Yokoyama 2009). Interestingly, Sayegh & Lajtha (1989) showed that protein synthesis in brain is approximately 3 times higher than in muscle in all species examined (goldfish, bullfrog, white Leghorn chicken, mouse, and Tokay lizard). That study and another study by Lajtha & Sershen (1975) also showed that protein metabolic rates increase with temperature in the brain of fishes and lizards. The increase in Hsp70 in the brain during thermal stress may be

necessary due to the high amount of proteins there and their higher metabolic rate. In muscle, which is an organ of low protein turnover rate (Loughna & Goldspink 1985), the cytoprotective efforts may be directed towards the maintenance of existing proteins.

The fact that Hsp70 and ubiquitin were correlated in muscle but not in brain tissue may support this hypothesis of tissue-specific protein expression and metabolism. Histopathological results did not show any gross lesions in muscle, only atrophy of some muscle bundles at higher temperatures (34°C and CTmax). In fact, some degree of atrophy of muscle fibers at higher temperatures has also been shown in other organisms including mammals (Wang & Chen 1999). In addition, Garcia de la serrana et al. (2012) found persistent effects of developmental temperature on muscle growth patterns during early life stages of *S. aurata* (20% more fibers of lower average diameter at low temperature, i.e. 17.5−18.5°C, than at high temperature, i.e. 21−22°C; myogenesis and gene expression patterns are not fixed during growth). Furthermore, the responses in brain and muscle may be linked to the loss of equilibrium. This loss of righting reflex may indicate that nerve impulse transmission is disturbed (Aslanidi et al. 2008). As described above, brain started to show signs of stress at 30°C and muscle at 32−34°C. The combination of brain damage with an abnormal amount of aberrant proteins in the muscle plus fiber atrophy led to the loss of equilibrium at 35−36°C. This explains why the CTmax value was 35.5°C.

Hepatopancreas and intestine showed equivalent amounts of Hsp70 but showed different response patterns, as intestine had a significant increase at CTmax while hepatopancreas did not show any increase. In addition, hepatopancreas had lower total ubiquitin levels. These results somewhat match the results of Feidantsis et al. (2013), who failed to find significant changes in Hsp70 in the liver of *S. aurata* exposed to seasonal temperature changes. However, these same authors reported changes in Hsp90, so perhaps other chaperones are more important in the liver. Moreover, Hsp70 and Hsp90 expression is tissue- and species-specific, which may explain the different responses (see Deane & Woo 2003). Nevertheless, liver and pancreatic acini started to show histopathological lesions at 26−28°C, with more significant alterations (inflammation and necrosis) at 32−34°C. Additionally, intact hepatocytes showed some degree of hypertrophy and eosinophilia, which may be regarded as a change in function, reflecting an alteration of regular metabolic pathways.

Hsps confer thermal tolerance (Moseley 1997). Still, this adaption is of short duration (hours to days) and undergoes a fast decline when Hsp contents start to decrease (Moseley 1997, Kregel 2002). Therefore, our results may indicate that *S. aurata* is particularly sensitive to prolonged exposures to elevated water temperatures (i.e. ≥28−30°C). This result is supported by other studies (e.g. Feidantsis et al. 2009), in which *S. aurata* started to show signs of stress at temperatures above 20°C and were unable to acclimate to temperatures beyond 26°C. These results were based on the analysis of several chaperones, kinases, dehydrogenases, and citrate synthase. The authors also concluded that 30°C is the critical threshold for this species (yielding a high increase in mortality, lactate accumulation, high Hsp70 levels, and behavioral alterations). Even though the study of Feidantsis et al. (2009) was a 10 d experiment, their

results somewhat match our study. Nevertheless, Requena et al. (1997) stated that after a temperature rise from 20 to 28°C (1°C d−1), the basal metabolism (oxygen consumption) of *S. aurata* was compensated within 2 wk. However, other studies (Madeira et al. 2012a, Vinagre et al. 2012a,b,c, 2013a,b) concerning different species (i.e. *Dicentrarchus labrax, Solea senegalensis*, and *Diplodus* sp.) also showed no acclimation to temperatures of 27−28°C, potentially rendering environmental heat waves detrimental to juvenile fish. Thus, there still might be some controversy about the ability of warm-temperate fish to acclimate to high temperatures. It is also reasonable to consider that fish are very mobile organisms and thus may escape stressful conditions. Nonetheless, considering that the growth of juveniles occurs mainly in estuaries or lagoons, they might be more restrained to these shallow warm habitats during this phase, limiting their escape possibilities (as shown in other species of comparable size, life cycle, and ecological requirements, see Vinagre et al. 2008, 2011). In the Tagus estuary (the largest in Western Europe), mean water temperature during the winter is 12°C, increasing to 24°C during the summer (Madeira et al. 2012a). The seasonal variation in temperature is thus 12°C, and the fish may be exposed to extreme temperatures during heat wave events. In a climate warming scenario, water temperature is expected to increase 2°C by 2100. During current heat waves, water temperature attains 28°C in several locations (e.g. Portuguese waters: Vinagre et al. 2012a; Mediterranean: Damianidis & Chintiroglou 2000), which means that by 2100, the seawater temperature may remain at 30°C for several weeks. As mentioned above, at this temperature, *S. aurata* juveniles show significant signs of stress, with adverse consequences to their health, growth, and therefore individual and populational fitness. In addition, as *S. aurata* is a very important commercial species, fishing pressure may also contribute to the negative impact upon *S. aurata* populations. Moreover, this species is one of the most important in saline and hyper saline aquaculture in the Mediterranean area (Feidantsis et al. 2009, 2013). It can be farmed in coastal ponds and lagoons, with extensive and semi-intensive methods, or in land-based installations and in sea cages, with intensive farming systems. In many cases, the fish are kept in low volumes of water and shallow depths, which makes the animals rather susceptible to warming. This potentially affects the health status of the fish and the quality of the end-product, with obvious impacts on this economic activity.

It is also relevant to mention that thermal windows may differ between life stages (Portner & Farrell 2008). According to these authors, juveniles have the greatest aerobic thermal window. Thus, eggs, larvae, and adults may experience physiological and molecular changes earlier than juveniles. Moreover, Polo et al. (1991) showed that eggs of *S. aurata* did not hatch at high temperatures such as 30°C, and outside the range of 16−22°C, mortality and abnormalities increased significantly. Georgakopoulou et al. (2010) also detected a significant effect of water temperature on the development of several deformities in *S. aurata*, such as inside folded gill-cover, hemal lordosis, and malformations of the caudal and dorsal fin-supporting elements. Therefore, it is important to evaluate other life stages in relation to biochemical and physiological alterations resulting from exposure to increased temperature.

5. Conclusions

The current work demonstrates that it is extremely important to know the level of impact of rising seawater temperature, either due to natural environmental change or anthropogenic disturbances, on *Sparus aurata*, since it has great commercial value. From this perspective, studies considering other life stages of *S. aurata* are currently being performed by our team, and these may be crucial to understand the population dynamics, recruitment success, survival rates, fitness, and ultimately the vulnerability and resilience of the species to climate and anthropogenic change. Our results indicate that *S. aurata* undergoes protein damage, inflammation, changes in the immune system, cell atrophy and cell death when exposed to high temperatures for short periods (**Fig. 4.9**). These results may be important from an environmental management perspective considering a bottom-up approach. In detail, subcellular (e.g. proteins) and intermediate-level biomarkers (e.g. histopathological alterations) may serve as early warning signals of stress and may help to understand the health status of the individual. Responses occurring at higher levels of biological organization (population, ecosystem) can ultimately be linked to biomarkers. Integrating biomarker information for several life stages can thus be important in predicting impacts at an ecological level. All together, the information can influence decision makers to improve marine resource sustainability by constraining catch limits (in all or specific life stages) and applying financial penalties to those with unsustainable practices, or giving incentives to those with sustainable ones (see Roberts & Brink 2010 for more detailed information on marine resources management).

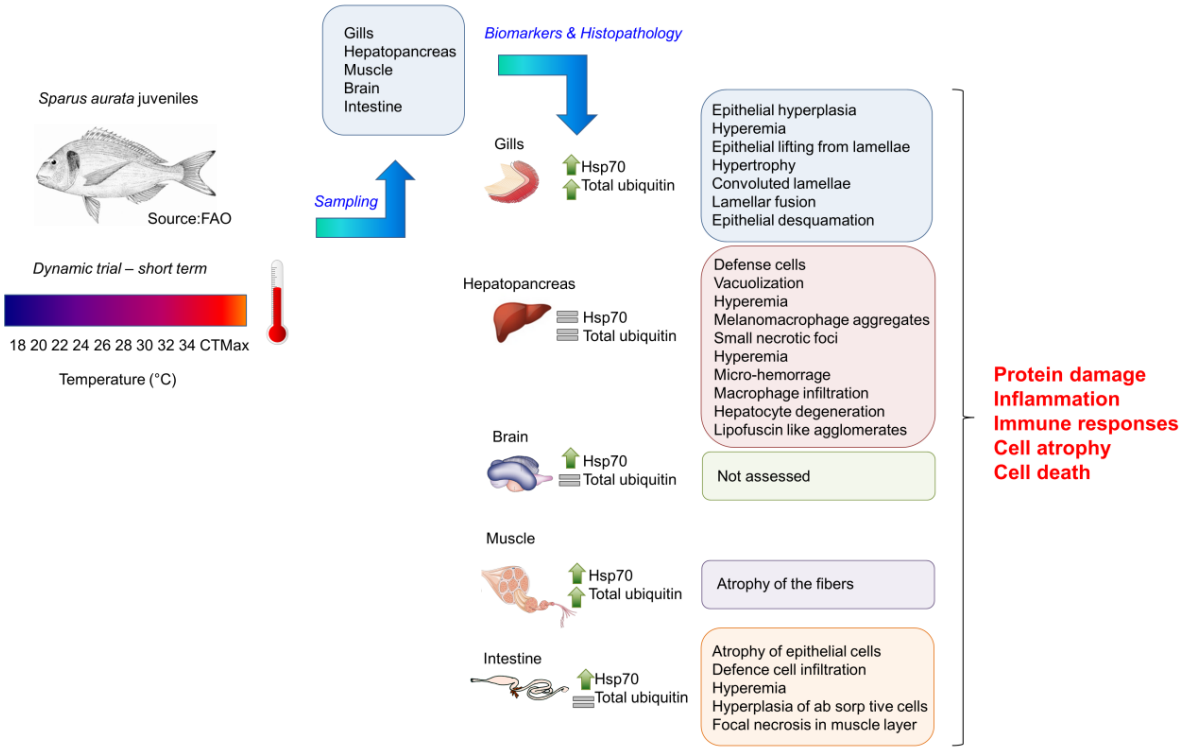

Figure 4.9 Summary of the study. *Sparus aurata* juveniles were subjected to a temperature trial from 18ºC until the Critical Thermal Maximum. Several organs were sampled for quantification of heat shock protein 70 kDa and total ubiquitin. Following, histopathological analyses were carried out. Results showed

that *S. aurata* is subjected to protein damage, inflammation, changes in immune responses, cell atrophy and cell death when exposed to high temperatures for short periods. Green arrows (↑) indicate an increase in the targeted biomarker when fish are exposed to increasing temperatures and = indicates no change in relation to the control temperature (18ºC). Species drawing retrieved from FAO. Organ icons by Sinauer Associates Inc, Shutterstock and Ariel Altaba.

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CHAPTER 5. ARE FISH IN HOT WATER? EFFECTS OF WARMING ON OXIDATIVE STRESS METABOLISM IN THE COMMERCIAL SPECIES *Sparus aurata*[1]

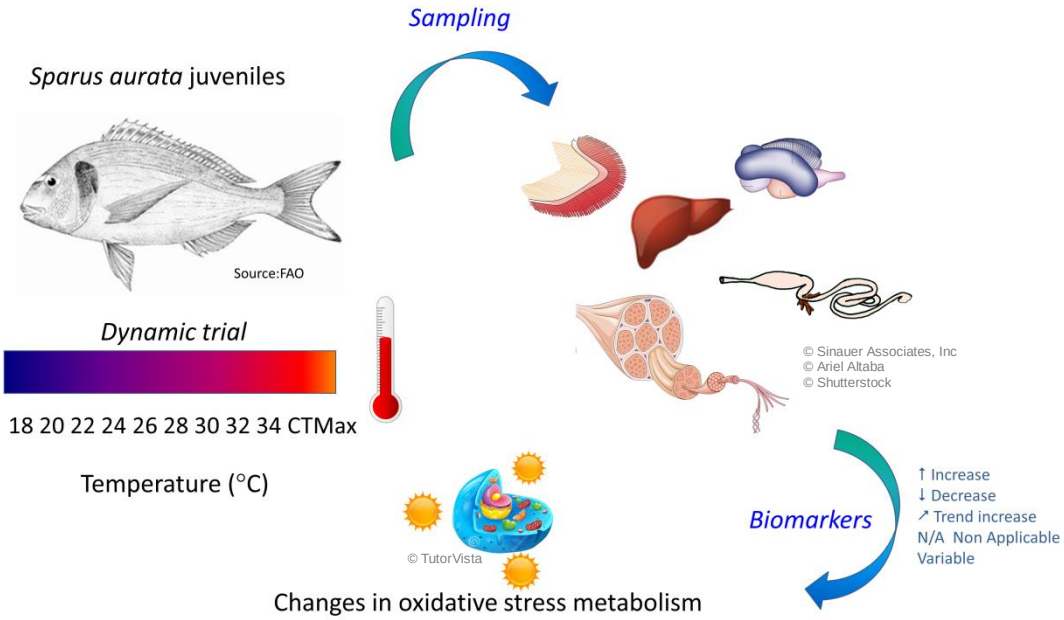
[1]Madeira D, Vinagre C, Diniz MS (2016) Ecological indicators 63, 324-331. DOI: 10.1016/j.ecolind.2015.12.008.

Abstract

Climate change is disturbing marine biological processes, and impacting goods and services provided to society. Physiological studies are a major contributor to the improvement of biological forecasting in the context of climate change. Oxidative stress biomarkers are useful tools to assess the metabolic status and health of organisms, improving management of wild and cultured populations. The aims of this study were to assess the health status and vulnerability of *Sparus aurata* juveniles toward ocean warming and heat wave events by 1) exposing fish to a thermal ramp from 18ºC until their Critical Thermal Maximum (≈35ºC) and 2) quantifying oxidative stress biomarkers in several organs, i.e. lipid peroxidation (LPO), catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferase (GST), and cytochrome CYP1A. Fish showed signs of oxidative stress in every tissue tested (gills, muscle, liver, brain and intestine), the most affected being muscle and liver, which showed greater increases in LPO. In general, antioxidant enzymes increased their activity: CAT increased in every organ tested, GST increased in every organ except brain (no change) and SOD increased in every organ except intestine (no change) and brain (decrease, probably due to enzyme denaturation). Muscle showed the greatest stress response with a massive increase in GST. Hepatic CYP1A decreased upon warming suggesting that temperature influences detoxifying mechanisms and may affect fish health. These results are significant in the context of climate change and associated impacts on fisheries and aquaculture because over-induction of oxidative stress due to warming can induce health problems, mortality and shortened lifespan.

Key words: anti-oxidant enzymes, oxidative damage, biomarkers, thermal stress, sea bream, climate change

Graphical abstract



Changes in oxidative stress metabolism

	CAT	GST	SOD	CYP1A	LPO
Gills	↑8.5fold (34ºC)	↑2.4fold (30, 34ºC, CTMax)	↑2.6fold (CTMax)	N/A	Variable
Muscle	↑12.4fold (CTMax)	↑43.9fold (34ºC)	↗	N/A	↑17.7fold (34ºC)
Liver	↑2.9fold (34ºC)	↑3.2fold (34ºC)	↑1.9fold (34ºC)	↓4.9fold (28, 30, 32ºC)	↑3.3fold (36ºC)
Intestine	↑7.7fold (32ºC)	↑2.5fold (24ºC)	-	N/A	Variable
Brain	↑8.5fold (CTMax)	-	↓1.7fold (24, 34, CTMax)	N/A	↗

1. Introduction

One of the main concerns of contemporary society is climate change. Climate projections predict significant changes in ocean chemistry and increases in air and ocean temperature by 2100 (IPCC 2001, 2007, 2014; Santos et al., 2002). This has led to a great interest in thermal eco-physiology because temperature is an extremely important factor in aquatic environments, especially considering that most of the inhabiting organisms are ectothermic and cannot regulate their body temperatures. Temperature starts to exert its influence at the kinetic level, impelling biochemical reactions and metabolic rates (Kordas et al. 2011). Its effect builds up to the upper levels of biological organization (i.e. community and ecosystem functioning) by affecting the energy available for growth, foraging and reproduction, which ultimately sets ecological patterns which are related to distribution, abundance of species and biological interactions (Hutchins 1947; Hochachka and Somero 2002; Kordas et al. 2011).

In fact, changes in communities and ecosystems have already been detected and attributed to climate change (Walther et al. 2002; Perry et al. 2005; Peck et al. 2012). According

to IPCC (2001), southern Europe is one of the regions where warming is expected to be most severe, with predicted air temperature increases up to 4 to 7ºC by 2100 in the Iberian Peninsula (Santos et al. 2002 – SIAM Project). More specifically, climate change models predict a +2-3ºC increase in Portuguese waters (Miranda et al. 2002) and +3-4ºC in the Mediterranean (Fischer and Schär 2010). In this context, species inhabiting these areas, which are already warm, will be exposed to even higher temperatures, especially considering that heat waves will also increase in frequency, duration and intensity (IPCC 2007). Therefore, it is relevant to understand the physiological and molecular responses deployed by fish to cope with ocean warming.

Several parameters and end-points have been used in the field of eco-physiology to detect changes on the health of wild and cultured fish. According to Blier (2014) oxidative stress is an emerging concept used to assess the metabolic status and health of organisms, which in turn are valuable tools in management and conservation of wild populations. These authors also state that such assessments are useful for the management and conservation of wild populations. Oxidative stress results from the excessive production of reactive oxygen species (ROS), in a way that the buffering effect of antioxidant agents is not enough to prevent the damaging effects of ROS, leading to a disturbance in cell homeostasis and possible cell death (Halliwell and Gutteridge 1999; Ahmed 2005; Heise et al. 2006; Blier 2014). In marine organisms, high temperature increases ROS production due to higher respiratory rates and hypoxia followed by re-oxygenation of tissues after temporary stressful events (Halliwell and Gutteridge 1999; Abele et al. 2002; Freire et al. 2011). Several studies in marine organisms have shown that oxidative stress products change in response to temperature and in order to cope with such stress, animals produce antioxidant enzymes that neutralize ROS e.g. catalase, superoxide dismutase and glutathione-S-transferase and peroxidase (Padmini et al. 2009; Madeira et al. 2013, 2014a; Vinagre et al. 2014a, 2014b). However, when these defenses are not strong enough and stress is severe, organisms can experience the deleterious effects of oxidative damage such as DNA degradation, lipid peroxidation and protein carbonylation (see Abele and Puntarulo 2004; Lesser 2006), which can be exacerbated under climate change scenarios and heat wave events. Such warming events and associated oxidative stress may have serious impacts on organismal health, potentially affecting biological processes such as spawning, mortality rates and recruitment success (e.g. Randall and Szmant 2009; Ross et al. 2013; Jeffries et al. 2014; Hemmer-Brepson et al. 2014). This is especially relevant in organisms inhabiting shallow waters (i.e. little thermal inertia) like juvenile fish that inhabit nursery grounds such as estuaries and coastal lagoons. Thus, the model species chosen for this study was the sea bream *Sparus aurata* (*Linnaeus* 1758). This is a commercial species, highly captured and cultured in Europe (see FAO, FishStat *Sparus aurata*, 2015). It has a subtropical distribution, occurring in Eastern Atlantic (British Isles to Cape Verde), and the Mediterranean and Black Seas (Froese and Pauly 2006; Sola et al. 2007). This species lives in sandy bottoms and seagrass beds, with a demersal juvenile phase occurring in shallow waters (down to 30m deep). Larvae/juveniles migrate to nursery grounds, passing through several

thermal regimes, with varying temperatures (mean of 15-16ºC in coastal waters to 24-26ºC in nursery grounds), which can be stressful to young fish. Considering this background, the aims of the present work were to assess the health status and vulnerability/resistance of *Sparus aurata* juveniles to ocean warming and heat wave events by 1) exposing fish to a thermal ramp until Critical Thermal Maximum is reached and 2) quantifying oxidative stress biomarkers in several organs, including oxidative damage products i.e. lipid peroxidation (LPO), and antioxidant enzymes i.e. catalase, superoxide dismutase, glutathione-S-transferase, and cytochrome CYP1A. This is especially relevant considering that thermal-induced oxidative damage can affect capture production, as well as mortality and meat quality in fish farms.

2. Materials and methods

2.1 Ethical statements

This study was carried out in strict accordance with the recommendations of the Portuguese legislation for animal experimentation following the approval of Direcção Geral de Alimentação e Veterinária. Moreover, the three authors have a level C certification by FELASA (Federation of European Laboratory Animal Science Associations). All efforts were made to minimize animal suffering.

2.2 Thermal ramp trial

Juvenile fish of *Sparus aurata* (mean±SD total length of 92.1±8.1 mm and 12.2±2.9 g in weight) were obtained from a fish farm (MARESA, Spain) and transported to the laboratory in 100L plastic boxes with oxygen pumps. *S. aurata* is a protandric hermaphrodite species, maturing first as a male (first or second year of age) and after as a female (second or third year). Considering that the collected juveniles were less than one year of age, the sex was still undetermined as fish had not yet ripened. The fish were placed in a re-circulating system with 70L white plastic tanks (with environmental enrichment i.e. sand and rocks) with aerated sea water (95-100% O_2), a constant temperature of 18°C and a salinity of 35‰ (same conditions of the fish farm). The fish acclimated for one week and their welfare was assessed (i.e. wounds or any disease symptoms). They were fed with commercial food pellets, under a regime of period feeding (twice a day). The food pellets contained cereals, fish protein, shrimp, amphipod, squid, refined soybean oil, fish oil, vitamins, vitamin C, chelated organic materials, yeast extract, minerals and antioxidants (Aquasoja, Portugal) mixed with cyanobacterium *Spirulina* sp. (Tropical®, Poland). The fish were starved for 24h before the experiments. The organisms were subjected to a thermal ramp (constant rate of water-temperature increase of $1°C.h^{-1}$) until they reached their Critical Thermal Maximum (CTmax), already estimated elsewhere via loss of equilibrium (Madeira et al., 2014b). Dynamic trials such as this are considered by several authors as accurate predictors of the responses of organisms to natural conditions (Bennett et al., 1997; Bennett and Judd, 1992). Even though $1°C.h^{-1}$ may not occur in semi-confined waters,

juvenile fish concentrate in low depth areas of the estuary (<10m), being subjected to wide environmental fluctuations occurring during the day, especially during summer.

Fish were randomly placed in 40.5 l white plastic tanks (n=5 tanks, 30 × 30 × 45cm with water from the home tank) which were transferred to a thermostatized bath, connected to a heated/refrigerator circulator (MultiTemp III, Pharmacia Biotech). Each tank had a lid to prevent evaporation and was equipped with an aeration system (10-11 fish.tank^{-1}) (**Fig. 5.1**).

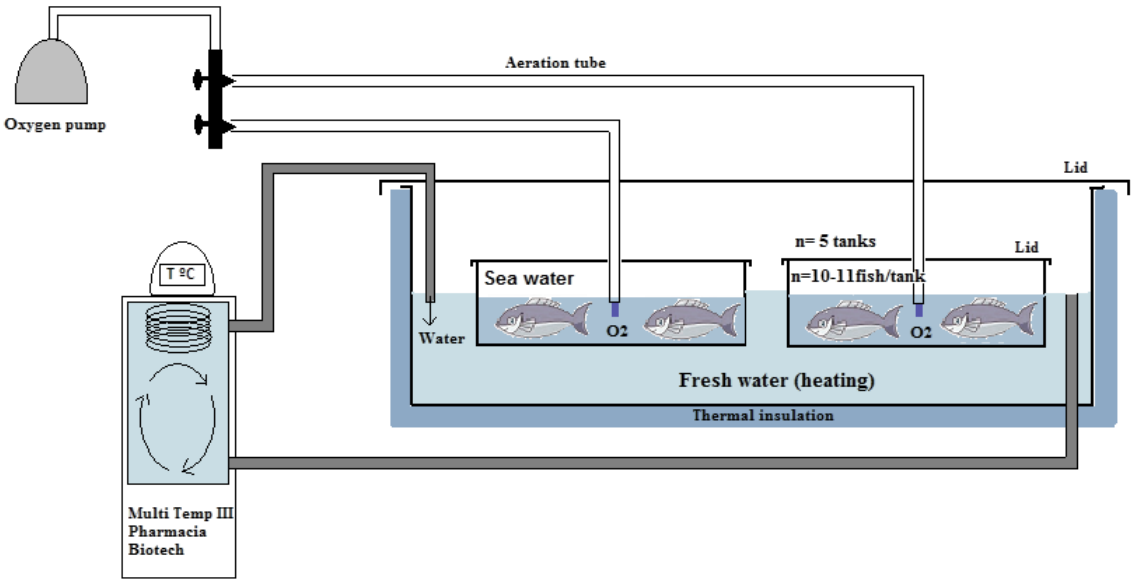


Figure 5.1 Experimental setup: the fish were randomly placed in 40.5 l white plastic tanks (n=5 tanks, 30 × 30 × 45cm with water from the home tank), which were transferred to a thermostatized bath, connected to a heated/refrigerator circulator (MultiTemp III, Pharmacia Biotech). Each tank had a lid to prevent evaporation and was equipped with an aeration system (10-11 fish.tank^{-1}). Note: not to scale. Constructed in Paint. Fish icon by Shutterstock.

The organisms were observed continuously until CTmax. The fish were euthanized with cervical transection and samples (brain, gills, intestine, liver, muscle) from 6 individuals were taken at 18ºC (control), 22ºC, 24ºC, 26ºC, 28ºC, 30ºC, 32ºC, 34ºC, CTmax (35.5±0.5ºC) and were immediately frozen in liquid nitrogen. The total number of individuals was 54. The experiments were carried out in shaded day light (15 L; 09 D). To prevent any additional handling stress, the total length and weight of all individuals was measured at the end of each trial using an icthyometer and a scale, respectively.

2.3 Protein extraction

Samples (200-250mg of gills, muscle, intestine, brain, liver) were homogenized in 1 mL of phosphate buffered saline solution (0.14 M NaCl, 0.003 M KCl, 0.01 M Na_2HPO_4, 0.002 M KH_2PO_4, pH 7.4) to extract cytosolic proteins, using a glass/teflon Potter Elvejhem tissue grinder, in ice-cold conditions. The crude homogenates were then centrifuged for 15 min at

10,000 × g. The supernatant was collected, transferred to new microtubes (1.5 mL) and frozen immediately (-80ºC).

2.4 Enzymatic assays

2.4.1 Catalase

The enzymatic assay of Catalase *(EC 1.11.1.6)* was carried out according to the procedures described previously (Beers and Sizer 1952; Aebi 1983; Li and Schellhorn 2007). Activity from a standard bovine catalase solution of 1523.6 $U.mL^{-1}$ was used as a positive control. Catalase activity was calculated using a molar extinction coefficient (at 240nm) for H_2O_2 of 0.04 ε^{mM} ($mM^{-1}cm^{-1}$).

2.4.2 Glutathione S-transferase

The enzymatic assay of glutathione-S-transferase (GST) activity *(EC 2.5.1.18)* was adapted from Habig et al. (1974) and optimized for 96-well microplates. The substrate CDNB (1-chloro-2,4-dinitrobenzene) was used to react with the enzyme. After reading the absorbance at 340 nm GST activity was calculated using a molar extinction coefficient for CDNB of 5.3ε^{mM} ($mM^{-1}cm^{-1}$) after correction for the microplate wells path length.

2.4.3 Superoxide dismutase

The enzymatic assay of superoxide dismutase (SOD) activity (*EC 1.15.1.1)*, using nitroblue tetrazolium (NBT) and xanthine oxidase (XOD), was carried out according to Sun et al (1988). After reading the absorbance at 560nm, SOD activity was calculated using the equation for the %inhibition:

((Abs$_{560}$/min negative control – Abs$_{560}$/min sample) / (Abs$_{560}$/min negative control)) x 100

2.4.4 Cytochrome P450 1A

The quantification of CYP450 1A (cytochrome P450 1A) was carried out in liver's microsomal fraction. Its quantification was achieved through an enzyme linked immunosorbent assay (ELISA), following Nilsen et al. (1998). First, 300 µL of every sample (processed as described in section 2.2) were centrifuged at 100,000 × g during 60 min in an ultracentrifuge (Beckman Coulter Optima^{TM} LE-80K, rotor 70Ti). Secondly, the pellets were re-suspended in 100 µL of sodium phosphate buffer (97 mM Na_2HPO_4, 0.15 M KCl, 1.27mM EDTA, 9.98 M DTT, 87% (v/v) glycerol, pH 7.4). Then, samples were diluted 1:10 in 0.05 M carbonate-bicarbonate buffer (Sigma-Aldrich, USA). This dilution was chosen to give optimum signal. Three replicates of 100 µL were taken from each diluted sample, transferred to the microplate wells and incubated overnight at 4ºC. The microplate was washed (3×) in PBS (phosphate buffered saline) containing 0.05% Tween-20 and then blocked by adding 200 µL of 1% BSA (Bovine Serum Albumin, Sigma-Aldrich, USA). The microplate was incubated at 37°C for 90 min. After microplate washing, the primary antibody (rabbit anti-fish CYP1A, Biosense Laboratories, Norway), diluted to 1:100 in 1% BSA in PBS, was added to the microplate wells (100 µL each). Then the microplates were incubated for 90 min at 37°C. After another washing stage, the

secondary antibody (anti-rabbit IgG, alkaline phosphatase conjugate, Sigma-Aldrich, USA) was diluted (1:1000 in 1% BSA in PBS) and added (100 µL) to each well followed by incubation at 37°C for 90 min. After the washing stage, 100µL of substrate (SIGMA *FAST*^{TM} p-Nitrophenyl Phosphate Tablets, Sigma-Aldrich, USA) was added to each well and incubated for 30 min at room temperature. Fifty µL of stop solution (3 N NaOH) was added to each well and the absorbance was read in a 96-well microplate reader at 405nm (BIO-RAD, Benchmark, USA). CYP1A quantification was relative (in comparison to controls) as there were no protein standards for an absolute quantification. For normalization purposes, the results were divided by the total amount of protein in the microsomal fraction, calculated through the Bradford method (Bradford 1976), to obtain CYP1A in absorbance units.mg^{-1} of total protein.

2.5 Oxidative damage products - lipid peroxidation

The lipid peroxides assay was adapted from the thiobarbituric acid reactive substances (TBARS) protocol (Uchiyama and Mihara 1978). Five µL of each sample, already processed as previously described were added to 45 µL of 50 mM monobasic sodium phosphate buffer. Then 12.5 µL of SDS 8.1%, 93.5 µL of trichloroacetic acid (20%, pH=3.5) and 93.5 µL of thiobarbituric acid (1%) were added to each microtube. To this mixture, 50.5 µL of Milli-Q grade ultrapure water were added. Then the microtubes were put in a vortex for 30 s and incubated in boiling water for 10 min. To stop the reaction, they were placed on ice for a few minutes and 62.5 µL of Milli-Q grade ultrapure water and 312.5 µL of n-butanol pyridine (15:1, v/v) were added. Then the microtubes were placed in a vortex and centrifuged at 10,000 × g for 5 min. Duplicates of 150 µL of the supernatant of each reaction were put into a 96-well microplate and absorbance was read at 530 nm. To quantify the lipid peroxides, an eight-point calibration curve (0-0.3 µM TBARS) was calculated using malondialdehyde bis(dimethylacetal) standards (Merck Millipore, Portugal).

2.6 Statistical analysis

The enzymes' activities and lipid peroxidation throughout the temperature trial were either analyzed via parametric ANOVAs by the *F* statistic or via the non-parametric Kruskal-Wallis *H* statistic, depending on the normality and homoscedasticity of the data. Either Tukey's post-hoc tests (following parametric ANOVA) or multiple comparisons of mean ranks (see Siegel and Castellan 1988) (following non-parametric Kruskal-Wallis) were applied whenever the null hypotheses were rejected (α=0.05). A factor analysis (principal components as extraction method) was carried out to detect if oxidative stress metabolism differed between tested organs. This analysis was carried out taking into consideration all temperatures tested, however only tissues were labeled for image simplicity. Statistics were carried out using the software Statistica (Version 10.0 StatSoft Inc, USA) and an α level of 0.05 was considered in all analyses.

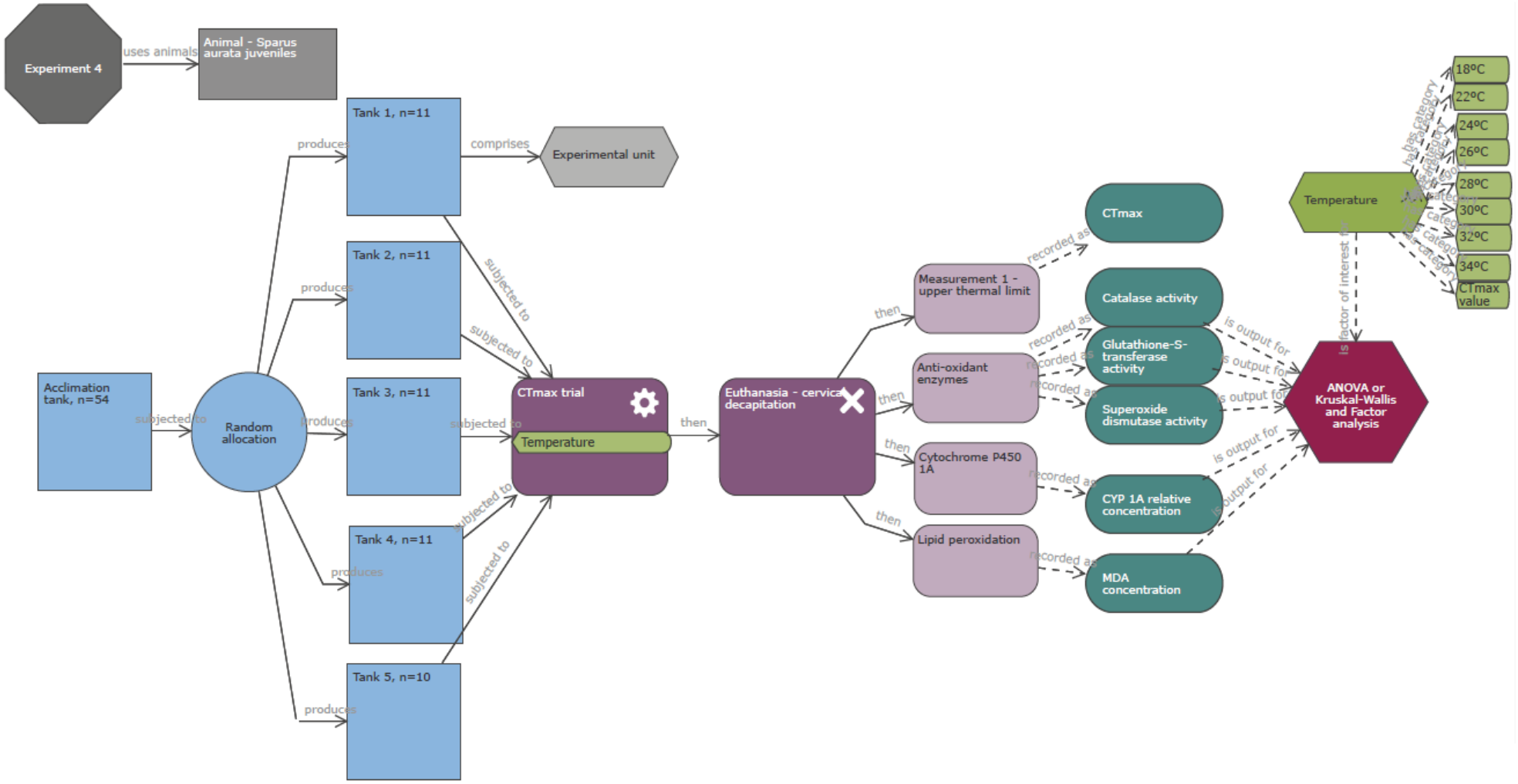


Figure 5.2 Experimental approach (diagram constructed using Experimental Design Assistant, https://eda.nc3rs.org.uk/).

3. Results

To simplify figure interpretation, all statistical differences highlighted correspond only to significant differences found in the temperature treatments when compared to the control group (18ºC).

3.1 Catalase activity

The statistical analyses detected significant differences in catalase activity among temperature groups in every targeted organ (**Table 5.1, Fig. 5.3a**). The results showed significant increases from control in gills (starts to increase at 30ºC; peaks at 34ºC, +8.5-fold, Post-hoc 18 vs 34 p=0.011), muscle (starts to increase at 32ºC; peaks at CTmax, +12.4-fold, Post-hoc 18 vs CTmax p=0.002), brain (peak at CTmax, +8.5-fold, Post-hoc 18 vs CTmax p=0.012), liver (peak at 34ºC, +2.9-fold, Post-hoc 18 vs 34 p=0.031) and intestine (peak at 32ºC, +7.7-fold, Post-hoc 18 vs 32 p=0.001).

3.2 GST activity

GST showed increased activity in liver (peak at 34ºC, +3.2-fold, Post-hoc 18 vs 34 p=0.0009), muscle (starts to increase at 32ºC; peaks at 34ºC, +43.9-fold, Post-hoc 18 vs 34 p=0.002), gills (starts to increase at 26ºC and peaks at 30ºC, 2.43-fold and at 34ºC and CTmax, 2.36-fold and 2.47-fold, respectively; Post-hocs: 18 vs 30 p=0.002, 18 vs 34 p=0.003, 18 vs CTmax p=0.003) and intestine (starts to increase at 22ºC, Post-hoc p=0.04; peaks at 24ºC, +2.5-fold, Post-hoc p=0.001 and then decreases 5-fold and maintains low activity levels until the end of the experiment). Brain showed no significant differences in GST activity throughout the trial (**Table 5.1, Fig. 5.3b**).

3.3 SOD activity

SOD showed increased activity in comparison to controls in liver (peak at 34ºC, +1.9-fold, Post-hoc 18 vs 34 p=0.04) and gills (peak at CTmax, +2.6fold, Post-hoc 18 vs CTmax p=0.03). In muscle, SOD activity was significantly higher at 34 and CTmax when compared to 24, 26 and 28ºC (Post hocs: 24 vs 34, p=0.002; 24 vs CTmax, p=0.011; 26 vs 32, p=0.02). However, no differences were found in relation to controls (18º) but the tendency was to increase throughout the trial. In brain, SOD activity decreased 1.7-fold in relation to the controls at 18ºC (Post-hocs: 18 vs 24 p=0.02, 18 vs 34 p=0.01, 18 vs CTmax p=0.01). In intestine, it showed no significant differences (**Table 5.1, Fig. 5.3c**).

Table 5.1 One-way ANOVA results for oxidative stress biomarkers for juvenile *Sparus aurata* exposed to high temperatures. Significant results are marked with an asterisk (*). GST – glutathione-S-transferase; CAT – catalase; SOD – superoxide dismutase; LPO – lipid peroxidation; CYP450 1A – cytochrome P450 1A.

Organ	Biomarker	Test statistic	df	p-value
Gills	GST	F=2.67	8	0.021*
	CAT	H=23.96	8	0.002*
	SOD	H=17.35	8	0.026*
	LPO	H=16.99	8	0.030*
Brain	GST	H=5.55	7	0.594
	CAT	F=5.99	7	0.004*
	SOD	H=15.49	7	0.030*
	LPO	F=3.74	7	0.013*
Liver	GST	F=6.56	8	0.0004*
	CAT	H=22.18	8	0.005*
	SOD	H=15.64	8	0.048*
	LPO	H=28.29	8	0.0004*
	CYP450 1A	H=17.42	8	0.026*
Intestine	GST	H=25.74	8	0.001*
	CAT	H=19.19	8	0.014*
	SOD	F=1.54	8	0.180
	LPO	H=25.83	8	0.001*
Muscle	GST	H=21.29	8	0.006*
	CAT	H=20.71	8	0.008*
	SOD	H=32.05	8	0.001*
	LPO	H=15.61	8	0.048*

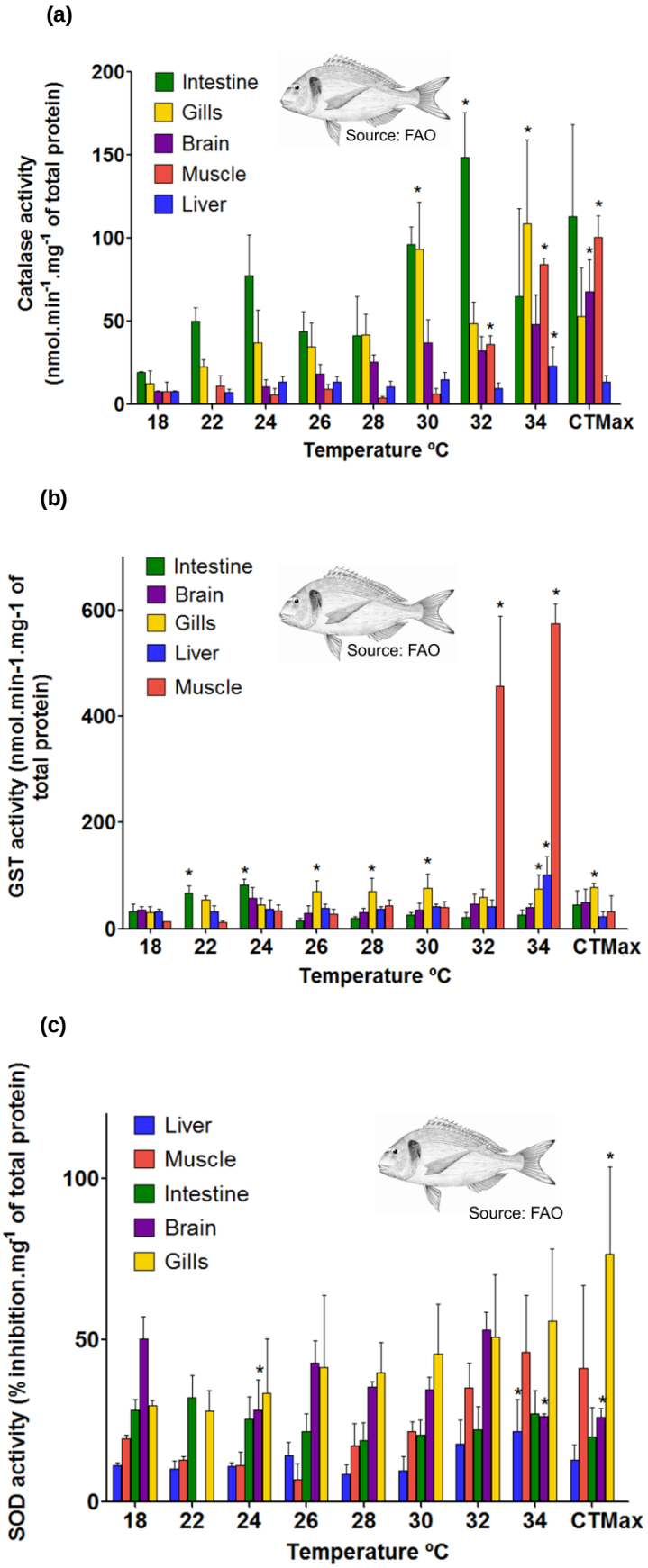


Figure 5.3 Levels (mean + SD) of antioxidant enzymes measured in several organs of juvenile *Sparus aurata* exposed to a temperature ramp of 1ºC.h^{-1} ranging from control conditions (18ºC) to the Critical Thermal Maximum (CTmax 35.5±0.5ºC). **(a)** Catalase activity, **(b)** Glutathione-S-transferase (GST) activity,

(c) Superoxide dismutase (SOD) activity. Six individuals were sampled at each temperature point (except 22°C for brain tissue). Groups significantly different from controls are marked with an asterisk ($p < 0.05$).

3.4 CYP450 1A quantification

CYP450 1A was quantified in liver and it showed a 4.9-fold decrease starting at 28ºC (Post-hoc p=0.003), recovering to control levels at 34ºC (**Table 5.1, Fig. 5.4**).

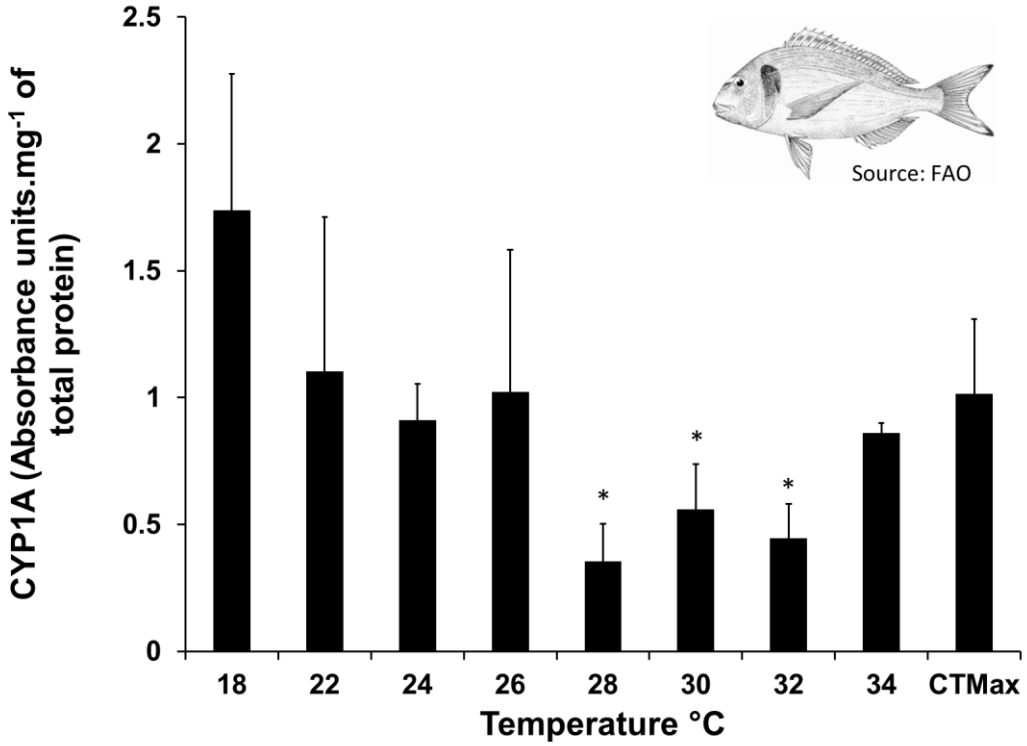


Figure 5.4 Levels (mean + SD) of cytochrome P450 1A (CYP P450 1A) measured in the liver of juvenile *Sparus aurata* exposed to a temperature ramp of 1ºC.h^{-1} ranging from control conditions (18ºC) to the Critical Thermal Maximum (CTmax 35.5±0.5ºC). Six individuals were sampled at each temperature point. Groups significantly different from controls are marked with an asterisk ($p < 0.05$).

3.5 Lipid peroxidation (LPO)

Statistical analyses showed that LPO varied significantly between temperature groups in all organs tested (**Table 5.1, Fig. 5.5**). It increased significantly in liver and muscle, starting at 34ºC. Liver showed its peak at CTmax (Post-hoc 18 vs CTmax, p=0.000), with a 3.3fold increase whereas muscle showed its peak at 34ºC, with a 17.7-fold (Post-hoc 18 vs 34, p=0.03) increase in comparison to the controls (18ºC). In gills and intestine, there was some variability along the thermal ramp but there were no differences in relation to control temperature (18ºC). In brain, lipid peroxidation levels were higher at 34ºC when compared to 24ºC and 26ºC, thus showing a tendency to increase with temperature (Post-hocs: 24 vs 34, p=0.026; 26 vs 34, p=0.011).

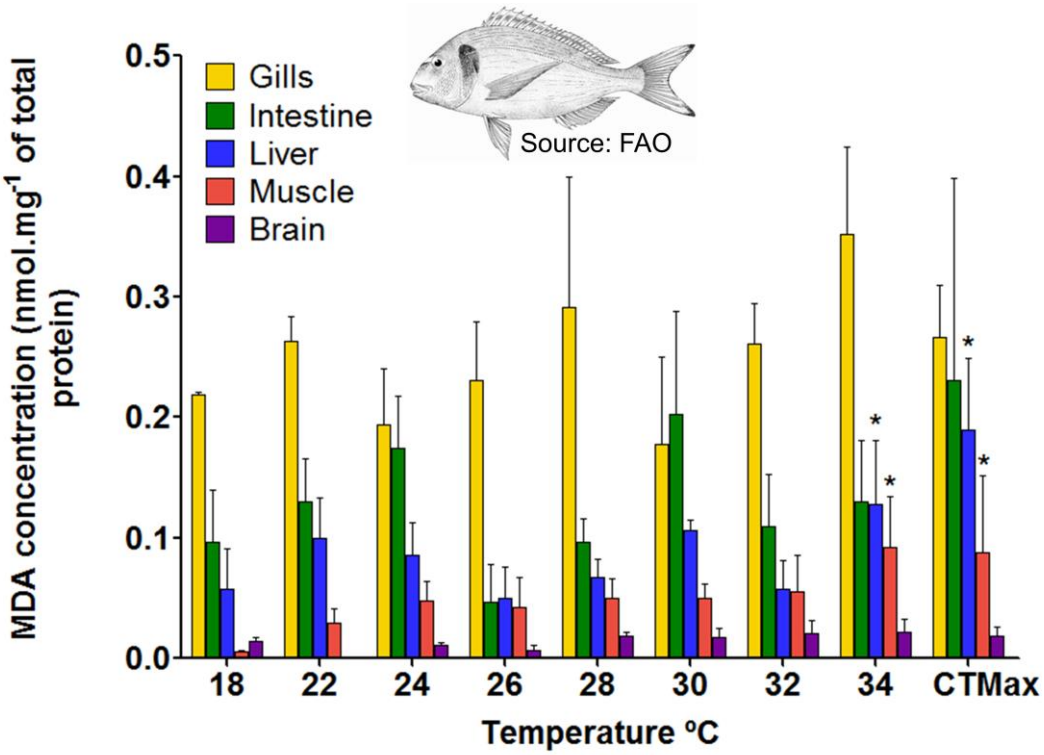


Figure 5.5 Levels (mean + SD) of lipid peroxidation (LPO) measured in juvenile *Sparus aurata* exposed to a temperature ramp of 1ºC.h^{-1} ranging from control conditions (18ºC) to the Critical Thermal Maximum (CTmax 35.5±0.5ºC). Six individuals were sampled at each temperature point. Groups with LPO levels significantly different from controls are marked with an asterisk ($p < 0.05$).

3.6 Factor analysis

Factor analysis (principal components as extraction method) was applied to statistically define the differences in oxidative stress metabolism between the several tested organs (**Fig. 5.6**). The results show that oxidative stress metabolism is very similar between organs even though muscle and gills slightly separate from other organs, especially when exposed to high temperatures. Results show that factor 1 and 2 explain 41.53% and 24.87% of the variance, respectively (cumulative value of 66.40%). According to the analysis, gills differentiate along factor 1 (temperatures of 34 and 36ºC), for which SOD activity was the main contributor (factor loading of -0.75). CAT and LPO also contributed to factor 1, showing a factor loading of -0.66 and -0.72, respectively. Muscle slightly differentiates from other organs along factor 2 (temperatures of 32 and 34ºC), for which the main contributor was GST activity.

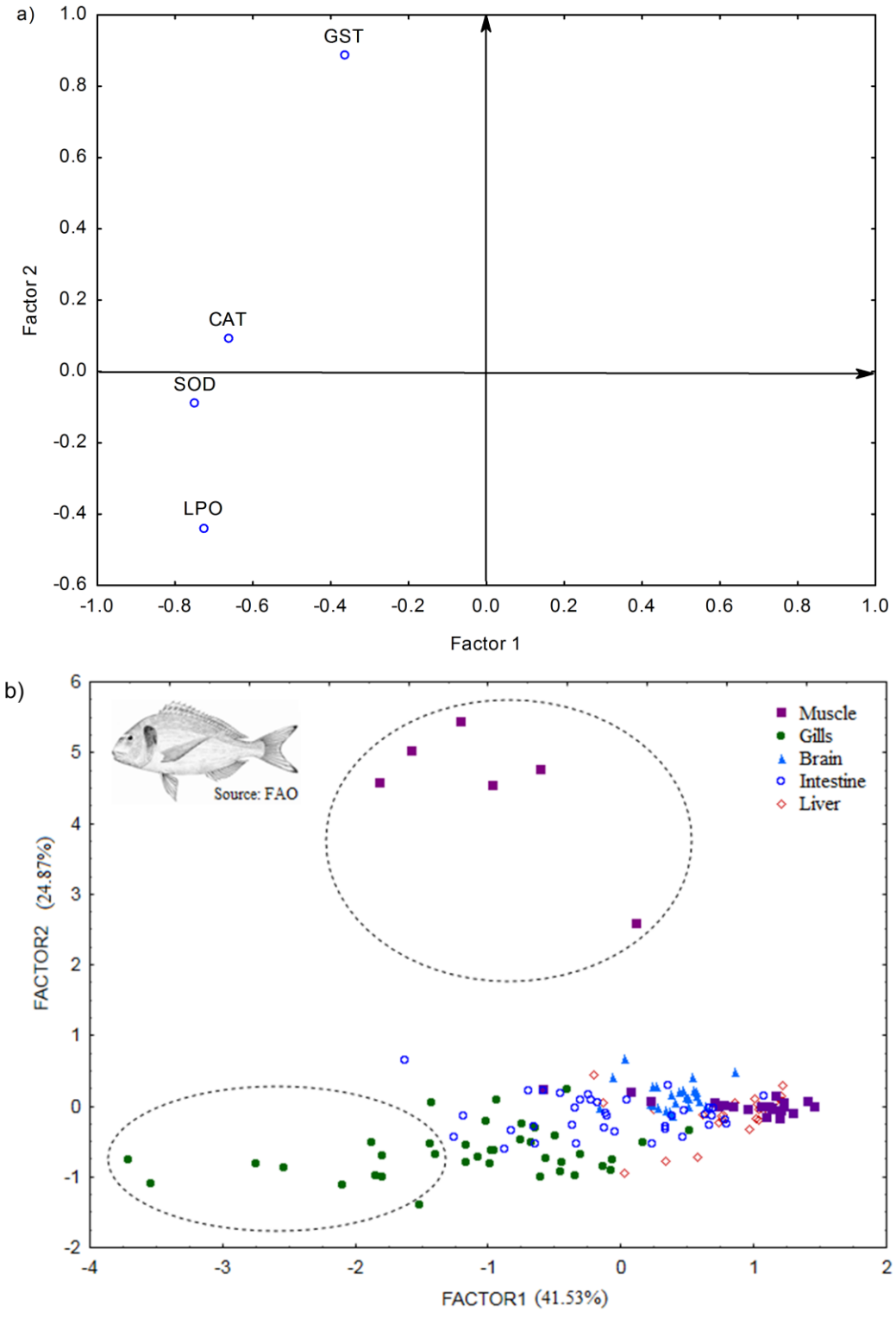


Figure 5.6 (a) Projection of the biomarkers on the factor plane: representation of the contribution of each biomarker to factor 1 and 2 of the factor analysis. The biomarkers were measured in response to temperature in different organs of *Sparus aurata*. **(b)** Factor analysis (principal components as extraction method) of all investigated biomarkers in the different tissues analyzed in *Sparus aurata* along the temperature trial (all temperatures were included in the analysis). The dashed circles represent the groups that differentiate along the factors: gills differentiate along factor 1 (temperatures of 34 and 36ºC), for which SOD activity was the main contributor; and muscle differentiates along factor 2 (temperatures of 32 and 34ºC), for which the main contributor was GST activity.

4. Discussion

In the present study, juveniles of *Sparus aurata* showed an increased oxidative stress response in several tissues, especially muscle and liver. This is in accordance with other studies that state that increased temperature levels lead to oxidative stress in ectothermic organisms (e.g. Bagnyukova et al. 2007; Madeira et al. 2013; Vinagre et al. 2014b). As expected, antioxidant enzymes generally increased their activities to counterbalance the effects of oxidative damage, being an important component of the stress response (Parihar et al. 1997).

Lipid peroxidation increased (in comparison to controls 18ºC) 17.7-fold in muscle and 3.3-fold in liver, suggesting that these organs are highly sensitive to ROS production. In brain, lipid peroxidation levels also tended to increase (although not in relation to controls), being significantly higher at 34ºC than at 24ºC and 26ºC (Table 5.1). These organs, especially brain and liver, perform vital functions. As such, they probably possess effective mechanisms to eliminate toxic products originated from oxidative stress. In liver, all antioxidant enzymes analyzed (catalase, glutathione-S-transferase and superoxide dismutase) increased their activity in the order of 2-3 fold. However, brain only showed increased CAT (8.5-fold), no change in GST and a decrease in SOD activity. According to Matozzo et al., (2013), when animals are exposed to ROS-generating stressful conditions, they can either induce or inhibit antioxidant enzymes. This seems to be the case in brain, in which a mixed situation of induction and inhibition occurred. The decrease in SOD, which occurred mainly at the end of the trial (i.e. extremely high temperatures near the CTmax) is probably associated with denaturation of the enzyme or inability of the cells to synthetize it. Enzymes are proteins held together by hydrogen bounds, which can be weakened when temperatures exceed a threshold value, leading to denaturation and a decrease in their catalyst activity (Kordas et al. 2011). This increase in LPO levels at 34ºC in brain may also be related to the loss of equilibrium and CTmax at 35.5±0.5ºC.

Considering muscle, antioxidant enzymes also increased in response to increasing temperatures, with CAT showing a +12.4-fold change and GST undergoing a major increase in its activity (+43.9-fold). SOD activity was also elevated but not in relation to controls, it was significantly higher at 34 and 36ºC when compared to 24, 26 and 28ºC. These results indicate that muscle has a potent antioxidant system and is very responsive to temperature. Interestingly, the factor analysis showed differentiation of the muscle along factor 2 (correlated with GST activity), showing that its oxidative stress metabolism is slightly different from the other organs when exposed to high temperatures, mainly due to the massive increase in GST activity. The factor analysis also showed a slight differentiation of gills along factor 1, for which SOD activity was the main contributor, followed by CAT and LPO. Even though LPO did not show a tendency to increase with temperature in gills, its absolute levels were always quite elevated throughout the trial. This may be related to the position of gills in the body, as they are in direct contact with the environment and are thus continuously exposed to the physical and chemical conditions of the water. This has also been observed in other studies in which gills presented high constitutive levels of biomarkers but were unresponsive to temperature (e.g. Vinagre et al. 2014a). Reid et al. (1998) stated that desensitization toward a stressor may also occur when

this stressor is frequently found, which can partially explain such results. Nevertheless, the antioxidant system was still activated in gills, as all enzymes showed significant increases in their activity (+8.5-fold in CAT and approximately +2-3-fold in GST and SOD), suggesting that gills were exposed to oxidative stress. With respect to intestine, lipid peroxidation showed no clear trend to increase with temperature but CAT and GST increased their activity indicating some degree of oxidative stress.

In addition to the main antioxidant enzymes tested, CYP 1A was also quantified in the liver, which is the most important organ expressing this cytochrome. This enzyme is important in oxidative stress, being a component of the xenobiotics metabolism as well as a regulator of endogenous compounds (Somnuek et al. 2012). In this work, CYP 1A was significantly decreased (-4.9-fold) upon warming, suggesting that temperature may affect detoxifying mechanisms in fish, influencing their health. All of these results are especially relevant considering that juvenile fish inhabit shallow environments such as estuaries and coastal lagoons. They migrate to these habitats as early metamorphosing larvae or juveniles in order to get shelter and food (e.g. Arabaci et al., 2010; Verdiell-Cubedo et al. 2013). However, these habitats have less thermal inertia than the open sea, being more prone to warming and heat wave events, increasing the probability of exposure to high temperatures. Several authors have stated the importance of 'event effects' in the dynamics of communities and ecosystems (e.g. Jentsch et al. 2007; Brierley and Kingsford 2009; Rose et al. 2012). This is even more emphasized in the context of climate change because extreme weather events will increase in frequency, duration and intensity, with the potential to affect marine species. Off course climate change is highly heterogeneous in space (Walther et al. 2002), but Southern Europe, where *Sparus aurata* occurs, is expected to undergo severe temperature increases (Santos et al. 2002 - SIAM Project). Currently, the mean summer temperature of several Portuguese estuaries is approximately 24ºC (e.g. Vinagre et al. 2012), reaching 28ºC during heat waves. By 2100, it is predicted to reach 30ºC during these extreme events, which is a very deleterious temperature to *Sparus aurata* as shown by other studies (Feidantsis et al. 2009; Madeira et al. 2014b). Altogether, the present and other studies show that *Sparus aurata* undergoes protein damage, oxidative stress, organ inflammation, atrophy, and lactate accumulation under elevated temperatures. Thus, coastal species will have to cope with such changes, depending on their capacity to adapt. This capacity to adapt is dependent on their tolerance limits, phenotypic plasticity, dispersal capacity, and generation time. Species with broad thermal tolerance ranges, large acclimation capacity, reduced generation times and increased dispersal will be the ones with the greatest potential for adaptation. Considering these parameters for *S. aurata*, its thermal limits range approximately from 12ºC to 35ºC (Ibarz et al., 2003; Madeira et al., 2014b), being unable to acclimate to temperatures beyond 26ºC (Feidantsis et al. 2009). Minimum population doubling time of *S. aurata* has been recorded as 1.4 to 4.4 years showing medium resilience (Froese and Pauly, 2006). They have some dispersal capacity since they are a mobile species with a planktonic larval phase. Nevertheless, some studies suggest that *S. aurata* populations can undergo genetic differentiation, so evidence for large dispersal is mixed

(Chaoui et al., 2009; Franchini et al., 2012). Considering these facts, *S. aurata* populations may have some capacity to adapt but further studies are necessary to reach any congruent conclusions.

As stated above, *S. aurata* is highly mobile and as such it can try to escape unfavorable conditions. However, juvenile fish are confined to shallow waters of estuaries and coastal lagoons (for instance the depth of Tagus estuary nursery grounds is <10 m – Costa and Bruxelas 1989; Cabral and Costa 1999; Vinagre 2007) and might not be able to move to more suitable conditions. This is especially true considering that heat waves can increase water temperature down to 20 m deep (Rose et al. 2012), which can be detrimental to juvenile fish. This is relevant not only in wild populations but also in farmed fish. *Sparus aurata* is one of the most cultured fish in Southern Europe, either in offshore and inshore cages or on-land ponds. The impact of heat waves will depend on the type of farming but main effects are related to increased disease, eutrophication and increased salinity due to evaporation and reduced rainfall, leading to changes in water quality (De Silva and Soto 2009; Rosa et al. 2012). In fact, physiological effects can also be expected from the temperature rise and consequent low water quality. Whereas a mild increase in temperature might favor growth, heat waves will likely lead to decreased quality of fish products through decreased health and/or increased mortality events. This is in accordance with studies that predict that climate change will negatively affect aquaculture in temperate zones, because as the temperature increases, it can exceed the optimal temperature range of organisms cultured (De Silva and Soto 2009). However, these impacts depend on the capacity of aquaculture industry to adapt to climate change (Doubleday et al. 2013).

5. Conclusions

Several authors have reported that climate change effects are disturbing marine biological processes from genes to ecosystems, impacting goods and services provided to society (e.g. Brierley and Kingsford 2009). Therefore, scientists have to face the challenge of improving the predictive power of ecological forecasting (Kordas et al. 2011). Physiology plays a crucial role in biological up scaling because it integrates mechanisms from the molecular level to the individual level and further on (Cooke et al. 2014). In this context, oxidative stress biomarkers are extremely relevant to assess the health of organisms. The commercial species *Sparus aurata* showed signs of oxidative stress upon exposure to warming in every organ tested (gills, muscle, liver, brain, intestine). In general, antioxidant enzymes increased their activities (**Table 5.2**) at temperatures around 30ºC in order to counterbalance the effects of ROS, measured by lipid peroxidation, which increased especially in muscle, liver and brain. These results are significant in the context of climate change and its impacts on fisheries and aquaculture because over-induction of oxidative stress due to warming can induce health problems, mortality and shortened lifespan (e.g. Blier 2014). Nevertheless, further studies should evaluate thresholds-for-effect in wild populations, to unravel the limit at which negative changes occur in performance and fitness, potentially leading to ecological alterations. This is of

major importance, considering that shifts that compromise health in the wild are still largely unknown.

Table 5.2 Fold-changes induced by high temperature in oxidative stress biomarkers measured in several organs of *Sparus aurata* juveniles. CAT – catalase; GST – glutathione-S-transferase; SOD – superoxide dismutase; CYP1A – cytochrome 1A; LPO – lipid peroxidation. ↑ and ↓ indicate significant fold-changes in relation to controls (18ºC) – temperatures at which these fold-changes occurred are provided below the fold-change; ↗ indicates that the biomarker significantly increased in relation to other groups but not in relation to controls; - indicates no change; N/A non applicable (not performed); variable – there was some variation in biomarker levels but no clear up or down-regulation was detected.

	CAT	GST	SOD	CYP1A	LPO
Gills	↑8.5fold (34ºC)	↑2.4fold (30, 34ºC, CTmax)	↑2.6fold (CTmax)	N/A	Variable
Muscle	↑12.4fold (CTmax)	↑43.9fold (34ºC)	↗	N/A	↑17.7fold (34ºC)
Liver	↑2.9fold (34ºC)	↑3.2fold (34ºC)	↑1.9fold (34ºC)	↓4.9fold (28, 30, 32ºC)	↑3.3fold (36ºC)
Intestine	↑7.7fold (32ºC)	↑2.5fold (24ºC)	-	N/A	Variable
Brain	↑8.5fold (CTmax)	-	↓1.7fold (24, 34, CTmax)	N/A	↗

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CHAPTER 6. MOLECULAR PLASTICITY UNDER OCEAN WARMING: INTEGRATING PROTEOME CHANGES WITH ORGANISM-LEVEL INDICATORS UNVEILS THERMAL TOLERANCE OF FISH[1]

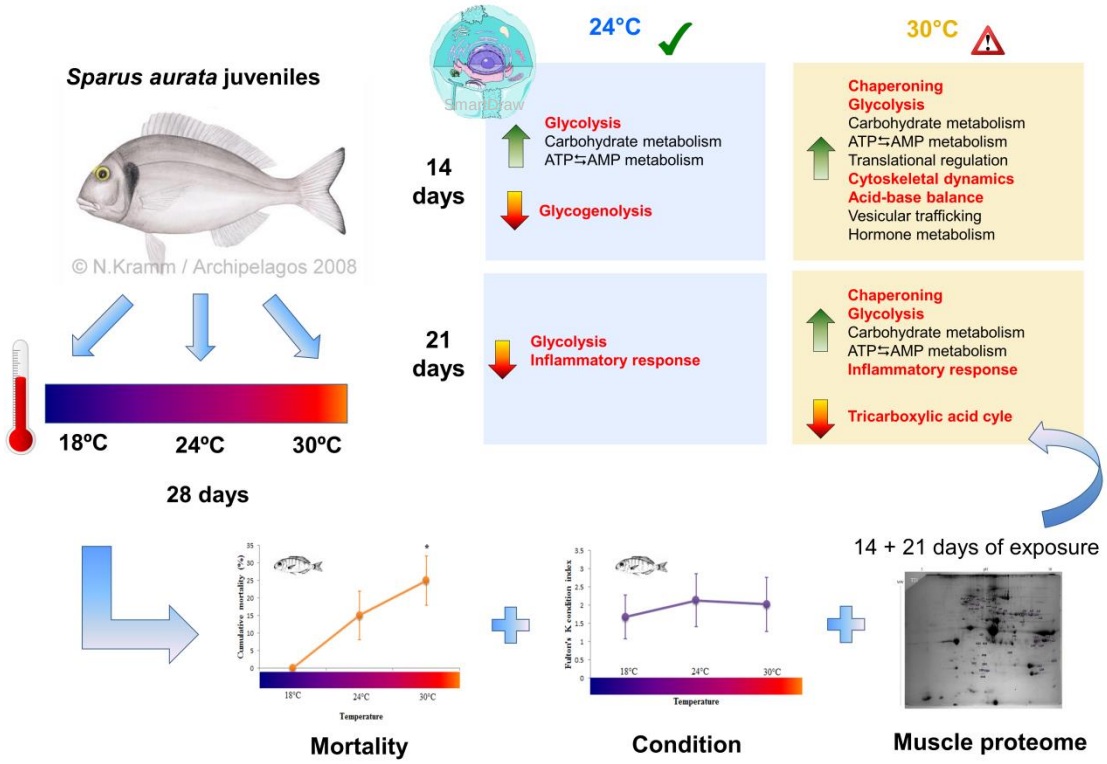
[1]Madeira D, Araújo JE, Vitorino R, Costa PM, Capelo JL, Vinagre C, Diniz MS. Submitted.

Abstract

Ocean warming is known to alter biodiversity patterns albeit the proteome underpinnings of organisms' thermotolerance are largely unknown. In this one-month experiment, we assessed the vulnerability of a key fisheries species, *Sparus aurata*, to ocean warming (control 18ºC, nursery ground temperature 24ºC and heat wave 30ºC). Fitness was impaired after 28 days, mainly due to increased mortality at 30ºC although fishes' condition was unaltered. Muscle proteome modulation was assessed at 14 and 21 days, showing that protein expression profiles were similar between fish exposed to 18ºC and 24ºC, differing from fish exposed to 30ºC. Fish subjected to 24ºC showed an enhanced glycolytic potential and decreased glycogenolysis mainly at 14 days of exposure. Fish exposed to 30ºC also showed enhanced glycolytic potential and up-regulated proteins related to gene expression, cellular stress response and homeostasis (mostly cytoskeletal dynamics, acid-base balance, chaperoning). However, inflammatory processes were elicited at 21 days along with a down-regulation of the tricarboxylic acid cycle. Thus, juvenile fish seem able to acclimate to 24ºC but possibly not to 30ºC, which is the predicted temperature for estuaries during heat waves by the year 2100. Consequently, recruitment of *S. aurata* may be in jeopardy, highlighting the need for improved management plans for fish stocks.

Key words: fish, proteomics, temperature, climate change, adaptation, phenotypic plasticity

Graphical abstract



1. Introduction

Climate change has been associated with ecological variation, including alterations in biodiversity, abundance and distribution of species, and phenology (Parmesan and Yohe, 2003; Poloczanska et al., 2013). Within Europe, the Iberian Peninsula and Mediterranean area have been considered regions with an elevated rate of warming, with a predicted increase between 4 and 7ºC in air temperature by 2100 (IPCC, 2001; Santos and Miranda, 2006). Under such scenario, Portuguese coastal waters and the Mediterranean Sea will be 2 to 3ºC and 3 to 4ºC warmer, respectively (Miranda et al., 2002; Fischer and Schär, 2010). The impacts of such temperature changes, coupled with the effects of other anthropogenic activities may exert strong influences on ecosystem services, affecting economic activities such as fisheries and aquaculture. Climate change is predicted to affect such activities not only through effects of temperature on species physiology but also through changes on fish farming practices (see Brander, 2007; Cochrane et al., 2009; Doubleday et al., 2013). Therefore, it is imperative to evaluate and understand biological responses of commercial fish species to the rise in temperature. This will allow researchers to identify the vulnerability of key species, and stakeholders to develop appropriate mitigation plans to reduce costs to society and environment (Poloczanska et al. 2013).

At a physiological level, temperature affects individual's metabolic rate, aerobic metabolism, performance, growth, reproduction, and survival with potential effects on population sustainability (Kordas et al., 2011; Dowd et al., 2015). Moreover, changes at the individual level are dependent upon the capacity to modulate gene expression under environmental variation (see Logan and Somero, 2011). Indeed, changes in cellular stress proteins (such as heat shock proteins and anti-oxidant enzymes) have been detected in marine organisms exposed to acute and chronic heat stress (e.g. Buckley and Hofmann, 2002; Tomanek, 2010; Madeira et al., 2013, 2014). Nevertheless, these studies have only focused on a few protein biomarkers. Additionally, research incorporating high throughput tools is highly skewed towards transcriptomics and therefore proteomics approaches are still scarce in marine biology. Proteomics examines the ultimate product of gene expression, i.e. proteins, which are the functional units mediating stressor-biological system interactions and its subsequent responses. Thus, it can revolutionize our understanding of adaptation processes and may be the basis of an integrative, systems biology approach (Karr, 2008), enabling a fast assessment of stress response networks activated by environmental cues. Currently, several pathways are known to be regulated by acute and chronic exposure to elevated temperature. When organisms are exposed to heat, hypoxaemia seems to set the first level of thermal limitation (Pörtner, 2010). Gradually, temperature will lead to other pervasive effects such as oxidative stress and protein damage. Accordingly, organisms usually modulate protein levels a few degrees above their acclimation temperature, in order induce a cellular stress response to protect cellular components from damage and/or meet potentially higher energetic demands caused by elevated metabolic rates. According to several 'omics' studies, modulated proteins play roles in chaperoning activity, energetic metabolism, oxidative stress metabolism, immune response,

cytoskeletal dynamics, transcriptional regulation, intracellular transport, protein synthesis and turnover, and signal transduction (see López et al., 2002; Gardeström et al., 2007; Stillman and Tagmount, 2009; Tomanek, 2014; Fields et al., 2012). By examining the full range of protein changes, the cells' phenotype can be assessed and directly related to the health and fitness of the organism and adaptation mechanisms (Dupont et al., 2007; Diz et al., 2012; Dalziel and Schulte, 2012). Consequently, predictions of cascading effects on populations can be improved, up-grading biogeographic forecasting, risk assessment and prediction of potential costs to society.

The aim of this study was to assess the vulnerability and acclimation capacity of a commercially important demersal fish species, *Sparus aurata*, to ocean warming and extreme events by i) unraveling proteome changes in the muscle of juvenile fish exposed to warming, ii) relating proteome plasticity to fitness measures (mortality and condition index) and iii) inferring on the possible ecological consequences of warming for *S. aurata* populations. Global capture production of *S. aurata* is 6,000-10,000 tonnes per year and global aquaculture production reached 73 062 tonnes in 2013 (FAO, 2015). Thus, this fish is used as proxy of high-importance sea bream species for fisheries and aquaculture, whose vulnerability towards climate change will have a direct impact on society. We hypothesize that fish exposed to chronic warming have higher energetic demands elicited by the need to maintain homeostasis and thus regulate a set of proteins involved in energetic and pro-survival pathways (e.g. protein turnover, glycolysis, cytoskeleton adjustments, and regulation of gene expression) providing cellular thermo-tolerance and viability, and preventing organismal death. Moreover, exposure to high temperatures may involve the down-regulation of proteins to reduce energy expenditure, especially at extreme temperatures. If fish can fully compensate temperature increases with proteome changes, then no decreases in condition and survival should be detected.

2. Methods

2.1 Ethical Statement

This study was approved by *Direcção Geral de Alimentação e Veterinária* and followed the recommendations of the Portuguese legislation for animal experimentation. Four authors have a level C certification by FELASA (Federation of European Laboratory Animal Science Associations).

2.2 Temperature data collection

Temperature data were obtained from several sources including 1) studies throughout Portuguese coastal waters and estuaries (Costa, 1990; Coutinho, 2003; Azevedo et al., 2006; Cabral et al., 2007; Madeira et al., 2012); 2) 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 to

2015); and 3) the Marine and Environmental Sciences Centre (MARE) database (data from several estuaries including Tagus' temperatures obtained from measurements carried out with thermometers and YSI loggers from 1978 to 2006).

2.3 Housing and husbandry of fish

Juvenile fish (n=36, mean±sd total length of 8.93±1.16 cm and 12.76±4.60 g weight) were obtained from a fish farm (MARESA, Spain) and transported to the laboratory in 100 L opaque polyvinyl tanks with constant aeration and stable temperature conditions (18.0±0.5ºC).

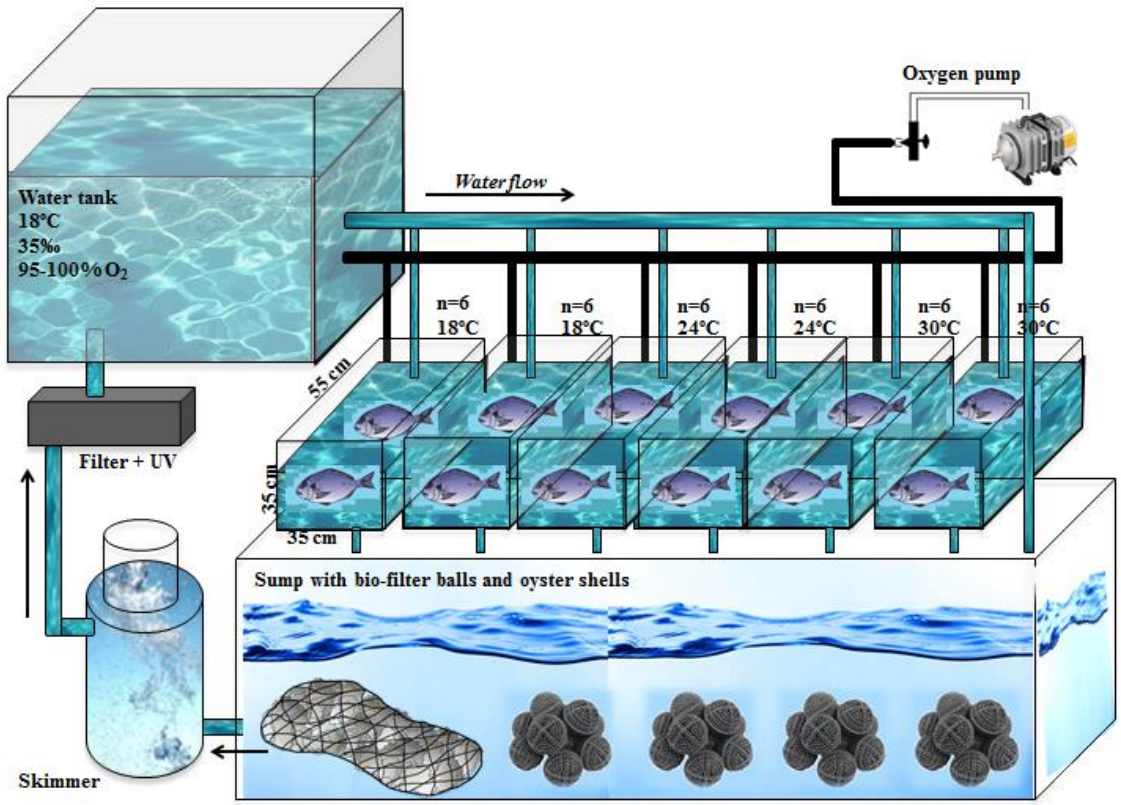
Fish were placed in a re-circulating system consisting of approximately 500 L white polyvinyl tanks (120 × 45 × 95 cm) (n=36 individuals in one tank). The tank was filled with clean and aerated sea water (95-100% O_2), with a constant temperature of 18.0±0.5°C, salinity 35‰ and pH 8.00±0.01 (same conditions of the fish farm). The fish acclimated for one week and their welfare was assessed (i.e. wounds or any disease symptoms). During the acclimation and experimental trial juveniles were fed with commercial food pellets once a day, containing cereals, fish protein, shrimp, amphipod, squid, refined soybean oil, fish oil, vitamins, vitamin C, essential element chelated organic materials, yeast extract, minerals and antioxidants (Aquasoja, Portugal) mixed with cyanobacterium *Spirulina* sp. (Tropical®, Poland).

2.4 Experimental setup

Following the acclimation period, fish were randomly transferred to experimental tanks (**Fig. 6.1a**). This system consisted of a re-circulating structure (total volume of 2,000 L) fitted with a skimmer plus biological, mechanical and UV filter. Six 70 L white polyvynil tanks (35 × 35 × 55 cm) with n=6 individuals.tank^{-1} composed the experimental system. Inflow of clean water in each tank was 300 mL.min^{-1}. All the tanks were filled with clean and aerated sea water (95-100% O_2), with a constant temperature of 18.0±0.5°C, salinity 35‰ and pH 8.00±0.01. All tanks were also provided with a filter (ELITE Underwater Mini-Filter Hagen, 220L.h^{-1}) to remove debris from feeding and excretion. Before the trial started, the temperature was gradually increased (during 2 days – 6ºC.day^{-1}) until the experimental temperatures were reached (control 18.0±0.5ºC; experimental temperatures 24.0±0.5ºC and 30.0±0.5ºC; n=2 tanks for each temperature). Temperatures were maintained using thermostats (TetraTec® HT 100, 100-150L, Tetra Werke, GmbH, Melle, Germany). The sampling scheme (**Fig. 6.1b**) consisted of euthanizing fish trough cervical transection at days 14, 21 and 28 (at 18ºC, 24ºC and 30ºC) for the collection of skeletal muscle. At each time point, four fish were randomly sampled at each temperature (two from each tank) and muscle was removed. Muscle tissue was chosen for several reasons, including 1) muscle proteome has been systematically characterized in *S. aurata* and is therefore well known in this species (Addis et al., 2010; Piovesana et al., 2016), 2) muscle is a highly energized tissue and its activity accounts for a great part of the organism's energy consumption, 3) muscular activity is crucial to dictate organismal functions such as locomotion and thus the capacity to escape unfavorable conditions and forage, determining the performance of the organism. Experiments were carried out in shaded day light (15L; 09D). To

keep environmental parameters constant throughout the experiment, a monitoring plan was employed. Temperature was monitored daily using a digital thermometer and salinity (≈35‰), pH (8.00±0.01), ammonia (≈0 $mg.L^{-1}$) and nitrites (≤0.3 $mg.L^{-1}$), were monitored twice a week using a hand-held 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), respectively. Total length and weight of all individuals were determined upon sampling.

(a)



(b)

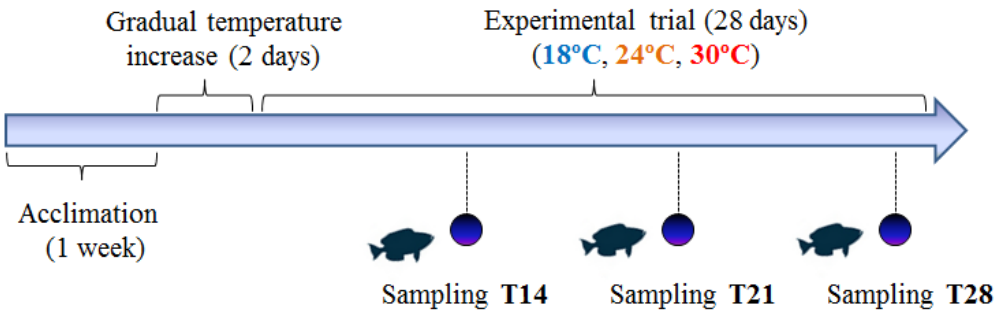


Figure 6.1 Experimental system and sampling, **a)** Experimental setup (not to scale). Re-circulating system (total of 2,000L) with six 70L white polyvinyl tanks (35 × 35 × 55 cm) for juveniles of *Sparus aurata* (n=6 individuals.$tank^{-1}$). Inflow of clean water in each tank was 300 $mL.min^{-1}$. All the tanks were filled with clean and aerated sea water (95-100% O_2), with a constant temperature of 18.0±0.5°C, 24.0±0.5°C and

30.0±0.5°C (n=2 tanks for each temperature). Salinity was kept at 35‰ and pH at 8.00±0.01. All tanks were provided with a filter (ELITE Underwater Mini-Filter Hagen, 220L.h^{-1}). **b)** Timeline and sampling scheme of the experiment. The fish were euthanized through cervical transection at day 14, 21 and 28 for collection of muscle. At each time point, four individuals were randomly sampled (2 from each tank). T – Timepoint in days (constructed using PowerPoint tools; fish icons by Shutterstock).

2.5 Fitness assessment

Mortality rates were calculated at the end of the experiment. Fulton's K condition index was calculated using the formula:

$K = 100\ M_t/L_t^3$ (1)

Where M_t is the total wet mass (mg) and L_t is the total length (mm) (Ricker, 1975). Briefly, this index assumes that heavier fish for a given length are in better condition.

2.5.1 Statistical analysis of fitness measures

Data was tested for normality (Shapiro-Wilk's test) and homoscedasticity (Levene's test). As Fulton's K data did not meet both assumptions, a non-parametric Kruskal-Wallis test was applied. Mortality data was analysed through Student's *t*-test (for independent samples, by groups), applying the Bonferroni correction. All analyses were carried out in Statistica v10 (StatSoft Inc., USA), using a significance level of 0.05.

2.6 Protein extraction

Muscle samples (approximately 200 mg) were homogenized in 1 mL of phosphate buffered saline (pH 7.4) to extract proteins, using a Tissue Master 125 homogenizer (Omni International, Kennesaw, USA) on ice-cold conditions. The crude homogenates were then centrifuged for 15 min at 10,000 × *g*, after which the clear supernatant was frozen immediately (-80ºC) until further analyses.

2.7 Proteomic analysis

2.7.1 Sample preparation

The homogenized and centrifuged samples (as previously described in section 'protein extraction') were precipitated through the DOC/TCA (Na-deoxycholate/trichloroacetic acid) method. Briefly, for each 100 µL of sample, 1 µL of 2% (w/v) DOC was added and samples were incubated 30 min on ice. Then, 18 µL of 100% (w/v) TCA was added to the mixture and microtubes were incubated overnight on ice. Following, samples were centrifuged at 14,000 ×*g* for 20 min at 4ºC. 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 4ºC). This washing step was performed twice. Subsequently, pellets were re-suspended 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 method of Bradford (1976), using a microplate spectrophotometer (model LT-4000, Labtech, United Kingdom).

2.7.2 Two Dimensional Gel electrophoresis (2-DE)

Samples containing 200 µg of muscle protein were loaded onto IPG strips (pH 3-10, 7cm, Bio-Rad) for separation according to their isoelectric point (pI). IPG strips had been previously rehydrated overnight with 7 M urea, 2 M 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 for protein reduction (6 M urea, 75 mM Tris-HCl, 20 % v/v glycerol, 2 % w/v SDS – sodium dodecyl sulphate, 0.002 % bromophenol blue, 2 % w/v DTT) for 15 min with continuous shaking, and then equilibration buffer II for protein alkylation (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 sulphate, 10 % w/v orthophosphoric acid, 20 % methanol). Following, gels were de-stained with milli-Q grade water in several washes. Two gels were carried out for each individual to ensure gel reproducibility.

2.7.3 Gel image analysis

Gel imaging was carried out with the PropicII-robot (Genomic Solutions™, Cambridgeshire, UK) and digitalized images of the gels were analysed with Progenesis SameSpots software (version 4.0, NonLinear Dynamics, Totallab, UK). Two image analyses were carried out, one for each time point, comparing 18, 24 and 30ºC at 14 days of exposure (T14) and then at 21 days of exposure (T21). A master gel was automatically defined in each analysis 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 compared treatments via a one-way analysis of variance to detect proteins that were differentially expressed. Following, Tukey's post-hocs (annex 3, **supplementary Table S6.1**) were carried out in StatPages.net (http://statpages.org/anova1sm.html) with a significance level of 0.05. These analyses were performed using mean±sd of log normalized volumes retrieved from SameSpots.

2.7.4 Protein digestion

The spots of interest were manually excised from gels and transferred to 0.5 mL Lo-bind tubes. Gel spots were washed with water and de-stained twice with 50% acetonitrile/ 25 mM Ambic (ammonium bicarbonate) and then dehydrated with 100 % acetonitrile. Posteriorly, 15 µL of trypsin (Sigma-Aldrich, USA) (0.02 $\mu g.\mu L^{-1}$ in Ambic 12.5 mM / 2% acetonitrile) was added to the gel spots and incubated for 60 min on ice. 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 37ºC. 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 37ºC. The supernatants were collected once again to the tubes and 25 µL of 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 -20ºC until MS and MS/MS analyses.

2.7.5 Tandem mass spectrometry

Tryptic peptides were ressuspended 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 samples (0.5µL) 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.

2.7.6 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. Either NCBI *Sparus aurata* database or Swiss-Prot nonredundant protein sequence database (October 2014) under the taxonomy Chordata were used for searches. 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. Positive identifications were accepted up to 95% of confidence level - MASCOT score >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. Highest MASCOT score and peptide representativity within prospected taxa were taken as main matching criteria.

2.7.7 Cluster analysis and pathway network

A two-way hierarchical clustering analysis was carried out using Cluster 3.0 plus Java TreeView. Normalized (spot volume/total spot volume of all proteins in a gel image) average spot volumes were used for this analysis, which followed the criteria i) adjustments to data: log transformation, center genes (mean), normalize genes (row-wise standardized expression values) ii) hierarchical analysis: cluster genes and arrays, iii) similarity metrics: correlation (uncentered) for genes and Euclidean distance for arrays, iv) clustering method: complete linkage. Moreover, a Venn diagram was constructed (using Venny 2.0, http://bioinfogp.cnb.csic.es/tools/venny/index.html) to illustrate shared and exclusively regulated proteins between fish collected at 24ºC and 30ºC after 14 and 21 days of exposure to warming. A protein network analysis was also performed using ClueGo+CluePedia 2.1.7 plugin (from Cytoscape v3.2 platform), to depict interactions between the differentially expressed proteins after 14 and 21 days of exposure to high temperatures. The analysis was performed using the following criteria: species: *Danio rerio* (phylogenetic proximity to *Sparus aurata*); biological process ontology release date 07092015; GO Tree interval from 3 to 8; GO term pathway selection includes at least 1 gene *per* cluster; kappa score 0.4.; significant pathways (p 0.05 to < 0.0005 was considered).

2.7.8 Categorization of identified proteins into functional classes

In order to assess the general categories of gene ontology, identified proteins were characterized using STRAP v1.5 according to biological process, cellular component and molecular function. In addition, to assess the proteins' function in detail, an independent search was carried out using gene ontology tools (i.e. UniProt, GeneCards, neXtprot beta, InterPro and Qiagen – Gene Globe Pathways).

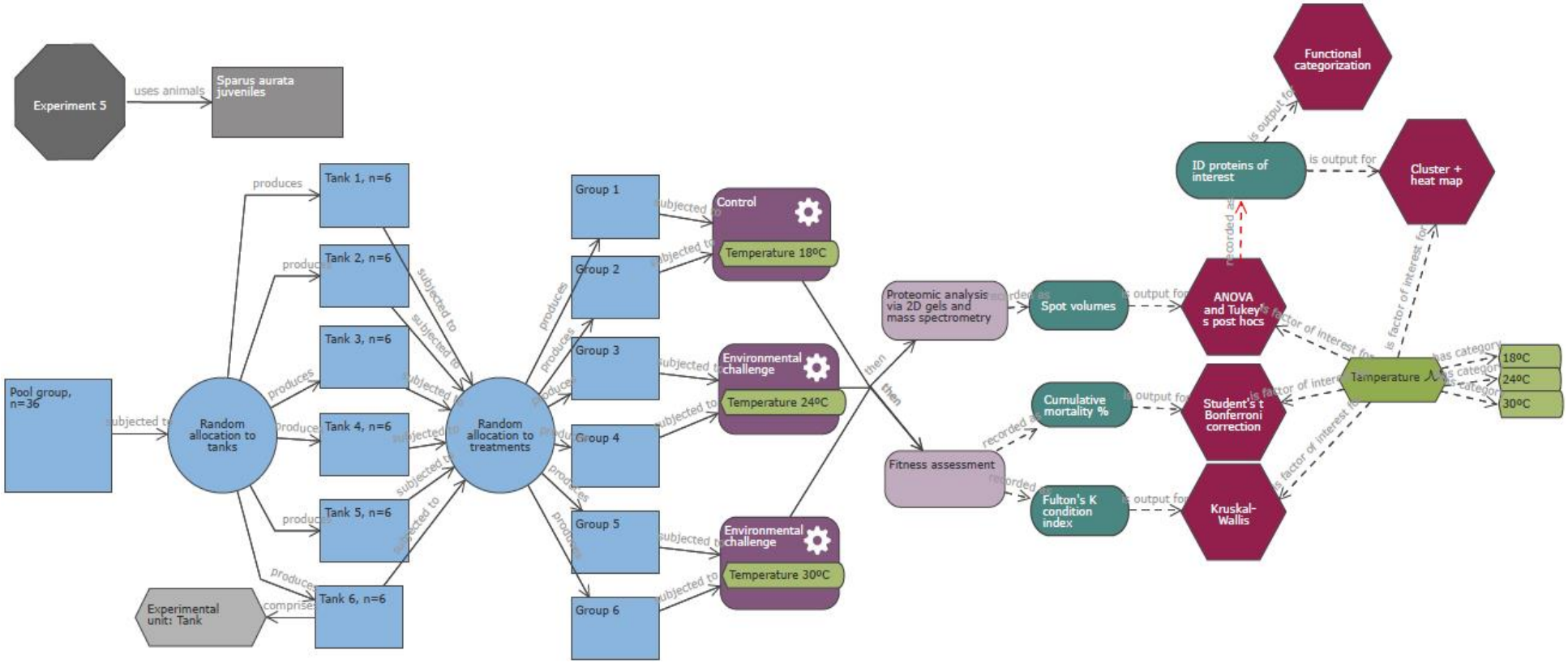


Figure 6.2 Experimental approach (diagram constructed using Experimental Design Assistant, https://eda.nc3rs.org.uk/).

3. Results

3.1 Temperature data

Water temperatures in the Portuguese coastal area range approximately from 10-16ºC during the winter and 15 to 20ºC during the summer. Temperatures are usually lowest in February (average of 13.9ºC, minimum of 12.2ºC and maximum of 15.2ºC taking into account the last 5 years) and highest in September (average of 19.3ºC, minimum of 17.2ºC and maximum of 21.8ºC taking into account the last 5 years). Coastal water temperatures predicted for 2100 (based on a 2-3ºC increase – Miranda et al., 2002) are in the range of 13-19ºC and 18-23ºC during the winter and summer, respectively. Nevertheless, such temperatures can also occur in present days for a limited period of time (days or weeks) during extremely hot summers, for instance the summer of 2010 (see Madeira et al., 2012a). Estuarine temperatures range approximately from 10-12ºC during the winter to 20-24ºC during summer. During heat waves (at least five days in a row with a maximum air temperature of ≥ 35ºC – Santos and Miranda, 2006), estuaries can reach maximum temperatures between 25 and 28ºC, and persisting for over 2 weeks (e.g. see Costa, 1990; Azevedo et al., 2006; Cabral et al., 2007; Madeira et al., 2012a). Following the scenario of a 2-3ºC increase in Portuguese waters by 2100 (Miranda et al., 2002), estuaries' mean temperature during summer would be in the range of 22-27ºC, reaching 30ºC during heat waves.

3.2 Fitness assessment

3.2.1 Mortality rates and Fulton's K condition index

At the end of the experimental period no mortality was registered for *S. aurata* juveniles exposed to 18ºC. However, a cumulative mortality of 15±7% was observed for fish exposed to 24ºC and 25±7% for fish exposed to 30ºC (**Fig. 6.3a**). No significant differences were detected between cumulative mortalities at 18ºC and 24ºC (t=-3; df=2; p=0.095). Nevertheless, juveniles exposed to 24ºC started dying after 26 days of exposure whereas juveniles exposed to 30ºC started dying after 25 days of exposure. Statistical analysis detected significant differences between cumulative mortality at 18ºC and 30ºC (t=-5; df=2; p=0.037). Due to mortality rates, proteomic analyses were only carried out for the first two sampling points: 14 and 21 days of exposure. No analyses were carried out at 28 days of exposure due to reduced sample size. Following the same rationale, the Fulton's K condition index was calculated after 21 days of exposure. This index did not vary between fish exposed to 18, 24 and 30ºC (Kruskal-Wallis, H=1.28, p=0.526) (**Fig. 6.3b**).

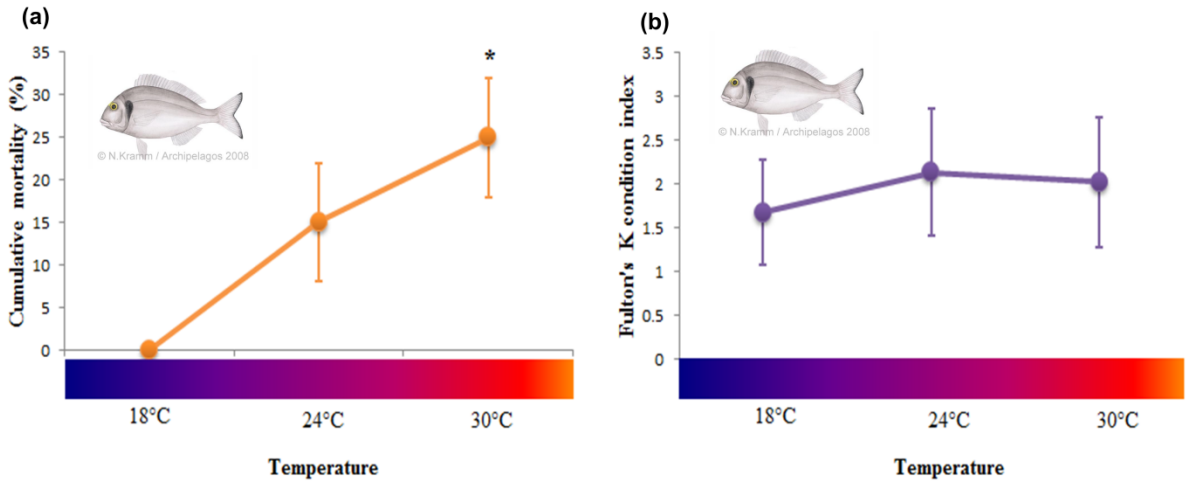
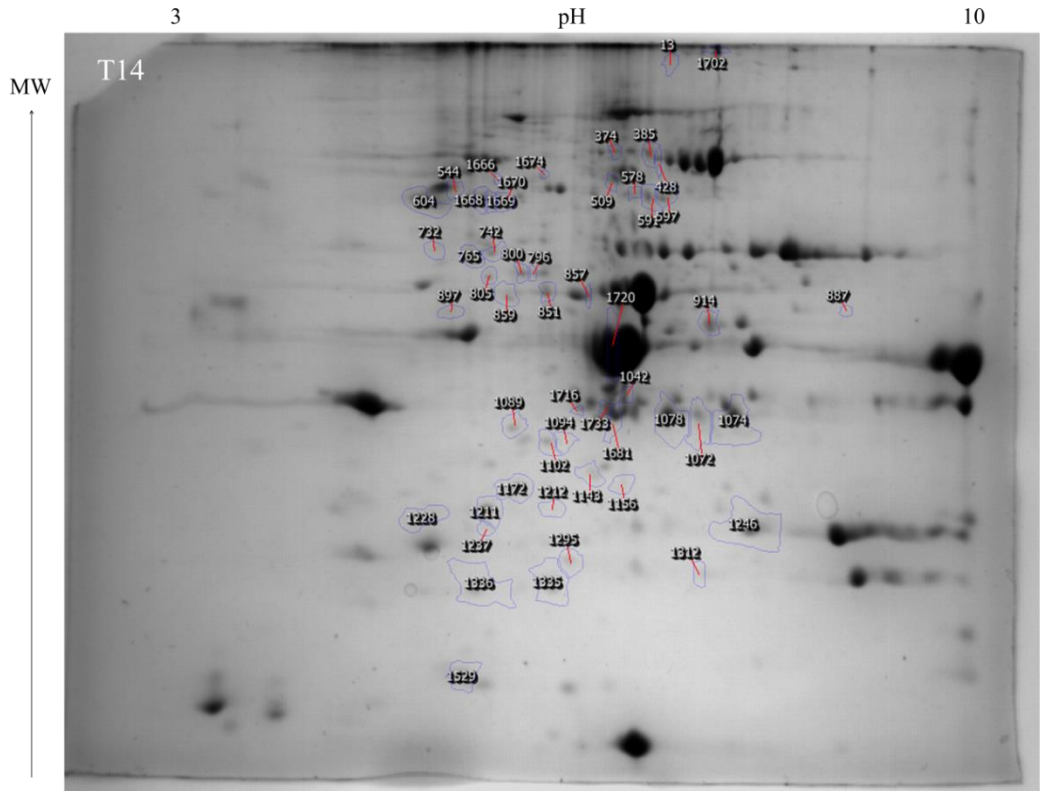


Figure 6.3 Fitness assessments of *Sparus aurata* juveniles exposed to 18ºC, 24ºC and 30ºC **(a)** Cumulative mortalities after 28 days of exposure. Data (mean±SD) were analysed through Student's *t* tests, applying Bonferroni correction (significance level of 0.05). Asterisks mark significant differences from the control group (18ºC); **(b)** physiological condition (measured through Fulton's K condition index, mean±SD) of juvenile *Sparus aurata* after 21 days of exposure to 18, 24 and 30ºC. Data was compared via a Kruskal-Wallis test (significance level of 0.05). No significance differences were detected for Fulton's K condition index among treatments. Species drawings by N. Kramm, Archipelagos Wildlife Library.

3.3 Proteome modulation

After 14 days of exposure to 18, 24 and 30ºC, the ANOVA showed that 52 spots were differentially expressed between temperature groups ($p<0.05$) (**Fig. 6.4a**, and **supplementary Table S6.1** and **S6.2**, annex 3), of which 21 were successfully identified (40.4%) (**Table 6.1**, and **supplementary Table S6.3**, annex 3). After 21 days of exposure, 42 spots were differentially expressed and of these, 14 were successfully identified (33.3%) (**Fig. 6.4b, Table 6.2** and **supplementary Table S6.1, S6.3** and **S6.4**, annex 3).

(a)



(b)

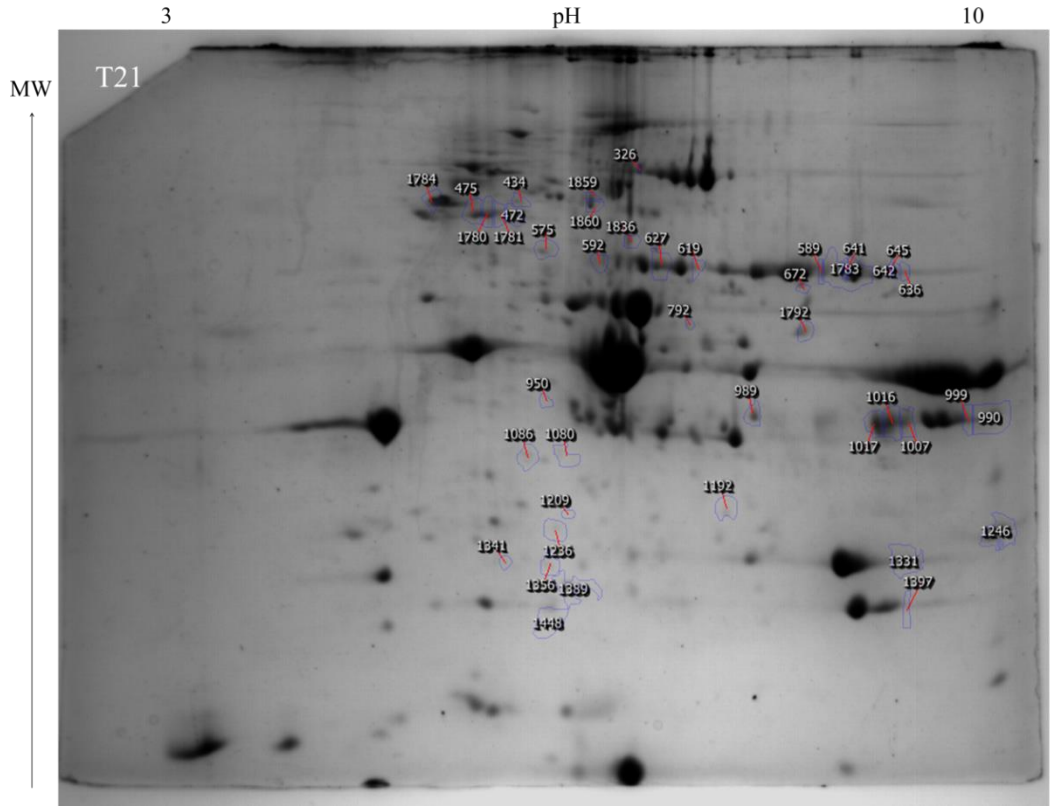


Figure 6.4 Representative image of the master gel depicting the protein spots detected in *Sparus aurata* juveniles. Annotated spots were those that were differentially expressed between temperature groups (18, 24 and 30ºC; n=4 individuals in each group, 2 per tank; 2 technical replicates; ANOVA $p<0.05$) **(a)** time-point 14 days of exposure (T14) and (**b)** time-point 21 days of exposure (T21).

Table 6.1 Spots differentially expressed between temperature treatments at 14 days of exposure in the muscle of juvenile *Sparus aurata.*

Spot no.	Protein Name	*Species*	Accession number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
859	Rab GDP dissociation inhibitor beta	*Canis familiaris*	GDIB_CANFA	50289.74	6.11	2	111	100
1529	Neuroendocrine convertase 1	*Homo sapiens*	NEC1_HUMAN	84099.28	5.66	5	64	96.67
851	Alpha-enolase	*Xenopus laevis*	ENOA_XENLA	47474.28	5.92	12	159	100
1733	Creatine kinase M-type	*Bos taurus*	KCRM_BOVIN	42961.82	6.63	7	132	100
1246	Triosephosphate isomerase B	*Danio rerio*	TPISB_DANRE	26810.80	6.45	8	138	100
1042	Creatine kinase M-type	*Homo sapiens*	KCRM_HUMAN	43073.89	6.77	5	78.9	99.89
1720	Creatine kinase M-type	*Homo sapiens*	KCRM_HUMAN	43073.89	6.77	10	213	100
1716	Creatine kinase M-type	*Sus scrofa*	KCRM_PIG	43031.85	6.61	14	78.1	99.87
1670	Heat shock 70 kDa protein 1	*Oryzias latipes*	HSP71_ORYLA	70307.01	5.47	16	370	100
1668	Heat shock cognate 71	*Cricetulus griseus*	HSP7C_CRIGR	70761.12	5.24	3	87.6	99.98

	kDa protein							
1312	Adenylate kinase isoenzyme 1	*Cyprinus carpio*	KAD1_CYPCA	21475.34	6.64	2	90.4	99.99
1681	Creatine kinase M-type	*Canis familiaris*	KCRM_CANFA	43125.89	6.77	5	126	100
1295	Adenylate kinase isoenzyme 1	*Cyprinus carpio*	KAD1_CYPCA	21475.34	6.64	4	88.5	99.98
857	Alpha-enolase	*Pongo abelii*	ENOA_PONAB	47167.36	7.57	10	108	100
1674	Actin, alpha cardiac muscle 2	*Xenopus tropicalis*	ACT2_XENTR	42005.89	5.23	9	115	100
428	Glycogen phosphorylase, brain form	*Ovis aries*	PYGB_SHEEP	96253.39	6.57	10	93.9	99.99
897	Eukaryotic initiation factor 4A-II	*Macaca fascicularis*	IF4A2_MACFA	46387.79	5.4	12	146	100
509	Creatine kinase M-type	*Homo sapiens*	KCRM_HUMAN	43073.89	6.77	8	156	100
1143	Creatine kinase, testis isozyme	*Oncorhynchus mykiss*	KCRT_ONCMY	42976.76	6.2	3	57.5	85.14

1211	Carbonic anhydrase 1	*Chionodraco hamatus*	CAH1_CHIHA	28324.92	5.58	2	106	100
1669	Heat shock 70 kDa protein 1	*Oryzias latipes*	HSP71_ORYLA	70307.01	5.47	11	149	100

Table 6.2 Spots differentially expressed between temperature treatments at 21 days of exposure in the muscle of juvenile *Sparus aurata*.

Spot no.	Protein Name	Species	Accession number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
1781	Heat shock 70kDa protein 1	*Oryzias latipes*	HSP71_ORYLA	70307.00	5.47	9	171	100
1389	Adenylate kinase isoenzyme 1	*Cyprinus carpio*	KAD1_CYPCA	21475.34	6.64	2	91.8	99.99
475	Heat shock cognate 71 kDa protein	*Ictalurus punctatus*	HSP7C_ICTPU	71296.14	5.19	12	148	100
1331	Triosephosphate isomerase (Fragments)	*Mesocricetus auratus*	TPIS_MESAU	20294.37	5.49	1	83.5	99.96
1017	Glyceraldehyde-3-phosphate dehydrogenase	*Danio rerio*	G3P_DANRE	35761.30	8.2	3	62.4	95.19
989	Glyceraldehyde-3-phosphate dehydrogenase (Fragment)	*Meleagris gallopavo*	G3P_MELGA	24836.69	7.22	3	123	100
1086	Sterile alpha motif domain-containing protein 9-like	*Homo sapiens*	SAM9L_HUMAN	184415.39	8.25	8	67.4	98.48

999	Glyceraldehyde-3-phosphate dehydrogenase	*Danio rerio*	G3P_DANRE	35761.30	8.2	2	163	100
1007	Glyceraldehyde-3-phosphate dehydrogenase	*Danio rerio*	G3P_DANRE	35761.30	8.2	2	146	100
1016	Glyceraldehyde-3-phosphate dehydrogenase	*Danio rerio*	G3P_DANRE	35761.30	8.2	2	70.4	99.24
1780	Heat shock 70 kDa protein	*Oncorhynchus tshawytscha*	HSP70_ONCTS	70931.89	5.42	6	118	100
1397	Adenylate kinase isoenzyme 1	*Cyprinus carpio*	KAD1_CYPCA	21475.34	6.64	2	108	100
1783	Glucose-6-phosphate isomerase	*Bos taurus*	G6PI_BOVIN	62815.04	7.33	5	75.8	99.78
672	Glutamate dehydrogenase, mitochondrial	*Chaenocephalus aceratus*	DHE3_CHAAC	55359.09	7.34	2	84.4	99.97

At both time time-points, juvenile fish had similar expression profiles at 18ºC and 24ºC, while such expression noticeably changed at 30ºC (**Fig. 6.5a, b**). This pattern was mainly linked to cluster 1 at both time-points, in which the main biological processes identified were energy metabolism and chaperoning (common to both time-points) and cytoskeletal dynamics, peptide hormone metabolism, vesicular trafficking and CO_2 transport (only at T14).

At both T14 and T21, the significant changes observed between 18ºC and 24ºC were related to energetic metabolism. While at T14, there was an up-regulation of triose phosphate isomerase and a down-regulation of glycogen phosphorylase (**Fig. 6.5a**; see supplementary **Table S6.1** for Tukey's post hocs in annex 3), at T21 there was a down-regulation of glucose-6-phosphate isomerase and one isoform of adenylate kinase. Moreover, at T21, a protein related to inflammatory processes was down-regulated from 18ºC to 24ºC (sterile alpha motif domain-containing protein 9-like). At 30ºC all of these proteins were significantly up-regulated with the exception of glycogen phosphorylase which returned to control levels (**Fig 6.5**; see supplementary **Table S6.1** for Tukey's post hocs in annex 3).

Moreover, the other proteins involved in energy balance metabolism were up-regulated at 30ºC at both T14 and T21, with the exception of one isoform of creatine kinase at T14 and glutamate dehydrogenase at T21, which were down-regulated. At 30ºC, proteins involved in chaperoning (both T14 and T21), CO_2 transport, cytoskeletal dynamics, peptide hormone metabolism and vesicular trafficking (T14) were also up-regulated (in comparison to 18ºC and/or 24ºC) (**Fig. 6.5a,b**). Proteins involved in translational regulation were also detected at T14, mostly increasing from 24ºC to 30ºC.

In summary, the majority of proteins regulated after 14 and 21 days of exposure to warming were related to energetic metabolism (61% at T14 and 72% at T21) and chaperoning (14% at T14 and 21% at T21), while other categories were less represented (**Fig. 6.5c**, supplementary **Table S6.5** and **S6.6** in annex 3). The Venn diagram showed that proteins common to both temperatures were triosephosphate isomerase, adenylate kinase 1 (up-regulated at 24 and 30ºC), sterile alpha motif domain containing protein 9-like and glucose 6 phosphate isomerase (down-regulated at 24ºC and up-regulated at 30ºC) whereas proteins common to both time-points were heat shock 70kDa protein 1, heat shock cognate 71kDa protein, adenylate kinase isoenzyme 1 and triosephosphate isomerase (which were all up-regulated at 30ºC at both exposure times) (**Fig. 6.6a**).

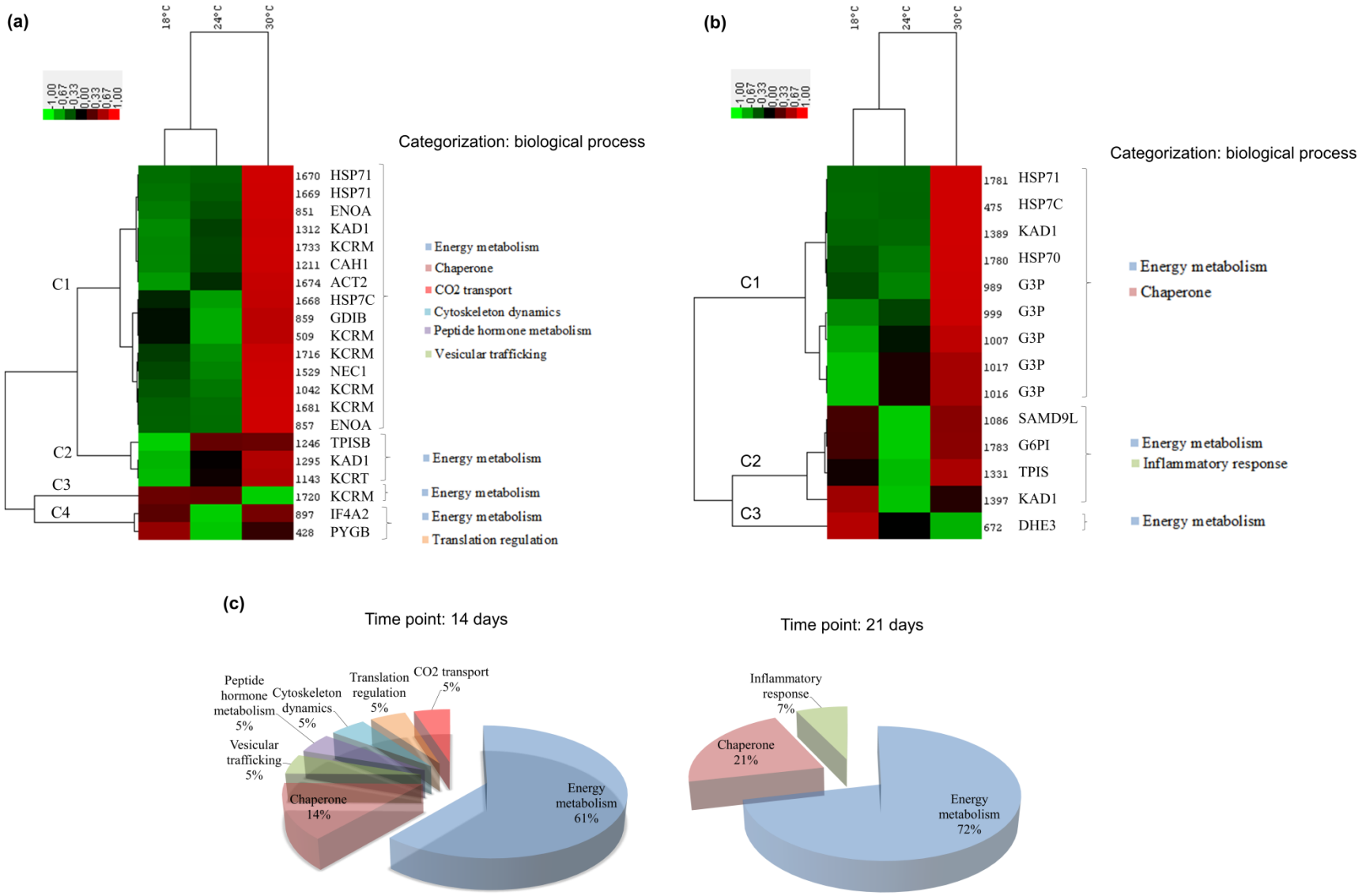
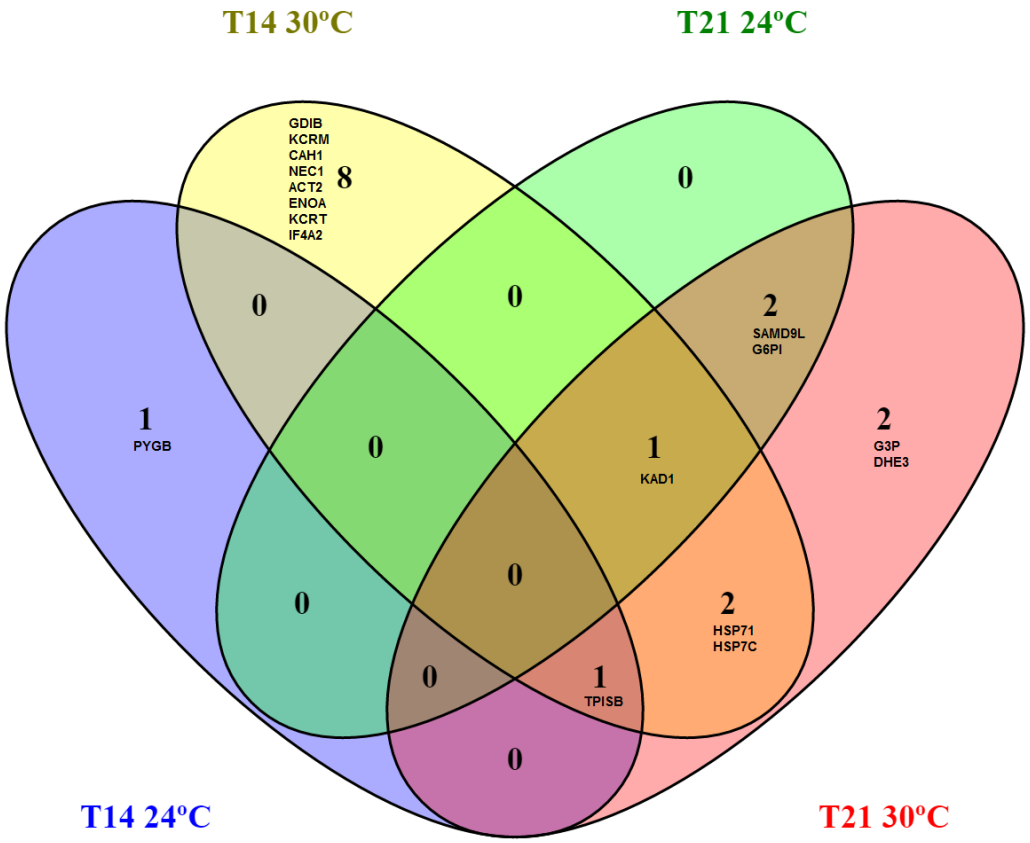

(a)
18°C
24°C
30°C
-1,00
-0,67
-0,33
0,00
0,33
0,67
1,00
C1
C2
C3
C4
1670 HSP71
1669 HSP71
851 ENOA
1312 KAD1
1733 KCRM
1211 CAH1
1674 ACT2
1668 HSP7C
859 GDIB
509 KCRM
1716 KCRM
1529 NEC1
1042 KCRM
1681 KCRM
857 ENOA
1246 TPISB
1295 KAD1
1143 KCRT
1720 KCRM
897 IF4A2
428 PYGB
Categorization: biological process
Energy metabolism
Chaperone
CO2 transport
Cytoskeleton dynamics
Peptide hormone metabolism
Vesicular trafficking
Energy metabolism
Energy metabolism
Energy metabolism
Translation regulation
(b)
18°C
24°C
30°C
-1,00
-0,67
-0,33
0,00
0,33
0,67
1,00
C1
C2
C3
1781 HSP71
475 HSP7C
1389 KAD1
1780 HSP70
989 G3P
999 G3P
1007 G3P
1017 G3P
1016 G3P
1086 SAMD9L
1783 G6PI
1331 TPIS
1397 KAD1
672 DHE3
Categorization: biological process
Energy metabolism
Chaperone
Energy metabolism
Inflammatory response
Energy metabolism
(c)
Time point: 14 days
Peptide hormone metabolism 5%
Cytoskeleton dynamics 5%
Translation regulation 5%
CO2 transport 5%
Vesicular trafficking 5%
Chaperone 14%
Energy metabolism 61%
Time point: 21 days
Inflammatory response 7%
Chaperone 21%
Energy metabolism 72%

Figure 6.5 Two-way hierarchical clustering analysis and functional categorization of proteome data from *Sparus aurata* juveniles subjected to 18ºC, 24ºC and 30ºC. Heat map representation of the clustered data matrix in which cells denote the Log2 values of protein normalized volumes. The color scale ranges from green (low expression) to red (high expression). Columns represent different temperatures while rows represent different proteins. Biological functions were listed for proteins in each cluster. **(a)** Time-point: 14 days of exposure. Two proteins were significantly regulated at 24ºC i.e. increase in triose phosphate isomerase (TPISB) and a decrease in glycogen phosphorylase (PYGB). At 30ºC, all identified proteins were significantly up-regulated when compared to 18ºC and/or 24ºC with the exception of glycogen phosphorylase, which returned to control levels and one isoform of creatine kinase, which was significantly down-regulated at 30ºC (see supplementaryTable S6.1 for Tukey's post-hocs). **(b)** Time-point: 21 days of exposure. Three proteins showed significant differences in expression levels between 18ºC and 24ºC (decrease of glucose-6-phosphate isomerase – G6PI – and one isoform of adenylate kinase – KAD1) and inflammatory response (decrease of sterile alpha motif domain-containing protein 9-like – SAMD9L). At 30ºC, these proteins were significantly up-regulated in comparison to 24ºC. Additionally, all of the other identified proteins were significantly up-regulated at 30ºC, when compared to 18ºC and/or 24ºC (see supplementary Table S6.1 for Tukey's post hocs). HSP71 - Heat shock 70 kDa protein 1; ENOA - Alpha-enolase; KAD1 - Adenylate kinase isoenzyme 1; KCRM - Creatine kinase M-type; CAH1 - Carbonic anhydrase 1; ACT2 - Actin, alpha cardiac muscle 2; HSP7C - Heat shock cognate 71 kDa protein; GDIB - Rab GDP dissociation inhibitor beta; NEC1 - Neuroendocrine convertase 1; TPISB - Triosephosphate isomerase B; KCRT - Creatine kinase, testis isozyme; IF4A2 - Eukaryotic initiation factor 4A-II; PYGB - Glycogen phosphorylase; HSP70 - Heat shock 70 kDa protein; G3P - Glyceraldehyde-3-phosphate dehydrogenase; SAMD9L - Sterile alpha motif domain-containing protein 9-like; G6PI - Glucose-6-phosphate isomerase; TPIS - Triosephosphate isomerase (Fragments); DHE3 - Glutamate dehydrogenase, mitochondrial. **(c)** Comparison between 14 and 21 days of exposure in terms of percent distribution of proteins into functional classes (at 14 days: total of 21 proteins; at 21 days: total of 14 proteins).

From the comparative analysis of the impact of 14 and 21 days of exposure, a predominance of biological processes related to guanosine tetraphosphate metabolic process, response to hypotonic stress and carbon dioxide transport was verified at T14 (red nodes, **Fig. 6.6b**) whereas gluconeogenesis and cellular carbohydrate biosynthetic process were more prevalent at T21 (green nodes, **Fig. 6.6b**). Carbonic anhydrase was the protein associated with response to hypotonic stress in the network analysis. Research evaluating the influence of hyperthermia in CO_2 transport in fish is scarce as opposed to the effect of other environmental factors such as salinity and pollutants. Therefore, as the network analysis is particularly reliant on the existing database for the freshwater model fish *Danio rerio*, a connection between carbonic anhydrase and salinity stress was expected.

For general functional categories regarding biological process, molecular function and cellular component see supplementary **Fig. S6.1** in annex 3.

(a)


T14 30ºC
T21 24ºC
GDIB
KCRM
CAH1
NEC1
ACT2
ENOA
KCRT
IF4A2
8
0
0
0
2
SAMD9L
G6PI
1
PYGB
0
1
KAD1
2
G3P
DHE3
0
0
2
HSP71
HSP7C
0
1
TPISB
0
T14 24ºC
T21 30ºC

(b)

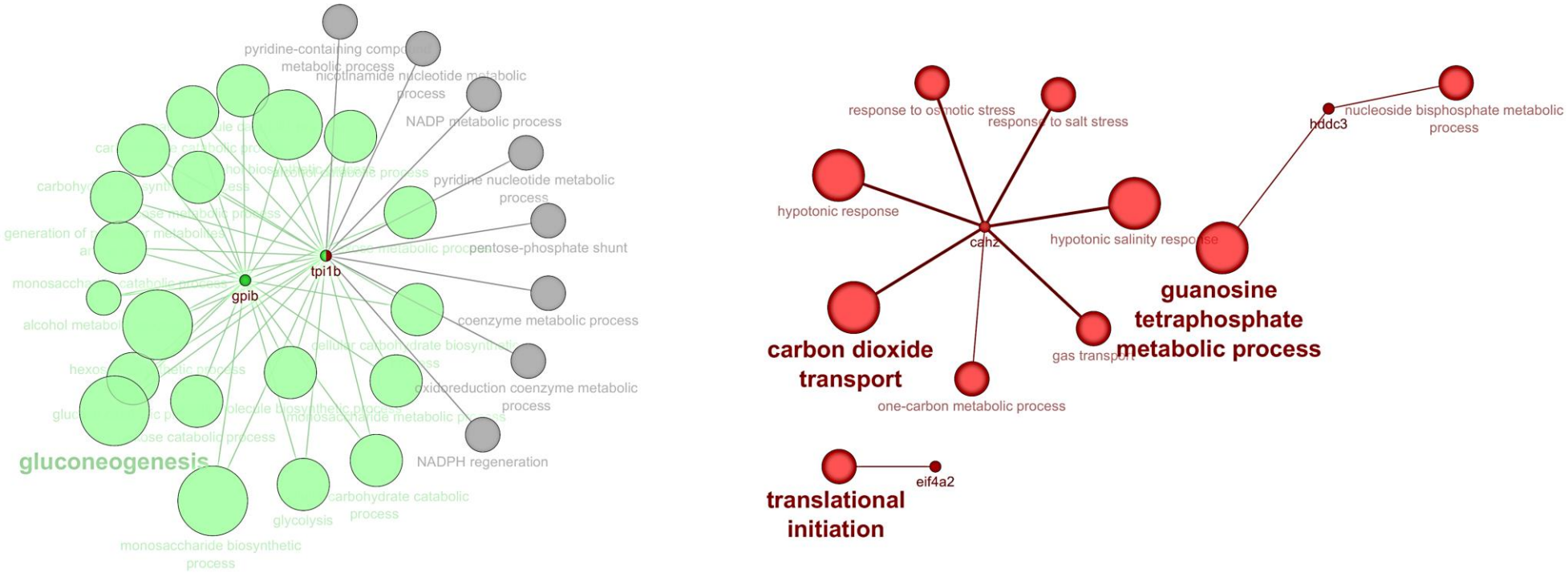


Figure 6.6 (a) Venn diagram showing shared and exclusively regulated proteins among temperatures and exposure times (14 days – T14, and 21 days – T21). Proteins common to both temperatures were TPISB, KAD1 (up-regulated at 24 and 30ºC), SAMD9L and G6PI (down-regulated at 24ºC and up-regulated at 30ºC). Proteins shared between exposure times were HSP71, HSP7C, KAD1and TPISB (which were all up-regulated at 30ºC at both exposure times). NOTE: All redundancies were eliminated in this analysis. HSP71 - Heat shock 70 kDa protein 1; ENOA - Alpha-enolase; KAD1 - Adenylate kinase isoenzyme 1; KCRM - Creatine kinase M-type; CAH1 - Carbonic anhydrase 1; ACT2 - Actin, alpha cardiac muscle 2; HSP7C - Heat shock cognate 71 kDa protein; GDIB - Rab GDP dissociation inhibitor beta; NEC1 - Neuroendocrine convertase 1; TPISB - Triosephosphate isomerase B; KCRT - Creatine kinase, testis isozyme; IF4A2 - Eukaryotic initiation factor 4A-II; PYGB - Glycogen phosphorylase; HSP70 - Heat shock 70 kDa protein; G3P - Glyceraldehyde-3-phosphate dehydrogenase; SAMD9L - Sterile alpha motif domain-containing protein 9-like; G6PI - Glucose-6-phosphate isomerase; TPIS - Triosephosphate isomerase (Fragments); DHE3 - Glutamate dehydrogenase, mitochondrial. **(b)** Protein network analysis carried out using ClueGo+CluePedia 2.1.7 plugin, to depict interactions between the differentially expressed proteins after 14 and 21 days of exposure to high temperatures (species: *Danio rerio*; biological process ontology release date 07092015; GO Tree interval from 3 to 8; GO term pathway selection includes at least 1 gene *per* cluster; kappa score 0.4; significant pathways (p 0.05 to < 0.0005 were considered). Red nodes represent proteins unique to T14 and green nodes represent proteins unique to T21. Grey nodes represent shared proteins between T14 and T21. Node size relates to significance and number of genes associated to that biological process.

4. Discussion

In this study, *Sparus aurata* juveniles modulated muscle protein levels under chronic exposure to elevated temperatures. Overall, most changes occurred in fish exposed to 30ºC while fish exposed to 24ºC only regulated a couple of proteins, mostly related to energy metabolism. These results coupled to a lack of a cellular stress response at 24ºC suggest that this temperature is not very stressful to juvenile fish; this may be explained by the fact that juveniles inhabit low depth waters (i.e. estuaries and coastal lagoons) that can reach such temperatures quite often in the summer (Froese and Pauly, 2006; Madeira et al., 2012). However, 30ºC was sufficiently warm to elicit the cellular stress response, indicated by the up-regulation of chaperones (heat shock proteins) and cytoskeletal re-arrangements, corroborating other acute and chronic experiments performed in aquatic animals (including *S. aurata*) (Feidantsis et al., 2009; Madeira et al., 2014; Tomalty et al., 2015; Jayasundara et al., 2015).

Chaperones are cytoprotective proteins that stabilize denatured proteins and have a selective value (Hofmann 2005) since they confer stress tolerance and delay thermal injury (Horowitz, 2001; Bahrndorff et al., 2009; Basu et al., 2002; Feder, 1996; Sorte and Hofmann, 2005). Moreover, several authors have reported a link between molecular chaperones and cytoskeletal components (Liang and MacRae, 1997; Mounier and Arrigo, 2002; Tomanek and Zuzow, 2010), suggesting that cytoskeletal proteins are very sensitive to temperature and chaperones may therefore play a decisive role on their modulation, function, and protection. In fact, cytoskeleton re-modelling may be essential to stabilize cytoskeletal components and maintain homeostatic balance upon challenging conditions (Buckley et al., 2006; Garlick and Robertson, 2007). This may be crucial not only to preserve cellular activities related to motility, signaling and organelle movement but also to adjust ATP turn-over rates (see Garland et al., 2015) and maintain muscle contraction.

Besides up-regulating the cellular stress response, fish exposed to 30ºC had significantly higher mortality rates. Increased mortality induced by temperature has also been observed in larval stages of *Sparus aurata* (Madeira et al., unpublished) and throughout the life cycle of other fish species (e.g. Houde, 1989; Hinch and Martins, 2011). However, thermotolerance, survival and the potential for acclimation of each individual may depend on several factors such as thermal history, sex, nutritional condition, diseases, age and reproductive status (e.g. Becker and Genoway, 1979; Lutterschmidt and Hutchison, 1997; Underwood et al., 2012; Bruneaux et al., 2014). In accordance with higher mortality levels, fish exposed to 30ºC presented signs of an inflammatory process or changed immune response, indicated by the up-regulation of sterile alpha motif domain-containing protein 9-like (from 24ºC to 30ºC). Thermal stress is known to cause damage to cellular components and tissues in aquatic organisms, including *S. aurata* (e.g. Madeira et al., 2014, 2015; Raina et al., 2015). As such, proteins or transcripts involved in the inflammatory process and immune response are commonly regulated upon exposure to thermal stress (e.g. Thorne et al., 2010; Windisch et al., 2014; Tomalty et al., 2015; Basu et al., 2015; Garland et al., 2015).

Interestingly, no changes were detected in Fulton's K condition index. Stressful environments likely lead to higher energy demands, thus fish subjected to heat stress could have lower condition due to weight loss. However, juvenile fish did not seem to experience significant weight loss, despite the detected changes in energetic metabolism. Proteins related to energetic pathways were both regulated at 24ºC and 30ºC. Overall, most of these proteins were up-regulated upon warming. This indeed suggests a higher energy demand at higher temperatures, which is in accordance with other studies that show that metabolic rates increase in fish chronically exposed to high temperatures (e.g. Vinagre et al., 2014; Rosa et al., 2014). This is especially relevant in muscle, which has high energetic demands and has to provide energy during long-periods or increase the rate of energy production in response to explosive contractions (Westerblad et al., 2010), in order to maintain locomotion. In fact, adjustments in energy metabolism may be essential for fish to be able to move to thermal refugia or keep their activities of foraging.

Overall, changes in energetic metabolism suggest an enhanced glycolytic potential and increased carbohydrate metabolism mainly at 30ºC but also, to some extent, at 24ºC. This finding is supported by the up-regulation of alpha-enolase, triose phosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase. Moreover, the up-regulation of creatine kinase and adenylate kinase indicates the regulation of ATP and AMP levels by producing ATP from phosphocreatine and ADP and converting 2 ADP ⇆ ATP + AMP, respectively. These reactions contribute to the maintenance of ATP levels and regulate glycolytic enzymes (Berg et al., 2002; Fischer et al., 2013). Changes in glycolytic enzymes have also been detected in other studies of acute (Garland et al., 2015) and chronic exposure to temperature (Jayasundara et al., 2015), usually increasing at higher temperatures. However, Garland et al. (2015) also detected that such enzymes could undergo antagonist changes, with enzymes at the preparatory phase decreasing and enzymes involved in the pay-off phase increasing. Moreover, there seemed to be a decrease in glycogenolysis at 24ºC after 14 days of exposure indicated by the down-regulation of glycogen phosphorylase. This suggests that fish can maintain some glycogen reserves if the temperature increase is mild. After 21 days of exposure, glucose-6-phosphate isomerase was also down-regulated at 24ºC suggesting a decrease in the inter-conversion of glucose-6-phosphate to fructose-6-phosphate (glycolysis/gluconeogenesis). This indicates that after 21 days of exposure to 24ºC there is no longer the need for an enhanced glycolytic potential which points out that fish may be acclimating to this temperature. However, after 21 days of exposure to 30ºC there seemed to be a decline in the tricarboxylic acid cycle (or related anaplerotic reactions), possibly suggesting a decrease in aerobic performance. This finding is supported by the down-regulation of glutamate dehydrogenase. Some studies suggest that exposure to high temperature induces a metabolic shift towards carbohydrate metabolism and the formation of glycogen stores (Brodte et al., 2006; Windisch et al., 2011; 2014), because these metabolic mechanisms are able to function under hypoxaemia, which is generally induced by hyperthermia (oxygen limited thermal tolerance, Pörtner and Farrell 2008). Entering a state of hypoxaemia means a shift from aerobic to anaerobic metabolism at some point and this

threshold for anaerobiosis is dependent on factors such as species and acclimation history (e.g. see Burleson and Silva, 2011). However, in this case, it is not entirely clear if pyruvate obtained from increased glycolysis is being used to maintain aerobic metabolism or is being converted to lactate in anaerobic conditions. Still, the down-regulation of glutamate dehydrogenase suggests that long-exposure to extreme temperatures decreases aerobic performance, potentially eliciting a transition to anaerobic metabolism.

Physiological adjustments to stress were influenced not only by temperature but also by the duration of exposure. While some mechanisms activated were common to both time-points (e.g. chaperoning), there seemed to be a transition in the predominant processes of response from T14 to T21. At T14, transcriptional/translational regulation, amino acid homeostasis and acid-base balance seemed more relevant while at T21, the shifting of the energetic metabolism was predominant and related to gluconeogenesis and cellular carbohydrate biosynthetic processes, which may be crucial to sustain metabolic performance under chronic stress.

Indeed, at T14, exposure to extreme temperature seems to have elicited the activation of endocrine processes and these may be responsible for the activation of molecular adaptive cascades, in which vesicular trafficking and translational regulation could play an important role. This finding is supported by the up-regulation of neuroendocrine convertase 1, eukaryotic initiation factor 4A-II and Rab GDP dissociation inhibitor beta in the muscle of juvenile fish exposed to 30ºC. Several studies have shown that a number of hormones dictate responses to stress in fish and other organisms (e.g. Moalic et al., 1989; Barton & Iwama, 1991; Iwama, 1999; Barton, 2002; Horowitz, 2002; Pepels et al., 2004) and that modulation of translation and transcription is crucial to deal with acute and chronic thermal stress in fish (e.g. Long et al., 2012; Windisch et al., 2014; Tomalty et al., 2015). Moreover, the regulation of acid-base status may be an important mechanism for homeostatic balance in fish as suggested by the up-regulation of carbonic anhydrase 1 at T14 (30ºC). However, the influence of hyperthermia in acid-base balance in fish is not well known, as opposed to the effect of hypothermia (Pörtner et al., 1998) and other environmental factors such as salinity and pollutants (e.g. de Polo et al., 2014).

5. Conclusion

Molecular information, coupled with performance measurements, reflects the health status of fish and their ability to successfully acclimate to new environmental conditions. In this study, *Sparus aurata* juveniles mildly responded to 24ºC but showed a stronger response to 30ºC. While exposure to 24ºC only elicited energetic adjustments (higher glycolytic potential and regulation of ATP levels, maintenance of glycogen reserves), 30ºC seems to induce much more molecular changes. At this temperature, the maturation of peptide hormones probably led to the activation of molecular cascades which potentially triggered energetic adjustments (enhanced glycolytic potential and regulation of ATP levels) necessary to induce the cellular stress response (increased chaperoning, cytoskeletal re-arrangements, and regulation of acid-base balance). Such changes are known to contribute to cellular homeostasis and muscle

functioning. Nevertheless, the data indicate that aerobic performance may be compromised (down-regulation of the tricarboxylic acid cycle) and an inflammatory process could be ongoing in animals subjected to 30ºC, which probably contributed to higher levels of mortality (**Fig. 6.7** – summary figure). Overall, the molecular information coupled with mortality rates suggests that juvenile fish are able to acclimate to 24ºC, but may not be able to acclimate to 30ºC. *S. aurata* is an eurythermal subtropical species (with an upper thermal limit of 35.5ºC – Madeira et al., 2014) that inhabits shallow habitats, in which a temperature of 24ºC is fairly common during summer. However, 30ºC (predicted future heat wave condition in Portuguese estuaries) seems somewhat deleterious to these sea breams suggesting that their health may be hindered if heat waves increase in frequency and intensity, as predicted by IPCC (2013). Therefore, recruitment and population levels of *S. aurata* may be affected by ocean warming, highlighting the need for improvement of management plans for fish stocks. Nevertheless, the ecological consequences of a warming ocean are dependent on several factors, including phenotypic plasticity throughout ontogeny, local adaptation (and thus larval dispersal), transgerational plasticity (e.g. Munday et al., 2014), and trophic-web cascading effects (e.g. Nagelkerken and Connell, 2015), warranting further research.

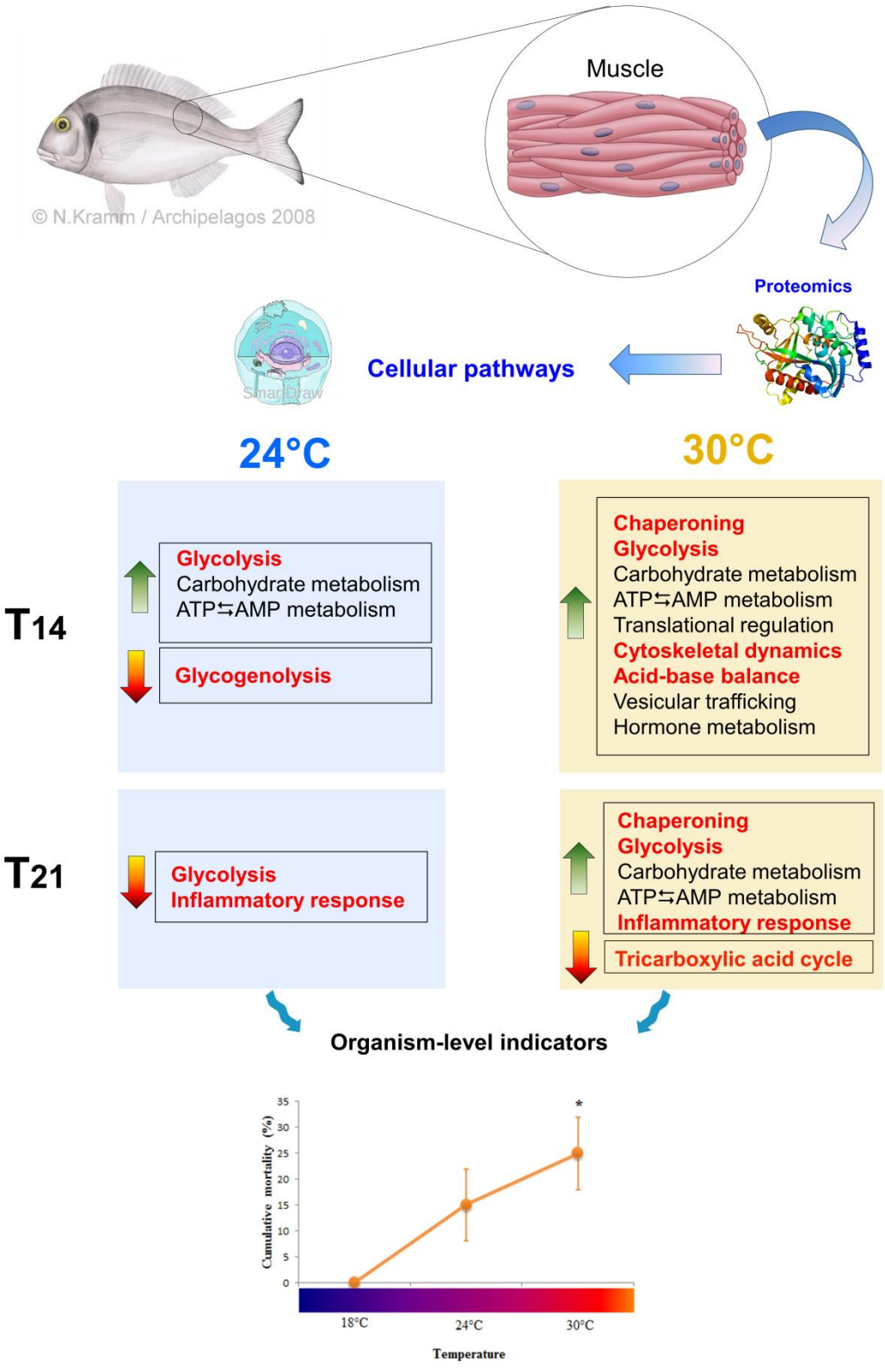


Figure 6.7 Summary of the study. *Sparus aurata* juveniles exposed to warm temperatures (control 18ºC *vs* 24ºC and 30ºC) modulated about 3% of their muscle proteins. This molecular plasticity was mainly related to energetic processes, chaperoning, cytoskeletal dynamics, acid-base balance, peptide hormone metabolism, vesicular trafficking and inflammatory processes. Fish seemed more able to acclimate to 24ºC, as opposed to 30ºC, as shown by significantly higher levels of cumulative mortality at this temperature. The mechanisms hypothesized to have the greatest influence on fitness outcomes are highlighted in red. Asterisks indicate significant differences from control ($p<0.05$). Green arrows indicate

up-regulation (↑) and red arrows indicate down-regulation (↓). T14 – 14 days of exposure; T21 – 21 days of exposure Constructed using SmartDraw and PowerPoint. Species drawing by N. Kramm.

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7. Data accessibility

Accession numbers: GDIB_CANFA; NEC1_HUMAN; ENOA_XENLA; KCRM_BOVIN; TPISB_DANRE; KCRM_HUMAN; KCRM_PIG; HSP71_ORYLA; HSP7C_CRIGR; KAD1_CYPCA; KCRM_CANFA; ENOA_PONAB; ACT2_XENTR; PYGB_SHEEP; IF4A2_MACFA; KCRT_ONCMY; CAH1_CHIHA; HSP7C_ICTPU; TPIS_MESAU; G3P_DANRE; G3P_MELGA; SAM9L_HUMAN; HSP70_ONCTS; G6PI_BOVIN; DHE3_CHAAC.

See supplementary information (annex 3) for additional data concerning Tukey's post-hocs (Table S6.1), protein expression levels (Table S6.2 and S6.4), masses and sequences of peptides obtained for each spot (Table 6.3), general categories of gene ontology (Fig. S6.1), detailed functional categorization (Table S6.5, and S6.6).

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CHAPTER 7. LIFE CYCLE CLOSURE IS HAMPERED BY FUTURE WARMING: DEVELOPMENTAL STAGE DETERMINES THE VULNERABILITY OF COASTAL FISH[1]

[1]Madeira D, Madeira C, Costa PM, Vinagre C, Pörtner HO, Diniz MS. Submitted.

Abstract

Climate change is one of the greatest environmental concerns faced by society. Fish species are one of the taxonomic groups at high risk of impact under business-as-usual CO_2 emission scenarios. However, accurate predictions of population changes are hampered due to the lack of eco-physiological approaches that integrate all stages of the life cycle. Here we tested the vulnerability of a highly commercial fish, *Sparus aurata*, towards ocean warming, throughout its life cycle. We combined a set of organism level indicators (mortality and Fulton's K condition index) with cellular (histopathological analyses) and molecular markers (heat shock proteins, total ubiquitin, anti-oxidant enzymes and lipid peroxidation). The vulnerability of life cycle stages can be ranked as larvae > adults > juveniles. Larvae were very sensitive to warming probably due to reduced biochemical acclimation which led to damage to muscle and kidneys and elevated mortality rates, suggesting that life cycle closure may be hampered in a warmer ocean. Juvenile fish were the most resistant, having a greater response capacity and being more able to maintain the integrity of vital organs than larvae and adults. Adults were sensitive to warming because vital organs were less responsive and showed greater tissue harm (damage to lipids and proteins, inflammation, atrophy, loss of glycogen storages), suggesting low plasticity. We conclude that the protection effort against thermal challenge is differentially allocated by distinct organs depending on developmental stage and that the larval phase is the key developmental stage that will determine life cycle closure of the sea bream *S. aurata* under projected ocean warming scenarios.

Key words: temperature, *Sparus aurata*, lifecycle, biomarkers, climate change

1. Introduction

Climate change is currently one of the greatest environmental concerns faced by society. Average global temperature has increased by 0.85ºC since 1880 and is predicted to increase by 2-6ºC in the next century, at an average rate of 0.2ºC per decade (IPCC, 2007, 2013; Hansen et al., 2010). More than 60% of this net energy increase will be stored in the upper ocean (IPCC, 2013). Thus, marine ecosystems, where net productivity is concentrated, will be vulnerable to change under ocean warming scenarios, with direct impacts on human activities. In recent reviews, fish have been pointed out as one of the main taxonomic groups with a high risk of impact under business-as-usual CO_2 emission scenarios (Gattuso et al., 2015; Nagelkerken and Connell, 2015). This risk may be even higher in exploited fish populations, such as demersal coastal species (e.g. sea breams and similar predators), which are key species in temperate and subtropical fisheries and aquaculture. In fact, marine biota have already experienced distributional shifts, abundance changes, altered migration patterns, lower recruitment success, and changes in trophic cascades, with serious impacts for carnivores (Walther et al., 2002; Sims et al., 2004; Rijnsdorp et al., 2009; Nagelkerken and Connel 2015). Such effects are not surprising considering that temperature is a major driver of physiological processes, performance and survival in ectotherms (e.g. Brett, 1971). Physical properties of molecules, metabolic rates, immune responses, growth, reproduction, foraging and performance are all dependent on temperature (Ettinger-Epstein et al., 2007; Pörtner and Farrell, 2008; Pittman et al., 2013). In order to cope with environmental change and maintain homeostasis, organisms can modify their gene expression patterns and regulate physiological functions (Hofmann and Todgham, 2010; Logan and Somero, 2011). However, it is not entirely clear if demersal fish are capable of evolutionary adaptation and acclimation (i.e. phenotypic plasticity) throughout their life cycles to meet the higher energetic demands of a warming ocean. This issue is especially relevant in commercial species considering that fishing reduces genetic variability and alters the structure and thus reproductive capacity of the population (Caddy and Agnew, 2003; Ottersen et al., 2006; Anderson et al., 2008), leading to increased sensitivity of fish stocks to adverse climate conditions (Planque and Frédou, 1999; Ottersen et al., 2006). In this context, the scientific community is faced with the challenge of predicting the effect of climate forcing on fish species which are already under the pressure of exploitation. As such, testing the vulnerability of commercial species towards ocean warming and the underlying stress resistance mechanisms is of major importance.

Despite the vast literature on thermal eco-physiology, most studies have focused on a specific life stage, hampering accurate predictions of climate change impacts on fish populations. Successive life stages have different requirements (habitat, food, physiology, size, form, behavior, thermal niche) and therefore climate change may differently affect eco-physiological traits of fish throughout their life cycles (Rijnsdorp et al., 2009; Petitgas et al., 2013), especially considering that an ontogenetic shift in temperature tolerance is expected (Pörtner and Farrell, 2008; Rijnsdorp et al., 2009). Based on aerobic scope, Pörtner and Farrell (2008) specified that thermal window widths are narrower for eggs, larvae and spawners, while

increasing in growing adults and juveniles. In addition, life history stages are functionally linked as the environmental experience of one stage may influence the performance and fitness of succeeding stages (Moran and Emlet, 2001; Phillips, 2002; Giménez and Anger, 2003; Podolsky, 2003; Giménez 2004, 2006). Consequently, determining the life history stage(s) most critical for life cycle closure under a warming ocean is essential not only for understanding the consequences and selection pressures imposed by climate change upon organisms (Kingsolver et al., 2011; Petitgas et al., 2013; Bozinovic and Pörtner, 2015), but also to design adequate conservation guidelines (Radchuk et al, 2013) to preserve the sustainability of fish stocks. Nonetheless, virtually no eco-physiological studies have addressed the effects of ocean warming on the whole life-cycle of environmentally-relevant species.

Therefore, the main aim of this study was to compare the vulnerability of larvae, juveniles and growing adults toward ocean warming, taking a common demersal fish, the commercial sea bream *Sparus aurata*, as model organism Specifically it is aimed at i) performing a comparative assessment of vulnerability changes along the life cycle, ii) understanding the physiological mechanisms toward thermal challenge in each life cycle stage and iii) analyze the biochemical changes by which the physiological responses are modulated and if these are surrendered into metabolic priorities that shift through development.

2. Methods

2.1 Ethical statement

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

2.2 Assessment of *Sparus aurata*'s thermal environments

S. aurata is a subtropical species, occurring in the Mediterranean and Black Seas, Eastern Atlantic, and from the British Isles to Cape Verde (Froese and Pauly,2006; Sola et al., 2007). Adults reproduce in the open sea from late autumn to early spring and larvae travel in ocean currents to settle in nursery grounds such as estuaries and coastal lagoons (Froese & Pauly, 2006; Dimitriou et al., 2007; Basurco et al., 2011; Kissil et al., 2011; Mylonas et al., 2011; Ibarra-Zatarain & Duncan, 2015). Juvenile sea breams remain in such habitats until they reach sexual maturity (at approximately two years of age – Froese and Pauly, 2006) and migrate to the open ocean to reproduce. Out of the reproduction season, adults can be found in coastal or estuarine waters (for more details about *S. aurata* see chaper 1 **Table 1.1**). Based on this background, temperature data were obtained for both coastal and estuarine waters. Data were collected from several sources including 1) studies throughout Portuguese coastal waters and estuaries (Costa, 1990; Coutinho, 2003; Azevedo et al., 2006; Cabral et al., 2007; Madeira et al., 2012); 2) 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 to 2015); and 3) the Marine and Environmental Sciences Centre (MARE) database (data from several estuaries including Tagus' temperatures obtained from measurements carried out with YSI loggers considering a time-series from 1978 to 2006).

2.3 Housing and husbandry of fish

Sparus aurata (35d post-hatch larvae, n=180, 10-15 mm; juveniles, n=75, mean±sd TL of 8.93±1.16 cm and 12.76±4.60 g weight; and adults, n=60, mean±sd TL of 13.15±1.40 cm and 45.78±12.25 g weight,) were obtained from a fish farm (MARESA, Spain) and transported to the laboratory in i) 10 L opaque plastic constainers (larvae) or ii) 100 L opaque plastic boxes (juveniles and adults) with constant aeration and stable temperature conditions (18±0.5ºC). Larvae were produced from a brood stock of 50 males and 25 females. Sample sizes were calculated following a power analysis based on previous studies. Since high natural mortalities were expected for larvae, a greater sample size was necessary. For detailed life-history traits see chapter 1 Table 1.1.

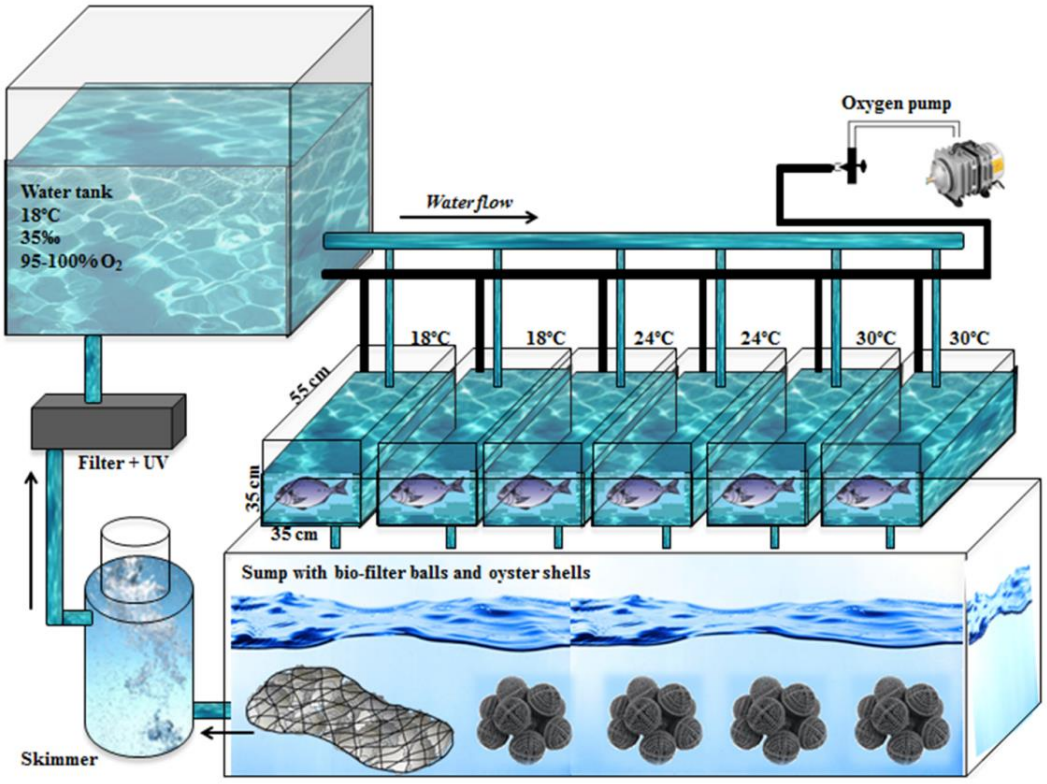
Fish were randomly placed in a re-circulating system (total of 2,000 L, **Fig. 7.1**) as follows:

i) Larvae were placed in six transparent polyvinyl containers (17.5 × 17.5 × 15 cm, approximately 4.5 L; n=30 larvae.tank^{-1}), each positioned inside a 70L tank with water flowing through small punctures (this allowed for a gentle water flow, preventing physical stress).

ii) Juveniles and adults were placed in 70L white plastic tanks (35 × 35 × 55 cm) (juveniles, n=13 individuals.tank^{-1}, 6 tanks; and adults, n=10 individuals.tank^{-1}, 6 tanks).

All tanks were filled with clean aerated sea water (95-100% O_2), with a constant temperature of 18.0±0.5°C, salinity 35‰ and pH 8.00±0.1 (same conditions of the fish farm). Inflow of clean sea water in each individual tank was 300 mL.min^{-1}. All individual tanks were provided with a filter (ELITE Underwater Mini-Filter Hagen, 220L.h^{-1}). Fish were acclimated (juveniles and adults: one week; larvae: one day - a longer acclimation period was not feasible because it would interfere with the objective of studying larval stage) and their welfare was assessed (i.e. wounds or any disease symptoms). During the acclimation and experimental trial larvae were fed under a regime of period feeding (every 6h) with *Artemia salina* metanauplii and two different grain-sized feeds (0.3-0.6 mm and 0.6-1.0 mm). Juveniles and adults were fed with commercial food pellets once a day (BRM3, Aquasoja, Portugal) mixed with cyanobacterium *Spirulina* sp. (Tropical®, Poland).

(a)



(b)

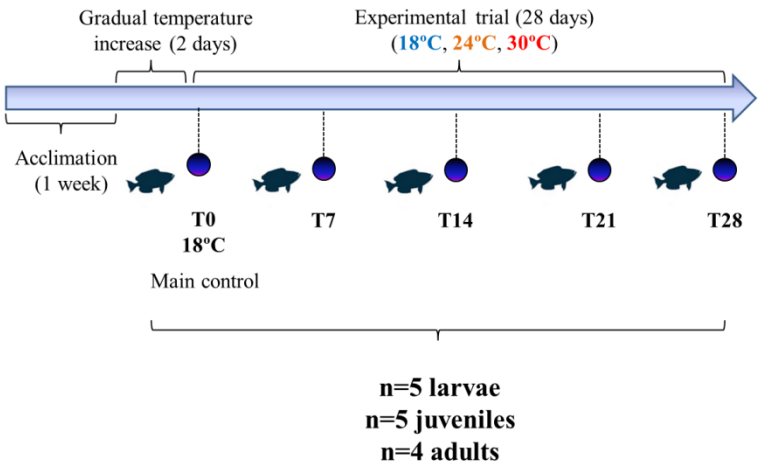


Figure 7.1 (a) Experimental setup (not to scale). Re-circulating system (total volume of 2,000L) with six 70L white polyvinyl tanks (35 × 35 × 55 cm). Inflow of clean water in each tank was 300mL.min^{-1}. All the tanks were filled with clean and aerated sea water (95-100% O_2), with a constant temperature of 18.0±0.5°C, 24.0±0.5°C and 30.0±0.5°C (n=2 tanks for each temperature). Salinity was kept at 35‰ and pH at 8.00±0.01. All tanks were provided with a filter (ELITE Underwater Mini-Filter Hagen, 220L.h^{-1}). **(b)** Timeline and sampling scheme of the experiment. The fish were euthanized through cervical transection at day 0, 7, 14, 21 and 28 for collection of samples. T – Timepoint in days. Constructed in PowerPoint. Fish icons by Shutterstock.

2.4 Experimental setup

After the acclimation period, temperature was gradually increased (for two days) until the experimental temperatures were reached (control 18.0±0.5ºC; experimental temperatures 24.0±0.5ºC and 30.0±0.5ºC; n=2 tanks for each temperature). Following, temperatures were maintained for 28 experimental days using thermostats (TetraTec® HT 100, 100-150L, Tetra Werke, GmbH, Melle, Germany). The sampling design (supplemental material, **Fig. 7.1b**) consisted of euthanizing fish trough cervical transection at day 0 (only 18ºC), 7, 14, 21 and 28 (all temperatures). At each time point, five (larvae and juveniles) or four (adult) individuals were randomly sampled. In larvae, the entire body was collected for biochemical analyses. In juveniles and adults, several organs were collected separately i.e. brain, gills, intestine, liver and muscle. The experiments were carried out in shaded day light (15L; 09D). Trials were run sequentially. To prevent any additional handling stress, the total length and weight of all juvenile and adult individuals was measured at the end of each sampling using an icthyometer and a scale, respectively. The total length of larvae was obtained by re observing and measuring individuals under a magnifying glass.

2.5 Temperature effects on *S. aurata* throughout its life cycle

2.5.1 Mortality and condition index

Mortality rates were calculated at the end of the experiment in each tank. Fulton's K condition index was calculated using the formula:

$$K = 100\ M_t/L_t^3 \qquad (1)$$

Where M_t is the total wet mass (mg) and L_t is the total length (mm) (Ricker, 1975). This index was calculated for fish in each temperature treatment after 21 days of exposure (due to the prediction of elevated mortality rates by the end of the experiment – 28 days – which could significantly decrease sample number).

2.6 Biomarkers

Molecular markers were chosen based on their role in the cellular stress response (CSR): i) heat shock proteins are chaperones with an adaptive value, repairing denatured proteins upon thermal stress (e.g Feder and Hofmann, 1999; Coles and Brown, 2003; Sørensen et al., 2003; Hofmann 2005; Hofmann and Todgham, 2010; Madeira et al., 2012, 2014; Narum and Campbell, 2015), ii) ubiquitin targets irreversibly damaged proteins for proteasome degradation (e.g. Hofmann and Somero, 1995; Logan and Somero, 2011; Madeira et al., 2014; Tang et al., 2014), iii) anti-oxidant enzymes neutralize ROS (reactive oxygen species) and oxidation products (e.g. lipid peroxides) that arise due to higher metabolic rates at higher temperatures (e.g. Lushchak and Bagnyukova, 2006; Heise et al., 2006; Bagnyukova et al., 2007; Vinagre et al., 2012). Multi-parameter and biomarker approaches are useful to detect metabolic alterations that constitute early signs of impairment in individuals and populations (Colin et al., 2016).

2.6.1 Sample treatment

The entire larvae or approximately 150-200mg samples of tissue (brain, gills, intestine, liver and muscle) of juveniles and adults were homogenized in 0.5 and 1 mL (respectively) 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 $\times$g) and the supernatant fractions were stored at -80ºC until further analysis.

2.6.2 Biochemical analyses

Total protein content was determined through the Bradford method (Bradford, 1976) for data normalization and followed the protocol described by Madeira et al. (2014).

Heat Shock Protein 70 (Hsc70/Hsp70) was quantified using an indirect Enzyme Linked Immunosorbent Assay (ELISA) (Njemini et al., 2005) with 96-well microplates and followed the protocol described by Madeira et al (2014).

The quantification of total ubiquitin (Tub) was achieved through a direct Enzyme Linked Immunosorbent Assay (ELISA) with 96-well microplates (Nunc-Roskilde, Denmark) and followed the protocol described by Madeira et al. (2014).

The enzymatic assay of Catalase (CAT) (EC 1.11.1.6) was adapted from Johansson and Borg (1988) and performed as described in Vinagre et al. (2014). The enzymatic assay of glutathione S-transferase (GST) activity (EC 2.5.1.18), using the substrate CDNB (1-Chloro-2,4-dinitrobenzene), was carried out according to Habig et al. (1974) and as described by Vinagre et al. (2014). The enzymatic assay of superoxide dismutase (SOD) activity was adapted from Sun et al. (1988) and performed using nitroblue tetrazolium (NBT) and xanthine oxidase (XOD), as described in Vinagre et al. (2014).

The lipid peroxides assay was adapted from the thiobarbituric acid reactive substances (TBARS) protocol (Uchiyama and Mihara, 1978) to quantify malondialdehyde bis(dimethylacetal) (MDA) following the procedure described by Vinagre et al (2014).

For more details concerning these analyses see annex 4, supplementary **Table S7.1**.

2.7 Histopathological procedures

Histological sections were obtained from whole-body sections of larvae and tissue sections of juveniles and adults fixed in Bouin's solution for 24 h or 48 h at room temperature and embedded in paraplast. Sections (5 µm thick) were stained with several histological and histochemical techniques, namely Haematoxylin and Eosin (H&E) for general histopathological screening, Periodic Acid-Shiff's (PAS) plus Haematoxylin (for glycogen detection and general structural analysis) and a trichrome stain using Weigert's Iron Haematoxylin and van Giesons' dye (Acid Picrofuchsin) in larvae to assist differentiation of multiple structures, since these animals were fixed and processed without dissection. More details may be found in Martins et al. (2015). Histological sections were obtained with a Jung RM2035 rotary microtome and

analysed with a DMLB model microscope equipped with a DFC480 camera, all from Leica Microsystems (Germany). The histopathological analyses were qualitative and based on the presence/absence of lesions, as well as type and extent of the identified lesions.

2.8 Statistical analysis

All data (concerning mortality, condition index and biochemical biomarkers) were firstly tested for normality (Shapiro-Wilk's test) and homoscedasticity (Levene's test).

2.8.1 Mortality and condition index

Mortality data were analysed through a two-way ANOVA (temperature and life stage as categorical predictors and mortality as dependent variable). Tukey's post-hocs were carried out to detect differences between all groups. Fulton's K condition index was analysed via a one-way ANOVA in juveniles (18ºC *vs* 24ºC *vs* 30ºC) and a Mann-Whitney U test in adults (18ºC *vs* 24ºC; mortality at 30ºC). All analyses were carried out in Statistica v10 (StatSoft Inc., USA), using a significance level of 0.05.

2.8.2 Biomarkers

Parametric ANOVAs or non-parametric Kruskal-Wallis (depending on whether the data met the assumptions of normality and homoscedasticity) were carried out to detect significant differences between biomarker levels throughout the experiment at different temperatures. For simplicity, the results only show significant differences from control (18ºC) at each time-point. These analyses were carried out in Statistica v10 (StatSoft, USA).

A discriminant function analysis was carried out in order to detect which biomarkers (considering all organs) contribute to discriminate between temperature treatments (18, 24 and 30ºC), exposure times (T7, T14, T21, T28) and life stages (larvae, juveniles, adults). Moreover, Principal Component Analysis (PCA) was also carried out to detect biomarkers that correlate with temperature and time and that contribute to explain the variance in the dataset within each targeted organ in larvae, juveniles and adults. To detect which life stage and tissue was more prone to warming, the Integrated Biomarker Response (IBR) was calculated according to Beliaeff and Burgeot (2002) (details can be found in annex 4, IBR computations). This index was constructed for ecotoxicological studies and assumes that higher biomarker levels indicate more damage to tissues. In the context of warming, it is also important to consider that organs with high biomarker levels can be more responsive and organs with biomarker levels below control (especially in extreme temperature regimes) can be less responsive due to exhaustion (i.e. denaturation of proteins at high temperatures). These analyses were carried out in Statistica v10 (StatSoft, USA). A significance level of 0.05 was used in all analyses.

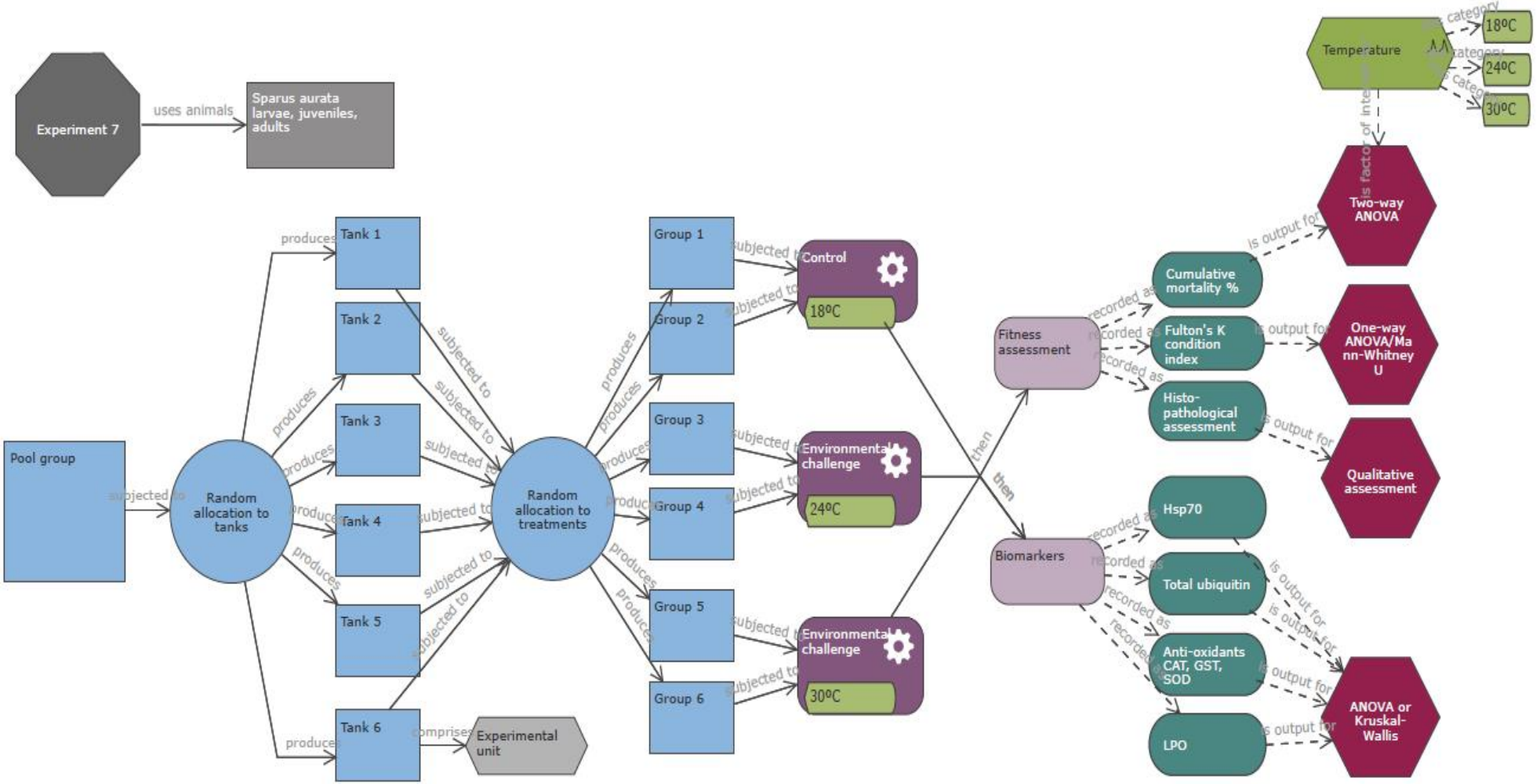


Figure 7.2 Experimental approach (diagram constructed using Experimental Design Assistant, https://eda.nc3rs.org.uk/).

3. Results

3.1 Assessment of *Sparus aurata*'s thermal environments

Water temperatures in the Portuguese coastal area range approximately from 10 to 16ºC during the winter and from 15 to 20ºC during the summer. Seasonal variation is high, with the lowest temperatures occurring during February (average of 13.9ºC, minimum of 12.2ºC and maximum of 15.2ºC considering the last 5 years). Coastal water temperatures are highest during September (average of 19.3ºC, minimum of 17.2ºC and maximum of 21.8ºC considering the last 5 years). According to regional predictions (Miranda et al. 2002), Portuguese water temperatures will rise 2-3ºC until 2100. Therefore, the expected temperatures for 2100 are in the range of 13 to 19ºC and 18 to 23ºC during the winter and summer, respectively (**Fig. 7.3a**). Nevertheless, such temperatures can already occur in present days for a limited period of time (days or weeks) during extremely hot summers (e.g. summer of 2010, see Madeira et al., 2012a). Present estuarine temperatures range approximately from 10 to 14ºC during the winter to 20 to 24ºC during summer. During heat waves (at least five days in a row with a maximum air temperature of ≥ 35ºC – Santos and Miranda, 2006), estuaries can reach maximum temperatures between 25 and 28ºC, and persisting for over 2 weeks (e.g. see Costa, 1990; Coutinho, 2003; Azevedo et al., 2006; Cabral et al., 2007; Madeira et al., 2012a). Following the scenario of a 2-3ºC increase in Portuguese waters by 2100 (Miranda et al., 2002), estuaries' mean temperature during summer would be in the range of 22 to 27ºC, reaching 30ºC during heat waves (**Fig. 7.3b**).

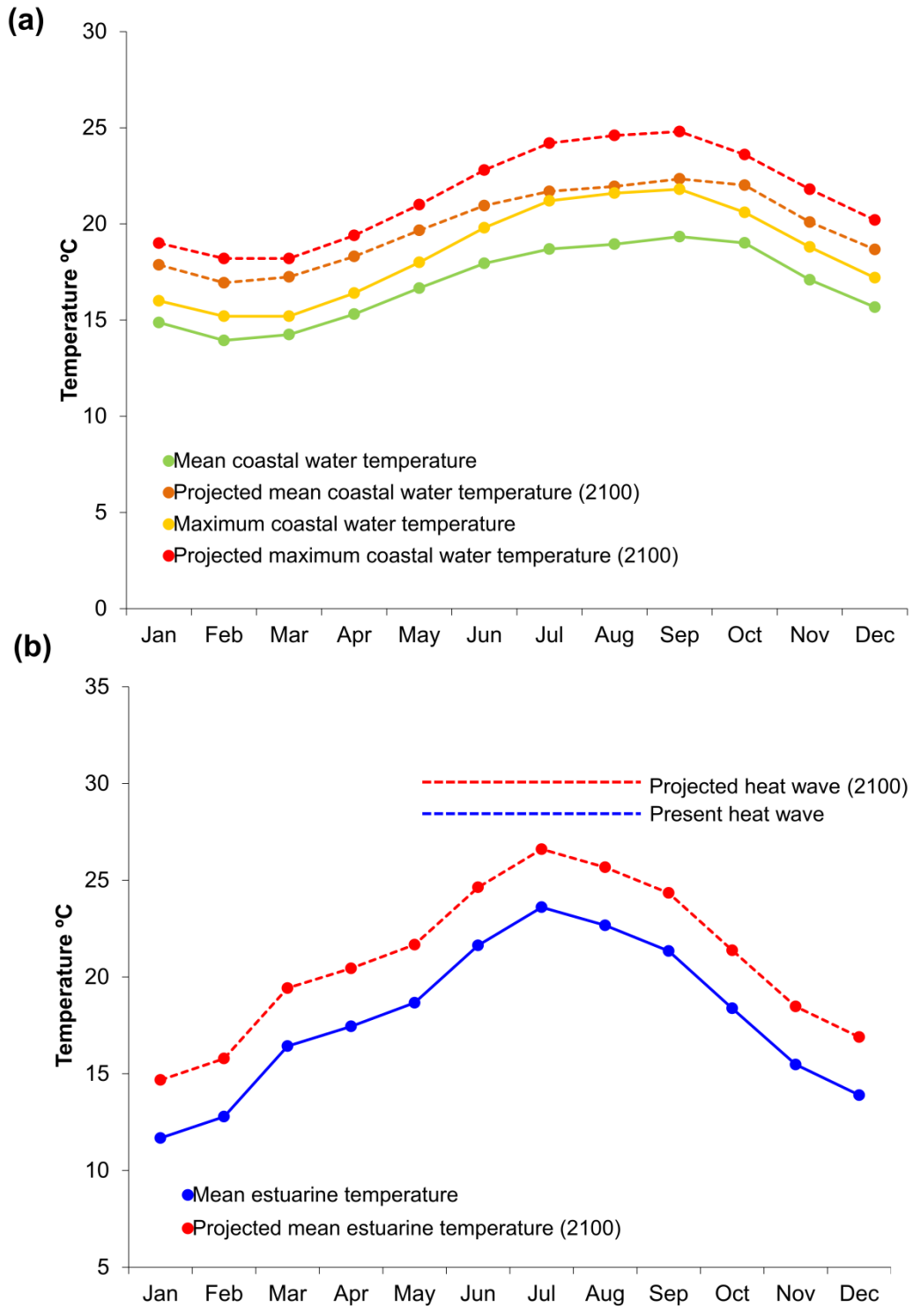


Figure 7.3 Present and projected temperatures for 2100 (+3ºC) in **(a)** Portuguese coastal waters (monthly average sea surface temperature for the main coastal cities from 2011-2015) and **(b)** estuaries (based on data from the Tagus estuary considering monthly average temperatures collected from 1978 to 2006).

3.2 Temperature effects on *S. aurata* throughout its life cycle

3.2.1 Mortality and condition index

Cumulative mortalities of *Sparus aurata* larvae were 67±5% at 18ºC, 100±0% at 24ºC and 100±0% at 30ºC; cumulative mortalities of juveniles were 0±0% at 18ºC, 14±3% at 24ºC and 28±11% at 30ºC; and cumulative mortalities of adults were 0±0% at 18ºC, 0±0% at 24ºC and 100±0% at 30ºC (**Fig. 7.4**). All larvae exposed to 30ºC died within five days, while all larvae exposed to 24ºC died within nine days (which prevented the comparison of Fulton's K condition index in larvae). Juveniles exposed to 24ºC started dying after 26 days of exposure whereas the ones exposed to 30ºC began to die after 25 days. Adults exposed to 30ºC were all dead within 12 days of exposure (which prevented the estimation of Fulton's K index for adults exposed to 30ºC). Statistical analysis detected significant differences between cumulative mortalities among temperature treatments (F=286.8; p=0.000) and life stages (F=572.9; p=0.000). Additionally, a significant interaction was detected between temperature and life stage (F=101.7; p=0.000). Fulton's K condition index did not vary with temperature in any of the developmental stages tested (juveniles, 18 *vs* 24 *vs* 30ºC: Kruskal-Wallis, H=1.28, p=0.526; adults, 18 *vs* 24ºC: Mann-Whitney, U=7, p=0.88).

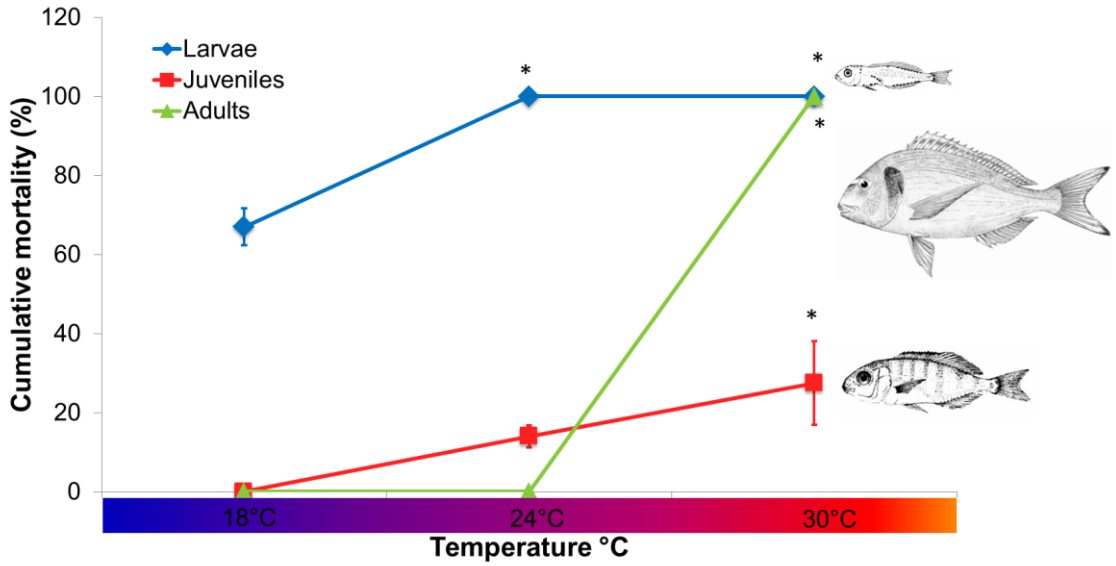


Figure 7.4 Cumulative mortalities (mean±SD) of *Sparus aurata* larvae, juveniles and adults exposed to 18ºC, 24ºC and 30ºC for a period of 28 days. Asterisks mark significant differences from the control group (18ºC). Species illustrations were retrieved from Arias and Drake (1990) and FAO (Food and Agriculture Organization of the United Nations), with permission.

3.3 Biomarkers

3.3.1 Analysis of variance

Overall, *Sparus aurata* was more responsive to a temperature increase after seven days of exposure, with juveniles and adults also responding on day 14 and 21, depending on organ and biomarker. Generally, juvenile fish responded to 30ºC within seven days of exposure and to 24ºC within 14 days of exposure while adult fish responded to 24ºC and 30ºC within seven days of exposure. Larvae also responded to 24ºC after seven days of exposure by increasing the activity of GST and SOD (the other biomarkers showed no significant change – **Table 7.1**, and supplementary **Table S7.2** in annex 4). At 30ºC, no biomarkers could be assessed because all larvae had died within five days of exposure. Similarly, no biomarkers could be assessed in larvae subjected to 24ºC from 14 days onwards because 100% mortality was attained within nine days of exposure.

Juveniles showed a response to 24ºC at T14 and T21 but neither at T7 nor T28. At T14, changes were detected in muscle (increased LPO and SOD), gills (increased GST) and brain (increased Hsp70 and CAT). At T21, there was a significant decrease of LPO in muscle. At 30ºC biochemical changes were detected at T7, T14 and T21. At T28, mortality was too high and no biomarkers were assessed due to reduced sample size. At T7, all tissues responded except brain, in which no biomarker changes were detected. In muscle, there was a significant increase in Hsp70, Tub and GST; in gills, there were significant increases in the activity of SOD and CAT; in liver, all biomarkers tested increased significantly (Hsp70, Tub, LPO, SOD, GST) except for CAT, which showed no changes. The intestine showed increased levels of Tub and reduced CAT activity. At T14, liver showed no changes as all biomarkers returned to control levels. Muscle showed increases in Hsp70, LPO, SOD and CAT, gills showed increased LPO, intestine still showed decreased CAT and brain showed significant increases in Hsp70 and CAT. By day 21, only muscle sustained biomarker changes such as increased Hsp70 and SOD (**Table 7.1**, and supplementary **Table S7.2** in annex 4).

Adults responded to 24ºC on day 7, 14, 21 and 28. On day 7, only muscle, gills and intestine showed alterations, namely increased LPO and CAT in muscle, and decreased Hsp70 in gills and intestine. At T14 these tissues did not respond, contrary to liver and brain which showed increased GST and CAT, respectively. By day 21, gills and liver showed increased LPO, whereas intestine yielded increased LPO and SOD and decreased CAT. By day 28, only brain showed a response, consisting of increased CAT and decreased SOD activity. Biochemical alterations were also detected in adult fish exposed to 30ºC, on day 7. Beyond that day no biomarkers could be assessed due to mortality levels. Nevertheless, on T7, changes were detected in all tissues except for brain. Muscle showed increased Hsp70 and decreased Tub; gills showed increased Tub, SOD and LPO; liver showed increased LPO; and intestine showed decreased Hsp70 and increased GST (**Table 7.1**, and supplementary **Table S7.2** in annex 4).

Table 7.1 Variation in biomarker's levels in comparison to control (18ºC) within each time point (T7 – seven days of exposure; T14 – fourteen days of exposure; T21 - twenty-one days of exposure). Green arrows (↑) indicate up-regulation and red arrows (↓) indicate down-regulation; hyphen (-) indicates no change in comparison to control (18ºC within that time-point). Hsp70 – heat shock protein 70 kDa; Tub – total ubiquitin; CAT – catalase; GST – glutathione-S-transferase; SOD – superoxide dismutase; LPO – lipid peroxidation.

Life stage	Organ	T7 18ºC	T7 24ºC	T7 30ºC	T14 18ºC	T14 24ºC	T14 30ºC	T21 18ºC	T21 24ºC	T21 30ºC	T28 18ºC	T28 24ºC	T28 30ºC
Larvae	**Whole body**	control	↑SOD ↑GST	Dead	control	Dead	Dead	End of experiment due to mortality					
Juveniles	**Muscle**	control	-	↑Hsp70 ↑Tub ↑GST	control	↑LPO ↑SOD	↑Hsp70 ↑LPO ↑SOD ↑CAT	control	↓LPO	↑Hsp70 ↑SOD	control	-	Dead
	Gills	control	-	↑SOD ↑CAT	control	↑GST	↑LPO	control	-	-	control	-	Dead
	Liver	control	-	↑Hsp70 ↑Tub ↑LPO ↑SOD ↑GST	control	-	-	control	-	-	control	-	Dead
	Brain	control	-	-	control	↑Hsp70	↑Hsp70	control	-	-	control	-	Dead

						↑CAT	↑CAT						
	Intestine	control	-	↑Tub ↓CAT	control	-	↓CAT	control	-	-	control	-	Dead
Adults	**Muscle**	control	↑LPO ↑CAT	↑Hsp70 ↓Tub	control	-	Dead	control	-	Dead	control	-	Dead
	Gills	control	↓Hsp70	↑Tub ↑SOD ↑LPO	control	-	Dead	control	↑LPO	Dead	control	-	Dead
	Liver	control	-	↑LPO	control	↑GST	Dead	control	↑LPO	Dead	control	-	Dead
	Brain	control	-	-	control	↑CAT	Dead	control	-	Dead	control	↑CAT ↓SOD	Dead
	Intestine	control	↓Hsp70	↓Hsp70 ↑GST	control	-	Dead	control	↑LPO ↓CAT ↑SOD	Dead	control	-	Dead

3.3.2 Discriminant and principal components analyses

Discriminant analysis showed that the biomarkers which significantly contributed to discriminate between life stages were CAT (in muscle and brain), GST (in muscle, gills and liver), SOD (in liver), lipid peroxidation (in muscle and brain), Hsp70 (in gills and brain) and total ubiquitin (in muscle and brain) (**Table 7.2**). Thus, differences between life stages depend mainly on how biomarkers are expressed in vital organs (brain, liver and muscle).

The biomarkers that significantly contributed to discriminate between temperature treatments were GST (in gills and liver), SOD (in gills and intestine), LPO (in muscle), Hsp70 (in muscle) and total ubiquitin (in muscle and brain) (**Table 7.2**). Finally, the biomarkers that significantly contributed to discriminate between sampling times were GST (in muscle, gills, liver and brain), SOD (in liver and intestine) and total ubiquitin (in muscle, gills and brain) (**Table 7.2**).

Table 7.2 Discriminant function analysis to detect which biomarkers (considering all organs) contribute to discriminate between life stages (larvae, juveniles, adults), temperature treatments (18, 24 and 30ºC) and exposure times (T7, T14, T21, T28). m – muscle; b – brain; g – gills; l – liver; i – intestine; Hsp70 – heat shock protein 70 kDa; Tub – total ubiquitin; CAT – catalase; GST – glutathione-S-transferase; SOD – superoxide dismutase; LPO – lipid peroxidation.

Grouping Variable	Model Wilks' Lambda	F	p	Significant biomarkers	Biomarker Wilks' Lambda	Biomarker p-value
Life stage	0.003	45.0	0.000	CATm	0.0036	0.002
				CATb	0.0035	0.003
				GSTm	0.0045	0.000
				GSTg	0.0036	0.001
				GSTl	0.0033	0.037
				SODl	0.0035	0.002
				LPOm	0.0036	0.001
				LPOb	0.0037	0.000
				Hsp70g	0.0030	0.026
				Hsp70b	0.0033	0.034
				Tubm	0.0039	0.000
				Tubb	0.0034	0.006
Temperature	0.075	6.9	0.000	GSTg	0.0825	0.038
				GSTl	0.0880	0.003
				SODg	0.1014	0.000
				SODi	0.0883	0.003

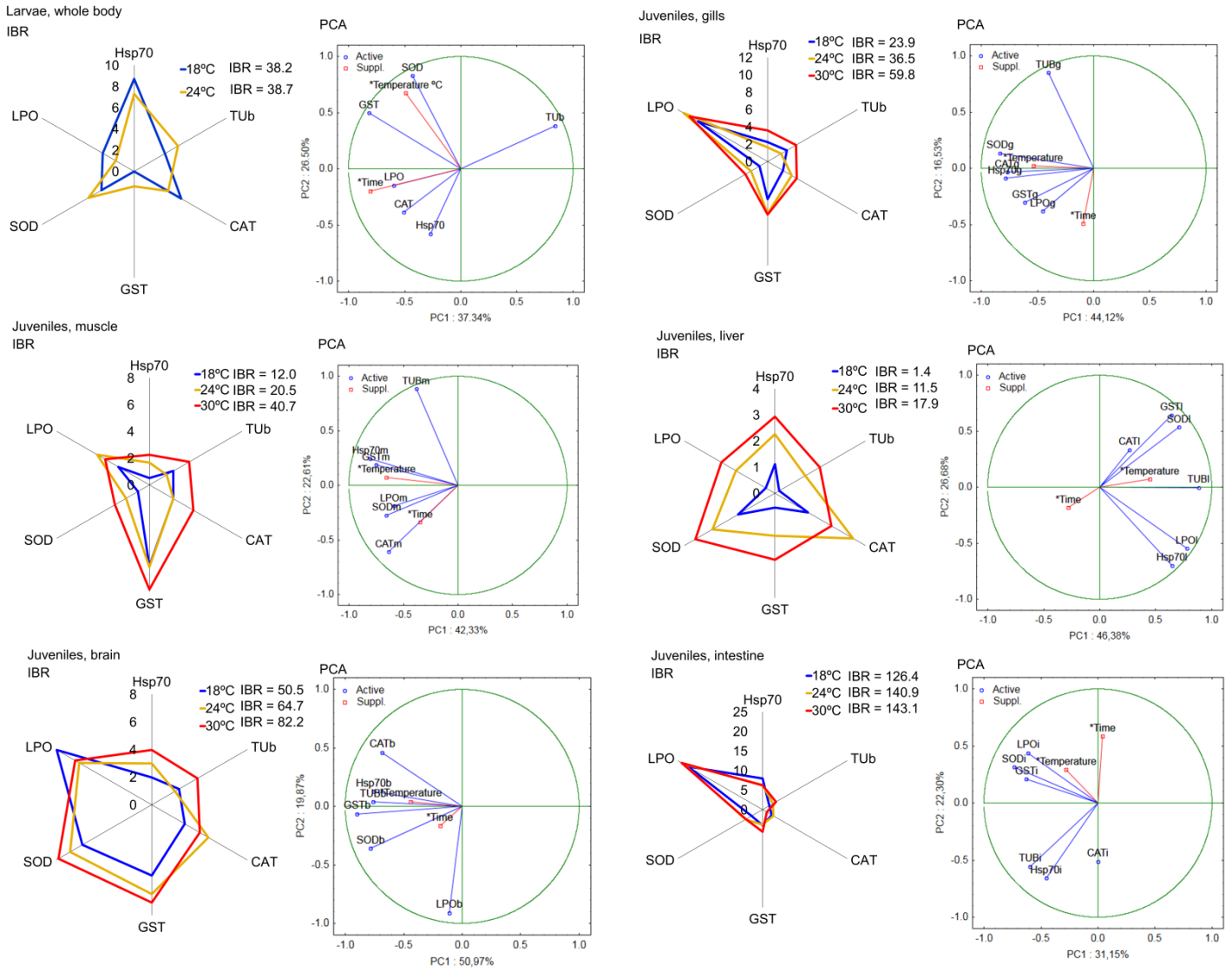
				LPOm	0.0840	0.019
				Hsp70m	0.1401	0.000
				Tubm	0.0834	0.024
				Tubb	0.0833	0.027
Time	0.00573	3.2	0.000	GSTm	0.0079	0.017
				GSTg	0.0079	0.015
				GSTl	0.0083	0.007
				GSTb	0.0112	0.000
				SODl	0.0087	0.004
				SODi	0.0088	0.003
				Tubm	0.0081	0.012
				Tubg	0.0078	0.024
				Tubb	0.0083	0.009

Moreover, principal component analysis showed that stress response mechanisms vary throughout the life cycle and depend on the organ tested (**Fig. 7.5a, b**). For instance, in larvae, SOD was more correlated with temperature, and LPO with time (PCA, **Fig. 7.5a**). In juveniles, temperature was related to SOD, CAT and Hsp70 in gills; GST and Hsp70 in muscle; SOD and Tub in liver; Hsp70, Tub and GST in brain; and LPO in intestine (PCAs, **Fig. 7.5a**). In adults, temperature was related to LPO and Tub in gills; CAT and Hsp70 in muscle; GST in liver; LPO and CAT in brain; and LPO, Tub and SOD in intestine (PCAs, **Fig. 7.5b**). The proportion of variance explained (goodness-of-fit) by the PCA in larvae was 63.8% (**Fig. 7.5c**). In juveniles, the proportion of variance explained was highest in liver (73.1%), brain (70.8%) and muscle (64.9%). On the contrary, in adults, the organs with the least proportion of explained variance were precisely liver, muscle and brain whilst the organs with the highest proportion of variance explained were gills (65.5%) and intestine (64.1%) (**Fig. 7.5c**). Thus, the most responsive organs (considering the biomarkers tested) varied with life stage.

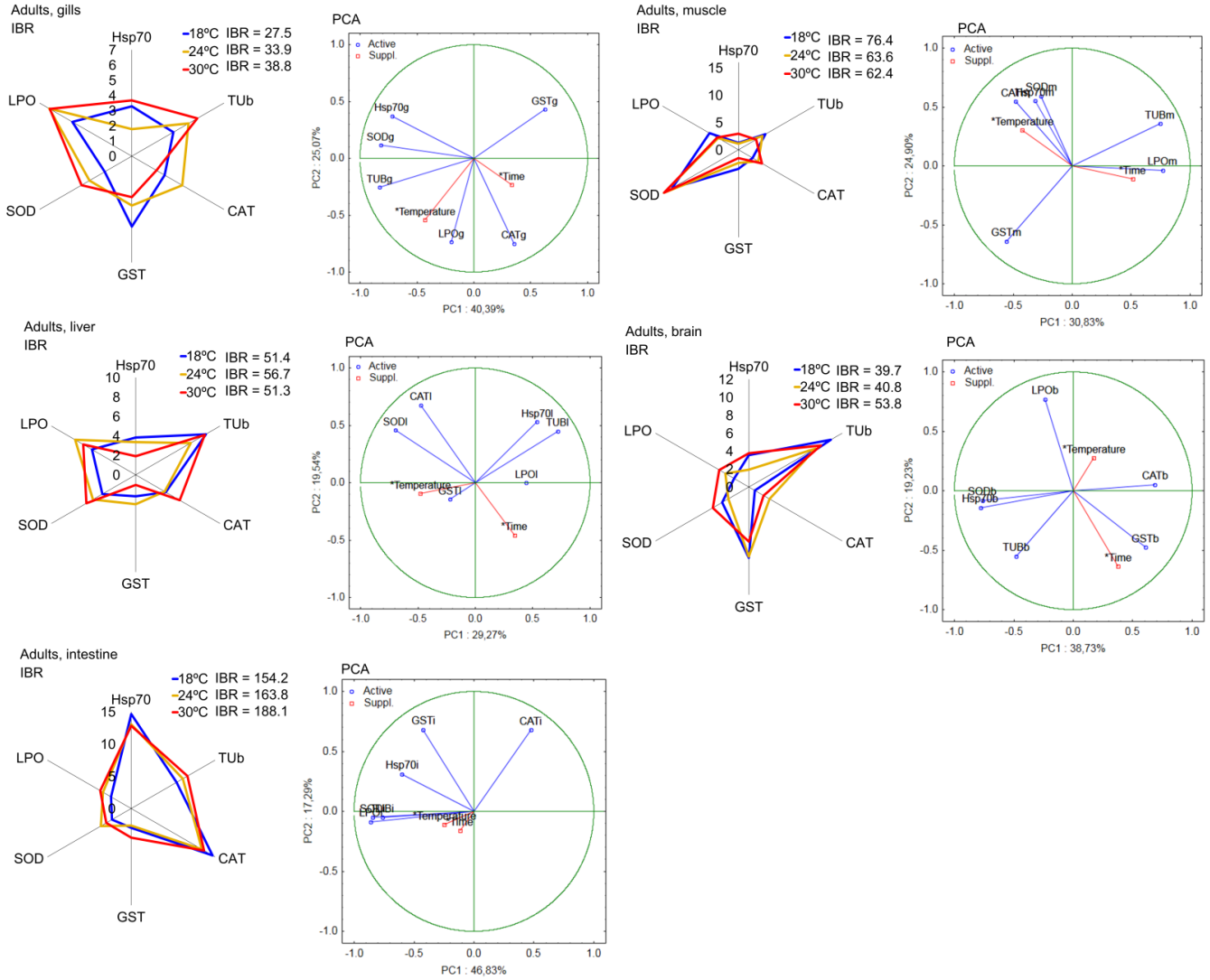
3.3.3 Integrated Biomarker Response (IBR)

IBR was similar in larvae exposed to 18ºC and 24ºC (**Fig. 7.5a**). In juveniles, IBR was always higher at 24ºC and 30ºC when compared to 18ºC (**Fig. 7.5a**). In adults, IBR was higher at 24ºC and 30ºC in all organs except muscle and liver (**Fig. 7.5b**). In muscle, the IBR of both 24ºC and 30ºC was lower than that of 18ºC and in liver, the IBRs of 18ºC and 30ºC were very similar and lower than 24ºC (**Fig. 7.5b**).

(a)


Larvae, whole body
IBR
Hsp70
LPO
TUb
SOD
CAT
GST
18ºC IBR = 38.2
24ºC IBR = 38.7
PCA
Active
Suppl.
SOD
*Temperature ºC
GST
TUb
*Time
LPO
CAT
Hsp70
PC1 : 37.34%
PC2 : 26.50%
Juveniles, gills
IBR
18ºC IBR = 23.9
24ºC IBR = 36.5
30ºC IBR = 59.8
PCA
TUBg
SODg
*Temperature
CATg
Hsp70g
GSTg
LPOg
*Time
PC1 : 44,12%
PC2 : 16,53%
Juveniles, muscle
IBR
18ºC IBR = 12.0
24ºC IBR = 20.5
30ºC IBR = 40.7
PCA
TUBm
Hsp70m
GSTm
*Temperature
LPOm
SODm
*Time
CATm
PC1 : 42,33%
PC2 : 22,61%
Juveniles, liver
IBR
18ºC IBR = 1.4
24ºC IBR = 11.5
30ºC IBR = 17.9
PCA
GSTi
SODi
CATi
*Temperature
TUBI
*Time
LPOi
Hsp70i
PC1 : 46,38%
PC2 : 26,68%
Juveniles, brain
IBR
18ºC IBR = 50.5
24ºC IBR = 64.7
30ºC IBR = 82.2
PCA
CATb
Hsp70b
TUBb
*Temperature
GSTb
*Time
SODb
LPOb
PC1 : 50,97%
PC2 : 19,87%
Juveniles, intestine
IBR
18ºC IBR = 126.4
24ºC IBR = 140.9
30ºC IBR = 143.1
PCA
*Time
LPOi
SODi
*Temperature
GSTi
TUBi
Hsp70i
CATi
PC1 : 31,15%
PC2 : 22,30%

(b)


Adults, gills
IBR
Hsp70
LPO
TUb
SOD
CAT
GST
18ºC IBR = 27.5
24ºC IBR = 33.9
30ºC IBR = 38.8
PCA
Active
Suppl.
Hsp70g
GSTg
SODg
TUBg
*Time
*Temperature
LPOg
CATg
PC2 : 25,07%
PC1 : 40,39%
Adults, muscle
IBR
Hsp70
LPO
TUb
SOD
CAT
GST
18ºC IBR = 76.4
24ºC IBR = 63.6
30ºC IBR = 62.4
PCA
Active
Suppl.
TUBm
LPOm
*Temperature
*Time
GSTm
PC2 : 24,90%
PC1 : 30,83%
Adults, liver
IBR
Hsp70
LPO
TUb
SOD
CAT
GST
18ºC IBR = 51.4
24ºC IBR = 56.7
30ºC IBR = 51.3
PCA
Active
Suppl.
CATl
SODl
Hsp70l
TUBl
LPOl
*Temperature
GSTl
*Time
PC2 : 19,54%
PC1 : 29,27%
Adults, brain
IBR
Hsp70
LPO
TUb
SOD
CAT
GST
18ºC IBR = 39.7
24ºC IBR = 40.8
30ºC IBR = 53.8
PCA
Active
Suppl.
LPOb
*Temperature
CATb
GSTb
TUBb
*Time
PC2 : 19,23%
PC1 : 38,73%
Adults, intestine
IBR
Hsp70
LPO
TUb
SOD
CAT
GST
18ºC IBR = 154.2
24ºC IBR = 163.8
30ºC IBR = 188.1
PCA
Active
Suppl.
GSTi
CATi
Hsp70i
*Temperature
*Time
PC2 : 17,29%
PC1 : 46,83%

(c)

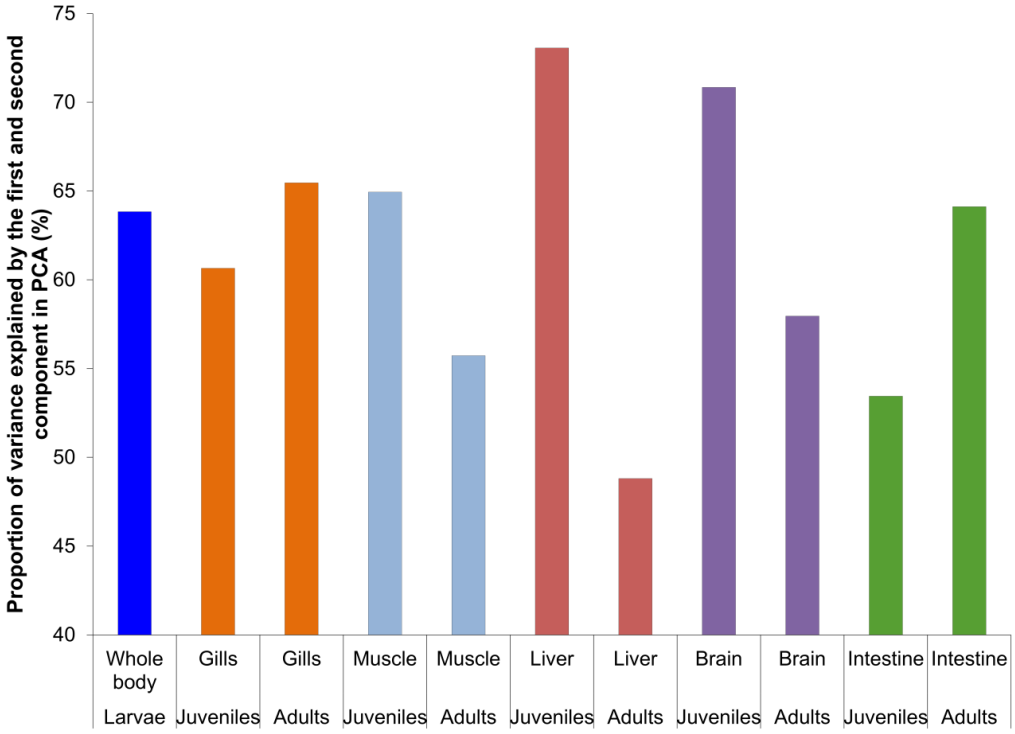


Figure 7.5 Integrated biomarker response index and principal components analysis carried out for each sampled tissue of *Sparus aurata* **(a)** larvae (whole body) and juveniles (gills, muscle, liver, brain, intestine) and **(b)** adults (gills, muscle, liver, brain, intestine) exposed to 18ºC, 24ºC and 30ºC, considering all sampling times (7, 14, 21, 28 days); **(c)** proportion of variance explained in the first and second components of the principal components analysis carried out for each sampled tissue of each life stage of *Sparus aurata* considering all temperatures (18ºC, 24 and 30ºC) and sampling times (0, 7, 14, 21, 28 days).

3.4 Histopathology

Skeletal muscle yielded changes in all three age groups, from larvae (**Fig. 7.6A**) to juveniles and adults (**Fig. 7.6B**), with a clear trend to increase with time and temperature. However, muscular dystrophies were more severe and diffuse in adults and juveniles, where infiltration of inflammatory cells accompanied focal (towards diffuse in animals subjected to 24ºC and higher) autolytic processes of muscle bundles and atrophy of connective tissue. The brain was seemingly affected only in adults (**Fig. 7.6C**) exposed to 24ºC or higher for longer periods of time. However histopathological changes were circumscribed to vacuolation of glial cells in the medulla. The liver (hepatopancreas) was the most affected organ in adults and overall the organ that yielded the most severe histopathological changes, affecting both hepatic and pancreatic tissue, the latter of which presented diffuse dystrophy of acini in animals exposed to 30ºC, with a clear time-dependent trend. The main alterations hitherto observed were fat vacuolation, inflammation (revealed by foci of infiltrating inflammatory cells and hyperaemia, see **Fig. 7.6D**, **inset**), loss of glycogen storage disclosed by PAS reaction and loss of zymogen granules in acinar cells, which appeared degenerated in shape and size, with loss of acinar structure. Control animals presented the normal structure of the organ throughout the

experiment (**Fig. 7.6E**). Gills did not reveal any significant changes that could be pinpointed to thermal stress in any of the age groups. Histopathological traits were solely related to benign infections, mostly by *Chlamidia*-like bacteria that were not affected by the experiment (**Fig. 7.6F**). No histopathological changes were observed in the digestive tract of any of the surveyed animals. Larvae subjected to higher temperatures presented minor alterations to kidneys, namely cuboidal cell vacuolation and loss of tubular shape (not shown). Overall, muscle was the organ that was most consistently sacrificed in all life stages.

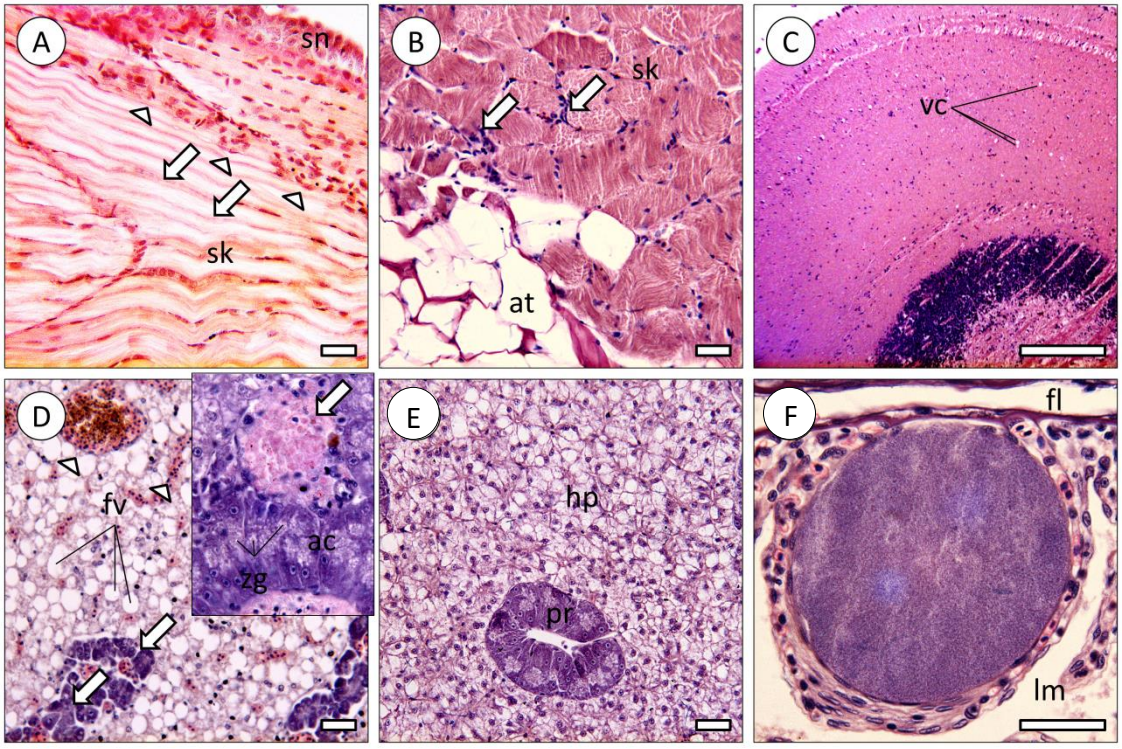


Figure 7.6 Histopathological sections of multiple organs from larval, juvenile and adult *Sparus aurata* subjected to different temperatures (18, 24 and 30ºC). **(A)** Muscle of a larva subjected to 24 ºC for seven days. Note the infiltration of inflammatory cells along the junction between skeletal muscle segments (arrowheads). Bundles of disorganized (atrophied) muscle bundles are also visible (arrows). sn) Skin. Staining: Weigert's Iron Haemtoxylin + van Gieson. **(B)** Skeletal muscle of an adult subjected to 24 ºC for 21 days, revealing diffuse atrophy of skeletal muscle (sk) bundles and inflammatory foci (H&E). The subcutaneous adipose tissue (at) was frequently observed to infiltrate affected muscle (H&E). **(C)** Section through the optic lobe of an adult fish exposed to 24 ºC for 21 days, revealing low-moderate diffusion of vacuolation (vc) in the medullar area, likely affecting glial tissue (H&E). **(D)** Liver (hepatopancreas) of an adult fish subjected to 30 ºC for seven days, with diffuse fat vacuolation (fv) plus inflammation, indicated by hyperaemia and infiltration of inflammatory cells into hepatic tissue (arrow heads). The pancreatic acini are severely affected (arrows), revealing loss of zymogen granules (H&E). Inset: Focus of inflammatory cells (likely melanomacrophages) in the vicinity of pancreatic acini in an adult exposed to 24 ºC for 14 days. Here the acini (ac) still presented a normal structure. Note zymogen granules (zg). PAS-Haematoxylin. **(E)** Hepatopancreas of a control (18 ºC) adult fish at 21 days, for comparative purposes. These animals presented the normal architecture of hepatic (hp) and pancreatic tissue (pr), similar to juveniles (H&E). **(F)** Benign bacterial infection by *Chlamydia*-like bacteria in the interlamellar space in gills of a control fish collected at 14 days of exposure. fl) filament; lm) lamella. Note the absence of inflammation. These benign infections were present in virtually all animals, regardless of test of age class (H&E). Scale bars: 25 µm except C (250 µm).

4. Discussion

Accurate predictions of the impacts of climate change on populations and ecosystems require the assessment of thermal tolerance of all life-history stages (see Zeigler, 2013). Thermal tolerance has two components that can be crucial to mitigate the impacts of climate change, including an adaptive (genotype dependent) and an acclimation component (physiological responses to environmental change; phenotypic plasticity) (Yampolsky et al., 2014; Bay and Palumbi, 2015). In this study, we showed that *S. aurata* larvae lack the ability to acclimate to chronic elevated temperature, as opposed to juveniles and to some extent adults. This finding is supported by the high mortality rates endured by larvae at both 24ºC and 30ºC, confirming that high temperatures lead to a bottleneck effect in early life stages of fish as shown by other studies (e.g. Houde, 1989; Faria et al., 2011; Bartolini et al., 2013).

Overall, the juvenile and adult phases had lower mortality rates when compared to larvae, but adults were also more sensitive than juveniles, especially at 30ºC. While juveniles only responded to 24ºC after 14 days of exposure with mild biochemical changes, adults responded to 24ºC within seven days of exposure. At 30ºC both the juvenile and adult phases responded within seven days of exposure. Overall, this suggests that 1) adults are more sensitive to high temperature than juveniles (in accordance with the predictions of Pörtner and Farrell, 2008) and 2) intensity of stress and duration of exposure are key components that determine the onset of biochemical and physiological adjustments, and fitness changes (in accordance with other studies e.g. Buckley and Hofmann, 2004; Schulte, 2014). Moreover, for higher levels of stress intensity, the biochemical changes occurred faster, suggesting a greater need for the cellular stress response. Interestingly, no variations were detected in physiological condition indexes between treatments for any of the developmental stages tested suggesting that fish were not losing weight, potentially maintaining foraging activity and food conversion efficiency.

Molecular mechanisms underpinning *S. aurata* thermal tolerance varied throughout its lifecycle. Anti-oxidant enzymes (SOD and GST) were mostly associated with temperature during the larval phase and damage to lipids was mostly associated with duration of exposure. These results suggest that anti-oxidant defenses can play an important role during thermal stress but are not enough to promote survival, leading to cellular damage and general metabolic failure (probably time-dependent). Moreover, IBRs were similar between larvae exposed to 24ºC and 30ºC corroborating the lack of a strong biochemical response. Even though it was impossible to separate organs for biochemical analyses (in larvae), histo-pathological observations indicate that the most affected organs were muscle and visceral organs such as kidneys (albeit these were deprived of a specific pattern), suggesting possible alterations in muscle contraction and osmoregulatory imbalances that could account for the elevated mortality rates of larvae. Temperature can narrow the range of salinities tolerated by organisms by influencing their osmoregulation capacity (e.g. Lemaire et al., 2002; Albuquerque et al., 2009; Madeira et al., 2014b; Tang et al., 2014). Therefore, elevated temperature could affect the ability of larvae to move and settle into nursery grounds due to metabolic failure and a potential

decrease in osmoregulation capacity. Larvae are born in the open ocean and then migrate to settle in estuaries and coastal lagoons as late larvae/early juveniles. In this transition, they have to change from colder saline coastal waters to warmer and brackish estuarine waters. Such event may thus be especially stressful for cohorts of larvae born in early spring, when temperatures are warmer, especially considering that an increase of 2-3ºC for Portuguese waters (Miranda et al., 2002) is expected, along with more intense and frequent heat waves (IPCC, 2013). A proteomic study carried out by our team on *S. aurata* larvae (in preparation) also showed that elevated temperatures induce further changes such as cytoskeleton reorganization and the up-regulation of the chaperone Hsp90 and of the proteasome, indicating that protein damage may contribute to decreased fitness. Moreover, crucial cellular processes seemed to be impaired by warming, including a slowdown of cargo transporting and decreased oxygen transport. Therefore, a lowered aerobic performance is expected for larvae exposed to heat, suggesting a mismatch between oxygen supply and demand, as described by the oxygen and capacity limited thermal tolerance hypothesis (Pörtner, 2010). Ultimately, all of these processes may have contributed to the low survival of larvae. The cumulative Critical Thermal Maximum (CTmax) calculated for *S. aurata* larvae is 30ºC (100% of larvae attained lethargy - Madeira et al., in preparation) but sub-lethal effects can arise at temperatures as low as 22ºC (Madeira et al., in preparation; Polo et al., 1991). This supports the finding that future coastal (~16-23ºC during spring and summer) and estuarine thermal regimes (~18-24ºC during spring and summer; heat waves 30ºC) will impose temperatures beyond the thermal scope of larvae, negatively affecting sea bream populations through high mortality and lowered recruitment success. Even if a portion of larvae cohorts survive short-term exposure to suboptimal temperatures, such an event during early life history stages could translate into downstream effects in juveniles and adults (developmental domino effect), possibly affecting growth rate, fecundity, maturation age and tolerance to other stressors (Pechenik, 2006; O'Connor et al., 2014). These latent carry-over effects are known to occur in marine organisms exposed to different types of stress, including fish and invertebrates (e.g. Wendt and Johnson, 2006; Araki et al., 2009; Calado et al., 2010; Rey et al., 2015).

In both juveniles and adults, the most responsive organs (considering the tested biomarkers) differed. This suggests that the effort to protect against thermal challenge is differentially allocated by distinct organs, depending on developmental stage. According to the proportion of variance explained by the PCA, the most responsive organs in the juvenile phase were liver, brain and muscle. Interestingly, in adults, these organs seemed to be less responsive (also shown by the lower IBRs at higher temperatures), as opposed to gills and intestine, which showed the highest proportions of explained variance. Histopathological observations corroborated these results, as no alterations were detected in gills and intestine but relevant modifications and potential loss of function were detected in adults' muscle, liver and brain. Thus, it seems that adults expend more energy (with loss of glycogen storage) and experience function loss in vital organs due to inflammation, dystrophy, damage to lipids (mainly in muscle and liver despite increases in anti-oxidant enzymes) and damage to proteins (mainly

in muscle; potentially reversible as increases were only detected in Hsp70 but not in Tub). Feidantsis et al. (2009) also detected an increase in Hsp in adult *S. aurata* exposed to warming for 10 days as well as the activation of MAPK (Mitogen Activated Protein Kinases) signaling cascades. MAPK signaling is known to be involved in the activation of Hsp70 as shown by Feidantsis et al., (2012) in an acute thermal stress experiment using *S. aurata* red blood cells. However, it can also be linked to inflammation and cell death (Dorion and Landry, 2002; Zarubin and Han, 2005) which could be more likely during chronic extreme warming, potentially explaining our results.

Juveniles also showed some histo-pathological alterations in muscle, but these were not as severe as in adults. Additionally, in the juveniles' muscle, liver and brain, temperature was both related to protein turnover and anti-oxidant defenses (see the PCAs, Fig. 3), suggesting that juveniles are more able to prevent cytotoxic accumulations of damaged proteins and oxidative damage when reared in extreme temperature. This finding is supported by the lower mortality rate of juveniles exposed to 30ºC. It is also noteworthy that a severe decline in fitness occurred between 24ºC and 30ºC for adults as mortality rates went from 0% at 24ºC to 100% at 30ºC. Thus, it seems that loss of performance is abrupt, as opposed to juveniles in which the changes were more gradual. Furthermore, adult fish presented histo-pathological alterations in the brain (as opposed to juveniles), which could also explain their higher sensitivity and indicate less phenotypic plasticity to survive chronic warming.

Juvenile *S. aurata* inhabit shallow semi-confined waters which are prone to warming due to low thermal inertia. These habitats can frequently attain elevated water temperatures during summer, down to a depth of 20m (e.g. see Madeira et al., 2012; Rose et al., 2012). This implies that juveniles do not have many thermal refugia within these semi-confined habitats and thus must rely on plastic responses to cope with such temperatures (taking advantage of the higher food availability and shelter). Thus, juvenile *S. aurata* seem somewhat able to acclimate to chronic elevated temperatures (mainly to 24ºC and to some extent 30ºC) even though sub-lethal effects could take place (protein damage, tissue atrophy), especially if the initial rate of warming is acute, as shown by Madeira et al. (2014). Projected heat waves for 2100 will lead to water temperatures around 30ºC in estuaries and coastal lagoons. In this case even juvenile fish could be subjected to significantly higher mortalities (see Fig. 1), with potential deleterious consequences in performance and fitness. Nevertheless, juveniles have approximately a 70% chance of survival when chronically exposed to water temperatures predicted for 2100 during heat waves, contrasting with a 0% chance of survival for larvae and adults in the same conditions. Yet, the probability of exposure to these conditions will be significantly lower for adults when compared to juveniles and larvae. Adults have higher mobility and they usually inhabit both coastal and estuarine habitats. Hence, behavioral thermoregulation and the higher availability of thermal refugia are crucial factors that will play a role in adult survival. Behavioral thermoregulation has been demonstrated in both freshwater and marine fish (Pulgar et al., 1999; Ward et al., 2010; Thums et al., 2012) and has been shown to provide benefits related to survivorship and the control of metabolic processes (e.g. Coutant, 1987; Golovanov, 2006;

Gräns et al., 2010). However, there are also associated costs for fish that move farther from the coast, including lower food availability and feeding efficiency, higher predation risk and competition. These factors will eventually determine the fate of adult sea breams and the consequent fitness of the offspring, which can be lowered or improved depending on the parents' environment (e.g. Harrison et al., 2011; Betini et al., 2014; Parker et al., 2015).

Larval migration to warm estuarine waters should be the most crucial phase of this species life-cycle, since larvae have been shown in the present work to be quite sensitive to the temperatures that will be attained by estuarine waters in the future. Failure to migrate will leave them in coastal waters where food and refuge is less abundant. Mortality during immigration will surely result in lower recruitment. Both options will be very disruptive for the life-cycle of this and other demersal fish species with similar life-cycles and that whose larvae, juveniles and adults evolved in the same thermal regime. This will result in lower abundance of demersal fish, which are the main target of traditional fisheries in southern Europe and the base of the Mediterranean diet, with impacts in both ecosystems and human society.

5. Conclusions

We showed, for the first time, that the larval phase is the key developmental stage that will determine life cycle closure of the sea bream *S. aurata* under projected ocean warming scenarios. The lack of biochemical acclimation, damage to muscle and kidneys and the extremely elevated mortality rates of larvae (at both 24 and 30ºC) suggest that life cycle closure may be hampered in a warmer ocean (**Fig. 7.7**, summary figure). Several factors will determine the viability and dynamics of *S. aurata* populations including the success of the first spawning events (autumn/winter), possible changes in phenology, and the success of migration towards nursery grounds. Moreover, the influence of anthropogenic disturbances (e.g. overfishing, habitat destruction and pollution), all of which are liable to produce additive and synergistic deleterious effects, will also play a relevant role in the ability of fish populations to acclimate and adapt.

Temperature also had pronounced effects on adult fitness. Vital organs (muscle, liver, brain) were less responsive and showed the greatest tissue injury (damage to lipids and proteins, inflammation, atrophy), suggesting a response to damage rather than a plastic response. However, adult fish may rely on their swimming ability and available thermal refugia to counterbalance such effects. Juvenile fish, which are confined to estuaries, showed the greatest survival and acclimation potential suggesting a plastic response to temperature, mainly based on effective protein turnover and antioxidant defenses of vital organs. Overall, the susceptibility of *S. aurata* to thermal challenge can be ranked as: larvae > adults > juveniles.

Mitigation of the impacts of climate change on fish stocks and future fish production will ultimately depend on cooperation between scientists and stakeholders so that information on vulnerability can be translated into marine protection frameworks and management strategies. Additionally, for ecological forecasting to be accurate several questions still need to be addressed, particularly if (1) transgenerational plasticity could ameliorate the impacts of ocean

warming on fish stocks?, (2) what is the potential for evolutionary adaptation (dependent on larval dispersal, and genetic variability) in wild populations?, (3) do wild populations have higher acclimation capabilities and higher fitness when compared to farmed ones, allowing them to cope better with change?, (4) if so, what will be the impact of farm escapees on wild populations with regards to vulnerability/resilience to climate change?. Answering all of these questions will be essential for effective conservation and future sustainability of demersal fish stocks.

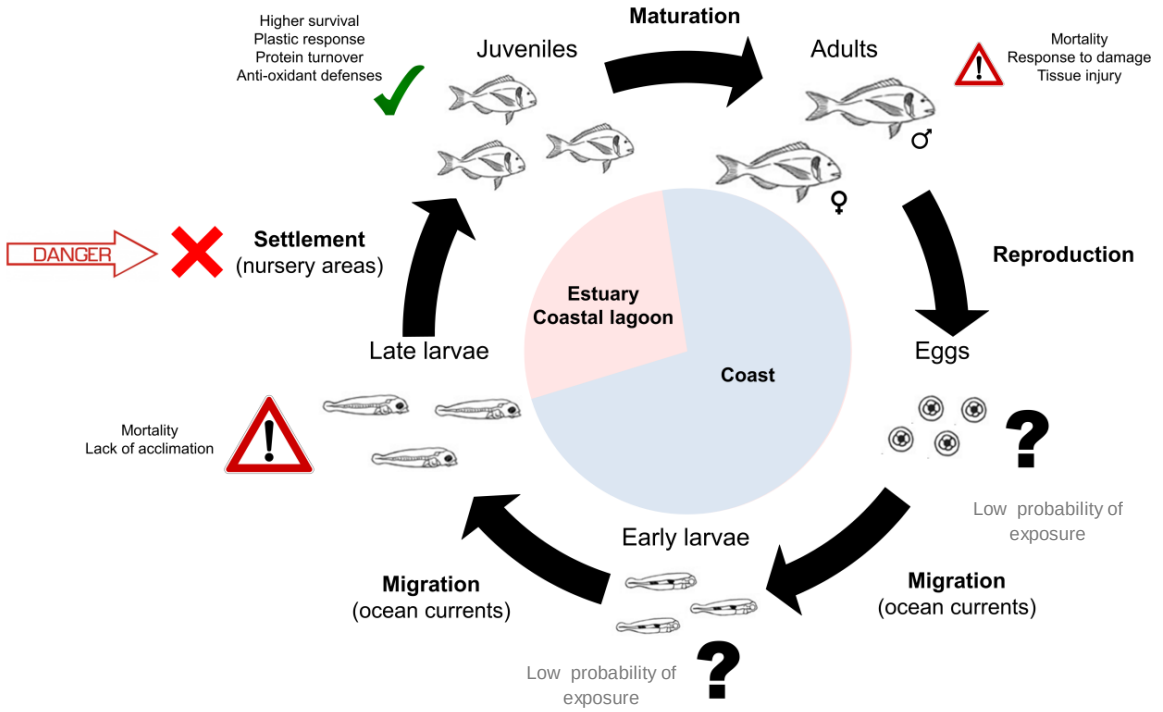


Figure 7.7 Summary figure. The effects of chronic warming (28 days at 18ºC, 24ºC or 30ºC) were investigated in *S. aurata* throughout several stages of its life cycle (larvae, juveniles and adults). The larval stage was the most sensitive as larvae showed extremely elevated mortality rates and lack of biochemical acclimation at both 24ºC and 30ºC, compromising the settlement into nursery areas and recruitment's success in a warming ocean. The juvenile stage showed the most plastic response and higher levels of survival probably due to effective protein turnover and anti-oxidant defenses. The adult phase was able to cope with 24ºC but was extremely sensitive to 30ºC, showing tissue injury and no acclimation potential (confirmed by 0% survival). Nevertheless, as adults inhabit coastal waters they may take advantage of their greater mobility to seek thermal refugia. Moreover, eggs and early larval stages (although not tested) inhabit the open sea and thus have low probability of exposure to elevated temperatures. Therefore, late larval stage is the key developmental stage that will determine the viability of *S. aurata* populations in a warming ocean. Note: *S. aurata* drawings were retrieved from FAO.

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CHAPTER 8. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Climate change is a major environmental problem that is expected to affect all ecosystem services and sectors of society. Among these, food production services (i.e. fisheries, aquaculture) are extremely relevant and could suffer serious losses due to the coupled effect of warming, ocean acidification, exploitation pressure and environmental contamination on the physiology of fish. The present work contributed to the assessment of the vulnerability/resistance of the highly commercial sea bream, *Sparus aurata*, to ocean warming. Most importantly, such assessment was quite novel in the field because it integrated several life cycle stages of this fish and combined parameters at different levels of biological organization, including proteomics, which has been seldom applied to marine biology research. This integrative, systems biology approach allowed realistic predictions of the impacts of climate change on *S. aurata* populations.

The experimental work revealed that the vulnerability of *S. aurata* life cycle stages to ocean warming can be ranked as larvae > adults > juveniles. Firstly, upper thermal limits of *S. aurata* shift throughout ontogeny, with larvae having the lowest CTmax value (30ºC, measured as cumulative CTmax with lethargy as end-point). Larvae were extremely vulnerable to both acute and chronic heat stress (Chapter 2, 3 and 7), showing increased levels of mortality and low acclimation capacity at temperatures above 22ºC. When exposed to acute stress, larvae showed muscle disorganization and protein denaturation, which probably led to lethargy and mortality. Oxidative stress did not seem to play an important role in this process. Moreover, exposure to chronic thermal stress induced histo-pathological changes not only in muscle but also in kidneys, suggesting that osmotic regulation and swimming ability may be impaired at temperatures of 24ºC and beyond. Larvae exposed to chronic warming also relied on protein turnover mechanisms, gene expression regulation, cytoskeletal re-arrangements, anti-oxidant enzymes and a fined-tuned regulation of cargo transporting to promote homeostasis. However, oxygen transport was compromised and larvae seemed to lack the ability to make the energetic adjustments needed to survive chronic thermal stress (although this hypothesis needs further research). Thus, a rise in water temperature may act as a strong selection pressure for larvae, either leading to evolutionary adaptation or bottleneck effects at early life stages, compromising recruitment's success of key fisheries species due to disturbances of biochemical homeostasis, emphasizing the risks of rising global temperatures for larval fishes.

Juvenile fish (Chapter 4, 5, 6, 7) were the most resistant when compared to larvae and adults, with a CTmax value of 35.5±0.5ºC (loss of equilibrium as end-point). Juveniles seem to have the greatest plastic response and capacity to enhance protective mechanisms. However, the threshold temperature at which negative effects were detected in the acute exposure trial was about 26-28ºC. Tissue injury started to occur at 24ºC in liver and pancreas and became more pronounced from 28ºC onwards in every organ tested (gills, liver, pancreas, intestine and muscle), indicating high levels of inflammation. Protein damage was detected at temperatures beyond 30ºC and was more relevant in muscle, brain, gills and intestine while oxidative damage to lipids was detected only beyond 34ºC solely in liver and muscle despite an enhanced anti-oxidant potential. Additionally, detoxifying mechanisms in juvenile fish seem to be impaired by

acute warming (CYP1A), which could impact the ability of fish to cope with environmental pollution.

Chronic warming also affected juvenile fish, although to a lesser extent when compared to larvae and adults. When subjected to chronic warming, juvenile fish showed a greater response capacity and were more able to maintain the integrity of vital organs than larvae and adults. Only mild biochemical changes were detected at 24ºC, suggesting that chronic warming may not be as damaging as acute warming. Nevertheless, chronic exposure to 30ºC elicited significant biochemical changes. Juvenile fish adjusted their muscle energy metabolism when exposed to chronic warming, showing an enhanced glycolytic potential which probably contributed to maintain cellular defenses (cytoskeleton adjustments, gene expression regulation, chaperoning, acid-base balance). However, inflammatory processes (potentially related to some protein damage and oxidative stress) were still detected at 30ºC, suggesting that juveniles could suffer if exposed to future heat waves in nursery areas. While it was not possible to estimate the CTmax of adult fish due to size constraints of the experimental system, biochemical and histo-pathological analyses showed that adults were more sensitive to temperature (Chapter 7) than juveniles. While juveniles could protect vital organs to some extent, vital organs of adult fish (muscle, liver and brain) had the lowest capacity to respond to stress, and showed the greatest tissue harm (damage to lipids and proteins, inflammation, atrophy) and loss of glycogen storages, suggesting that this energy expenditure and failure to respond was at least partially responsible for the elevated mortality rates, mainly at 30ºC.

In summary, the main conclusions of this work are:

1. There is an ontogenetic shift in upper thermal limits of *S. aurata*.

2. The effort to protect against thermal challenge is differentially allocated by distinct organs, depending on developmental stage.

3. Larvae are more sensitive to warming than juveniles and adults, being the key life cycle stage that will determine recruitment's success and life cycle closure and thus the viability of *S. aurata* populations to ocean warming

4. Despite being more resistant, juvenile fish will be frequently exposed to heat waves (as opposed to adults for example), which could affect juvenile survival and recruitment's success

5. Acute exposure induces faster and greater inflammation of tissues than chronic exposure, which means that sudden heat waves with fast rates of warming will be especially dangerous for fish stocks

6. The capacity to detoxify contaminants may be affected by ocean warming, which could pose a threat to juvenile fish living in coastal and estuarine nursery areas (subjected to elevated input of contaminants)

7. Cytoskeletal proteins, translational regulators, glycolitical enzymes and inflammation-related proteins could be further used as markers of stress

8. There is a need to develop improved stock management plans that take into account the vulnerability of species towards climate change (considering the different requirements and vulnerabilities of different life cycle stages), contributing to the sustainability of fish stocks

Eco-physiological studies under a background of climate change can provide valuable information that will help scientists and stakeholders to improve and adapt conservation practices for exploited species. This is in fact one of the great challenges of society considering that fisheries and aquaculture provide crucial food resources to the world's population. The acclimation capacity and range of temperatures appropriate for larval migration and survival, juvenile growth and reproduction should be determined for the main commercial species and should be incorporated into environmental protections efforts (fish quotas, marine protected areas, legal size at catch) in order to promote the sustainability of fish stocks. Moreover, the combined effect of environmental variables should be evaluated in wild populations under relevant ecological contexts and time-frames since organisms can respond differently when exposed (acutely or chronically) to one or more stress factors (additive, synergistic or antagonistic effects can be expected).

Another key aspect to be addressed is related to genome sequencing of non-model organisms. In order to apply 'omics' techniques to marine ecology research the genomes of marine species should be decoded and made available to the scientific community. The lack of genome sequencing has been one of the major draw-backs in the application of high throughput techniques in other scientific fields other than human health. This is of major importance considering that 'omic' studies provide information that can generate new hypotheses and truly advance our knowledge concerning the molecular underpinnings of the stress response.

In addition, several other issues remain to be addressed concerning the capacity of fish to survive and thrive in warmer oceans. The capacity for evolutionary adaptation is one of them. Despite the potential for acclimation, the maintenance and viability of populations is dependent upon the capacity to adapt, which could help species to cope with environmental stress and even explore new ecological niches. Species with the greatest and the lowest potential for adaptation should be identified across taxa and this information should also be considered in management plans. Factors such as the evolvability of the stress response network (and consequently mutation rates), generation time, genetic flow (larval dispersal), eury- or steno-thermality may be crucial to determine the adaptation potential of species. Moreover, epigenetic effects (e.g. parental effects) should be highlighted since they can confer transgenerational plasticity, ameliorating the effects of climate change on wild populations.

ANNEXES

ANNEX 1. THESIS OUTPUTS

Articles in international peer-reviewed journals

1. Madeira D, Vinagre C, Costa PM, Diniz MS (2014) Histopathological alterations, physiological limits and molecular responses of a juvenile eurythermal fish (*Sparus aurata*) to thermal stress. Marine Ecology Progress Series 505, 253-266.

2. Madeira D, Vinagre C, Diniz MS (2016) Are fish in hot water? Effects of warming on oxidative stress metabolism in the commercial species *Sparus aurata*. Ecological Indicators 63, 324-331.

3. Madeira D, Costa PM, Vinagre C, Diniz MS (2016) When warming hits harder: survival, cellular stress and thermal limits of *Sparus aurata* larvae under global change. Marine Biology 163, 91-104.

4. Madeira D, Araújo JE, Vitorino R, Capelo JL, Vinagre C, Diniz MS (2016) Ocean warming alters cellular metabolism and induces mortality in fish early life stages: a proteomic approach. Environmental Research 148, 164-176.

5. Madeira D, Araújo JE, Vitorino R, Costa PM, Capelo JL, Vinagre C, Diniz MS. Molecular plasticity under ocean warming: integrating proteome changes with organism-level indicators unveils thermal tolerance of fish. Submitted.

6. Madeira D, Madeira C, Costa PM, Vinagre C, Pörtner HO, Diniz MS. Life cycle closure is hampered by future warming: developmental stage determines the vulneravility and physiological priorities of coastal fish. Submitted.

Conference proceedings

1. Madeira D, Diniz MS, Rosa R, Vinagre C, Costa PM, Costa MH, Caeiro S, Santos HM, Nuñez C, Oliveira E, Castro L, Peres I, Lodeiro C, Capelo JL (2012) Sea warming affects bream (*Sparus aurata*) tissues and stress proteins (Hsp70)? Microscopy and Microanalysis 19 (Suppl 4), 83-84. doi:10.1017/S1431927613001037

Results presented in scientific meetings (national and international)

1. Madeira D, Diniz MS, Rosa R, Vinagre C, Costa PM, Costa MH, Caeiro S, Santos HM, Nuñez C, Oliveira E, Castro L, Peres I, Lodeiro C, Capelo JL (2012) Sea warming affects bream (*Sparus aurata*) tissues and stress proteins (Hsp70). Poster presented at the XLVI SPMicros annual congress “Microscopy: a tool for the advancement of science”, 24-25Sept, in Lisbon, Portugal.

2. Madeira D, Vinagre C, Diniz MS (2013) Thermal stress response and potential effects of global warming at a cellular and molecular level on the commercial species gilthead seabream (*Sparus aurata*). Poster presented at Marine Sciences Symposium, 17-18Jun, in Lisbon, Portugal.

3. Madeira D, Dias M, Roma J, Cabral HN, Diniz MS, Narciso L, Vinagre C (2013) Critical thermal maximum of coastal organisms of the Portuguese coast. Poster presented at Marine Sciences Symposium, 17-18Jun, in Lisbon, Portugal.

4. Diniz MS, **Madeira D**, Costa PM, Repolho T, Rosa R (2013) Sea warming and acidification effects on bream (*Sparus aurata*) livers and oxidative stress markers. Poster presented at "Microscopy In Research – SPMicros Congress", 9-10 Dec, in Monte da Caparica, Portugal.

5. Madeira D, Madeira C, Larguinho M, Costa PM, Vinagre C, Diniz MS (2014) *Sparus aurata* larvae sensitivity to global warming: oxidative stress responses. Poster presented at CLIMA 2014: IV National Congress on Climate Change, 4-5 Dec, in Aveiro, Portugal.

6. Madeira D, Madeira C, Costa PM, Vinagre C, Matos APA, Diniz MS (2015) The potential effects of global warming on *Sparus aurata* larvae: clues from oxidative stress responses and histopathology. Poster presented at "From Basic Sciences to Clinical Research", 1st International Congress, 27-28 November, in Caparica, Portugal.

7. Diniz MS, Larguinho M, **Madeira D**, Costa PM, Repolho T, Rosa R (2015) Response of oxidative stress enzymes and Hsp70 in Sea bream juveniles: effects of exposure to temperature increase and different pH. Poster presented at "From Basic Sciences to Clinical Research", 1st International Congress, 27-28 November, in Caparica, Portugal.

8. Madeira D, Vinagre C, Rosa R, Repolho T, Costa PM, Castro L, Costa MH, Cabral H, Peres I, Diniz MS (2013) Ocean temperature effects on the gilthead seabream: thermal stress proteins (Hsp70) and oxidative stress biomarkers. 14th National Meeting of Ecology SPECO/ Spring Meeting APEP, 17-19Jun, in Bragança, Portugal. Oral presentation.

9. Diniz MS, **Madeira D**, Costa PM, Repolho T, Rosa R (2014). Stress oxidative responses in gilthead seabream (*Sparus aurata*) juveniles as a result of sea warming and acidification. ECSA 54 Conference - Coastal systems under change: tuning assessments and management tools. May 12-16, Sesimbra, Portugal. Oral presentation.

10. Madeira D, Vinagre C, Costa PM, Diniz MS (2014) Biochemical and physiological changes induced by thermal stress in juvenile *Sparus aurata*: short-term versus long-term trials. CLIMA 2014: IV National Congress on Climate Change, 4-5 Dec, in Aveiro, Portugal. Oral presentation.

11. Larguinho M, **Madeira D**, Costa PM, Repolho T, Rosa R, Diniz MS (2014) Impact of exposure to sea warming and acidification in *Sparus aurata* juveniles: oxidative stress responses. CLIMA 2014: IV National Congress on Climate Change, 4-5 Dec, in Aveiro, Portugal. Oral presentation.

ANNEX 2. SUPPLEMENTARY INFORMATION FOR CHAPTER 3

Table S3.1 Masses and sequences of peptides obtained for each spot.

SPOT nº								
546	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1130.548	1130.552	4	201	210	GYSFVTTAER	28.76	67.475
	1130.548	1130.552	4	201	210	GYSFVTTAER		
	1516.703	1516.695	-5	364	376	QEYDESGPSIVHR		
	1790.892	1790.893	1	243	258	SYELPDGQVITIGNER	69.49	99.997
	1790.892	1790.893	1	243	258	SYELPDGQVITIGNER		
	1959.927	1959.903	-12	73	88	YPIEHGIITNWDNMEK		
	3182.594	3182.586	-2	152	181	TTGIVLDSGDGVSHNVPIYEGYALPHAIMR		
860	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1019.505	1019.504	-1	888	895	ENAMRDRK		
	1091.533	1091.526	-7	502	510	SQEVEDKTR		
	1171.683	1171.691	7	205	214	SHSIFLINIK		
	1487.651	1487.612	-26	642	653	SLTDYMQNMEQK		
	1489.786	1489.787	1	215	227	QENVETEKKLSGK		
	1681.887	1681.85	-22	532	545	ELSQLQELSNHQKK		
	1803.902	1803.944	23	447	461	QQMLDQDELLASTRR		
	1821.866	1821.921	30	702	716	ALEQQMESHREAHQK		
	1837.929	1837.889	-22	749	763	LSSDYNKLKIEDQER		
	1837.995	1837.989	-3	355	369	TLKNVIQHLEMELNR	37.67	86
	1857.882	1857.894	6	372	388	NGEAVPEDEQISAKDQK		
	1917.951	1917.924	-14	813	831	VKKSVELDNDDGGGSAAQK		
	2174.121	2174.034	-40	93	111	THTMEGKLHDPQLMGIIPR		
	2342.159	2342.083	-33	619	639	MNASERELAACQLLISQHEAK		
	2344.233	2344.182	-22	52	72	VLPPNTTQEQVYNACAKQIVK		

2358.178	2358.086	-39	427	446	QLDDKDDEINQQSQLAEKLK
2384.293	2384.258	-15	258	280	SLSALGNVISALAEGTKTHVPYR
3348.655	3348.636	-6	816	846	SVELDNDDGGGSAAQKQKISFLENNLEQLTK

502	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1088.552	1088.536	-15	249	256	DYLHYHIK		
	1343.707	1343.703	-3	238	248	DNTINLIHTFR	37.21	92.428
	1343.707	1343.703	-3	238	248	DNTINLIHTFR		
	1450.755	1450.746	-6	191	203	ASHTAPQVLFSHR	44.14	98.465
	1450.755	1450.746	-6	191	203	ASHTAPQVLFSHR		
	1606.856	1606.84	-10	190	203	RASHTAPQVLFSHR		
	2184.097	2184.096	-1	211	230	DTDAAVGDNIGYITFVLFPR	46.17	99.038
	2184.097	2184.096	-1	211	230	DTDAAVGDNIGYITFVLFPR		
	2257.209	2257.209	0	191	210	ASHTAPQVLFSHREPPLELK		
	2413.241	2413.249	3	238	256	DNTINLIHTFRDYLHYHIK		

283	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1677.833	1677.897	38	266	279	QVEKEEEFQEAIAK		
	1810.029	1809.978	-28	450	464	NLRLAVDREETRPLK		
	1810.029	1809.978	-28	450	464	NLRLAVDREETRPLK		
	1899.023	1899.044	11	438	452	VQVDELMRQELRNLR		
	2091.955	2092.032	37	314	332	FPDYPDEASGGSYLIFADK		
	2674.461	2674.503	16	540	563	QNIALYGVLRLGSHDIHTMAPLVR		
	2691.353	2691.377	9	391	413	SIPVINAGHEEFTSIWKSRYDNK		
	2691.353	2691.377	9	391	413	SIPVINAGHEEFTSIWKSRYDNK		
	2695.457	2695.383	-27	386	409	VLPSKSIPVINAGHEEFTSIWKSR		
	2749.251	2749.268	6	270	293	EEEFQEAIAKTHESLTETEGPDMK		

	3794.924	3794.923	0	2	34	SEGTYQRLWEASHATLEEVLEKEPSPLEPVLTR
	3814.098	3813.965	-35	540	575	QNIALYGVLRLGSHDIHTMAPLVRSILLVGPSGMGK

664	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1255.595	1255.617	17	119	129	AGFEDYVEGLR	43.57	99.126
	1255.595	1255.617	17	119	129	AGFEDYVEGLR		
	1293.662	1293.678	12	62	72	VAYNQIADIMR		
	2407.12	2407.162	17	37	56	VEFSADQIEDYREAFGLFDR		

775	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1551.803	1551.738	-42	34	45	KYIPEEQRHSHK		
	1609.797	1609.801	3	30	41	DFERKYIPEEQR		
	1667.84	1667.871	19	150	163	NYGLLACFKKDMHK		
	1838.896	1838.963	36	160	174	DMHKVETYLKVMNCK		
	1882.041	1881.966	-40	13	29	AVHRAQHLHLVAAETTK		
	2363.141	2363.223	35	138	158	FDGNLSEEALMKNYGLLACFK		
	3202.762	3202.686	-24	70	96	SDRELLLYSLLLIQSWLNPIQNLSAFR		
	3809.064	3808.916	-39	73	104	ELLLYSLLLIQSWLNPIQNLSAFRTSDRVYDK		

867	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1077.584	1077.564	-19	181	188	RNEVWFVK		
	1193.653	1193.614	-32	62	72	LLKYVGGSNDK		
	1407.795	1407.797	1	151	163	LKTALGSEAAYRK		
	2239.161	2239.099	-28	1	19	MLGMIKNSLLSTVETWPYR		
	2691.348	2691.297	-19	73	99	GAGMGMTAPVSITAFPAEDGSLQQKVK		
	2716.413	2716.342	-26	98	121	VKVYLRIPNQFQASPPCPSDESIK		
	2839.382	2839.325	-20	35	61	ECEGGQFAVVEVTGKPFDEASKEAALK		

	3331.594	3331.606	4	122	150	IEERQGMTIYSTQFGGYAKEVDYVNYAAK		
875	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1195.683	1195.718	29	69	77	FRLNLYELK		
	1234.679	1234.715	29	71	80	LNLYELKEGR		
	1590.766	1590.833	42	86	98	TFMSMVSNLLYER		
	1778.936	1778.96	13	100	115	FGPYYIEPVIAGLDPK	40.86	98.716
	1778.936	1778.96	13	100	115	FGPYYIEPVIAGLDPK		
	1905.044	1905.112	36	49	66	LYIGLAGLATDVQTVSQR	92.59	100
	1905.044	1905.112	36	49	66	LYIGLAGLATDVQTVSQR		
	1935.038	1935.094	29	99	115	RFGPYYIEPVIAGLDPK		
359	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1000.59	1000.559	-31	685	692	KRNDNLLK		
	1008.507	1008.5	-7	884	891	ELPDYLMK		
	1186.606	1186.634	24	508	518	LKGSENGQPEK		
	1198.705	1198.7	-4	729	739	AVFPSIVGRPR		
	1480.789	1480.752	-25	673	684	EKKYLEDIESVK		
	1483.681	1483.646	-24	532	543	ELENFMAIEEMK		
	1515.749	1515.742	-5	785	795	IWHHTFYNELR		
	1516.703	1516.71	5	1060	1072	QEYDESGPSIVHR		
	1633.862	1633.81	-32	878	891	LDLAGRELPDYLMK		
	1639.686	1639.648	-23	64	77	WCRHCFPCCRGSGK		
	1691.871	1691.835	-21	653	666	EEIAMLRLELDTMK		
	1790.892	1790.899	4	939	954	SYELPDGQVITIGNER	64.4	99.995
	1790.892	1790.899	4	939	954	SYELPDGQVITIGNER		
	1960.965	1960.94	-13	364	380	QMLKISSENSNPEQDLK		

1994.912	1994.975	31	482	498	QMPKYSSENSNPEQDLK
2246.089	2246.069	-9	499	518	LTSEEESQRLKGSENGQPEK
2811.328	2811.345	6	486	509	YSSENSNPEQDLKLTSEEESQRLK
3350.657	3350.617	-12	602	629	QFCEEQNTGILHDEILIHEEKQIEVVEK

877	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1130.548	1130.586	34	200	209	GYSFVTTAER	14.42	0
	1130.548	1130.586	34	200	209	GYSFVTTAER		
	1198.705	1198.733	23	32	42	AVFPSIVGRPR	35.89	96.244
	1198.705	1198.733	23	32	42	AVFPSIVGRPR		
	1354.623	1354.687	40	54	65	DSYVGDEAQSKR		
	1515.749	1515.781	21	88	98	IWHHTFYNELR		
	1516.703	1516.755	35	363	375	QEYDESGPSIVHR		
	1627.844	1627.886	26	200	213	GYSFVTTAEREIVR		
	1790.892	1790.947	31	242	257	SYELPDGQVITIGNER	85.39	100
	1790.892	1790.947	31	242	257	SYELPDGQVITIGNER		
	1954.065	1954.116	26	99	116	VAPEEHPVLLTEAPLNPK		
	3182.594	3182.68	27	151	180	TTGIVLDSGDGVSHNVPIYEGYALPHAIMR		

190	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1081.568	1081.565	-2	349	357	LLQDFFNGK		
	1197.663	1197.674	10	459	469	FELTGIPPAPR		
	1215.669	1215.679	8	160	171	DAGTISGLNVLR		
	1228.628	1228.635	5	26	36	VEIIANDQGNR		
	1235.624	1235.626	1	237	246	MVNHFIAEFK		
	1253.616	1253.641	20	302	311	FEELNADLFR		
	1451.797	1451.811	10	329	342	AQVHDIVLVGGSTR		

1473.686	1473.714	19	37	49	TTPSYVAFTDSER	50.5	99.901
1473.686	1473.714	19	37	49	TTPSYVAFTDSER		
1480.754	1480.776	15	300	311	ARFEELNADLFR		
1691.726	1691.76	21	221	236	STAGDTHLGGEDFDNR	94.4	100
1691.726	1691.76	21	221	236	STAGDTHLGGEDFDNR		
1787.99	1787.998	5	172	188	IINEPTAAAIAYGLDKK		
1825.959	1825.984	13	326	342	MDKAQVHDIVLVGGSTR		
1952.06	1952.101	21	452	469	DNNLLGKFELTGIPPAPR	75.8	100
1952.06	1952.101	21	452	469	DNNLLGKFELTGIPPAPR		
2009.009	2009.054	22	138	155	SINNAVITVPAYFNDSQR	86.9	100
2009.009	2009.054	22	138	155	SINNAVITVPAYFNDSQR		
2774.327	2774.403	28	424	447	QTQTFTTYSDNQPGVLIQVYEGER		
2983.516	2983.538	7	129	155	EIAEAYLGKSINNAVITVPAYFNDSQR		

841	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1156.657	1156.69	28	169	178	LVIIEGDLER		
	1243.653	1243.686	26	92	101	IQLVEEELDR		
	1268.623	1268.662	31	150	160	EAKHIAEEADR		
	1271.684	1271.706	17	38	48	QLEDDLVALQK		
	1284.752	1284.792	31	168	178	KLVIIEGDLER		
	1308.618	1308.658	31	234	244	EAETRAEFAER		
	1399.779	1399.791	8	38	49	QLEDDLVALQKK		
	1416.794	1416.815	14	106	118	LATALTKLEEAEK		
	1446.718	1446.761	29	77	90	KATDAEGDVASLNR		
	1474.725	1474.772	32	78	91	ATDAEGDVASLNRR		
	1486.811	1486.829	12	36	48	SKQLEDDLVALQK		
	1552.738	1552.769	20	252	264	TIDDLEDELYAQK	104.97	100

1552.738	1552.769	20	252	264	TIDDLEDELYAQK
1602.82	1602.859	25	77	91	KATDAEGDVASLNRR
1614.906	1614.934	17	36	49	SKQLEDDLVALQKK
1671.891	1671.925	20	169	182	LVIIEGDLERTEER
1688.801	1688.851	29	214	226	YSQKEDKYEEEIK
1727.892	1727.937	26	92	105	IQLVEEELDRAQER
1727.892	1727.937	26	92	105	IQLVEEELDRAQER
1758.822	1758.888	38	269	284	AISEELDHALNDMTSI
1793.917	1793.929	7	252	266	TIDDLEDELYAQKLK
1799.986	1800.053	37	168	182	KLVIIEGDLERTEER
1815.898	1815.958	33	153	167	HIAEEADRKYEEVAR
1815.898	1815.958	33	153	167	HIAEEADRKYEEVAR
1883.993	1884.036	23	91	105	RIQLVEEELDRAQER
1962.987	1963.044	29	134	149	AMKDEEKMELQEIQLK
2175.114	2175.192	36	106	125	LATALTKLEEAEKAADESER
2175.114	2175.192	36	106	125	LATALTKLEEAEKAADESER
2416.257	2416.308	21	169	189	LVIIEGDLERTEERAELSESK

117	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1235.601	1235.619	14	476	486	DQVANSAFVER		
	1348.664	1348.681	12	304	314	HFSVEGQLEFR	67.84	99.997
	1348.664	1348.681	12	304	314	HFSVEGQLEFR		
	1513.786	1513.788	1	363	376	GVVDSEDLPLNISR		
	1527.744	1527.777	22	291	303	SLTNDWEDHLAVK		
	1833.781	1833.825	24	276	290	NPDDITNEEYGEFYK	33.72	91.013
	1833.781	1833.825	24	276	290	NPDDITNEEYGEFYK		
	2441.21	2441.272	25	466	486	HIYYITGETKDQVANSAFVER		

285	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1410.774	1410.77	-3	85	96	QLFHPEQLITGK		
	1487.879	1487.891	8	230	243	LISQIVSSITASLR		
	1715.921	1715.991	40	65	79	AVFVDLEPTVIDEIR		
	1718.882	1718.936	32	216	229	NLDIERPTYTNLNR		
	1756.963	1756.992	16	265	280	IHFPLATYAPVISAEK		
	1807.883	1807.938	30	374	390	AVCMLSNTTAIAEAWAR		
	1824.985	1824.97	-8	353	370	VGINYQPPTVVPGGDLAK		
	1912.974	1912.968	-3	337	352	TKRSIQFVDWCPTGFK		
	2191.111	2191.152	19	371	390	VQRAVCMLSNTTAIAEAWAR		
	2409.209	2409.28	30	244	264	FDGALNVDLTEFQTNLVPYPR		
	2415.205	2415.281	31	85	105	QLFHPEQLITGKEDAANNYAR		
	2693.27	2693.376	40	281	304	AYHEQLSVAEITNACFEPANQMVK		
	3349.598	3349.691	28	125	156	LSDQCTGLQGFLVFHSFGGGTGSGFTSLLMER		

840	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1063.542	1063.57	26	250	257	QIFEEEIR		
	1066.516	1066.536	19	203	210	YEDEINKR		
	1079.512	1079.541	27	289	297	AQYEDIANR		
	1082.599	1082.621	20	126	134	FASFIDKVR	41.39	99.372
	1082.599	1082.621	20	126	134	FASFIDKVR		
	1277.71	1277.741	24	406	416	LALDIEIATYR		
	1308.654	1308.693	29	201	210	NKYEDEINKR		
	1317.691	1317.723	24	147	157	WSLLQNQTATR		
	1351.616	1351.675	44	265	276	DTSVVVEMDNSR		
	1399.79	1399.768	-16	377	388	IKDLEDALQRAK		

1405.805	1405.828	17	406	417	LALDIEIATYRK
1449.758	1449.798	27	238	249	LESLTDEINFLR
1475.825	1475.804	-14	497	509	SVVIKMIETKDGR
1680.918	1680.962	26	121	134	TLNNRFASFIDKVR
2130.024	2130.077	25	353	371	ANLENQIAEAEERGEMAVR
2178.071	2178.132	28	258	276	ELQSQIKDTSVVVEMDNSR
2807.451	2807.412	-14	135	157	FLEQQNKMLETKWSLLQNQTATR
2811.423	2811.412	-4	341	365	LQSEIDAVKGQRANLENQIAEAEER
2854.415	2854.388	-9	158	182	SNIDAMFEAYIANLRRQLDSLGNDK

Table S3.2 Detailed functional categorization of proteins. Information was retrieved from UniProt, GeneCards, neXtprot beta, InterPro and Qiagen.

Spot no.	Protein Name	Function	Main functional category
546	Actin, muscle	Cell motility, structure and integrity; Alpha, beta and gamma actin isoforms have been identified, with alpha actins being a major constituent of the contractile apparatus, while beta and gamma actins are involved in the regulation of cell motility. Actin has four is involved in four major functions 1) transport of vesicles, organelles and other cargo, 2) give mechanical support to cells and attach them to each other and the extracellular matrix, 3) muscle relaxation and contraction, 4) cell motility through polymerization and depolymerization of fibrils; ATP and ADP-binding; nucleotide-binding; protein binding; chromosome movement; cytoskeleton structure; Response to extracellular stimulus; Skeletal muscle fiber adaptation; Skeletal muscle fiber development; Skeletal muscle thin filament assembly.	**Cytoskeletal dynamics**
860	Kinesin heavy chain isoform	Microtubule-associated force-producing protein that play a role in organelle transport; mediates dendritic trafficking of mRNAs; ATP-binding; cytoskeleton dependent intracellular transport; protein localization; microtubule-based movement; organelle organization; axon guidance; metabolic process	**Intracellular transport**
502	Actin-related protein 2/3 complex subunit 2	Functions as actin-binding component of the Arp2/3 complex which is involved in regulation of actin polymerization and together with an activating nucleation-promoting factor (NPF) mediates the formation of branched actin networks. Seems to contact the mother actin filament; structural constituent of cytoskeleton; Arp2/3 complex-mediated actin nucleation; axon guidance; innate immune response; movement of cell or subcellular component; small GTPase mediated signal transduction;	**Cytoskeletal dynamics**
283	IQ and AAA domain-containing protein 1-like	Nucleotide-binding; ATP-binding; AAA ATPases (ATPases Associated with diverse cellular Activities) form a large protein family and play a number of roles in the cell including cell-cycle regulation, protein proteolysis and disaggregation, organelle biogenesis and intracellular transport. Some of them function as molecular chaperones, subunits of proteolytic complexes or independent proteases (FtsH, Lon). They also act as DNA helicases and transcription factors; The functional variety seen between AAA ATPases is in part due to their extensive number of accessory domains and factors, and to their variable organization within oligomeric assemblies, in addition to changes in key functional residues within the ATPase domain itself; The IQ motif, which can be present in one or more copies, serves as a binding site for different EF-hand proteins including the essential and regulatory myosin light chains, calmodulin (CaM), and CaM-like proteins; many IQ motifs are protein kinase C (PKC) phosphorylation sites.	**Diverse (cell-cycle regulation, proteolysis, intracellular transport, chaperoning, transcription factor activity, protein binding)**

668	Myosin light chain 1, skeletal muscle isoform	Calcium ion binding; motor protein; muscle contraction; myosins are a large family of motor proteins that share the common features of ATP hydrolysis, actin binding and potential for kinetic energy transduction.	**Cytoskeletal dynamics**
775	Somatotropin	Somatotropin is a hormone that plays an important role in growth control and is involved in the regulation of several anabolic processes. Implicated as an osmoregulatory substance important for seawater adaptation; metal-ion binding,	**Growth metabolism**
867	Heme-binding protein 1	May bind free porphyrinogens that may be present in the cell and thus facilitate removal of these potentially toxic compounds. Binds with a high affinity to one molecule of heme or porphyrins. It binds metalloporphyrins, free porphyrins and N-methylprotoporphyrin with similar affinities; signal transduction; peptide ligand-binding receptor	**Porphyrin metabolism**
875	Proteasome subunit beta type-3	The proteasome is a multicatalytic proteinase complex which is characterized by its ability to cleave peptides with Arg, Phe, Tyr, Leu, and Glu adjacent to the leaving group at neutral or slightly basic pH. The proteasome has an ATP-dependent proteolytic activity; Protein binding; Threonine-type endopeptidase activity; Cleavage of peptide bonds with very broad specificity; protein degradation; proteolysis involved in cellular protein catabolic process;	**Proteolysis**
359	POTE ankyrin domain family member F	Cell adhesion and migration; Actin Nucleation by ARP-WASP Complex**;** involved in PAK pathway (p21 protein activated kinase) **-** which are activated in response to extracellular signals and regulate cell shape and motility. PAKs regulate diverse cellular functions, including gene expression, cytoskeletal actin assembly; Ras pathway (Ras is a cellular and biochemical signaling molecule that displays dynamic nature of signal transduction across the membrane in determining cellular responses to external stimuli, and is associated to an ever increasing list of signaling pathways with immense gene expression strategies); cytoskeletal signaling; protein metabolism	**Regulation**
877	Actin, muscle-type	Cell motility, structure and integrity; Alpha, beta and gamma actin isoforms have been identified, with alpha actins being a major constituent of the contractile apparatus, while beta and gamma actins are involved in the regulation of cell motility. Actin has four is involved in four major functions 1) transport of vesicles, organelles and other cargo, 2) give mechanical support to cells and attach them to each other and the extracellular matrix, 3) muscle relaxation and contraction, 4) cell motility through polymerization and depolymerization of fibrils; ATP and ADP-binding; nucleotide-binding; protein binding; chromosome movement; cytoskeleton structure; Response to extracellular stimulus; skeletal muscle fiber adaptation; Skeletal muscle fiber development; skeletal muscle thin filament assembly	**Cytoskeletal dynamics**

190	Heat shock cognate 71 kDa protein	In cooperation with other chaperones, Hsp70s stabilize preexistent proteins against aggregation and mediate the folding of newly translated polypeptides in the cytosol as well as within organelles. These chaperones participate in all these processes through their ability to recognize nonnative conformations of other proteins. They bind extended peptide segments with a net hydrophobic character exposed by polypeptides during translation and membrane translocation, or following stress-induced damage. Positive regulator of PARK2 translocation to damaged mitochondria; ATP binding; protein binding; unfolded protein binding; protein refolding; stress response; MAPK signaling pathway; Regulation of HSF1-mediated heat shock response; spliceosome; Protein processing in endoplasmic reticulum; stress response	**Chaperoning**
841	Tropomyosin alpha-1 chain	Binds to actin filaments in muscle and non-muscle cells. Plays a central role, in association with the troponin complex, in the calcium dependent regulation of vertebrate striated muscle contraction. Smooth muscle contraction is regulated by interaction with caldesmon. In non-muscle cells is implicated in stabilizing cytoskeleton actin filaments; actin binding; structural constituent of cytoskeleton; cytoskeletal signaling; Cellular response to reactive oxygen species; Movement of cell or subcellular component; Positive regulation of cell adhesion; Positive regulation of stress fiber assembly; Sarcomere organization	**Cytoskeletal dynamics**
117	Heat shock protein HSP 90-alpha	Molecular chaperone that promotes the maturation, structural maintenance and proper regulation of specific target proteins involved for instance in cell cycle control and signal transduction. Undergoes a functional cycle that is linked to its ATPase activity. This cycle probably induces conformational changes in the client proteins, thereby causing their activation. Interacts dynamically with various co-chaperones that modulate its substrate recognition, ATPase cycle and chaperone function; chaperone-mediated protein complex assembly; positive regulation of nitric oxide biosynthetic process; protein folding; protein import into mitochondrial outer membrane; response to stress; involved in various cellular processes including signal transduction, protein folding, protein degradation and morphological evolution; The full functional activity of Hsp90 is gained in concert with other co-chaperones, playing an important role in the folding of newly synthesized proteins and stabilization and refolding of denatured proteins after stress; ATPase activity: ATP and GTP binding; protein binding; ion channel binding; mRNA binding; nucleotide binding; signal transduction; response to stress; Positive regulation of lamellipodium assembly; protein refolding	**Chaperoning**
285	Tubulin alpha-4A chain	Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha chain; structural constituent of cytoskeleton; 'de novo' posttranslational protein folding; cellular protein metabolic process; microtubule-based process; mitotic cell cycle; organelle organization; protein folding; protein polymerization; Development of Slit-Robo signaling; cell junction dynamics; Microtubule-dependent trafficking of connexons from Golgi to the plasma membrane; Post-chaperonin tubulin folding pathway	**Cytoskeletal dynamics**

840	Keratin, type II cytoskeletal 8	Intermediate filament protein; together with KRT19, helps to link the contractile apparatus to dystrophin at the costameres of striated muscle; structural molecule activity; this protein plays a role in maintaining cellular structural integrity and also functions in signal transduction and cellular differentiation; cytoskeletal signaling and remodeling	**Cytoskeletal dynamics**

ANNEX 3. SUPPLEMENTARY INFORMATION FOR CHAPTER 6

Table S6.1 Tukey's Post-hocs **(a)** 14 days of exposure, **(b)** 21 days of exposure.

(a) 14 days of exposure **p values ≤ 0.05 are marked with red**

Spot	Protein

1670 Heat shock 70 kDa protein 1

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=0.0200, 95%CI=-0.0812 to 0.1212, p=0.87

18ºC vs 30ºC: Diff=0.5000, 95%CI=0.3988 to 0.6012, p=0.000

24ºC vs 30ºC: Diff=0.4800, 95%CI=0.3788 to 0.5812, p=0.000

1669 Heat shock 70 kDa protein 1

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=0.0300, 95%CI=-0.1292 to 0.1892, p=0.88

18ºC vs 30ºC: Diff=0.5700, 95%CI=0.4108 to 0.7292, p=0.000

24ºC vs 30ºC: Diff=0.5400, 95%CI=0.3808 to 0.6992, p=0.000

859 Rab GDP dissociation inhibitor beta

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=-0.0700, 95%CI=-0.1542 to 0.0142, p=0.11

18ºC vs 30ºC: Diff=0.0800, 95%CI=-0.0042 to 0.1642, p=0.06

24ºC vs 30ºC: Diff=0.1500, 95%CI=0.0658 to 0.2342, p=0.0006

1668 Heat shock cognate 71 kDa protein

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=-0.0600, 95%CI=-0.1994 to 0.0794, p=0.53

18ºC vs 30ºC: Diff=0.1500, 95%CI=0.0106 to 0.2894, p=0.03

24ºC vs 30ºC: Diff=0.2100, 95%CI=0.0706 to 0.3494, p=0.003

1312 Adenylate kinase isoenzyme

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=0.0400, 95%CI=-0.0896 to 0.1696, p=0.72

18ºC vs 30ºC: Diff=0.1800, 95%CI=0.0504 to 0.3096, p=0.006

24ºC vs 30ºC: Diff=0.1400, 95%CI=0.0104 to 0.2696, p=0.03

1681 Creatine kinase M-type

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=-0.0100, 95%CI=-0.1177 to 0.0977, p=0.97

18ºC vs 30ºC: Diff=0.1200, 95%CI=0.0123 to 0.2277, p=0.03

24ºC vs 30ºC: Diff=0.1300, 95%CI=0.0223 to 0.2377, p=0.02

1733 Creatine kinase M-type

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=0.0400, 95%CI=-0.0789 to 0.1589, p=0.67

18ºC vs 30ºC: Diff=0.1600, 95%CI=0.0411 to 0.2789, p=0.007

24ºC vs 30ºC: Diff=0.1200, 95%CI=0.0011 to 0.2389, p=0.047

1211 Carbonic anhydrase 1

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=0.0300, 95%CI=-0.0881 to 0.1481, p=0.79

18ºC vs 30ºC: Diff=0.1500, 95%CI=0.0319 to 0.2681, p=0.01

24ºC vs 30ºC: Diff=0.1200, 95%CI=0.0019 to 0.2381, p=0.046

1716 Creatine kinase M-type

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=-0.0400, 95%CI=-0.1836 to 0.1036, p=0.76

18ºC vs 30ºC: Diff=0.1400, 95%CI=-0.0036 to 0.2836, p=0.05

24ºC vs 30ºC: Diff=0.1800, 95%CI=0.0364 to 0.3236, p=0.01

509 Creatine kinase M-type

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=-0.1000, 95%CI=-0.3071 to 0.1071, p=0.45

18ºC vs 30ºC: Diff=0.1500, 95%CI=-0.0571 to 0.3571, p=0.19

24ºC vs 30ºC: Diff=0.2500, 95%CI=0.0429 to 0.4571, p=0.02

1042 Creatine kinase M-type

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=-0.0400, 95%CI=-0.2470 to 0.1670, p=0.88

18ºC vs 30ºC: Diff=0.2000, 95%CI=-0.0070 to 0.4070, p=0.05

24ºC vs 30ºC: Diff=0.2400, 95%CI=0.0330 to 0.4470, p=0.02

1529 Neuroendocrine convertase 1

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=-0.0300, 95%CI=-0.1834 to 0.1234, p=0.88

18ºC vs 30ºC: Diff=0.1400, 95%CI=-0.0134 to 0.2934, p=0.07

24ºC vs 30ºC: Diff=0.1700, 95%CI=0.0166 to 0.3234, p=0.03

1295 Adenylate kinase isoenzyme 1

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=0.1100, 95%CI=-0.0500 to 0.2700, p=0.21

18ºC vs 30ºC: Diff=0.1800, 95%CI=0.0200 to 0.3400, p=0.02

24ºC vs 30ºC: Diff=0.0700, 95%CI=-0.0900 to 0.2300, p=0.52

1720 Creatine kinase M-type

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=0.0000, 95%CI=-0.0534 to 0.0534, p=NaN

18ºC vs 30ºC: Diff=-0.0600, 95%CI=-0.1134 to -0.0066, p=0.03

24ºC vs 30ºC: Diff=-0.0600, 95%CI=-0.1134 to -0.0066, p=0.03

1246 Triosephosphate isomerase B

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=0.1000, 95%CI=0.0027 to 0.1973, p=0.043
18ºC vs 30ºC: Diff=0.1000, 95%CI=0.0027 to 0.1973, p=0.043
24ºC vs 30ºC: Diff=0.0000, 95%CI=-0.0973 to 0.0973, p=NaN

1674 Actin, alpha cardiac muscle 2

Tukey HSD Post-hoc Test...
18ºC vs 24ºC: Diff=0.0800, 95%CI=-0.1012 to 0.2612, p=0.52
18ºC vs 30ºC: Diff=0.2100, 95%CI=0.0288 to 0.3912, p=0.02
24ºC vs 30ºC: Diff=0.1300, 95%CI=-0.0512 to 0.3112, p=0.19

857 Alpha-enolase

Tukey HSD Post-hoc Test...
18ºC vs 24ºC: Diff=0.0100, 95%CI=-0.2176 to 0.2376, p=0.99
18ºC vs 30ºC: Diff=0.2200, 95%CI=-0.0076 to 0.4476, p=0.05
24ºC vs 30ºC: Diff=0.2100, 95%CI=-0.0176 to 0.4376, p=0.0739

1143 Creatine kinase, testis isozyme

Tukey HSD Post-hoc Test...
18ºC vs 24ºC: Diff=0.0900, 95%CI=-0.0644 to 0.2444, p=0.33
18ºC vs 30ºC: Diff=0.1600, 95%CI=0.0056 to 0.3144, p=0.04
24ºC vs 30ºC: Diff=0.0700, 95%CI=-0.0844 to 0.2244, p=0.49

897 Eukaryotic initiation factor 4A-II

Tukey HSD Post-hoc Test...
18ºC vs 24ºC: Diff=-0.1300, 95%CI=-0.2690 to 0.0090, p=0.07
18ºC vs 30ºC: Diff=0.0100, 95%CI=-0.1290 to 0.1490, p=0.98
24ºC vs 30ºC: Diff=0.1400, 95%CI=0.0010 to 0.2790, p=0.048

851 Alpha-enolase

Tukey HSD Post-hoc Test...
18ºC vs 24ºC Diff=0.0200, 95%CI=-0.1065 to 0.1465, p=0.91
18ºC vs 30ºC: Diff=0.1300, 95%CI=0.0035 to 0.2565, p=0.04
24ºC vs 30ºC: Diff=0.1100, 95%CI=-0.0165 to 0.2365, p=0.096

428 Glycogen phosphorylase, brain form

Tukey HSD Post-hoc Test...
18ºC vs 24ºC: Diff=-0.2600, 95%CI=-0.4937 to -0.0263, p=0.03
18ºC vs 30ºC: Diff=-0.0600, 95%CI=-0.2937 to 0.1737, p=0.79
24ºC vs 30ºC: Diff=0.2000, 95%CI=-0.0337 to 0.4337, p=0.10

(b) 21 days of exposure **p values ≤ 0.05 are marked with red**

Spot	Protein

1781 **Heat shock 70kDa protein**

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=0.0000, 95%CI=-0.1707 to 0.1707, p=NaN

18ºC vs 30ºC: Diff=0.3800, 95%CI=0.2093 to 0.5507, p=0.000

24ºC vs 30ºC: Diff=0.3800, 95%CI=0.2093 to 0.5507, p=0.000

1780 **Heat shock 70 kDa protein**

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=0.0100, 95%CI=-0.1728 to 0.1928, p=0.99

18ºC vs 30ºC: Diff=0.3400, 95%CI=0.1572 to 0.5228, p=0.0004

24ºC vs 30ºC: Diff=0.3300, 95%CI=0.1472 to 0.5128, p=0.0005

475 **Heat shock cognate 71 kDa protein**

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=0.0100, 95%CI=-0.1295 to 0.1495, p=0.98

18ºC vs 30ºC: Diff=0.2000, 95%CI=0.0605 to 0.3395, p=0.005

24ºC vs 30ºC: Diff=0.1900, 95%CI=0.0505 to 0.3295, p=0.007

1086 **Sterile alpha motif domain-containing protein 9-like**

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=-0.1100, 95%CI=-0.1989 to -0.0211, p=0.01

18ºC vs 30ºC: Diff=0.0200, 95%CI=-0.0689 to 0.1089, p=0.84

24ºC vs 30ºC: Diff=0.1300, 95%CI=0.0411 to 0.2189, p=0.004

1389 **Adenylate kinase isoenzyme 1**

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=-0.0200, 95%CI=-0.1953 to 0.1553, p=0.96

18ºC vs 30ºC: Diff=0.2200, 95%CI=0.0447 to 0.3953, p=0.01

24ºC vs 30ºC: Diff=0.2400, 95%CI=0.0647 to 0.4153, p=0.007

999 **Glyceraldehyde-3-phosphate dehydrogenase**

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=0.0700, 95%CI=-0.1993 to 0.3393, p=0.79

18ºC vs 30ºC: Diff=0.3400, 95%CI=0.0707 to 0.6093, p=0.01

24ºC vs 30ºC: Diff=0.2700, 95%CI=0.0007 to 0.5393, p=0.04

1007 **Glyceraldehyde-3-phosphate dehydrogenase**

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=0.1300, 95%CI=-0.0816 to 0.3416, p=0.29

18ºC vs 30ºC: Diff=0.2900, 95%CI=0.0784 to 0.5016, p=0.006

24ºC vs 30ºC: Diff=0.1600, 95%CI=-0.0516 to 0.3716, p=0.16

1783 Glucose-6-phosphate isomerase

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=-0.1000, 95%CI=-0.1940 to -0.0060, p=0.03

18ºC vs 30ºC: Diff=0.0200, 95%CI=-0.0740 to 0.1140, p=0.85

24ºC vs 30ºC: Diff=0.1200, 95%CI=0.0260 to 0.2140, p=0.01

989 Glyceraldehyde-3-phosphate dehydrogenase (Fragment)

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=-0.0300, 95%CI=-0.1957 to 0.1357, p=0.89

18ºC vs 30ºC: Diff=0.1700, 95%CI=0.0043 to 0.3357, p=0.04

24ºC vs 30ºC: Diff=0.2000, 95%CI=0.0343 to 0.3657, p=0.01

1017 Glyceraldehyde-3-phosphate dehydrogenase

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=0.1500, 95%CI=-0.0324 to 0.3324, p=0.12

18ºC vs 30ºC: Diff=0.2000, 95%CI=0.0176 to 0.3824, p=0.03

24ºC vs 30ºC: Diff=0.0500, 95%CI=-0.1324 to 0.2324, p=0.77

672 Glutamate dehydrogenase, mitochondrial

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=-0.1500, 95%CI=-0.4113 to 0.1113, p=0.34

18ºC vs 30ºC: Diff=-0.2900, 95%CI=-0.5513 to -0.0287, p=0.03

24ºC vs 30ºC: Diff=-0.1400, 95%CI=-0.4013 to 0.1213, p=0.38

1016 Glyceraldehyde-3-phosphate dehydrogenase

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=0.1500, 95%CI=-0.0528 to 0.3528, p=0.17

18ºC vs 30ºC: Diff=0.2200, 95%CI=0.0172 to 0.4228, p=0.032

24ºC vs 30ºC: Diff=0.0700, 95%CI=-0.1328 to 0.2728, p=0.66

1331 Triosephosphate isomerase (Fragments)

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=-0.1400, 95%CI=-0.3996 to 0.1196, p=0.38

18ºC vs 30ºC: Diff=0.1300, 95%CI=-0.1296 to 0.3896, p=0.43

24ºC vs 30ºC: Diff=0.2700, 95%CI=0.0104 to 0.5296, p=0.04

1397 Adenylate kinase isoenzyme 1

Tukey HSD Post-hoc Test...

18ºC vs 24ºC: Diff=-0.2400, 95%CI=-0.4643 to -0.0157, p=0.03

18ºC vs 30ºC: Diff=-0.1100, 95%CI=-0.3343 to 0.1143, p=0.45

24ºC vs 30ºC: Diff=0.1300, 95%CI=-0.0943 to 0.3543, p=0.33

Table S6.2 Protein expression levels extracted from Same Spots concerning the proteomic analysis carried out in the muscle of *Sparus aurata* exposed to 18ºC, 24ºC and 30ºC for 14 days. Bold lines indicate spots identified through mass spectrometry.

Spot	Identification	Anova (p)	Fold	Average Normalised Volumes		
				18ºC	24ºC	30ºC
1670	**Heat shock 70 kDa protein 1**	**7,458e-012**	**3,1**	**1,672e+006**	**1,763e+006**	**5,250e+006**
1669	**Heat shock 70 kDa protein 1**	**1,534e-009**	**3,6**	**1,094e+006**	**1,203e+006**	**3,984e+006**
765		8,247e-007	1,6	2,292e+006	2,204e+006	3,496e+006
742		3,977e-004	1,4	1,624e+006	1,594e+006	2,270e+006
859	**Rab GDP dissociation inhibitor beta**	**8,210e-004**	**1,4**	**2,685e+006**	**2,299e+006**	**3,299e+006**
800		9,267e-004	1,4	1,025e+006	1,197e+006	1,453e+006
1335		9,464e-004	1,5	5,377e+006	5,081e+006	7,821e+006
1702		0,002	2,4	2,864e+006	2,105e+006	1,187e+006
1668	**Heat shock cognate 71 kDa protein**	**0,002**	**1,6**	**3,448e+006**	**2,930e+006**	**4,693e+006**
805		0,003	1,4	1,004e+006	9,106e+005	1,291e+006
1312	**Adenylate kinase isoenzyme**	**0,003**	**1,5**	**7,847e+005**	**8,606e+005**	**1,195e+006**
1089		0,004	1,3	2,022e+006	1,853e+006	2,467e+006
604		0,005	1,6	7,856e+006	9,104e+006	1,282e+007
1681	**Creatine kinase M-type**	**0,005**	**1,4**	**6,657e+006**	**6,574e+006**	**8,885e+006**
1733	**Creatine kinase M-type**	**0,005**	**1,4**	**2,181e+006**	**2,319e+006**	**3,022e+006**
1211	**Carbonic anhydrase 1**	**0,005**	**1,4**	**3,775e+006**	**4,042e+006**	**5,285e+006**
597		0,006	1,5	2,531e+006	1,843e+006	2,762e+006
1078		0,008	1,4	1,588e+007	1,565e+007	2,194e+007
591		0,008	1,4	3,636e+006	2,684e+006	3,530e+006
796		0,008	1,5	4,132e+005	5,257e+005	6,248e+005
1716	**Creatine kinase M-type**	**0,011**	**1,6**	**1,016e+006**	**9,177e+005**	**1,426e+006**
914		0,011	1,5	2,303e+006	2,615e+006	3,388e+006
509	**Creatine kinase M-type**	**0,013**	**1,7**	**1,608e+006**	**1,269e+006**	**2,186e+006**
1074		0,013	1,3	2,241e+007	1,920e+007	2,556e+007
1172		0,014	1,3	4,249e+006	3,593e+006	4,721e+006
1042	**Creatine kinase M-type**	**0,014**	**1,6**	**2,605e+006**	**2,488e+006**	**3,970e+006**
578		0,016	1,5	1,124e+006	7,283e+005	1,081e+006
374		0,016	1,8	1,246e+006	9,526e+005	1,695e+006
1156		0,017	1,3	9,904e+005	1,152e+006	1,329e+006
1094		0,018	1,8	8,947e+005	1,066e+006	1,641e+006
1529	**Neuroendocrine convertase 1**	**0,018**	**1,6**	**4,067e+006**	**3,746e+006**	**5,877e+006**
1228		0,020	1,3	3,820e+006	3,035e+006	3,180e+006
1666		0,020	1,9	1,757e+005	1,910e+005	3,384e+005
1295	**Adenylate kinase**	**0,021**	**1,6**	**1,108e+006**	**1,429e+006**	**1,805e+006**

Spot	Identification	Anova (p)	Fold	Average Normalised Volumes		
				18ºC	24ºC	30ºC
	isoenzyme 1					
13		0,022	1,8	1,699e+006	1,229e+006	9,353e+005
1072		0,022	1,4	7,265e+006	6,155e+006	8,706e+006
732		0,022	1,3	8,047e+005	9,315e+005	1,053e+006
385		0,022	1,6	3,797e+006	2,390e+006	3,318e+006
1720	**Creatine kinase M-type**	**0,023**	**1,1**	**4,170e+007**	**4,156e+007**	**3,692e+007**
1246	**Triosephosphate isomerase B**	**0,024**	**1,3**	**1,593e+007**	**2,021e+007**	**2,033+007**
544		0,025	1,8	1,730e+006	2,231e+006	3,074e+006
1674	**Actin, alpha cardiac muscle 2**	**0,026**	**1,6**	**1,660e+005**	**1,942e+005**	**2,737e+005**
1336		0,028	1,4	1,295e+007	1,018e+007	1,420e+007
857	**Alpha-enolase**	**0,029**	**1,6**	**1,028e+006**	**1,013e+006**	**1,666e+006**
1237		0,029	1,7	5,212e+005	4,214e+005	7,025e+005
1143	**Creatine kinase, testis isozyme**	**0,031**	**1,5**	**1,260e+006**	**1,566e+006**	**1,865e+006**
897	**Eukaryotic initiation factor 4A-II**	**0,034**	**1,4**	**1,438e+006**	**1,068e+006**	**1,478e+006**
851	**Alpha-enolase**	**0,046**	**1,3**	**2,173e+006**	**2,253e+006**	**2,872e+006**
1212		0,047	1,3	1,050e+006	9,366e+005	1,175e+006
1102		0,047	1,5	1,323e+006	1,377e+006	1,924e+006
887		0,048	1,6	2,532e+005	2,857e+005	3,958e+005
428	**Glycogen phosphorylase, brain form**	**0,049**	**2,0**	**1,725e+006**	**8,464e+005**	**1,418e+006**

Table S6.3 Masses and sequences of peptides obtained for each spot at **(a)**14 days of exposure, **(b)** 21 days of exposure.

(a) 14 days of exposure

SPOT nº	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
859	Rab GDP dissociation inhibitor beta	*Canis familiaris*	GDIB_CANFA	50289.73828	6.11	2	111	100
	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1993.947	1993.8823	-32	330	348	SDIYVCMISSAHNVAAQGK		
	2141.1064	2141.186	37	222	240	SPYLYPLYGLGELPQGFAR	99.63999939	100
	2141.1064	2141.186	37	222	240	SPYLYPLYGLGELPQGFAR		
	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
1529	Neuroendocrine convertase 1	*Homo sapiens*	NEC1_HUMAN	84099.28125	5.66	5	64	96.6738544
	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1994.9454	1994.8646	-40	90	105	LSDDDRVIWAEQQYEK		
	1994.9454	1994.8646	-40	90	105	LSDDDRVIWAEQQYEK		
	2268.0925	2268.0195	-32	618	637	RGVEKMVDPGEEQPTQENPK		
	2788.3884	2788.3342	-19	4	28	RAWSLQCTAFVLFCAWCALNSAKAK		
	2788.3884	2788.3342	-19	5	29	AWSLQCTAFVLFCAWCALNSAKAKR		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
851	Alpha-enolase	*Xenopus laevis*	ENOA_XENLA	47474	5.92	12	159	100

Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
1143.6157	1143.6514	31	184	193	IGAEVYHNLK		
1541.7642	1541.7538	-7	359	372	LAQSNGWGVMVSHR		
1554.7072	1554.729	14	257	269	YDLDFKSPDDPSR		
1804.944	1804.8773	-37	33	50	AAVPSGASTGIYEALELR	97.34999847	100
1804.944	1804.8773	-37	33	50	AAVPSGASTGIYEALELR		
1851.9191	1851.851	-37	90	105	IDKLMIEMDGTENKSK		
1960.9247	1960.9109	-7	203	221	DATNVGDEGGFAPNILENK		
2277.1357	2277.0425	-41	33	54	AAVPSGASTGIYEALELRDNDK		
2501.3721	2501.358	-6	307	330	FTAASGIQVVGDDLTVTNPKRIAK		
2508.2439	2508.2074	-15	68	89	YVNEFLGPALCTQNLNVVEQEK		
2566.2419	2566.2776	14	198	221	EKYGKDATNVGDEGGFAPNILENK		
2757.394	2757.35	-16	203	228	DATNVGDEGGFAPNILENKEALELLK		
3021.5903	3021.537	-18	133	162	HIADLAGNPEVILPVPAFNVINGGSHAGNK		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
1733	Creatine kinase M-type	*Bos taurus*	KCRM_BOVIN	42962	6.63	7	132	100

Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
1507.7023	1507.7299	18	117	130	GGDDLDPNYVLSSR		
1643.8176	1643.8443	16	224	236	SFLVWVNEEDHLR	59.79000092	99.982
1643.8176	1643.8443	16	224	236	SFLVWVNEEDHLR		

1785.9592	1785.9634	2	342	358	LGSSEVEQVQLVVDGVK		
1800.9524	1800.9608	5	366	381	KLEKGQSIDDMIPAQK		
1994.9414	1994.9568	8	321	341	GTGGVDTAAVGSVFDVSNADR	32.34999847	90.103
1994.9414	1994.9568	8	321	341	GTGGVDTAAVGSVFDVSNADR		
2151.0425	2150.9629	-37	320	341	RGTGGVDTAAVGSVFDVSNADR		
3761.8826	3761.7926	-24	321	358	GTGGVDTAAVGSVFDVSNADRLGSSEVEQVQLVVDGVK		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
1246	Triosephosphate isomerase B	*Danio rerio*	TPISB_DANRE	26810.80078	6.45	8	138	100

Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
1082.5781	1082.5544	-22	5	13	KFFVGGNWK		
1096.5898	1096.574	-14	90	98	WVILGHSER	84.80999756	100
1096.5898	1096.574	-14	90	98	WVILGHSER		
1252.6909	1252.6694	-17	90	99	WVILGHSERR		
1458.7223	1458.6913	-21	100	112	HVFGESDELIGQK		
1602.889	1602.835	-34	160	174	VVLAYEPVWAIGTGK		
1614.8234	1614.8153	-5	99	112	RHVFGESDELIGQK		
2034.0865	2034.0222	-32	131	148	LDEREAGITEKVVFAQTK		
2432.3071	2432.2161	-37	225	247	DLDGFLVGGASLKPEFIDIINAK		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
1042	Creatine kinase M-type	*Homo sapiens*	KCRM_HUMAN	43073.89844	6.77	5	78.90000153	99.89236808

Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
1267.6542	1267.6226	-25	97	107	HGGYKPTDKHK		
1643.8176	1643.7942	-14	224	236	SFLVWVNEEDHLR	55.06000137	99.958
1643.8176	1643.7942	-14	224	236	SFLVWVNEEDHLR		
2151.0425	2151.0168	-12	320	341	RGTGGVDTAAVGSVFDVSNADR		
3348.5933	3348.4941	-30	210	236	DWPDARGIWHNDNKSFLVWVNEEDHLR		
3808.9116	3808.7048	-54	183	215	EQQQLIDDHFLFDKPVSPLLLASGMARDWPDAR		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
1720	Creatine kinase M-type	*Homo sapiens*	KCRM_HUMAN	43073.89844	6.77	10	213	100

Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
1002.5002	1002.4893	-11	97	105	HGGYKPTDK		
1037.5197	1037.4858	-33	243	251	GGNMKEVFR		
1507.7023	1507.6552	-31	117	130	GGDDLDPNYVLSSR		
1553.8396	1553.7616	-50	253	265	FCVGLQKIEEIFK		
1643.8176	1643.8132	-3	224	236	SFLVWVNEEDHLR	112	100
1643.8176	1643.8132	-3	224	236	SFLVWVNEEDHLR		
1724.8378	1724.8068	-18	12	25	LNYKPEEEYPDLSK		
1785.9592	1785.9015	-32	342	358	LGSSEVEQVQLVVDGVK		
1994.9414	1994.9055	-18	321	341	GTGGVDTAAVGSVFDVSNADR	68.12000275	99.999
1994.9414	1994.9055	-18	321	341	GTGGVDTAAVGSVFDVSNADR		
2151.0425	2150.9807	-29	320	341	RGTGGVDTAAVGSVFDVSNADR		
3761.8826	3761.7659	-31	321	358	GTGGVDTAAVGSVFDVSNADRLGSSEVEQVQLVVDGVK		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
1716	Creatine kinase M-type	*Sus scrofa*	KCRM_PIG	43031.85156	6.61	14	78.09999847	99.87059787
	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1002.5002	1002.4925	-8	97	105	HGGYKPTDK		
	1037.5197	1037.5169	-3	243	251	GGNMKEVFR		
	1045.4884	1045.5146	25	1	9	MPFGNTHNK		
	1193.6017	1193.5699	-27	237	247	VISMEKGGNMK		
	1205.6062	1205.5931	-11	2	11	PFGNTHNKYK		
	1302.6359	1302.6357	0	370	381	GQSIDDMIPAQK		
	1507.7023	1507.7317	19	117	130	GGDDLDPNYVLSSR		
	1628.8074	1628.7775	-18	139	152	GYTLPPHCSRGERR		
	1643.8176	1643.848	18	224	236	SFLVWVNEEDHLR		
	1785.9592	1785.9655	4	342	358	LGSSEVEQVQLVVDGVK		
	1838.937	1838.8656	-39	1	15	MPFGNTHNKYKLNFK		
	1994.9414	1994.8635	-39	321	341	GTGGVDTAAVGSVFDVSNADR	39.20000076	99.38
	1994.9414	1994.8635	-39	321	341	GTGGVDTAAVGSVFDVSNADR		
	2151.0425	2150.9619	-37	320	341	RGTGGVDTAAVGSVFDVSNADR		
	3348.5933	3348.5507	-13	210	236	DWPDARGIWHNDNKSFLVWVNEEDHLR		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
1670	Heat shock 70 kDa protein 1	*Oryzias latipes*	HSP71_ORYLA	70307.00781	5.47	16	370	100
	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1017.5687	1017.532	-36	503	511	ITITNDKGR		

1109.5739	1109.576	2	351	359	LLQDFFNGR		
1193.5645	1193.5656	1	553	563	SSAQDDSLKDK		
1228.6281	1228.5903	-31	28	38	VEIIANDQGNR		
1235.6631	1235.6992	29	542	552	NSLESLAFNLK		
1434.7046	1434.7246	14	79	90	RFDEPVVQADMK		
1487.7013	1487.6521	-33	39	51	TTPSYVAFTDTER	81.08999634	100
1487.7013	1487.6521	-33	39	51	TTPSYVAFTDTER		
1501.7605	1501.7688	6	529	541	AEDEQQRDKIAAK		
1628.9039	1628.8523	-32	598	611	QKELEKVCNPIISK		
1659.8951	1659.8319	-38	174	189	IINEPTAAAIAYGLDK		
1675.7307	1675.6835	-28	223	238	ATAGDTHLGGEDFDNR	91.13999939	100
1675.7307	1675.6835	-28	223	238	ATAGDTHLGGEDFDNR		
1845.0116	1844.99271	-10	174	191	IINEPTAAAIAYGLDKGK		
1880.9501	1880.8909	-31	141	157	VSNAVITVPAYFNDSQR	149.2400055	100
1880.9501	1880.8909	-31	141	157	VSNAVITVPAYFNDSQR		
2286.2087	2286.15	-26	52	73	LIGDAAKNQVALNPSNTVFDAK		
2309.1885	2309.171	-8	141	161	VSNAVITVPAYFNDSQRQATK		
2981.3364	2981.3696	11	612	639	LYQGGMPSGSCREQARADSQGPTIEEVD		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
1668	Heat shock cognate 71 kDa protein	*Cricetulus griseus*	HSP7C_CRIGR	70761.11719	5.24	3	87.59999847	99.98548084

Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
1480.7543	1480.7427	-8	300	311	ARFEELNADLFR		
2774.3267	2774.2542	-26	424	447	QTQTFTTYSDNQPGVLIQVYEGER	72.94999695	99.999

				2774.3267	2774.2542	-26	424	447	QTQTFTTYSDNQPGVLIQVYEGER		
				2956.5051	2956.445	-20	129	155	EIAEAYLGKTVTNAVVTVPAYFNDSQR		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
1312	Adenylate kinase isoenzyme 1	*Cyprinus carpio*	KAD1_CYPCA	21475.33984	6.64	2	90.40000153	99.99238024

Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
1053.5364	1053.573	34	89	97	GYLIDGYPR		
1481.7383	1481.682	-38	32	44	YGYTHLSSGDLLR	81.72000122	100
1481.7383	1481.682	-38	32	44	YGYTHLSSGDLLR		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
1681	Creatine kinase M-type	*Canis familiaris*	KCRM_CANFA	43125.89844	6.77	5	126	100

Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
1037.5197	1037.5306	11	243	251	GGNMKEVFR		
1507.7023	1507.6902	-8	117	130	GGDDLDPNYVLSSR		
1657.8333	1657.8374	2	224	236	TFLVWVNEEDHLR	58.52999878	99.972
1657.8333	1657.8374	2	224	236	TFLVWVNEEDHLR		
1785.9592	1785.967	4	342	358	LGSSEVEQVQLVVDGVK	48.02000046	99.682
1785.9592	1785.967	4	342	358	LGSSEVEQVQLVVDGVK		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
1295	Adenylate kinase isoenzyme 1	*Cyprinus carpio*	KAD1_CYPCA	21475.33984	6.64	4	88.5	99.98819839

Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
1053.5364	1053.5208	-15	89	97	GYLIDGYPR		
1350.6899	1350.684	-4	98	108	EVKQGEEFEKK		
1481.7383	1481.6904	-32	32	44	YGYTHLSSGDLLR	79.58000183	100
1481.7383	1481.6904	-32	32	44	YGYTHLSSGDLLR		
1837.9807	1837.91957	-33	156	171	ATEPVIAYYETRGIVR		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
857	Alpha-enolase	*Pongo abelii*	ENOA_PONAB	47167.35938	7.57	10	108	100

Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
1143.6157	1143.614	-1	184	193	IGAEVYHNLK		
1434.7444	1434.7045	-28	331	343	AVNEKSCNCLLLK		
1554.7072	1554.6644	-28	257	269	YDLDFKSPDDPSR		
1804.944	1804.8762	-38	33	50	AAVPSGASTGIYEALELR	58.84000015	99.984
1804.944	1804.8762	-38	33	50	AAVPSGASTGIYEALELR		
1826.8556	1826.8005	-30	254	269	SGKYDLDFKSPDDPSR		
1901.0055	1900.942	-33	270	285	YISPDQLADLYKSFIK		
1960.9247	1960.9348	5	203	221	DATNVGDEGGFAPNILENK		
2277.1357	2277.193	25	33	54	AAVPSGASTGIYEALELRDNDK		
2501.3721	2501.2555	-47	307	330	FTASAGIQVVGDDLTVTNPKRIAK		
2743.3784	2743.317	-22	203	228	DATNVGDEGGFAPNILENKEGLELLK		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
1674	Actin, alpha cardiac muscle 2	*Xenopus tropicalis*	ACT2_XENTR	42005.89844	5.23	9	115	100

Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
1130.5476	1130.5159	-28	199	208	GYSFVTTAER		
1161.6184	1161.5804	-33	318	328	EITALAPSTMK		
1198.7054	1198.745	33	31	41	AVFPSIVGRPR		
1198.7054	1198.745	33	31	41	AVFPSIVGRPR		
1500.7078	1500.66	-32	362	374	QEYDEAGPSIVHR		
1515.7491	1515.6948	-36	87	97	IWHHTFYNELR		
1628.8027	1628.769	-21	362	375	QEYDEAGPSIVHRK		
1790.892	1790.8229	-39	241	256	SYELPDGQVITIGNER	56.70999908	99.975
1790.892	1790.8229	-39	241	256	SYELPDGQVITIGNER		
1956.0437	1955.972	-37	98	115	VAPEEHPTLLTEAPLNPK		
3793.9561	3793.89	-17	87	118	IWHHTFYNELRVAPEEHPTLLTEAPLNPKANR		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
428	Glycogen phosphorylase, brain form	*Ovis aries*	PYGB_SHEEP	96253.39063	6.57	10	93.90000153	99.99659638

Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
1053.5728	1053.5591	-13	643	650	VIFLENYR		
1145.5626	1145.5304	-28	162	170	YEFGIFNQK		
1355.743	1355.7541	8	400	410	HLDIIYAINQR		

	1442.6951	1442.7335	27	279	290	VLYPNDNFFEGK		
	1478.7737	1478.7134	-41	508	520	IGEDFLTDLSQLK		
	1829.8817	1829.8486	-18	171	185	IVNGWQVEEADDWLR	60.40999985	99.985
	1829.8817	1829.8486	-18	171	185	IVNGWQVEEADDWLR		
	1840.9227	1840.8696	-29	279	293	VLYPNDNFFEGKELR		
	1845.0051	1844.9572	-25	593	609	KDPTQAFVPRTVMIGGK		
	2512.3606	2512.2731	-35	571	590	IHEYKRQLLNCLHVVTLYNR		
	2691.2871	2691.2356	-19	774	796	VFADYEAYVACQAQVDQLYRNPK		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
897	Eukaryotic initiation factor 4A-II	*Macaca fascicularis*	IF4A2_MACFA	46387.78906	5.4	12	146	100
	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1065.551	1065.543	-8	314	321	DVIMREFR		
	1114.6831	1114.6416	-37	327	336	VLITTDLLAR		
	1157.6174	1157.6372	17	356	364	ENYIHRIGR		
	1174.658	1174.6939	31	285	293	RKVDWLTEK		
	1198.6791	1198.645	-28	102	112	ETQALVLAPTR		
	1394.691	1394.642	-35	71	84	GYDVIAQAQSGTGK		
	1483.6919	1483.693	1	180	192	MFVLDEADGMLSR		
	1539.7697	1539.732	-24	314	326	DVIMREFRSGSSR		
	1638.8784	1638.831	-29	164	176	VFDMLNRRYLSPK		
	1827.9388	1827.9525	7	48	63	GIYAYGFEKPSAIQQR	65.13999939	99.997
	1827.9388	1827.9525	7	48	63	GIYAYGFEKPSAIQQR		
	2144.1345	2144.1312	-2	337	355	GIDVQQVSLVINYDLPTNR	47.11000061	99.841

		2144.1345	2144.1312	-2	337	355	GIDVQQVSLVINYDLPTNR	
		2499.2773	2499.17641	-40	172	192	YLSPKWIKMFVLDEADGMLSR	

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
509	Creatine kinase M-type	*Homo sapiens*	KCRM_HUMAN	43073.89844	6.77	8	156	100
	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1189.6113	1189.5642	-40	2	11	PFGNTHNKFK		
	1507.7023	1507.7218	13	117	130	GGDDLDPNYVLSSR		
	1643.8176	1643.7619	-34	224	236	SFLVWVNEEDHLR	71.16999817	100
	1643.8176	1643.7619	-34	224	236	SFLVWVNEEDHLR		
	1785.9592	1785.9608	1	342	358	LGSSEVEQVQLVVDGVK		
	1994.9414	1994.955	7	321	341	GTGGVDTAAVGSVFDVSNADR	47.75	99.834
	1994.9414	1994.955	7	321	341	GTGGVDTAAVGSVFDVSNADR		
	2151.0425	2150.9948	-22	320	341	RGTGGVDTAAVGSVFDVSNADR		
	3348.5933	3348.4729	-36	210	236	DWPDARGIWHNDNKSFLVWVNEEDHLR		
	3808.9116	3808.866	-12	183	215	EQQQLIDDHFLFDKPVSPLLLASGMARDWPDAR		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
1143	Creatine kinase, testis isozyme	*Oncorhynchus mykiss*	KCRT_ONCMY	42976.76172	6.2	3	57.5	85.14265336
	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1638.8633	1638.8318	-19	35	47	VLTQDMYTKLRDR		
	1657.8333	1657.8588	15	226	238	TFLVWVNEEDHLR	45.29000092	99.424
	1657.8333	1657.8588	15	226	238	TFLVWVNEEDHLR		

	2691.4346	2691.408	-10	344	367	LGFSEVELVQMVVDGVKLLVEMEK		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
1211	Carbonic anhydrase 1	*Chionodraco hamatus*	CAH1_CHIHA	28324.91992	5.58	2	106	100
	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1607.8329	1607.8223	-7	113	125	YPAELHLVHWNTK	94.48000336	100
	1607.8329	1607.8223	-7	113	125	YPAELHLVHWNTK		
	2811.3274	2811.2783	-17	1	26	AHAWGYGPTDGPDKWVSNFPIADGPR		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
1669	Heat shock 70 kDa protein 1	*Oryzias latipes*	HSP71_ORYLA	70307.00781	5.47	11	149	100
	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1109.5739	1109.577	3	351	359	LLQDFFNGR	49.00999832	99.823
	1109.5739	1109.577	3	351	359	LLQDFFNGR		
	1435.7151	1435.7135	-1	239	249	MVNHFVEEFKR		
	1487.7013	1487.7169	10	39	51	TTPSYVAFTDTER		
	1628.9039	1628.914	6	598	611	QKELEKVCNPIISK		
	1659.8951	1659.8582	-22	174	189	IINEPTAAAIAYGLDK		
	1675.7307	1675.7623	19	223	238	ATAGDTHLGGEDFDNR		
	1880.9501	1880.9473	-1	141	157	VSNAVITVPAYFNDSQR	57.63000107	99.976
	1880.9501	1880.9473	-1	141	157	VSNAVITVPAYFNDSQR		
	2309.1885	2309.1375	-22	141	161	VSNAVITVPAYFNDSQRQATK		
	2801.4429	2801.4193	-8	105	128	IQVDYKGENKTFFPEEISSMVLVK		

2936.3267	2936.4236	33	223	248	ATAGDTHLGGEDFDNRMVNHFVEEFK
2981.3364	2981.4426	36	612	639	LYQGGMPSGSCREQARADSQGPTIEEVD

(b) 21 days of exposure

SPOT nº	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
1781	Heat shock 70 kDa protein 1	*Oryzias latipes*	HSP71_ORYLA	70307.00781	5.47	9	171	100
	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1109.5739	1109.5664	-7	351	359	LLQDFFNGR	40.29999924	97.175
	1109.5739	1109.5664	-7	351	359	LLQDFFNGR		
	1487.7013	1487.7029	1	39	51	TTPSYVAFTDTER		
	1501.7605	1501.7949	23	529	541	AEDEQQRDKIAAK		
	1628.9039	1628.9182	9	598	611	QKELEKVCNPIISK		
	1659.8951	1659.87	-15	174	189	IINEPTAAAIAYGLDK		
	1675.7307	1675.7318	1	223	238	ATAGDTHLGGEDFDNR		
	1845.0116	1844.9695	-23	174	191	IINEPTAAAIAYGLDKGK		
	1880.9501	1880.9434	-4	141	157	VSNAVITVPAYFNDSQR	80.33000183	100
	1880.9501	1880.9434	-4	141	157	VSNAVITVPAYFNDSQR		
	2309.1885	2309.1162	-31	141	161	VSNAVITVPAYFNDSQRQATK		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
1389	Adenylate kinase isoenzyme 1	*Cyprinus carpio*	KAD1_CYPCA	21475.33984	6.64	2	91.80000305	99.99447998
	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence	End Sequence	Sequence	Ion Score	Ion Score C.I.

			Position	Position			%
1053.5364	1053.5267	-9	89	97	GYLIDGYPR		
1481.7383	1481.7466	6	32	44	YGYTHLSSGDLLR	70.86000061	99.992
1481.7383	1481.7466	6	32	44	YGYTHLSSGDLLR		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
475	Heat shock cognate 71 kDa protein	*Ictalurus punctatus*	HSP7C_ICTPU	71296.14063	5.19	12	148	100
	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1197.6626	1197.6881	21	459	469	FELTGIPPAPR		
	1215.6692	1215.6469	-18	160	171	DAGTISGLNVLR		
	1252.6605	1252.6339	-21	127	137	MKEIAEAYLGK		
	1473.6857	1473.6835	-1	37	49	TTPSYVAFTDSER		
	1480.7543	1480.7748	14	300	311	ARFEELNADLFR		
	1691.7256	1691.7026	-14	221	236	STAGDTHLGGEDFDNR		
	1787.9901	1787.96	-17	172	188	IINEPTAAAIAYGLDKK		
	1807.9775	1807.8646	-62	343	357	IPKMEKLLQDFFNGK		
	1963.9371	1964.00272	33	78	93	FEDSVVQADMKHWPFK		
	2009.0087	2009.0255	8	138	155	SINNAVITVPAYFNDSQR		
	2774.3267	2774.3042	-8	424	447	QTQTFTTYSDNQPGVLIQVYEGER	52.68999863	99.809
	2774.3267	2774.3042	-8	424	447	QTQTFTTYSDNQPGVLIQVYEGER		
	2776.3635	2776.337	-10	358	384	ELNKSINPDEAVAYGAAVQAESSLGDK		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
1331	Triosephosphate isomerase	*Mesocricetus auratus*	TPIS_MESAU	20294.36914	5.49	1	83.5	99.96268003

(Fragments)

	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1602.889	1602.8638	-16	160	174	VVLAYEPVWAIGTGK	50.81999969	99.527
	1602.889	1602.8638	-16	160	174	VVLAYEPVWAIGTGK		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
1017	Glyceraldehyde-3-phosphate dehydrogenase	*Danio rerio*	G3P_DANRE	35761.30078	8.2	3	62.40000153	95.19225856

	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1495.8479	1495.8491	1	233	246	VPTPNVSVVDLTVR		
	1763.8024	1763.8037	1	308	321	LVTWYDNEFGYSNR	40.22999954	96.548
	1763.8024	1763.8037	1	308	321	LVTWYDNEFGYSNR		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
989	Glyceraldehyde-3-phosphate dehydrogenase (Fragment)	*Meleagris gallopavo*	G3P_MELGA	24836.68945	7.22	3	123	100

	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1697.8929	1697.879	-8	107	124	DGRGAAQNIIPASTGAAK		
	1749.7867	1749.8069	12	219	232	LVSWYDNEFGYSNR	98.09999847	100
	1749.7867	1749.8069	12	219	232	LVSWYDNEFGYSNR		

	2257.1467	2257.0627	-37	137	157	LTGMAFHVPTPNVSVVDLTCR		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
1086	Sterile alpha motif domain-containing protein 9-like	*Homo sapiens*	SAM9L_HUMAN	184415.3906	8.25	8	67.40000153	98.47965866
	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1127.6095	1127.6201	9	1471	1480	QASTLFYLGK		
	1127.6095	1127.6201	9	1471	1480	QASTLFYLGK		
	1551.9945	1551.8969	-63	1399	1411	LIQPLTTLKKQLR		
	1600.917	1600.943	16	1546	1560	IPVISVYSGPLRSGR	37.29000092	90
	1600.917	1600.943	16	1546	1560	IPVISVYSGPLRSGR		
	1842.0959	1841.9996	-52	1544	1560	IKIPVISVYSGPLRSGR		
	1874.9792	1874.9623	-9	1220	1235	ENELSKKHMVQFLSGK		
	1874.9792	1874.9623	-9	1220	1235	ENELSKKHMVQFLSGK		
	1954.8777	1954.938	31	1169	1184	QTDSKNYETENWSPQK		
	1954.8777	1954.938	31	1169	1184	QTDSKNYETENWSPQK		
	2783.3845	2783.3286	-20	894	917	SNFDETYIENVVRNILKGQDVDSK		
	2784.511	2784.4167	-34	1374	1398	KPMTNEKQNSILANIILSCLKPNSK		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
999	Glyceraldehyde-3-phosphate dehydrogenase	*Danio rerio*	G3P_DANRE	35761.30078	8.2	2	163	100
	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %

	1495.8479	1495.8875	26	233	246	VPTPNVSVVDLTVR	68.76000214	99.993
	1495.8479	1495.8875	26	233	246	VPTPNVSVVDLTVR		
	1763.8024	1763.84	21	308	321	LVTWYDNEFGYSNR	79.01999664	100
	1763.8024	1763.84	21	308	321	LVTWYDNEFGYSNR		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
1007	Glyceraldehyde-3-phosphate dehydrogenase	*Danio rerio*	G3P_DANRE	35761.30078	8.2	2	146	100
	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1495.8479	1495.851	2	233	246	VPTPNVSVVDLTVR	47.68999863	99.023
	1495.8479	1495.851	2	233	246	VPTPNVSVVDLTVR		
	1763.8024	1763.8396	21	308	321	LVTWYDNEFGYSNR	83.05000305	100
	1763.8024	1763.8396	21	308	321	LVTWYDNEFGYSNR		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
1016	Glyceraldehyde-3-phosphate dehydrogenase	*Danio rerio*	G3P_DANRE	35761.30078	8.2	2	70.40000153	99.23802433
	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1495.8479	1495.8795	21	233	246	VPTPNVSVVDLTVR	16.55999947	0
	1495.8479	1495.8795	21	233	246	VPTPNVSVVDLTVR		
	1763.8024	1763.8206	10	308	321	LVTWYDNEFGYSNR	31.29000092	20.464
	1763.8024	1763.8206	10	308	321	LVTWYDNEFGYSNR		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
1780	Heat shock 70 kDa protein	*Oncorhynchus tshawytscha*	HSP70_ONCTS	70931.89844	5.42	6	118	100
	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1109.5739	1109.5743	0	351	359	LLQDFFNGR	24.79000092	0
	1109.5739	1109.5743	0	351	359	LLQDFFNGR		
	1487.7013	1487.7036	2	39	51	TTPSYVAFTDTER		
	1675.7307	1675.7417	7	223	238	ATAGDTHLGGEDFDNR		
	1880.9501	1880.9432	-4	141	157	VSNAVITVPAYFNDSQR	53.93999863	99.783
	1880.9501	1880.9432	-4	141	157	VSNAVITVPAYFNDSQR		
	2309.1885	2309.1428	-20	141	161	VSNAVITVPAYFNDSQRQATK		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
1397	Adenylate kinase isoenzyme 1	*Cyprinus carpio*	KAD1_CYPCA	21475.33984	6.64	2	108	100
	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1053.5364	1053.5082	-27	89	97	GYLIDGYPR		
	1481.7383	1481.7144	-16	32	44	YGYTHLSSGDLLR	94.08999634	100
	1481.7383	1481.7144	-16	32	44	YGYTHLSSGDLLR		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
1783	Glucose-6-phosphate isomerase	*Bos taurus*	G6PI_BOVIN	62815.03906	7.33	5	75.80000305	99.78024389

	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1424.7029	1424.708	4	29	39	LFEGDRDRFNR		
	1586.8325	1586.8656	21	181	194	VWFVSNIDGTHIAK	66.27999878	99.997
	1586.8325	1586.8656	21	181	194	VWFVSNIDGTHIAK		
	1618.7609	1618.7562	-3	15	27	TWYEQHGSDLNLR		
	1655.8123	1655.751	-37	212	226	TFTTQETITNAETAK		
	1954.0062	1953.9714	-18	107	124	SNAPILVDGKDVMPEVNR		

	Protein Name	Species	Accession Number	Protein MW	Protein PI	Peptide Count	Protein Score	Protein Score C.I. %
672	Glutamate dehydrogenase, mitochondrial	*Chaenocephalus aceratus*	DHE3_CHAAC	55359.08984	7.34	2	84.40000153	99.9696652
	Calculated Mass	Observed Mass	Match Error PPM	Start Sequence Position	End Sequence Position	Sequence	Ion Score	Ion Score C.I. %
	1059.5331	1059.5177	-15	391	399	NLNHVSYGR		
	1652.8755	1652.8666	-5	427	442	QGGPIPVVPTADFQAR	68.02999878	99.989
	1652.8755	1652.8666	-5	427	442	QGGPIPVVPTADFQAR		

Table S6.4 Protein expression levels extracted from Same Spots concerning the proteomic analysis carried out in the muscle of *Sparus aurata* exposed to 18ºC, 24ºC and 30ºC for 21 days. Bold lines indicate spots identified through mass spectrometry.

Spot	Identification	Anova (p)	Fold	Average Normalised Volumes		
				18ºC	24ºC	30ºC
1781	**Heat shock 70kDa protein**	**4,928e-006**	**2,5**	**2,031e+006**	**2,043e+006**	**5,054e+006**
1780	**Heat shock 70 kDa protein**	**6,131e-005**	**2,2**	**3,516e+006**	**3,236e+006**	**7,058e+006**
475	**Heat shock cognate 71 kDa protein**	**0,001**	**1,6**	**4,290e+006**	**4,321e+006**	**6,701e+006**
1836		0,001	1,5	1,359e+006	1,216e+006	1,826e+006
1792		0,002	2,1	2,656e+006	1,759e+006	1,243e+006
1086	**Sterile alpha motif domain-containing protein 9-like**	**0,002**	**1,3**	**1,866e+006**	**1,479e+006**	**1,982e+006**
1246		0,002	3,3	4,404e+006	7,147e+006	1,437e+007
1389	**Adenylate kinase isoenzyme 1**	**0,002**	**1,8**	**2,476e+006**	**2,459e+006**	**4,337e+006**
627		0,003	1,6	4,882e+006	7,831e+006	7,149e+006
472		0,004	1,5	1,561e+006	1,721e+006	2,278e+006
1448		0,005	1,5	2,836e+006	2,378e+006	3,526e+006
999	**Glyceraldehyde-3-phosphate dehydrogenase**	**0,006**	**2,1**	**4,654e+006**	**5,416e+006**	**9,946e+006**
575		0,006	1,6	2,388e+006	1,703e+006	1,507e+006
641		0,006	1,6	1,350e+006	9,300e+005	1,475e+006
1007	**Glyceraldehyde-3-phosphate dehydrogenase**	**0,006**	**1,9**	**5,109e+006**	**6,707e+006**	**9,902e+006**
1783	**Glucose-6-phosphate isomerase**	**0,008**	**1,3**	**2,235e+007**	**1,810e+007**	**2,371e+007**
1080		0,008	1,4	1,670e+006	1,215e+006	1,152e+006
792		0,009	1,7	4,025e+005	2,336e+005	2,728e+005
990		0,009	2,0	1,635e+007	1,476e+007	2,988e+007
1859		0,009	1,6	3,945e+005	6,440e+005	5,598e+005
589		0,010	1,7	2,141e+006	1,777e+006	3,058e+006
326		0,010	2,1	6,542e+005	3,576e+005	3,147e+005
989	**Glyceraldehyde-3-phosphate dehydrogenase (Fragment)**	**0,010**	**1,6**	**3,466e+006**	**3,185e+006**	**5,145e+006**
1356		0,019	1,4	1,461e+006	1,019e+006	1,222e+006
1236		0,019	1,3	1,531e+006	1,210e+006	1,513e+006
1341		0,021	1,5	3,396e+005	3,266e+005	4,814e+005
642		0,021	2,4	1,036e+006	5,574e+005	4,344e+005
434		0,025	1,4	9,685e+005	1,368e+006	1,044e+006
1860		0,025	1,3	1,600e+006	1,884e+006	1,460e+006
1017	**Glyceraldehyde-3-phosphate dehydrogenase**	**0,025**	**1,6**	**6,087e+006**	**8,252e+006**	**9,962e+006**
672	**Glutamate**	**0,027**	**1,9**	**1,299e+006**	**9,467e+005**	**6,963e+005**

Spot	Identification	Anova (p)	Fold	Average Normalised Volumes		
				18ºC	24ºC	30ºC
	dehydrogenase, mitochondrial					
1016	**Glyceraldehyde-3-phosphate dehydrogenase**	**0,030**	**1,6**	**7,847e+006**	**1,048e+007**	**1,260e+007**
645		0,031	2,2	6,132e+005	3,914e+005	2,781e+005
1331	**Triosephosphate isomerase (Fragments)**	**0,032**	**2,0**	**1,500e+007**	**1,020e+007**	**1,996e+007**
619		0,033	1,4	1,210e+006	1,315e+006	1,649e+006
1397	**Adenylate kinase isoenzyme 1**	**0,034**	**1,7**	**2,612e+006**	**1,513e+006**	**2,156e+006**
950		0,039	2,0	5,009e+005	4,562e+005	9,268e+005
1192		0,039	1,5	1,772e+006	1,213e+006	1,783e+006
1209		0,043	1,7	2,281e+005	2,109e+005	3,523e+005
1784		0,044	1,4	2,974e+006	3,428e+006	2,412e+006
592		0,045	1,4	1,412e+006	1,716e+006	2,027e+006
636		0,046	2,0	1,099e+006	6,429e+005	5,587e+005

Table S6.5 Functional categorization of spots differentially expressed between temperature treatments at 14 days of exposure in the muscle of juvenile *Sparus aurata*. The information on this table was retrieved from neXtprot, GeneCards, InterPro, UniProt and Qiagen.

Spot no.	Protein Name	Function	Main functional category
859	Rab GDP dissociation inhibitor beta	Regulates the GDP/GTP exchange reaction of most Rab proteins by inhibiting the dissociation of GDP from them, and the subsequent binding of GTP to them; GTPase activator activity; Poly(A) RNA binding; Protein binding; Small GTPase binding; Positive regulation of GTPase activity; Protein transport; Signal transduction; Vesicle-mediated transport; GTPase activation; Vesicular trafficking	**Vesicular trafficking**
1529	Neuroendocrine convertase 1	Involved in the processing of hormone and other protein precursors at sites comprised of pairs of basic amino acid residues; Serine-type endopeptidase activity; Cell-cell signaling; Metabolic process; Peptide biosynthetic process; Proteolysis; Release of protein hormones, neuropeptides and renin from their precursors; Peptide hormone biosynthesis; Hydrolase; Protease	**Peptide hormone metabolism**
851	Alpha-enolase	Multifunctional enzyme that, as well as its role in glycolysis, plays a part in various processes such as growth control, hypoxia tolerance and allergic responses. May also function in the intravascular and pericellular fibrinolytic system due to its ability to serve as a receptor and activator of plasminogen on the cell surface of several cell-types such as leukocytes and neurons. Stimulates immunoglobulin production; DNA binding; GTPase binding; magnesium ion binding; Phosphopyruvate hydratase activity; Poly(A) RNA binding; Protein binding; DNA binding transcription activity; transcription corepressor activity; negative regulation of cell growth; negative regulation of transcription; glycolysis; gluconeogenesis; carbohydrate degradation; RNA degradation;	**Energetic metabolism**
1733	Creatine kinase M-type	Reversibly catalyzes the transfer of phosphate between ATP and various phosphogens (e.g. Creatine phosphate). Creatine kinase isoenzymes play a central role in energy transduction in tissues with large, fluctuating energy demands, such as skeletal muscle, heart, brain and spermatozoa; ATP binding; protein binding; phosphocreatine biosynthetic process; phosphorylation; arginine and proline metabolism; creatine metabolism; cellular nitrogen compound metabolic processes	**Energetic metabolism**
1246	Triosephosphate isomerase B	Carbohydrate metabolism; gluconeogenesis; glycolysis; pentose phosphate shunt; efficient energy production;	**Energetic metabolism**

1042	Creatine kinase M-type	✓	**Energetic metabolism**
1720	Creatine kinase M-type	✓	**Energetic metabolism**
1716	Creatine kinase M-type	✓	**Energetic metabolism**
1670	Heat shock 70 kDa protein 1	In cooperation with other chaperones, Hsp70s stabilize preexistent proteins against aggregation and mediate the folding of newly translated polypeptides in the cytosol as well as within organelles. These chaperones participate in all these processes through their ability to recognize nonnative conformations of other proteins. They bind extended peptide segments with a net hydrophobic character exposed by polypeptides during translation and membrane translocation, or following stress-induced damage (By similarity). Positive regulator of PARK2 translocation to damaged mitochondria; ATP binding; protein binding; unfolded protein binding; protein refolding; stress response; MAPK singnaling pathway; Regulation of HSF1-mediated heat shock response; spliceosome; Protein processing in endoplasmic reticulum	**Chaperone**
1668	Heat shock cognate 71 kDa protein	Acts as a repressor of transcriptional activation; chaperone; May have a scaffolding role in the spliceosome assembly as it contacts all other components of the core complex; Participates in the ER-associated degradation (ERAD) quality control pathway in conjunction with J domain-containing co-chaperones and the E3 ligase CHIP; ATP binding; ATPase activity; enzyme binding; Poly(A) RNA binding; Heat shock protein binding; ubiquitin protein ligase binding; unfolded protein binding; Chaperone mediated protein folding requiring cofactor; Negative regulation of transcription and fibril organization; Positive regulation of mRNA splicing; Regulation of cell cycle; response to unfolded protein; MAPK signaling; Spliceosome; Protein processing in endoplasmic reticulum; vesicle biogenesis; Regulation of HSF1-mediated heat shock response; GABA synthesis, release, reuptake and degradation; stress response	**Chaperone**
1312	Adenylate kinase isoenzyme 1	Catalyzes the reversible transfer of the terminal phosphate group between ATP and AMP. Also displays broad nucleoside diphosphate kinase activity. Plays an important role in cellular energy homeostasis and in adenine nucleotide metabolism; ATP binding; Nucleoside diphosphate kinase activity; ADP biosynthetic process; AMP and ATP metabolic process; cell cycle arrest; nucleoside phosphate processes; purine metabolism	**Energetic metabolism**
1681	Creatine	✓	**Energetic metabolism**

	kinase M-type		
1295	Adenylate kinase isoenzyme 1	✓	**Energetic metabolism**
857	Alpha-enolase	✓	**Energetic metabolism**
1674	Actin, alpha cardiac muscle 2	Highly conserved proteins that are involved in various types of cell motility; involved in the formation of filaments that are major components of the cytoskeleton. These filaments interact with myosin to produce a sliding effect, which is the basis of muscular contraction and many aspects of cell motility, including cytokinesis; muscle contraction	**Cytoskeleton dynamics**
428	Glycogen phosphorylase, brain form	Phosphorylase is an important allosteric enzyme in carbohydrate metabolism. Enzymes from different sources differ in their regulatory mechanisms and in their natural substrates. However, all known phosphorylases share catalytic and structural properties; nucleotide binding; protein binding; Pyridoxal phosphate binding; glycogen catabolic process; glycogenolysis; The activity of this enzyme is positively regulated by AMP and negatively regulated by ATP, ADP, and glucose-6-phosphate. This enzyme catalyzes the rate-determining step in glycogen degradation	**Energetic metabolism**
897	Eukaryotic initiation factor 4A-II	An ATP-dependent RNA helicase which is a subunit of the eIF4F complex involved in cap recognition and required for mRNA binding to ribosome; In the current model of translation initiation, IF4A unwinds RNA secondary structures in the 5'-UTR of mRNAs which is necessary to allow efficient binding of the small ribosomal subunit, and subsequent scanning for the initiator codon; ATP-dependent RNA helicase activity; Translation initiation factor activity; Poly(A) RNA binding; protein binding; Negative regulation of RNA-directed RNA polymerase activity; Activation of the mRNA upon binding of the cap-binding complex and eIFs, and subsequent binding to 43S; Deadenylation of mRNA; GTP hydrolysis and joining of the 60S ribosomal subunit; Ribosomal scanning and start codon recognition; protein biosynthesis	**Translation regulation**
509	Creatine kinase M-type	✓	**Energetic metabolism**
1143	Creatine kinase, testis isozyme	✓	**Energetic metabolism**

1211	Carbonic anhydrase 1	Reversible hydration of carbon dioxide. Can hydrate cyanamide to urea; Arylesterase activity; Carbonate dehydratase activity; Hydro-lyase activity; protein binding; zinc ion binding; one-carbon metabolic process; nitrogen metabolism; bicarbonate transport; Pathway erythrocytes take up carbon dioxide and release oxygen;	**CO_2 transport**
1669	Heat shock 70 kDa protein 1	✓	**Chaperone**

✓ Already described above

Table S6.6 Functional categorization of spots differentially expressed between temperature treatments at 21 days of exposure in the muscle of juvenile *Sparus aurata*. The information on this table was retrieved from neXtprot, GeneCards, InterPro, UniProt and Qiagen.

Spot no.	Protein Name	Function	Main functional category
1781	Heat shock 70kDa protein 1	In cooperation with other chaperones, Hsp70s stabilize preexistent proteins against aggregation and mediate the folding of newly translated polypeptides in the cytosol as well as within organelles. These chaperones participate in all these processes through their ability to recognize nonnative conformations of other proteins. They bind extended peptide segments with a net hydrophobic character exposed by polypeptides during translation and membrane translocation, or following stress-induced damage (By similarity). Positive regulator of PARK2 translocation to damaged mitochondria; ATP binding; protein binding; unfolded protein binding; protein refolding; stress response; MAPK singnaling pathway; Regulation of HSF1-mediated heat shock response; spliceosome; Protein processing in endoplasmic reticulum	**Chaperone**
1389	Adenylate kinase isoenzyme 1	Catalyzes the reversible transfer of the terminal phosphate group between ATP and AMP. Also displays broad nucleoside diphosphate kinase activity. Plays an important role in cellular energy homeostasis and in adenine nucleotide metabolism; ATP binding; Nucleoside diphosphate kinase activity; ADP biosynthetic process; AMP and ATP metabolic process; cell cycle arrest; nucleoside phosphate processes; purine metabolism	**Energetic metabolism**
475	Heat shock cognate 71 kDa protein	✓	**Chaperone**
1331	Triosephosphate isomerase (Fragments)	Glycolytic enzyme that catalyses the reversible interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate; plays an important role in several metabolic pathways and is essential for efficient energy production; protein binding; ubiquitin protein ligase binding; glycolysis/gluconeogenesis; organismal development; pentose-phosphate shunt; carbohydrate metabolism; inositol phosphate metabolism; fructose and mannose metabolism	**Energetic metabolism**
1017	Glyceraldehyde-3-phosphate dehydrogenase	It has both glyceraldehyde-3-phosphate dehydrogenase and nitrosylase activities, thereby playing a role in glycolysis and nuclear functions, respectively. Glyceraldehyde-3-phosphate dehydrogenase is a key enzyme in glycolysis that catalyzes the first step of the pathway by converting D-glyceraldehyde 3-phosphate (G3P) into 3-phospho-D-glyceroyl phosphate. Modulates the organization and assembly of the cytoskeleton. Also participates in nuclear events including transcription, RNA transport, DNA replication	**Energetic metabolism**

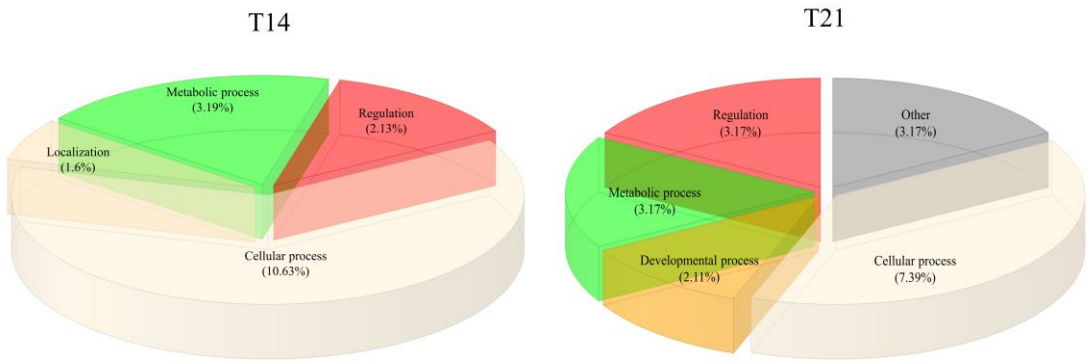
		and apoptosis. Nuclear functions are probably due to the nitrosylase activity that mediates cysteine S-nitrosylation of nuclear target proteins; microtubule binding and cytoskeleton organization; NAD and NADP binding; protein stabilization; apoptosis	
989	Glyceraldehyde-3-phosphate dehydrogenase (Fragment)	✓	**Energetic metabolism**
1086	Sterile alpha motif domain-containing protein 9-like	May play a role in the inflammatory response to tissue injury and the control of extra-osseous calcification, acting as a downstream target of TNF-alpha signaling; Involved in the regulation of EGR1 (early growth response protein 1 – transcriptional regulator), in coordination with RGL2 (ral guanine nucleotide dissociation stimulator-like 2); protein binding; endosomal vesicle fusion; cell differentiation and proliferation; apoptosis; regulation of protein catabolic processes	**Inflammatory response**
999	Glyceraldehyde-3-phosphate dehydrogenase	✓	**Energetic metabolism**
1007	Glyceraldehyde-3-phosphate dehydrogenase	✓	**Energetic metabolism**
1016	Glyceraldehyde-3-phosphate dehydrogenase	✓	**Energetic metabolism**
1780	Heat shock 70 kDa protein	✓	**Chaperone**
1397	Adenylate kinase	✓	**Energetic metabolism**

	isoenzyme 1		
1783	Glucose-6-phosphate isomerase	Functions as a glycolytic enzyme that interconverts glucose-6-phosphate and fructose-6-phosphate. Extracellularly, it functions as neurotrophic factor that promotes survival of skeletal motor neurons and sensory neurons, and as a lymphokine, which induces immunoglobulin secretion; glycolytic process/gluconeogenesis; carbohydrate degradation	**Energetic metabolism**
672	Glutamate dehydrogenase, mitochondrial	Converts L-glutamate into alpha-ketoglutarate. Plays a key role in glutamine anaplerosis by producing alpha-ketoglutarate, an important intermediate in the tricarboxylic acid cycle; ATP/ADP binding; GTP binding; identical protein binding; leucine binding; NAD+ binding; glutamate biosynthesis and catabolic process; glutamine metabolism; Oxidation-reduction; nitrogen metabolism; amino acid synthesis and transamination	**Energetic metabolism**

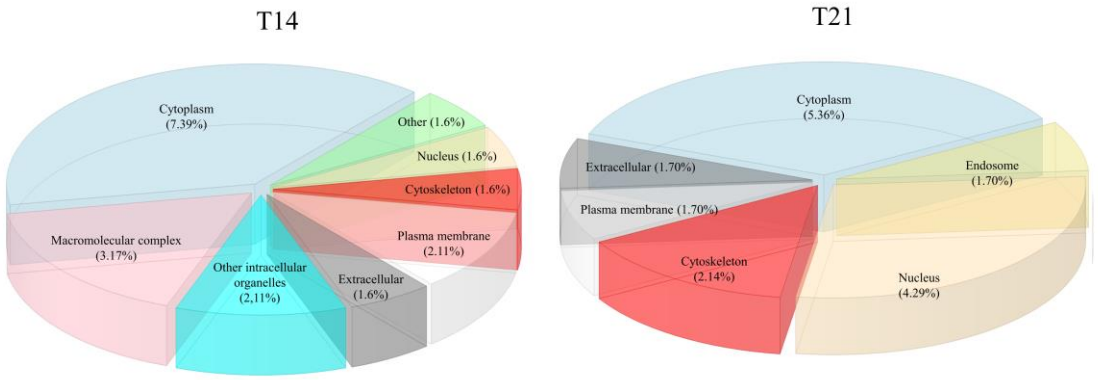
✓Already described above

Figure S6.1 General categories of gene ontology obtained from STRAP v1.5 (T14: 14 days of exposure to warming; T21: 21 days of exposure to warming), **(a)** biological process, **(b)** cellular component, **(c)** molecular function.

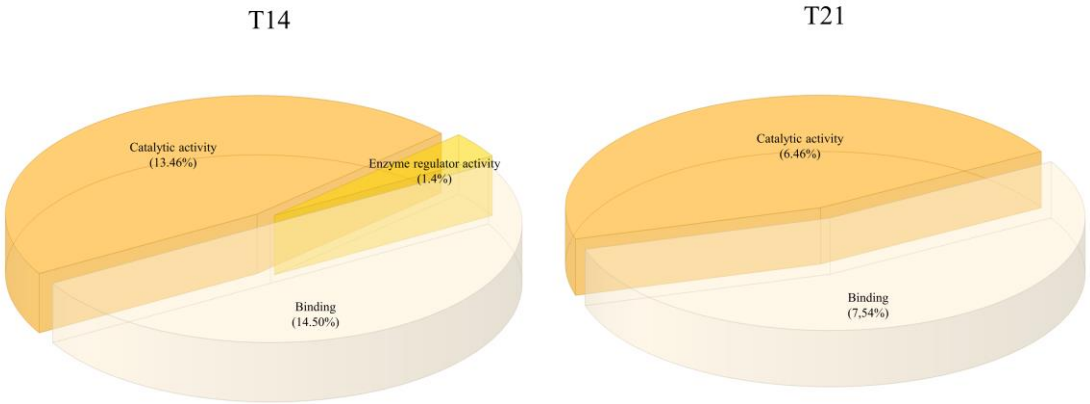
(a)



(b)



(c)



ANNEX 4. SUPPLEMENTARY INFORMATION FOR CHAPTER 7

Methodological details

Table S7.1 Methodology details for laboratorial procedures.

Biomarker	Method used	Detailed description
CAT	Enzymatic assay	Three replicates of 10 µL were taken from each diluted sample (1:10) and transferred to the microplates' wells. Then, 100 µL of assay buffer (100 mM potassium phosphate), 30 µL of methanol and 20 µL of 0.035 M hydrogen peroxide were added to each well, following 20 min incubation in the shaker. Another 30 µL of potassium hydroxide 10 M and 30 µL of Purpald 34.2 mM were added and samples were left to incubate again for 10 min in the shaker. Reactions were finalized with 10 µL of potassium periodate 65.2 mM and absorbance was read at 540 nm. Activity from a standard bovine catalase solution of 1523.6 $U.mL^{-1}$ was used as a positive control. Formaldehyde standards were used to produce a calibration curve. Catalase activity was calculated considering that one unit of catalase is defined as the amount that will cause the formation of 1.0 nmol of formaldehyde per minute at 25 °C.
LPO	TBARS method	Five microliters of each sample were added to 45 µL of 50 mM monobasic sodium phosphate buffer. Then 12.5 µL of SDS 8.1%, 93.5 µL of trichloroacetic acid (20%) and 93.5 µL of thiobarbituric acid (1%) were added to each microtube. To this mixture, 50.5 µL of Milli-Q grade ultrapure water was added and the microtubes were put in a vortex for 30 s. The microtubes' lids were punctured with a needle and the microtubes were incubated in boiling water for 10 min. Straight after, they were placed on ice for a few minutes to cool and 62.5 µL of Milli-Q grade ultrapure water and 312.5 µL of n-butanol pyridine (15:1, v/v) were added. Then the microtubes were placed in a vortex and centrifuged at 10000 ×g for 5 min. Duplicates of 150 µL of the supernatant of each reaction were put into a 96-well microplate and absorbancewas read at 530 nm. To quantify the lipid peroxides, an eight-point calibration curve (0–0.3 mM TBARS) was calculated using malondialdehyde bis (dimethylacetal) standards (from Merck).
GST	Enzymatic assay	Three replicates of 20 µLwere taken from each diluted sample (1:10) and transferred to the microplates' wells. In each well we added 180 µL of a reagent mix containing: 200 mM reduced L-glutathione, 100 mM CNDB and buffer Dulbecco (Sigma Aldrich, USA). After reading the absorbance at 340 nm, GST activity was calculated using a molar extinction coefficient for CDNB of 0.0053εmM.
SOD	Enzymatic assay	Three replicates of 10 µLwere taken from each diluted sample (1:10) and transferred to the microplates' wells. In each well we added 240 µL of a reagent mix containing: EDTA 3 mM, xanthine 3 mM, NBT 0.75 mM and XOD 100 um. Negative controls of mix without sample were included. After reading the absorbance at 560 nm, SOD activity was calculated using the equation for the % inhibition: Abs_{560} = ((Abs_{560}/min negative control – Abs_{560}/min sample) / (Abs_{560}/min negative control)) × 100

Hsp70 Tub	Indirect ELISA Direct ELISA	In both ELISAs, three replicates of 50 µLwere taken from each diluted sample (1:10), transferred to the microplates' wells and incubated overnight at 4°C. The microplates were washed (3×) in PBS 0.05% Tween-20 and then blocked by adding 200 µL of 1% BSA (Bovine Serum Albumin, Sigma-Aldrich, USA) in PBS. The microplates were then incubated at 37°C for 90 min. After microplate washing, the primary antibodies (anti-Hsp70/Hsc70, Acris, USA, in the first case and Ub P4D1, sc-8017, HRP conjugate, Santa Cruz, USA, in the second case), were diluted to 1 $\mu g.mL^{-1}$ and 0.5 $\mu g.mL^{-1}$, respectively, in 1% BSA, and added to the microplates' wells (50 µL each). Then the microplates were incubated for 90 min at 37 °C. After another washing stage: a) For Hsp quantification, the secondary antibody (anti-mouse IgG, fab specific, alkaline phosphatase conjugate, Sigma-Aldrich, USA) was diluted (1 $\mu g.mL^{-1}$ in 1% BSA) and added (50 µL) to each well followed by incubation at 37 °C for 90 min. After the washing stage, 100 µL of substrate (SIGMA FAST™ p-Nitrophenyl Phosphate Tablets, Sigma- Aldrich, USA) was added to each well and incubated for 30 min at room temperature. Fifty microliters of stop solution (3 N NaOH) was added to each well and the absorbance was read in a 96-well microplate reader at 405 nm (BIO-RAD, Benchmark, USA). b) For ubiquitin quantification, 100 µL of substrate (TMB/E, Temecula California, Merck Millipore) was added to each well and incubated for 30 min at room temperature. One hundred microliters of stop solution (1 N HCl) was added to each well and the absorbance was read in a 96-well microplate reader at 415 nm (BIO-RAD, Benchmark, USA). For quantification purposes, calibration curves were constructed using serial dilutions of purified HSP70 active protein (Acris, USA) and of purified ubiquitin (UbpBio, E-1100, USA), respectively, to give a range from 0 to 2 $\mu g.mL^{-1}$ of protein.
Total protein	Bradford method	200 µL of Bradford reagent (Comassie Blue G250, methanol, phosphoric acid, distilled water) were added to three replicates of 10 µL of diluted samples (1:10). Absorbance was read at 595 nm. BSA standards were used for a calibration curve. Total protein measurements were used to normalize all biomarker levels (Hsp70, total ubiquitin, antioxidant enzymes and LPO).

IBR calculations

The integrated biomarker response (IBR) was calculated according to Beliaeff and Burgeot (2002), posteriorly changed by Broeg and Lehtonen (2006) and described in detail in Ferreira et al (2015a and b). The IBR was calculated by summing up triangular star plot areas calculated for each two neighbouring data.

To calculate IBR for each temperature integrating all biomarkers, the general mean (*m*) and the standard deviation (*s*) of all data (including all sampling times) regarding a given biomarker was calculated, followed by a standardization to obtain *Y*, where $Y = (X - m)/s$, and *X* is the mean value for the biomarker at a given temperature (considering all sampling times). Then *Z* was calculated using $Z = -Y$ or $Z = Y$, in the case of a biological effect corresponding respectively to an inhibition or a stimulation. Regarding the biological effect, biomarkers can either increase or decrease depending on the intensity of the stressor, and with organisms' strategy as well. In theory, organisms tend to spend more energy in order to deal with a stressor, but an opposite strategy can also be used. As such:

i) The activity of the biomarkers CAT, GST and SOD can be induced in order to cope with the formation of lipid peroxides or inactivated by ROS-mediated denaturation (Reactive Oxygen Species);
ii) LPO usually increases under stress due to damage to cellular membranes caused by ROS, but it can decrease if anti-oxidant enzymes counteract the damaging effects of ROS;
iii) Hsp70 can be induced to prevent protein unfolding and aggregation, and Tub can be induced to target irreversibly denatured proteins to be degraded in the proteasome, or they can be downregulated if these cytoprotective systems are exhausted and there is a stress induced metabolic depression;

The score (*S*) was calculated by $S = Z + |Min|$, where $S \geq 0$ and $|Min|$ is the absolute value for the minimum value for all calculated *Y* in a given biomarker at all measurements made. Star plots were then used to display Score results (*S*) and to calculate the integrated biomarker response (IBR) as:

$$IBR = \sum_{t=1}^{n} A_i$$

$$A_i = \frac{S_i \times S_{i+1} \times sin\alpha}{2}$$

where S_i and S_{i+1} are two consecutive clockwise scores (radius coordinates) of a given star plot; *Ai* corresponds to the area of the connecting two scores; and $\alpha = 2\pi/n$ where *n* is the number of biomarkers used for calculations. The IBR is obtained by summing up all the A_i. The IBR

calculations were always performed with the same order of biomarkers for all temperatures taking into account their biological function (Hsp70, Tub, CAT, GST, SOD, LPO).

We provide an example of all calculations below.

IBR calculations for larvae (18 and 24ºC; 30ºC could not be included in the computations due to mortality):

The next two tables are the mean calculations for each biomarker at each sampling time

Time at 18ºC	Hsp70	Tub	CAT	GST	SOD	LPO
T0	3.154	0.302	17.007	68.45	39.83	0.091
T7	3.85	0.137	31.55	80.33	31.6	0.234
T14	3.94	0.07	29.16	48.22	81.38	0.56
T21	Samples not taken, larvae at 24ºC and 30ºC were all dead so the experiment did not continue					
T28						
mean	3.648	0.169667	25.90567	65.66667	50.93667	0.295
SD	0.430177	0.1194	7.798572	16.23494	26.6839	0.240377

Time at 24ºC	Hsp70	Tub	CAT	GST	SOD	LPO
T0	3.154	0.302	17.007	68.45	39.83	0.091
T7	3.46	0.166	24.6	101.66	92.01	0.284
T14	Samples not taken, larvae at 24ºC and 30ºC were all dead so the experiment did not continue					
T21						
T28						
mean	3.307	0.234	20.8035	85.055	65.92	0.1875
SD	0.216375	0.096167	5.369062	23.48302	36.89683	0.136472

From these two tables we then calculate

	Hsp	Ub	CAT	GST	SOD	LPO
18ºC	3.648	0.169667	25.90567	65.66667	50.93667	0.295
24ºC	3.307	0.234	20.8035	85.055	65.92	0.1875
30ºC	Dead					
mean	3.4775	0.201833	23.35458	75.36083	58.42833	0.24125
SD	0.241123	0.045491	3.607777	13.70962	10.59482	0.076014
Min*	1.546856	0.01695	7.422	65.77204	13.25212	0.035433

*Minimum of each biomarker (considering all times and temperatures)

Following, we compute the IBRs for each temperature:

Computation of IBR at 18ºC

	Hsp70	Tub	CAT	GST	SOD	LPO
	3.648	0.169667	25.90567	65.66667	50.93667	0.295
mean	3.4775	0.201833	23.35458	75.36083	58.42833	0.24125
sd	0.241123	0.045491	3.607777	13.70962	10.59482	0.076014

Y	0.707107	-0.70711	0.707107	-0.70711	-0.70711	0.707107
Z	0.707107	-0.70711	0.707107	-0.70711	-0.70711	0.707107
Min	1.546856	0.01695	7.422	65.77204	13.25212	0.035433
Min standardized	-8.00687	-4.06421	-4.41618	-0.69942	-4.26399	-2.70762
|Min|	8.00687	4.064213	4.416178	0.69942	4.263992	2.707617
S	8.713977	3.357106	5.123285	0.00769	3.556885	3.414724
A	12.66724	7.447562	0.01706	0.011844	5.259277	12.88465
IBR total	**38.28764**					

Computation of IBR at 24ºC

	Hsp70	Tub	CAT	GST	SOD	LPO
	3.307	0.234	20.8035	85.055	65.92	0.1875
mean	3.4775	0.201833	23.35458	75.36083	58.42833	0.24125
sd	0.241123	0.045491	3.607777	13.70962	10.59482	0.076014
Y	-0.70711	0.707107	-0.70711	0.707107	0.707107	-0.70711
Z	-0.70711	0.707107	-0.70711	0.707107	0.707107	-0.70711
Min	1.546856	0.01695	7.422	65.77204	13.25212	0.035433
Min standardized	-8.00687	-4.06421	-4.41618	-0.69942	-4.26399	-2.70762
|Min|	8.00687	4.064213	4.416178	0.69942	4.263992	2.707617
S	7.299763	4.771319	3.709071	1.406527	4.971098	2.00051
A	15.08162	7.663096	2.258988	3.027618	4.306196	6.323394
IBR total	**38.66091**					

Note: calculation of Sin α = sin (2 π/6) = 0.866025

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Table S7.2 Analysis of variance to detect significant differences between biomarker levels throughout the experiment at the different tested temperatures (18ºC, 24ºC, 30ºC). Significant results are in bold and marked with an asterisk (p<0.05).

Life stage	Organ	Biomarker	Statistic	df	p-value
Larvae	**Whole body**	Hsp70	F=2.8	3	0.080
		TUb	F=4.8	3	**0.020***
		CAT	F=1.16	3	0.368
		GST	F=13.3	3	**0.001***
		SOD	F=6.2	3	**0.015***
		LPO	H=10.3	3	**0.016***
Juveniles	**Muscle**	Hsp70	H=47.5	11	**0.000***
		TUb	H=34.7	11	**0.000***
		CAT	H=38.6	11	**0.000***
		GST	H=33.9	11	**0.000***
		SOD	H=41.9	11	**0.000***
		LPO	H=44.1	11	**0.000***
	Gills	Hsp70	H=23.2	11	**0.016***
		TUb	H=16.3	11	0.128
		CAT	H=37.6	11	**0.000***
		GST	H=38.7	11	**0.000***
		SOD	H=33.0	11	**0.001***
		LPO	H=35.4	11	**0.000***
	Liver	Hsp70	H=30.0	11	**0.002***
		TUb	H=42.7	11	**0.000***
		CAT	H=26.8	11	**0.005***
		GST	H=37.4	11	**0.000***
		SOD	H=27.0	11	**0.005***
		LPO	H=42.8	11	**0.000***
	Brain	Hsp70	H=29.9	11	**0.002***
		TUb	H=43.6	11	**0.000***
		CAT	H=43.7	11	**0.000***
		GST	H=32.9	11	**0.001***
		SOD	H=31.3	11	**0.001***

		LPO	H=35.8	11	**0.000***
	Intestine	Hsp70	H=30.1	11	**0.002***
		TUb	H=39.9	11	**0.000***
		CAT	H=36.9	11	**0.000***
		GST	H=17.1	11	0.105
		SOD	H=24.3	11	**0.012***
		LPO	H=30.0	11	**0.002***
Adults	**Muscle**	Hsp70	H=22.8	11	**0.006***
		TUb	H=30.3	9	**0.000***
		CAT	H=27.3	9	**0.001***
		GST	H=26.1	9	**0.002***
		SOD	H=15.8	9	0.070
		LPO	F=4.6	9	**0.000***
	Gills	Hsp70	H=26.6	9	**0.002***
		TUb	H=26.9	9	**0.002***
		CAT	F=2.6	9	**0.026***
		GST	F=2.4	9	**0.033***
		SOD	H=27.6	9	**0.001***
		LPO	F=3.9	9	**0.002***
	Liver	Hsp70	F=2.2	9	0.055
		TUb	H=17.0	9	**0.048***
		CAT	F=1.8	9	0.122
		GST	H=32.8	9	**0.000***
		SOD	F=4.6	9	**0.000***
		LPO	H=25.3	9	**0.003***
	Brain	Hsp70	H=15.0	9	0.089
		TUb	H=29.7	9	**0.001***
		CAT	H=23.5	9	**0.005***
		GST	F=5.3	9	**0.000***
		SOD	H=20.6	9	**0.015***
		LPO	H=13.3	9	0.149

Intestine	Hsp70	H=25.8	9	**0.002***
	TUb	H=18.1	9	**0.034***
	CAT	H=19.7	9	**0.019***
	GST	F=3.9	9	**0.003***
	SOD	H=27.7	9	**0.001***
	LPO	H=16.7	9	0.053