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Licenciada em Bioquímica

Physiological Responses to Ocean Warming and Acidification of *Diplodus cervinus*

Dissertação para obtenção do Grau de Mestre em
Bioquímica

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"I'd rather attempt to do something great and fail than to attempt nothing and succeed." – Robert H. Schuller

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Abstract

Climate change is one of the main global environmental threats, especially in the marine biosphere. The increased ocean temperatures and low pH can induce the generation of oxygen reactive species (ROS) which are the primary source of damage to marine species, forcing them to activate response mechanisms that allows them to survive under environmental stress conditions. Nevertheless, the studies focused on the impacts of these stressors on marine organism's physiological responses are still scarce and require further understanding. In this context, the main goals of the present work were to study, for the first time, the effects of ocean warming (+4 °C) and acidification (ΔpH=−0.4 units equivalent to ΔpCO_2~1000μatm) on the antioxidant enzyme activities [superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione peroxidase (GPx) and total antioxidant capacity (TAC)], lipid peroxidation (LPO), acetylcholinesterase (AChE), heat shock response (HSP70) and ubiquitin content (Ub)], animal fitness [hepatosomatic index (HSI), viscerosomatic index (VSI) and Fulton's condition index (K)] and haematological parameters (erythrocytes and leucocytes content) in different tissues (brain, muscle, liver and gills) of juvenile zebra seabream (*Diplodus cervinus*). Despite the distinct responses of each tissue to environmental stressors, the present study evidenced that fish antioxidant machinery and other mechanisms such as HSP70, Ub, AChE and LPO significantly responded to warming and acidification, acting alone or combined (e.g. CAT increased in liver and decreased in muscle; SOD activity is severely compromised and only significantly increases in the muscle when exposed to both stressors). The liver, being the main tissue responsible for ROS detoxification, revealed the highest expression for the measured biomarkers. Animal fitness was also significantly affected by warming, i.e. body weight increased, while HSI decreased. Haematological parameters showed that warming, alone or combined with acidification increases erythrocytes percentage and warming alone decreases the leucocytes percentage. Overall, the present findings demonstrated that the exposure to ocean warming and acidification may compromise the cellular adjustments and antioxidant responses of marine organisms and thus lead to physiological impairments.

Keywords: Climate Change; Ocean warming; Ocean acidification; Biomarkers; Zebra seabream; Oxidative Stress.

Resumo

As alterações climáticas são uma das principais ameaças ambientais globais, especialmente na biosfera marinha. O aumento da temperatura dos oceanos e o decréscimo do pH podem induzir a geração de espécies reativas de oxigénio (ROS), que são a principal fonte de danos celulares nos organismos marinhos, forçando-os a ativar mecanismos de resposta à exposição a condições de stress ambiental. No entanto, os estudos que se focam nos impactos destes fatores de stress nas respostas fisiológicas dos organismos marinhos ainda são pouco conhecidos e exigem maior compreensão. Neste contexto, o principal objetivo do presente trabalho foi estudar, pela primeira vez, os efeitos do aquecimento (+4 °C) e da acidificação (ΔpH=-0,4 unidades equivalentes a ΔpCO_2~1000 μatm) dos oceanos na atividade de várias enzimas antioxidantes [superóxido dismutase (SOD), catalase (CAT), glutationa S-transferase (GST), glutationa peroxidase (GPx) e capacidade antioxidante total (TAC)], peroxidação lipídica (LPO), atividade da acetilcolinesterase (AChE), resposta de choque térmico (HSPs) e teor de ubiquitina (Ub)], no fitness do animal [índice hepatosomático (HSI), índice viscerosomático (VSI) e índice de condição de Fulton (K)] e parâmetros hematológicos (conteúdo em eritrócitos e leucócitos) em diferentes tecidos (cérebro, músculo, fígado e brânquias) de sargos-veados juvenis (*Diplodus cervinus*). Apesar da resposta diferenciada de cada tecido aos fatores de stress ambiental, o presente estudo evidencia que o sistema antioxidante e outros mecanismos como a HSP70, Ub, AChE e LPO, respondem significativamente ao aquecimento e acidificação, atuando isoladamente ou combinados (e.x. CAT aumentou significativamente no fígado, mas diminuiu no músculo; a atividade da SOD está severamente comprometida e apenas aumenta significativamente no músculo, quando expostos a ambos os fatores de stress). O fígado, sendo o principal tecido responsável pela desintoxicação dos ROS, revelou uma maior expressão para os biomarcadores estudados. O fitness do animal também foi significativamente afetado pelo aquecimento, isto é o peso corporal aumentou, enquanto o HSI diminuiu. Os parâmetros hematológicos demonstraram que o aquecimento isolado ou combinado com a acidificação aumenta a percentagem de eritrócitos e que o aquecimento sozinho diminui a percentagem de leucócitos. De um modo geral, os resultados do presente estudo demonstraram que a exposição prolongada ao aquecimento e acidificação dos oceanos pode comprometer as respostas celulares e antioxidantes dos organismos marinhos e, assim, levar a distúrbios fisiológicos.

Palavras – Chave: Alterações Climáticas; Aquecimento; Acidificação; Biomarcadores; Sargo-veado; Stress Oxidativo.

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List of Abbreviations and Symbols

Ω	Saturation State
Ωara	Aragonite Saturation State
Ωcal	Calcite Saturation State
ºC	Degree Celsius
%	Percentage
µg	Micrograms
µatm	Micro-atmospheres
µL	Microliter
µm	Micrometre
µmol	Micromol
µM	Micromolar
Δ	Variation
ABTS	2,2′-Azino-bis (3-Ethylbenzothiazoline-6-Sulfonic Acid)
Acid	Acidification Treatment
Acid+Warm	Acidification + Warming Treatment
AChE	Acetylcholinesterase
ACTI	Acetylthiocholine
Ara	Aragonite
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
cal	Calcite
CAT	Catalase
$CaCO_3$	Calcium Carbonate
CDNB	1-Chloro-2,4-Dinitrobenzene
cm	Centimeter
CO_2	Carbon Dioxide
CO_3^{2-}	Carbonate Ions
CT	Total Carbon
D. cervinus	*Diplodus cervinus*
D. labrax	*Dicenthrachus labrax*
DO	Dissolved Oxygen
DNA	Deoxyribonucleic Acid
DTNB	5,5'-Dithio-bis-2-Nitrobenzoic Acid
E1	Ubiquitin-activating Enzyme
E2	Ubiquitin-conjugating Enzyme
E3	Ubiquitin-ligase Enzyme
EDTA	Ethylenediamine Tetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EPPO	Estação Piloto de Piscicultura de Olhão
ENAs	Erythrocyte Nuclear Abnormalities
g	Grams
GPx	Glutathione Peroxidase
GR	Glutathione Reductase

GSH	Reduced Glutathione
GST	Glutathione S-Transferase
GSSG	Oxidized Glutathione
H^+	Proton
HCO_3^-	Bicarbonate
H_2CO_3	Carbonic Acid
H_2O	Water
H_2O_2	Hydrogen Peroxide
HABs	Harmful Algae Blooms
$HO^{\bullet}$	Hydroxyl Radical
HSI	Hepatosomatic Index
HSP	Heat Shock Protein
HSR	Heat Shock Response
IPCC	Intergovernmental Panel on Climate Change
IPMA	Portuguese Institute for the Sea and Atmosphere
K	Fulton's condition index
KCl	Potassium Chloride
kg	Kilogram
KH_2PO_4	Potassium Phosphate Monobasic
L	Liter
LPO	Lipid Peroxidation
LOOH	Lipid Peroxide
LW	Liver Weight
m	Meter
M	Molar
mg	Milligram
$MgCl_2$	Magnesium Chloride
mL	Milliliter
mM	Millimolar
MDA	Malondialdehyde
MS	Methanesulfonate Solution
nmol	Nanomole
N_2O	Nitrous Oxide
Na_2HPO_4	Sodium Phosphate Dibasic
$NaC_2H_3O_2{\cdot}3H_2O$	Sodium Acetate
NaCl	Sodium Chloride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
$NaHCO_3$	Sodium Bicarbonate
NaOH	Sodium Hydroxide
NBT	Nitro Blue Tetrazolium
NH_3/NH_4^+	Ammonia
NO_2^-	Nitrite
NO_3^-	Nitrate
O_2	Molecular Oxygen
$O_2^{\bullet-}$	Superoxide Anion

ORBEA	Ethical Committee of the Faculty of Sciences, University of Lisbon
OCLTT	Oxygen and Capacity-limited Thermal Tolerance Hypothesis
P	Phosphorous
PBS	Phosphate Buffer Saline
$\boldsymbol{pCO_2}$	Partial Pressure of Carbon Dioxide
PnPP	4-Nitrophenyl Phosphate Disodium Salt Hexahydrate
Purpald	4-Amino-3-Hydrazino-5-Mercapto-1,2,4-Triazole
RCP	Representative Concentration Pathways
RAS	Recirculating Aquaculture Systems
ROH	Alcohol
ROOH	Hydroperoxide
ROS	Reactive Oxygen Species
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SOD	Superoxide Dismutase
TA	Total Alkalinity
TAC	Total Antioxidant Capacity
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substances
Tc	Critical Temperature
TCA	Trichloroacetic Acid
Td	Denaturation Temperatures
TL	Total Length
Topt	Optimum Temperature
Tp	*pejus* Temperature
Trolox	6-Hydroxy-2,5,7,8-Tetramethylchroman-2-Carboxylic Acid
TW	Total Weight
Ub	Ubiquitin
VW	Viscera Weight
VSI	Viscerosomatic Index
XOD	Xanthine Oxidase
Warm	Warming Treatment
$\boldsymbol{ZnSO_4.7H_2O}$	Zinc Sulfate Heptahydrate

1. INTRODUCTION

1.1 Climate Change

In the last decades, the impact of climate change has become one of the greatest concerns for marine ecosystems and their biota.[1] Since the pre-industrial era, the anthropogenic greenhouse gas emissions have been increasing. Moreover, from 1970 to 2010, about 78% of the total increase was attributed to the emissions of carbon dioxide (CO_2) from fossil fuels combustion and industrial processes.[1] Ocean acidification and increased seawater temperatures are examples of direct consequences of CO_2 successive accumulation.[2] Consequently, climate change alters the physical, chemical and biological properties of the ocean.[2]

The increased CO_2 atmospheric concentrations, are threatening the different marine ecosystems leading to additional ocean changes, such as rising of sea level due to thawing, increased ocean stratification, altering patterns of precipitation, ocean circulation, storms and freshwater input (Figure 1.1).[3,4] The projected alterations in ocean properties, like stratification will reduce nutrients supply for organisms. Anthropogenic CO_2 will lower the pH of the ocean surface and this penetration of acidified waters to depth will result in dissolution of calcium carbonate ($CaCO_3$) structures, such as bivalve' shells.[4] In addition, the presence of molecular oxygen (O_2) in depth waters will decrease as the waters progressively become deeper. Consequently, ocean biota and ecosystems will be affected by changes in the ocean physical and chemical properties of the seawater that are influenced by changes in storm activity and dust deposition.[3,5] All these changes produced in ocean circulation reduce subsurface oxygen concentrations, decreasing oxygen availability.[5]

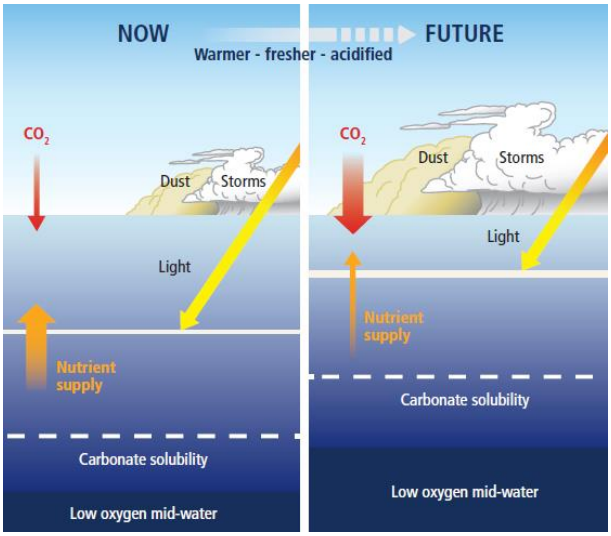

Figure 1.1 – Projected alteration in ocean circulation and possible stratification and atmospheric events due to changing climate in the coming decades *(Adapted from IPCC 2013, chapter 6, Ocean systems).*[6]

For the marine species these climate alterations produce not only changes on organism's physiological properties and behaviour, but also affect at a molecular level, the biochemical reactions.[7] The ecosystems changes will have consequences namely in growth, food availability, acid-base balance, metabolism, species fitness and survival.[8,9] Other consequences will occur, for instance the community structure and ecosystem function will suffer alterations that can lead to disruption of biological interactions and consequently in the propagation of climate signals from primary producers to upper-trophic-levels.[2]

1.2 Ocean Warming

Ocean temperature is rising at unforeseen rates and therefore the impacts on marine organisms and ecosystems are unknown. For 2100 is predicted an increase between 0.3 ºC and 4.8 ºC in temperature, represented by the scenarios RCP2.6 and RCP8.5 (RCP – Representative Concentration Pathways) in the IPCC report (Intergovernmental Panel on Climate Change), shown in Figure 1.2.[1]

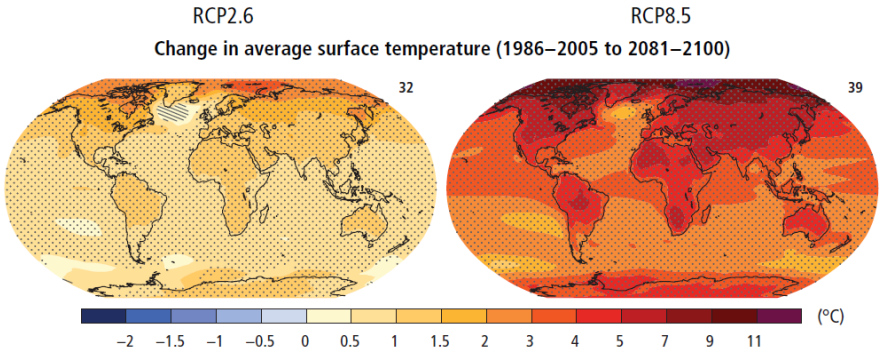

Figure 1.2 – Average change in surface temperature based in climate change projections for 2081-2100 relative to 1986-2005 under the RCP2.6 (left) and RCP8.5 (right) scenarios. *(Adapted from IPCC 2014, Synthesis Report, Summary for Policymakers).* [1]

Changes observed in temperature have an impact in biological functions and ecological processes in a wide range of marine ectothermic organisms.[10,11]

Species thermal range and development stage are variable, so the extent of effects can be extremely different. The predicted future ocean warming will impose an additional thermal stress to the organisms and will favour those that do not live close to their thermal limits. [12–14]

When individuals are subjected to environmental changes, they may adjust and adapt physiologically, making the new conditions tolerable (acclimation) or they may consider the conditions intolerable, promoting populations geographically dispersion (migration), or even death and local extinction.[15]

The acclimation process to warming might include a wide range of adjustments, such as phenotypic modifications, namely behavioural and physiological, that are determinant to species tolerance to environmental variability and change.[16] These processes have an optimal within a

narrow thermal tolerance window, setting of an optimum temperature (Topt) for which the performance levels are maximal. The pejus temperature (Tp; pejus = getting worse) is defined as the temperature limit from which organisms are incapable to induce compensatory mechanisms and the performance levels begins to decrease, so it is important for the organisms to stay within this limit (Figure 1.3, based on the Oxygen and capacity-limited thermal tolerance hypothesis - OCLTT).[17] This performance becomes progressively limited during cooling or warming. When the first low- and high-temperature thresholds (Tp) of the organisms are surpassed, they enter the time-limited tolerance. If the cooling or warming continues and transcends the next high thresholds (Tc, critical temperature), the reduction of oxygen supply will progressively lead to transition to anaerobic mitochondrial metabolism as a compensatory mechanism, that will function for a limited period and eventually lead to insufficient cellular energy levels. When the denaturation temperatures (Td) are achieved, the damaged is extreme and characterized by cells and proteins destruction.[17,18]

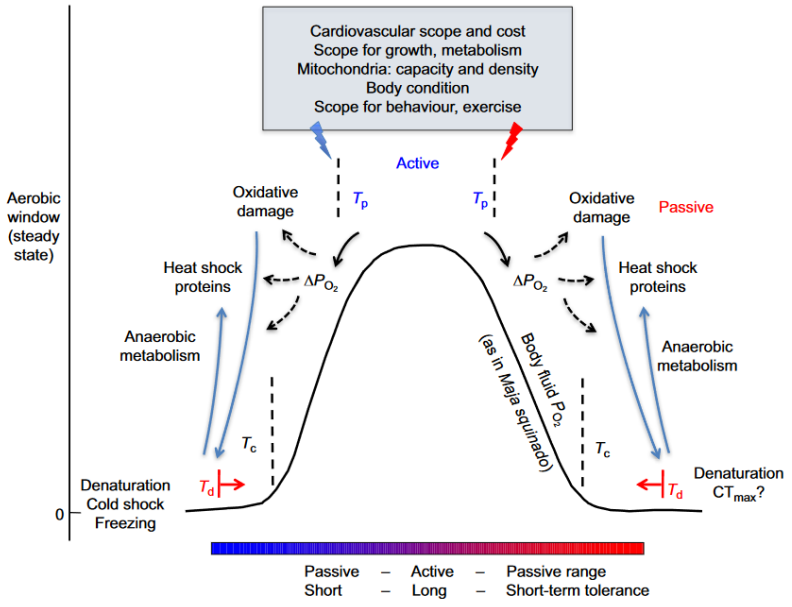

Figure 1.3- Thermal tolerance window of an organism, showing the limits for climate sensitivity, acclimatization and performance levels *(Adapted from IPCC 2013, chapter 6, Ocean systems).*[6]

Species with restricted climate and habitat requirements, limited dispersal abilities and ectothermal physiology will likely be in risk of local and global extinction. In order to survive, their only option is to adapt to climate changes via evolutionary processes. [19]

1.3 Hypoxia

Another potential consequence of climate change is the decrease in dissolved O_2 concentration in the ocean's (deoxygenation). The O_2 decreased solubility in warmer waters and the increase in the upper ocean stratification reduces the O_2 availability in a warming ocean.[5] The

subsequentially decrease in dissolved O_2 concentration will have extensive effects, since this compound is a key component in the biogeochemical cycling of carbon and nitrogen.[6,20] Ocean stratification, the lack of circulation and continuous consumption of O_2 by deep-dwelling organisms will decrease O_2 concentration in deep ocean. [21,22] Once O_2 levels achieve an hypoxic threshold, the organisms that are not very sensitive to O_2 levels will undergo a variety of stressors that ultimately can lead to death. These thresholds vary between marine taxa, being fish and crustaceans the most sensitive organisms.[22]

The hypoxia thresholds depend not only on O_2 levels, but also on levels of CO_2 and temperature, so it is important to consider the consequences of deoxygenation in warming oceans coupled with the effect of acidification.[23] When the dissolved oxygen content reaches very low concentrations, around 5 μmol kg^{-1}, a anaerobic respiration becomes to rise, making this ion the primary electron acceptor (suboxic conditions), but when the O_2 concentration decreases to zero, the water becomes anoxic.[6,24] The low levels of O_2 also affect the nitrogen cycling, more specifically the suboxia conditions will affect the nitrogen fixation in the ocean via denitrification, influencing the nitrate supply, consequently limiting the nutrient availability and, therefore, affecting global ocean productivity.[22,25,26] The global warming will be also potentially amplified by the production of nitrous oxide (N_2O), a powerful greenhouse gas, by oxic and suboxic pathways, that leads to drop in the oxygen content. Consequently, the recycling of marine phosphorous (P) from sediments will increase.[20,25,27,28]

Hypoxia has a negative impact in marine organisms, for example, at individual levels, the sublethal conditions of this stressor causes a huge variety of disturbances, altering the phenology, growth rates, reproductive success, increasing the vulnerability to diseases and parasites and also affects the locomotory activity. At the population and ecosystem levels, the hypoxia will alter the migration patterns, loss of biomass and species richness and trophic mismatch, which will displace energy flow toward microbial pathways to the detriment of higher trophic levels.[29]

1.4 Ocean Acidification

The increased accumulation of atmospheric CO_2 causes an increase in oceans CO_2 concentration and consequently is the main cause of the ocean acidification.[30] Oceans absorbs approximately 30% of the CO_2 produced by anthropogenic sources making it one of the most important carbon sinks.[21,31]

The ocean has a capacity to buffer changes in oceans chemistry, maintaining the pH level using the isotopic boron levels in fossilized carbonate shells in marine organisms. Changes in temperature, weathering and sedimentation, may also act as buffers.[32] Since the ocean's chemistry has been stable for over 400,000 years, the threats of ocean acidification have been neglected and the minimal change in the pH range will have considerable effects.[33] There are several chemical

changes that reflect ocean acidification such as pH decrease, elevated aqueous CO_2, total inorganic carbon, carbonate ion and calcium carbonate states.[4] By the year of 2100 it is estimated, by the scenarios RCP6.0 and RCP8.5 in the IPCC report, an increase up to 1000 µatm and a drop up to 0.4 units in the pH, if the rate of anthropogenic CO_2 emissions doesn't decrease (Figure 1.4).[1,6]

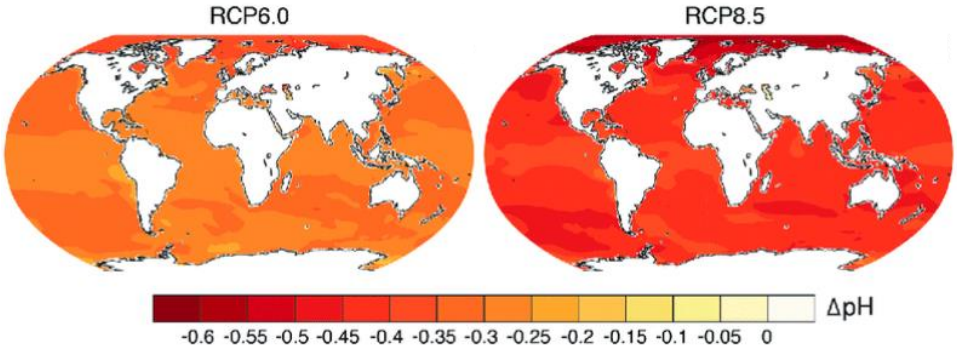

Figure 1.4- Average change in pH based in climate change projections for 2081-2100 relative to 1986-2005 under the RCP6.0 (left) and RCP8.5 (right) scenarios *(Adapted from IPCC report, Ocean Acidification).*[6]

The animal's thermal tolerance window may be narrowed due to exposure to ocean acidification and, as a result, intensify the impact of increasing sea surface temperature on biological processes and changes of marine ectothermic animals, such as growth, calcification, behaviour and metabolism. Marine organisms became more vulnerable when exposed to ocean acidification especially those that are already near their thermal tolerance limits.[23,34–38]

Calcium carbonate ($CaCO_3$) ions are an essential components of the seawater carbonate system (Equation 1) acting as ocean buffers and also, control the calcification rates of shell-building marine animals.[39]

$$CO_{2(atmos)} \leftrightarrow CO_{2(aq)} + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-} \quad (1)$$

When CO_2 atmospheric is dissolved in seawater it reacts forming carbonic acid (H_2CO_3), that can dissociate into bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions. These reactions are reversible and maintain the seawater near an equilibrium. The CO_2 concentration increase in seawater will also increase bicarbonate and hydrogen ions concentration, leading to a pH decrease (ocean acidification). Carbonate ions concentration also decreases with the increasing H^+ concentrations.[4]

Ocean climate change has direct consequences on calcifying organisms due to the reduced availability of $CaCO_3$ that alters the carbonate system. So, the main studies have been focused on these organisms and more recently have been performed studies on the impact on non-calcifying marine organisms.[36] These changes in CO_2 concentrations may lead to harmful effects, and the evidences have been growing for these perturbations, altering fundamental processes, like

reproduction and development, acid-base regulation, respiration, behaviour aspects and physiology of marine species, including some mollusks and fish species.[37,40–42]

It was thought by some authors that fish species were relatively resistant to the increase CO_2 concentrations, presenting a capacity to maintain their homeostasis, although energetically expensive, and also produce compensatory mechanisms for the extra intracellular pH disturbances.[35,40] However, CO_2 diffusion across gill epithelia into fish tissues and fluids may occur when pCO_2 in seawater increases and might lead to extracellular acidosis if not compensated by HCO_3^- accumulation and/or H^+ secretion (Figure 1.5).[35]

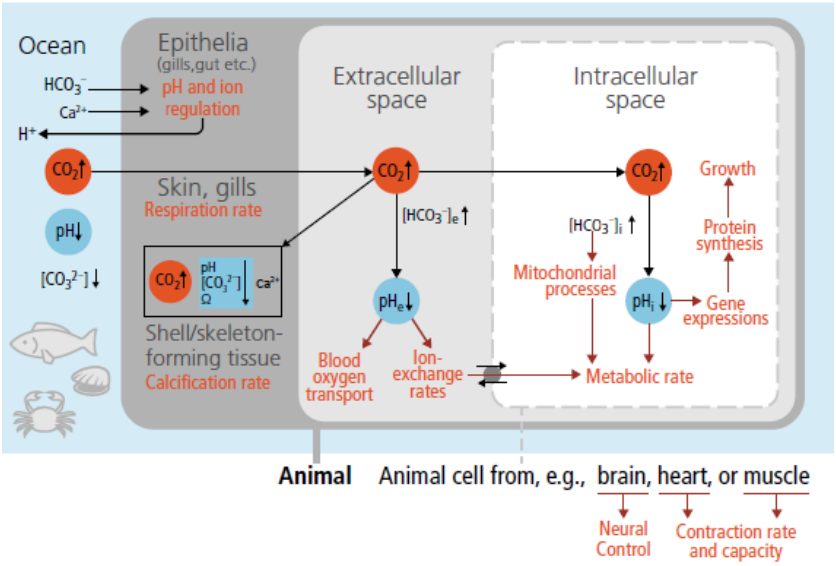

Figure 1.5- Schematic representation of marine animal to ocean acidification. The effects of CO_2 diffusion (black arrows) into body and cell compartments are represented, like the increase in pCO_2 (highlighted in red) and a drop in compartmental pH (highlighted in blue). The effects of pCO_2 increase and pH decreased are represented in red arrows, as well as on calcium carbonate saturation state (Ω) at calcification sites. *(Adapted from IPCC 2013, chapter 6, Ocean systems).*[6]

Additionally, the capacity of oxygen supply and delivery can be restricted by changes in plasma pH, causing hypoxia in tissues, affecting the oxygen necessary to maintain cell functions and progressively reducing activities beyond the required for basic maintenance.[23] Consequently, metabolic pathways will suffer adjustments reflecting the changes in aerobic scope and aerobic performance of the organisms and energy production mode can be also changed progressively due to these modifications.[22,42,43]

Nevertheless, recent studies reported fish vulnerability to ocean acidification even at early stages.[18,45,46]

1.5 Effects of Ocean Warming and Acidification on Marine Organisms

As it was referred in the last chapters, climate change alters the ocean structure and composition, affecting this way the marine ecosystems.

The demand in seafood has been growing, and for that reason, the need for aquacultures has also increased, so it important to understand the implications that these climate changes will have in the aquaculture sector and to the seafood quality and safety.[47]

The climate change impacts are countless and can be unexpected. Industrial sectors such as aquaculture as well as fisheries, human health and coastal cities are expected to be affected. Some of the predictable impacts by environmental parameters, as shown in Figure 1.6, are:

- Temperature - the species survival and productivity will be dependent on their optimal range of temperature and the upper thermal limit. The expected increase in water temperature can in one way, boost the metabolism and growth rate, consequently helping the overall production.[9,47]

- Eutrophication and harmful algae blooms – The eutrophication and pronounced stratification are consequences of these climate changes and lead to oxygen depletion. The combination of eutrophication process with nutrient enrichment decay leads to hypoxia and may possibly affect the species survival (fish and benthic invertebrates). Also, the combination of increased temperatures and eutrophication induce harmful algae blooms (HABs) and can enhance the occurrence of toxic tides, impacting negatively the aquaculture production (reducing O_2 content, as it was mentioned above) and threatened the human health. Indeed, the consumption of contaminated seafood with biotoxins can cause a series of illnesses in humans. These marine biotoxins are dangerous compounds that affect primarily the nervous system but can also lead to other diseases like fatal acute respiratory distress and immunological illnesses.[9,47]

- Sea level rise and melting mountain ice caps - Climate changes will have an impact in the water cycle and can lead to extreme events like heat waves, droughts or even floods, affecting the coastal aquaculture nutrient load and may also stimulate HABs. The continue rise of global temperatures will expand ocean water and melt mountain ice caps and glaciers, contributing this way to the rise of the sea level. This sea level rise in combination with saline water intrusion will affect the water quality and subsequently the aquaculture production. The combination of sea level rise coupled with changes in nutrient availability will affect species habitat and may lead to shifts in fish distribution and migration behavior. [9,47]

- Salinity – fish can maintain ionic and osmotic homeostasis among environmental salinities by using osmoregulatory mechanisms that are processes requiring high energy demand. Increase in salinity will generate compensatory mechanisms that will affect growth and development, the metabolism and may also force the species migration.[48,49]

- Acidification – The ocean acidification, as it was mentioned in the previous sub-chapter will affect the ocean chemistry, posing serious threats to marine organisms, particularly those with $CaCO_3$ shells and skeletons.[9,47]

- Diseases – Climate changes can influence the predominance of marine pathogens and biotoxins. The presence of parasites, bacteria, viruses and biotoxins can compromise seafood safety and, consequently human health. In fact, the increased temperature can stimulate the parasite growth and transmission, increasing host densities and making the species more vulnerable to infectious diseases.[9,47]

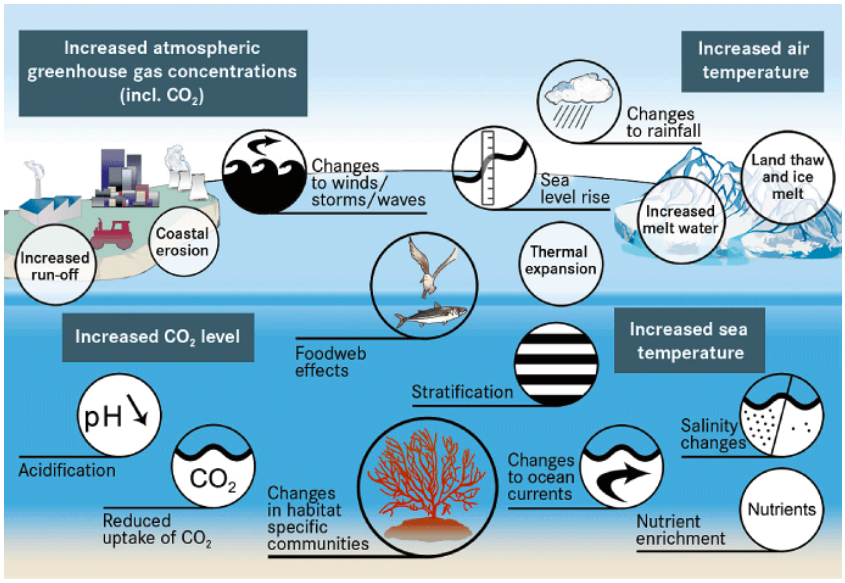

Figure 1.6- Effects of increased atmospheric CO_2 levels and air/ocean temperature in marine organisms (*Adapted from OSPAR 2010 Quality Status Report*).[47]

1.6 Molecular Mechanisms of Defence

1.6.1 Oxidative Stress and Cellular Damage

A variety of environmental stressors, including ocean warming and acidification, induce the production of reactive oxygen species (ROS).[50] ROS are naturally produced when molecular oxygen is reduced to reactive intermediates in aerobic respiration, such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$).[51] The production of ROS, within the cells (mostly in mitochondria), plays an important role in homeostasis and redox signalling.

Increased ROS levels raises oxidative stress, affecting cellular integrity and injuring cellular mechanisms. When ROS concentration surpasses the organism capacity of defence then, they became harmful, causing lipid peroxidation, damaging proteins and DNA.[50]

Marine organisms have developed diverse anti-oxidative mechanisms that neutralize, detoxify or remove this reactive species as a protective response. These mechanisms are dependent on a network of antioxidant enzymes that together can detoxify ROS.[50] These antioxidant defence system includes specific enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST) and glutathione peroxidase (GPx) and also specific biomarkers for ROS effects, like lipid peroxidation (LPO), acetylcholinesterase (AChE) and total antioxidant capacity (TAC).

The oxidative stress is considered the period of time between the generation and the neutralization of ROS within the organism by antioxidant mechanisms.[50] These antioxidant enzymes are found with higher activity in the liver of fish, although widely expressed through other organisms tissues (e.g. kidney, gills).[52] Thus, the antioxidant enzymes are usually used as biomarkers for oxidative stress, since they can indicate the antioxidant status of the organisms.[53] The effects caused by stressors (e.g. chemical, physical, etc.) are dependent on the intensity, the exposure time and the susceptibility of the exposed organism. Consequently, these stress agents can trigger different kinds of responses in the organisms, being the cellular antioxidant defence systems also susceptible to these stress factors.[54]

Climate change scenarios induce a significant variable antioxidant response to the oxidative stress depending on the different aquatic species. A higher expression of antioxidant enzymes can be considered as an adaptation of the organism to the environmental changes, allowing the species to survive. In opposition, a decrease in the expression, can suggest an organism's inability to respond to the stressors, becoming more susceptible to adverse effects. The study of several biomarkers of oxidative stress can be a better approach than a single antioxidant parameter to evaluate the total antioxidant status of an organism when exposed to stress agents. [54]

1.6.1.1 Superoxide Dismutase (SOD)

Superoxide dismutase (SOD) is one of the first lines of defence for oxidative stress and catalyses the dismutation of the superoxide anion ($O_2^{\bullet-}$), a highly reactive species, converting into hydrogen peroxide (H_2O_2) and oxygen (O_2) (Equation 2).[55]

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad (2)$$

The method typically used for determination of SOD activity is based on the spectrophotometric detection of nitroblue tetrazolium (NBT).[56] This method is based on the generation of a blue formazan dye. More specifically, xanthine oxidase (XOD) converts xanthine

to uric acid, producing important ions for the conversion of NBT in NBT-diformazan. The concentration of superoxide ions is lowered by SODs activity and at the same time the rate of NBT/NBT-diformazan conversion decreases as shown in Figure 1.7. SODs activity is determined by measuring absorbance of NBT-diformazan at 560 nm.[56]

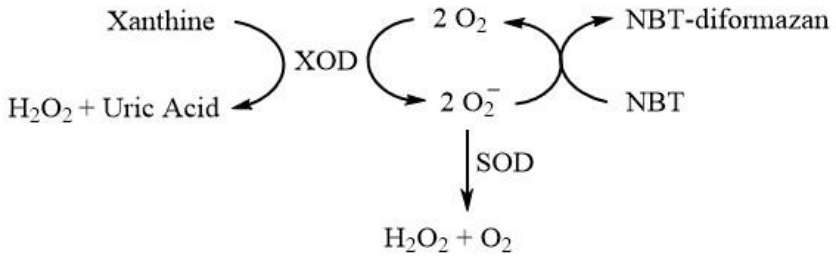

Figure 1.7 – Representation of reactions involving the enzymes, xanthine oxidase (XOD) and superoxide dismutase (SOD). XOD is involve in the transformation of xanthine to hydroxide peroxide and uric acid, coupling the transformation of molecular oxygen to reactive oxygen species and the conversion of NBT to the complex NBT-diformazan. SOD is involved in a parallel reaction, "stealing" the oxygen reactive species needed to form the NBT-diformazan complex, to hydrogen peroxide and water.[56]

Although SOD is one of the most important antioxidant agents, the production of H_2O_2 still poses a threat to the organism. The exposure to H_2O_2 can then cause significant damage, because of its highly diffusion capability within the cell and their ability to enter into numerous reactions.[55] This compound when is not decomposed can be converted into HO•, a very short-lived and aggressive ROS, being directly involved in tissue damage and apoptosis, acting as signal in ROS functions.[50,57]

The antioxidant network helps to continue the detoxification process, linking the different antioxidant enzymes that depend upon the activity of each other, and consequently controlling the ROS production. The remaining H_2O_2 produced will be removed by catalase (CAT), avoiding its accumulation in cells and tissues or will be used to oxidize substrates by glutathione peroxidase (GPx).[50] This array of antioxidant enzymes are complemented by small molecule antioxidant like glutathione and vitamins E, among others.[58]

1.6.1.2 Catalase

Catalase (CAT) is an enzyme present in most aerobic organisms and in many anaerobic organisms. It is considered part of oxidoreductases subclass, and catalysis the conversion of H_2O_2, a strong and potentially harmful oxidizing agent, to molecular oxygen and water (Equation 3), preventing its accumulation in cell and tissues.[59]

$$2H_2O_2 \xrightarrow{CAT} O_2 + 2H_2O \qquad (3)$$

CAT is considered one of the most efficient enzymes found in cells, being capable to decompose millions of H_2O_2 molecules per second and cannot be saturated by any H_2O_2

concentration. At low peroxide concentration, CAT can also act as peroxidase, using co-substrates, having a variety of metabolites and toxins donors of hydrogen such as alcohols, formic acid, formaldehyde or phenols. Catalase structure makes it more resistant to unfolding, to pH, thermal denaturation and resistant to proteolysis than most of other enzymes. Most of these enzymes are tetramers, being each subunit composed by a heme in active site, within its structure that is accessible from the surface through hydrophobic channels. CAT as a two steps mechanism to decompose H_2O_2, alternatively oxidizing and reducing the heme iron at the active site. The heme is oxidized to an oxyferryl species by one molecule of H_2O_2 in the first step. The second hydrogen peroxide molecule acts as a reductant, making the enzyme return to the resting state, producing water and oxygen.[60]

The method used to quantify CAT activity is based in the enzyme's reaction with methanol, in the presence of optimal hydrogen peroxide concentration, producing formaldehyde. The produced formaldehyde is measured with Purpald (4-amino-3-hydrazino-5mercapto-1,2,4-triazole) as a chromogen. This compound forms a purple complex when it is oxidized that can be followed spectrophotometrically.[59,61]

1.6.1.3 Glutathione S-Transferase (GST)

Glutathione-S-transferases (GSTs) are a large family of isoenzymes involved in phase II biotransformation reactions, varying in their substrate specificity, as electrophilic xenobiotics or endogenous products of oxidative stress. GSTs are responsible for xenobiotics transformation into more soluble conjugates, in association with reduced glutathione (GSH).[51] The conjugation of the glutathione thiol group to electrophilic xenobiotics confers protection to cells against mutagenic, carcinogenic, and toxic effects of the compounds. GST activity was detected in plants, yeast, bacteria and in most mammalian tissues, being present in higher concentrations in the liver, and playing a key role in detoxification processes.[58]

Although GSTs are not directly involved in ROS decomposition, they complement the first line of defence, protection against ROS. The highly efficient enzymes SOD, CAT and GPx, decompose the superoxide anion and H_2O_2, but there are some reactive species that can escape this line of defence and cause harmful effects to cells. Moreover, the generation of free radicals can also initiate the autocatalytic chain of lipid peroxidation. GSTs reduce the formation of hydroperoxides, preventing the lipid peroxidation (LPO) propagation and detoxifying the toxic end-products of this process.[64]

The GST assay uses CDNB (1-chloro-2,4-dinitrobenzene) as a substrate, that can be followed spectrophotometrically at 340 nm (Equation 4).[65]

$$GSH + CDNB \xrightarrow{GST} GS - DNB\ Conjugate + HCl \qquad (4)$$

1.6.1.4 Glutathione Peroxidase (GPx)

Glutathione peroxidase (GPx) is a tetrameric enzyme that has a key role in protecting the organism against oxidative stress damage. GPx is composed by four identical subunits, each containing a selenocysteine in active site, participating directly in the reduction of peroxide substrate with two-electron. This enzyme converts reduced glutathione (GSH) to oxidized glutathione (GSSG), using it as the final electron donor, reducing free H_2O_2 to water, and regenerating the reduced form of selenocysteine.[66]

GPx activity is determined indirectly through a coupled reaction with glutathione reductase (GR). In this assay the hydroperoxide is reduced by GPx and GSH is oxidized to GSSG (Equation 5). GR recycles the glutathione to its reduced state (GSH), coupling in the reaction the consumption/oxidation of NADPH to $NADP^+$ (Equation 6). GPx activity will be proportional to the decrease in NADPH and this can be measured at 340 nm.[67]

$$ROOH + 2GSH \xrightarrow{GPx} ROH + GSSG + H_2O \quad (5)$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+ \quad (6)$$

1.6.1.5 Total Antioxidant Capacity (TAC)

The total antioxidant capacity (TAC) is based on a substance capacity to inhibit oxidation. This method has in count the total antioxidant, enzymatic and non-enzymatic. The method based on Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalent antioxidant capacity, a compound analogue to vitamin E, is the most commonly used approach to determine the total antioxidant capacity. This method is based on the formation of ABTS radical, that exhibits a blue-green colour, when ABTS (2,2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid)) is oxidized by myoglobin and H_2O_2. The presence of antioxidants in a sample reduce the radical produced to its ground state, decreasing the blue-green colour. The decrease in colour is proportional to the antioxidant capacity.[68]

1.6.2 Neurotoxicity: Acetylcholinesterase (AChE)

Acetylcholinesterase (AChE) is an enzyme that catalyses the hydrolysis of the neurotransmitter acetylcholine to acetate ion and choline. This enzyme is usually a tetramer present in neuromuscular junctions and in chemical synapses of cholinergic receptors, were its purpose is to terminate synaptic function.[69] The break of acetylcholinesterase in the synaptic cleft allows the cholinergic receptors to return to is resting state. When AChE is inhibited, an accumulation of acetylcholine starts to occur in the synaptic cleft and subsequently an overstimulation of cholinergic receptors. These changes can be due to the different stressors and

may result on severe disruption of numerous organism's functions and ultimately death caused by respiratory failure. Low concentrations of acetylcholinesterase is also associated to exposure to pesticides and other contaminants.[70]

AChE activity determination is based in the thiocholine production rate through the hydrolysis of acetylthiocholine, an acetylcholine analogous. The thiocholine reacts with the Ellman reagent (DTNB, 5,5'-dithio-bis-2-nitrobenzoic acid) forming a yellow anion that absorbs at 415 nm (Equations 7 and 8).[71]

$$Acetylthiocholine \xrightarrow{AChE} Acetate + Thiocholine \quad (7)$$

$$Thiocholine + DTNB \longrightarrow yellow\ anion \quad (8)$$

1.6.3 Cellular Damage: Lipid Peroxidation (LPO)

Lipid peroxidation (LPO) is one of the most prevalent cellular injuries mechanisms that occurs when cells are exposed to ROS, constituting a constant threat to the stability of the phospholipidic cell membranes of aerobic organisms. The cellular membranes of the polyunsaturated fatty acids double bonds make them easily susceptible to oxidation by reactive species.[58,72]

The chain reactions that composed lipid peroxidation start by hydrogen capture from lipid molecules by free radicals, resulting in the formation of many lipid peroxides (LOOH) as it is represented in Figure 1.8.[72]

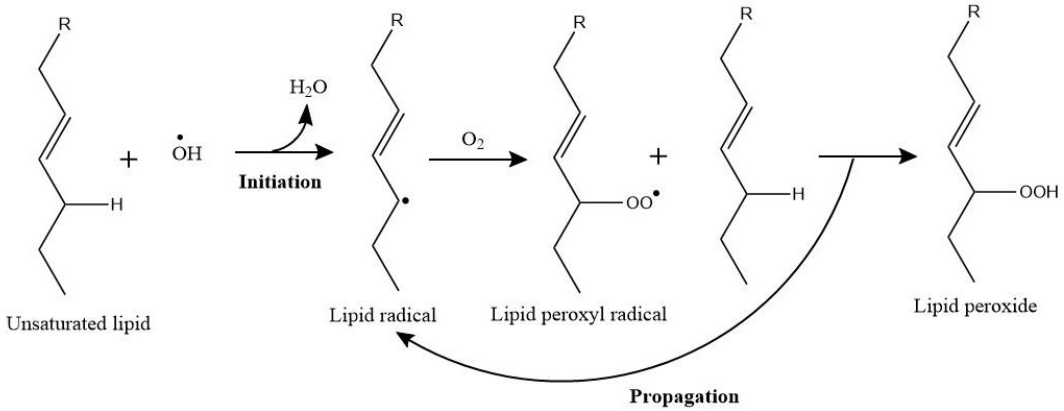

Figure 1.8- Lipid peroxidation chain reactions. Schematic representation of lipid peroxidation chain reactions, resulting in the formation of lipid radicals and lipid peroxides. Adapted from Young and McEnemy.[73]

LOOH can be decomposed into several reactive species, that are toxic themselves, activating mutagens, and acting as second messengers for radical damage.[39,72] The oxidative degradation process of lipids has a specific end-product, the malondialdehyde (MDA), which has been used as a marker of lipid peroxidation, that can be detected in the most widely used assay, the thiobarbituric acid reactive substance (TBARS) test.[58] Enzymes activity, ATP production and

apoptosis are the multiple effects in mitochondria that reveal the particularly cytotoxic impact of lipid peroxidation.[58]

1.6.4 Heat Shock Proteins (HSPs)

When exposed to environmental stressors, such as elevated water temperatures, aquatic organisms exhibit an heat shock response (HSR) inducing the expression of a specific group of proteins, the heat shock proteins (HSPs).[74] These proteins are molecular chaperones essential to aid proteins fold correctly and facilitate transport against membranes under non-stressful conditions.[75,76] Under stressful conditions, these chaperones stabilize, repair, refold and eliminate damaged or denatured proteins.[74] These molecular chaperones are classified in different families based on their molecular size (e.g. HSP70, HSP90) and are a mechanism of defence, maintaining cellular homeostasis. HSR is an important biochemical indicator of thermal stress and thermal tolerance limits and may also determine the degree of damage and protein unfolding that occurred in cell.[75]

Ocean warming and acidification induce stressful conditions that may have negative effects at different levels, changing metabolic processes, disturbing vital functions and subsequently affecting organism's survival, biological interactions and ultimately community and ecosystem's structure.

Generally, the stress response occurs at three levels:

a) Primary response – perception of an altered state and activation of the neuroendocrine response by a rapid production of stress hormones;[77]

b) Secondary response – adjustments at physiological and biochemical levels, regulated by stress hormones, like adrenaline and cortisol, and activation of the metabolic pathways that lead to haematological and biochemical alterations changes in cardiovascular, respiratory and immune functions.[78] During this stressful condition, organisms mobilize their energy stores in order to continue cellular functions by supply of energy to tissues;

c) Tertiary response – changes at organism and population levels begin to occur. If the organisms are unable to adapt or maintain homeostasis, changes may occur at the behavioural level, the resistance to disease can be affected and growth and reproduction capacity can be impaired.[77,78] An extended period of exposure to stress can lead alterations in population demographics and dynamics. A higher impact can occur in the larvae and juvenile stages because growth is of crucial importance to their fitness at these stages. More specifically, during stressful conditions, growth and reproduction become energetically compromised. The growth of species will be slower, which will increase the risk of predators, and consequently the first maturation will occur later reducing the investment in reproduction, and altering the abundance and diversity of species in a community.[77]

1.6.5 Ubiquitin (Ub)

Ubiquitins (Ub) are proteins composed by 76 aminoacids that bind covalently to other proteins and help their stability regulation, function or even modify then. Ubiquitins bind with low affinity to specific receptors making the bond highly dynamic, being able to mediate cell processes. These proteins are also involved in immune responses, transport through a membrane, in DNA repair, chromatin remodelling and protein degradation.[79]

The ubiquitination process is well-known and characterized through an enzymatic cascade involving three enzymes: ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-ligase (E3) (Figure 1.9).[79]

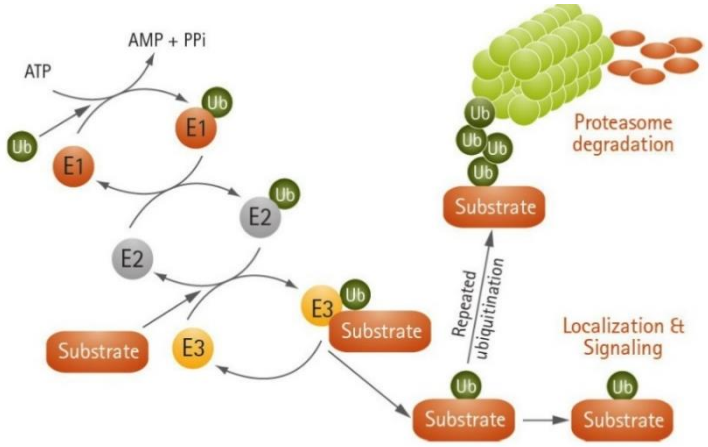

Figure 1.9- Ubiquitination cascade reactions.[80]

The first step it is an ATP-dependent reaction, where an ubiquitin molecule is transferred to E1, activating this way the cascade reaction. In the second step, E2 receives the ubiquitin molecule from E1. In the final step, E3 mediates the transfer process of ubiquitin from E2 to a protein substrate.[79]

1.7 Biological model: zebra seabream *Diplodus cervinus*

1.7.1 Distribution and Habitat

The zebra seabream (*Diplodus cervinus*) is a demersal species, that inhabits rocky substrate areas in depths between 80-300 m (Atlantic), being most abundant in 80 m depths. They can also inhabit sea floors that are muddy and a depth between 25-300 m (Mediterranean).[81]

This zebra seabream can be found in wide range in the Eastern Atlantic: in the Bay of Biscay and Mediterranean to the strait of Gibraltar and South Africa including Madeira and the Canary Islands. This species is absent in Cape Verde, Senegal and the Gulf of Guinea.[81]

In the Canary Islands, this fish species is overfished, needing a closer monitorization. Zebra seabream has shown an 85% decrease in its abundance and a reduction in the length at the first

maturity of 58% of the total catch.[82] In Portuguese waters restocking initiatives have already been started, but additional management will be essential to prevent further population decline.[82]

1.7.2 Biology

The zebra seabream (*Diplodus cervinus*) usually has a silver or gold colour and presents five characteristic bands over the lateral line. These bands are usually large, dark and are positioned in specific areas (i.e. over the eyes), as shown in Figure 1.10.[81] This species has a long and oval body, having also a conical mouth and thick lips. They usually achieve a length between 30 and 35 cm but can growth up to 55 cm and can live to at least 17 years. The zebra seabream is an omnivore fish, eating small invertebrates and seaweeds.[81,82]

Figure 1.10- *Diplodus cervinus*, by Vasco Ferreira in *OMARE* fish base.[83]

Diplodus cervinus is a protogynous hermaphrodite, meaning that the female can change to hermaphrodite, allowing the organism to switch between functional male or functional female.[82,84] The maturity is attained at different stages for the different sex, for instant males generally mature at about 32.7 cm and five years of age while females reach around 27.3 cm of the total length (TL) and four years of age.[82]

This species spawning season extends from spring to summer, peaking from May to June. The recruitment occurs from October to January and at depths between 0.5 m to 8.0 m. This species exhibits polygamy, where small groups are formed consisting of one dominant male and several females.[82]

2. OBJECTIVES

Since climate change has been the greatest environmental and long-term threat to the survival of marine organisms, it is crucial understanding their effects and impacts on these animals. Therefore, the main objectives of the present dissertation were to investigate the physiological responses of juvenile zebra seabream *Diplodus cervinus* (Lowe, 1838) to the interactive effects of ocean warming (ΔT ºC= +4 ºC) and acidification (ΔpCO_2 ~+1000 µatm, equivalent to ΔpH=0.3 units). More specifically, fish exposed to these two stressors, acting alone or combined, for 60 days and samples of different tissues (muscle, liver, brain and gills) were subsequently collected in order to evaluate:

i) Animal fitness (HSI – hepatosomatic index; VSI – viscerosomatic index and K - Fulton's condition index);
ii) Haematological parameters (percentage of erythrocytes and leucocytes);
iii) Molecular biomarkers for exposure of oxidative stress and other agents – total protein content, antioxidant activities (SOD – superoxide dismutase, CAT – catalase, GST – glutathione S-transferase, GPx – glutathione peroxidase and TAC – total antioxidant capacity), acetylcholinesterase activity, lipid peroxidation (LPO, using malondialdehyde, MDA levels as a biomarker), heat shock response (HSP70/HSC70 content) and ubiquitin content.

3. MATERIALS AND METHODS

3.1 Acclimation

Juvenile zebra seabream (*Diplodus cervinus*) with similar biometric characteristics (5.7±0.9 g total weight; mean±standard deviation) were reared until juvenile stage at the aquaculture pilot station of the Portuguese Institute for the Sea and Atmosphere (EPPO-IPMA, Olhão, Portugal). Fish were transported in thermal isolated containers, with constant aeration, to the Guia Marine Laboratory (MARE-FCUL, Cascais, Portugal) and randomly distributed in twelve rectangular incubating glass tanks (98x33x24.7 cm; 100 L total capacity each), each with independent recirculating aquaculture systems (RAS). Water temperature was independently and automatically controlled (Frimar, Fernando Ribeiro Lda, Portugal). Each RAS was equipped with biological filtration (model FSBF 1500, TMC Iberia, Portugal), physical filtration (protein skimmer; ReefSkimPro, TMC-Iberia, Portugal), UV disinfection (Vecton 300, TMC Iberia, Portugal) and pH control (model Profilux 3.1 N, GHL, Germany) via solenoid valves system. In each system (n=12) the pH was monitored with independent pH electrodes, connected to the Profilux system apparatus and adjusted by up and down regulation through a certified CO_2 gas mixture (Air Liquide, Portugal) via air stones or increased by tank aeration with CO_2-filters (using soda lime; Sigma-Aldrich, Germany). Daily seawater was partially replaced (around 20%) and faeces were removed. Seawater used in the RAS system was filtered (0.35 μm) and UV sterilized (Vecton 600, TMC Iberia, Portugal). Ammonia (NH_3/NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) were determined with a commercial colorimetric test kits (Tropic Marin, USA), and kept below 0.05 mg/L, 0.20 mg/L and 2.00 mg/L, respectively. Furthermore, seawater total alkalinity was measured in every tank on a weekly basis, following the protocol described by Sarazin *et al.*[85] and the combination of total alkalinity (TA) and pH was used to calculate carbonate system parameters (average values obtained for each treatment can be consulted in Annex A, Table A1).

Fish acclimation to laboratory conditions was performed for three weeks and a half before the start of the experiment. During this period the following abiotic conditions were maintained: dissolved oxygen (DO) above 5 mg/L; temperature = 19.10 ± 0.34 ºC; pH =8.02 ± 0.06; salinity = 35.0 ± 1.3 ‰ (WTW handheld Meter Multi 350i, Germany) and a photoperiod of 12h light and 12h dark (12L:12D).

One week before initiating the climate change exposure scenarios, seawater temperate was slowly raised (1 ºC per day), and the pH was slowly decreased 0.1 pH unit per day, until reaching 23 ºC and ~1000 μatm pCO_2 (equivalent to pH=7.7 units) in tanks simulating warming and acidification conditions respectively, according to the projection of the Intergovernmental Panel for Climate Change (scenario RPC8.5).[1,86]

3.2 Experimental Design

After the acclimation period, fish were exposed to four treatments for 60 days to understand the potential consequences to organisms under the current and future expected conditions for 2100 [i.e. seawater warming (ΔT ºC=+4 ºC) and acidification, (Δ*p*CO2 ~ +1000 µatm, equivalent to ΔpH=-0.3 units), according to IPCC projections scenario (RCP8.5)[1]]:

i. Control - Seawater temperature set at 19 ºC and pH at 8.0 (pCO_2 ~405 µatm; current conditions in juvenile zebra seabream rearing in Iberian Peninsula)[81];
ii. Acidification - Seawater temperature set at 19 ºC and pH set at 7.7 (pCO_2~1000 µatm);
iii. Warming - Seawater temperature set at 23 ºC and pH 8.0 (pCO_2 ~405 µatm);
iv. Acidification and Warming (Aci+Warm) - Seawater temperature set at 23 ºC and pH 7.7 (pCO_2~1000 µatm).

Each treatment comprised three independent replicate tanks (n=4 animals per replicate/tank, i.e. 12 animals per treatment). In each tank, fish were individualized in plastic boxes (5 L), with nets on the sides that allowed the water re-circulation (n=1 per box, n=4 per tank; Figure 3.1). During the experimental period, the fish were fed with 3% of the individual average body weight (divided in two meals per day). The ration (dry inert pellets) was manufactured by a specialized feed producing company (SPAROS Lda, Olhão, Portugal), considering the nutritional requirements for juvenile zebra seabream (detailed formulation and chemical composition can be consulted in Annex A, Table A2). Fish were weighed weekly to adjust feed rations in all treatments. The fish were fasted for 24h before the sampling to allow evacuation of faeces before bulk-weighing.

Figure 3.1- Fish facilities at Guia Marine Laboratory.

3.3 Samples Collection

After 60 days of the experiment period, eight fish per treatment were anaesthetized by immersion for 10 minutes in an overdosed tricaine methanesulfonate solution (2000 mg/L of MS-222, Sigma-Aldrich, USA) buffered with sodium bicarbonate ($NaHCO_3$, Sigma-Aldrich, USA) using a ratio of 1:1 to reduce fish stress. Fish trials were approved by the Ethical Committee of the Faculty of Sciences of the Lisbon University (ORBEA) and conducted according to legal regulations (EU Directive 2010/63). Immediately after a fraction of peripheral fish blood was collected with a syringe coated with 1 M EDTA (ethylenediamine tetraacetic acid; Riedel-de Haën, Germany) by puncture of the caudal vein, from eight fish of each treatment (n=32 in total).

Euthanized fish were dissected, the organs removed (muscle, liver, gills and brain; Figure 3.2) and stored ate -80 ºC. Approximately 100 mg of muscle, liver and gills and about 40 mg of brain were sampled for homogenization. Each tissue was individually homogenized (Ika T10 basic, Germany) on ice-cold conditions using 2.0 mL of a phosphate buffered saline solution (PBS; 140 mM NaCl, Panreac, Spain; 10 mM Na_2HPO_4, Sigma-Aldrich; 3 mM KCl, Merck, Germany; 2 mM KH_2PO_4, Sigma-Aldrich, Germany) at pH=7.4. The crude homogenates were then centrifuged in 1.5 mL microtubes, for 15 min at 10,000 *g*, at 4 ºC (VWR, model CT 15RE from Hitachi Koki Co., Ltd, Japan) and immediately frozen at -80 ºC until further analyses. Biometric data (total weight and total length) were registered for T60 (Table A3). All biochemical analyses (Table A4) were performed in duplicate using chemicals of pro analysis grade or higher.

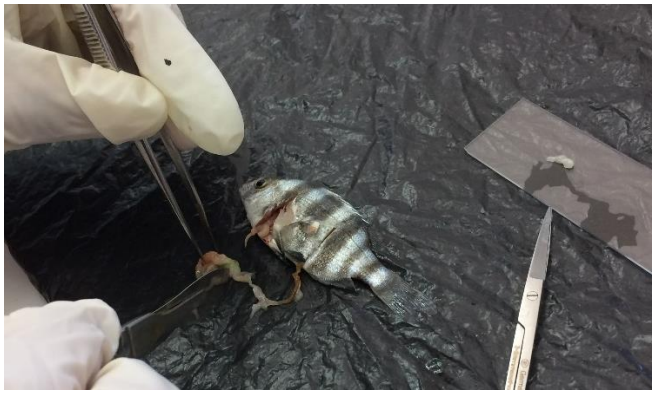

Figure 3.2- Representative image of *Diplodus cervinus* dissection.

3.4 Animal Fitness

The Fulton's condition index (K) was calculated to determine fish condition using directly the biometric data, according to the equation[87]**:**

$$K = \frac{TW}{TL^3} \times 100 \qquad (9)$$

where TW is the fish total weight (g, wet mass) and TL is the total length (cm).

In order to provide information on liver and viscera condition, the hepatosomatic index (HSI) and the viscerosomatic index (VSI) were calculated. These indexes were determined following the Equations 10 and 11, respectively, displaying the relationship between the fish total weight and the respective organ weight.[88,89]

$$HSI(\%) = \frac{LW}{TW} \times 100 \quad \textit{(10)}$$

$$VSI(\%) = \frac{VW}{TW} \times 100 \quad \textit{(11)}$$

where LW is the fish liver weight (g, wet mass), VW is the fish viscera weight (g, wet mass) and TW is the fish total weight (g, wet mass).

3.5 Haematological Parameters

Precleaned glass microscopy slides were used to prepare fish blood smears. The slides were stained following a method previously described by Kaplow.[90] After dried on air at room temperature for 60s, the slides were fixed with 10% formaldehyde-ethanol (Honeywell, USA) and washed gently for 15s in running tap water, previously incubated at room temperature for 30s in Coplin mixture, washed 5-10s in running water and left air dried. Afterwards, wet slides were incubated at room temperature for 30s in a Coplin jar containing an incubation mixture [30% ethanol (Honeywell, USA) + 0.3 g benzidine dihydrochloride (Riedel-Haën, Germany) + 0.132 M $ZnSO_4 \cdot 7H_2O$ (Merck, Germany) + 1.0 g $NaC_2H_3O_2 \cdot 3H_2O$ (Calbio-Chem, USA) + 3% H_2O_2 (Sigma-Aldrich, Germany) + 1.0 N NaOH (Panreac, Spain) + 2.0 g safranin O (Fluka, USA)]. Then, slides were washed 5-10s in running tap water and dried on air. Afterwards, microscope glass slides were mounted with DPX (Scharlau, Spain) and observed through optical microscopy (OPTIKA Microscopes Italy). For each sample, 10 slides were prepared. Each slide had a minimum of 100 cells when examined under the microscope (400× magnification) and the presence of erythrocytes and lymphocytes was determined. The classification previously described in Gallo *et al.* (2015) was used to count the presence of erythrocytes and white bloods cells (leucocytes = lymphocytes + macrophages).[91]

3.6 Biochemical Analyses

3.6.1 Total Protein Content

The total protein content in the samples was determined according to the method first described by Bradford.[92] This method is based on the binding ability of the acidic dye Coomassie Blue G-250 to proteins, which result an anionic blue form that can be detected spectrophotometrically at 595 nm, allowing the protein concentration estimation. [92]

The Bradford reagent was prepared by adding 100 mg of blue Coomasie G-250 (BIO-RAD, USA) in 95% ethanol (Honeywell, USA) and 100 mL of 85% phosphoric acid (Sigma-Aldrich, Germany). The solution was diluted with distilled water to one litre and filtered to remove precipitates before storing at 4 ºC.

A stock of bovine serum albumin (BSA; Nzytech, Portugal) was used to prepare the standard curve, within a range from 0 to 4 mg/L.

The assay was performed using a 96-well plate (Greiner Bio-one, Austria), where 20 µL of sample or standard and 180 µL of Bradford reagent were added to each well. The absorbance was measured using a microplate reader (BIO-RAD, Benchmark, USA) at 595 nm. The total protein content was determined using BSA as standard in the calibration curve and the results were expressed in mg total protein per mL.

3.6.2 Superoxide Dismutase Activity (SOD)

The superoxide dismutase assay (EC 1.15.1.1) based on the enzymes dismutation capability of superoxide radical into hydrogen peroxide, follows the nitroblue tetrazolium (NBT) method adapted from Sun *et al*..[93] In this assay, 200 µL of 50 mM phosphate buffer (pH 8.0, Sigma-Aldrich, Germany) were added to a 96-well microplate (Greiner Bio-one, Austria), followed by the addition of 10 µL of 3 mM EDTA (Riedel-de Haën, Germany), 10 µL of 3 mM xanthine (Sigma, Germany) and 10 µL of 0.75 mM NBT (Sigma-Aldrich, Germany) to each well. Afterwards, 10 µL of samples were transferred to the microplate wells.[94]

The addition of 10 µL of 100 mU xanthine oxidase (XOD, Sigma-Aldrich, Germany) initiated the reaction, and the absorbance was recorded at 560 nm every minute for 5 minutes, and then read at 10 and 15 minutes, using a microplate reader (BIO-RAD, Benchmark, USA). Negative controls (included all components except the sample) were also included and results were presented as the percentage of enzyme inhibition, using Equation 9. The SOD activity was then normalized for the total protein content expressed in percentage of inhibition per milligram of total protein.

$$\underset{Inibition}{\%} = \frac{\Delta\, Abs_{560\,nm}\,/\,min\ \ negative\ control - \Delta\, Abs_{560\,nm}/\,min\ samples}{\Delta\, Abs_{560\,nm}\,/\,min\ of\ \ negative\ control} \times 100 \quad \textit{(12)}$$

3.6.3 Catalase Activity (CAT)

Catalase (CAT) activity (EC 1.11.1.6) was carried out following the method described by Johansson and Borg[59] and adapted to 96-well microplate.

In this assay, 20 µL of each sample or standard, 100 µL of 25 mM potassium phosphate (Sigma-Aldrich, Germany; pH 7.0), 1 mM EDTA (Riedel-de Haën, Germany), 0.1% BSA (Nzytech, Portugal) and 30 µL methanol (Scharlau, Spain) were added to each microplate well

(Greiner Bio-one, Austria). The reaction was initiated by the addition of 20 µL of hydrogen peroxide (0.035 M, Sigma-Aldrich, Germany) and incubated for 20 minutes at room temperature. Afterwards, 30 µL of potassium hydroxide (10 M, Chem-Lab, Belgium), and 30 µL of Purpald (34.2 M in 0.5 M HCl, Aldrich, Germany), were added to each microplate well and incubated for 10 minutes at room temperature. Subsequently, 10 µL of potassium periodate (65.2 mM in 0.5 M KOH; Chem-lab, Belgium) were added and a final incubation was performed for 5 minutes. Enzymatic activity was determined spectrophotometrically at 540 nm using a microplate reader (BIO-RAD, Benchmark, USA).

Catalase activity was calculated considering that one unit of catalase is defined as the amount of enzyme that will cause the formation of 1.0 nmol of formaldehyde per minute at 25 ºC. The formaldehyde concentrations of the samples were determined based on a calibration curve ranging from 0 to 75 µM of formaldehyde (Sigma, Germany). The results were expressed in relation to the total protein content (nmol/min/mg total protein).

3.6.4 Glutathione S-Transferase Activity (GST)

Glutathione S-transferase (GST) activity (EC 2.5.1.18) was determined according to the method described by Habig *et al.*[65] and modified to a 96-well microplate. This procedure is based on the enzyme capability to conjugate xenobiotics in less toxic conjugates, that is the conjugation of glutathione (GSH) thiol group with CDNB (1-chloro-2,4-dinitrobenzene) substrate. The formation of this conjugate is proportional to the increase of GST activity and it is followed spectrophotometrically every minute for 6 minutes at 340 nm, using a microplate reader (BIO-RAD, Benchmark, USA).[65]

In this assay, 20 µL of each sample and 180 µL of substrate solution was added to each well (Greiner Bio-one, Austria). The substrate solution was composed by Dulbecco's phosphate buffered saline, 200 mM $_{L}$-glutathione reduced, and 100 mM of CDNB (all from Sigma-Aldrich, Germany).

The increase in absorbance per minute (ΔA_{340}) was estimated and the reaction rate at 340 nm was determined using the CDNB extinction coefficient of 0.0053 μM^{-1} cm^{-1} (Sigma Technical Bulletin, CS0410; Equation 10), using a microplate reader (BIO-RAD, Benchmark, USA).The results were expressed in relation to the total protein content of the samples, as nmol/min/mg total protein.

$$GST\ Activity\ (nmol/min/mL) = \frac{\Delta A_{340}/min}{0.0053} \times \frac{Total\ volume}{Sample\ Volume} \quad (13)$$

3.6.5 Glutathione Peroxidase Activity (GPx)

Glutathione peroxidase (GPx) activity (EC 1.11.1.9) was determined according to Lawrence and Burk[95], through an adaptation to 96-well microplates. Briefly, 120 µL of assay buffer was added to the sample wells and 140 µL for the negative control. The assay buffer is composed by 50 mM potassium phosphate buffer (pH 7.4, Sigma-Aldrich, Germany) and 5 mM EDTA (pH 7.6; Riedel-de Haën, Germany). Previously 50 µL of co-substrate mixture and 20 µL of sample were added to each microplate well (Greiner Bio-one, Austria). The co-substrate mixture is composed by 4 mM sodium azide (Sigma-Aldrich, Germany), 1 mM nicotinamide adenine dinucleotide phosphate (NADPH, Sigma-Aldrich, Germany), 4 U/mL glutathione reductase (GSSG-reductase, Sigma, Germany) and 4 mM reduced glutathione (GSH, Sigma, Germany). The reaction was initiated by the addition of 20 µL of 15 mM hydroperoxide cumene (Sigma-Aldrich, Germany) and the absorbance was read at 340 nm, every minute for 6 minutes using a microplate reader (BIO-RAD, Benchmark, USA). The decrease in absorbance per minute (ΔA_{340}) was estimated and the reaction rate was determined using the β-NADPH extinction coefficient of 3.73 mM^{-1} cm^{-1} (adapted to the solution path length in the microplate well, the actual extinction coefficient for NADPH at 340 nm is 6.22 mM^{-1} cm^{-1}; Sigma Technical Bulletin, CGP1; Equation 11). The results were expressed in relation to the total protein content of the samples, as nmol/min/mg total protein.

$$GPx\ Activity\ (nmol/min/mL) = \frac{\Delta A_{340}/min}{0.00373} \times \frac{Total\ volume}{Sample\ Volume} \quad (14)$$

3.6.6 Total Antioxidant Capacity (TAC)

Total antioxidant capacity (TAC) was determined according to the method described by Kambayashi *et al.*[68] In this assay, 10 µL of each sample, 10 µL of 90 µM myoglobin (Sigma, Germany), 150 µL of 600 µM ABTS (2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid, Alfa Aesar, Germany) were added to a 96-well microplate (Greiner Bio-one, Austria). The reaction started by the addition of 40 µL of 500 µM hydroxide peroxide (Sigma-Aldrich, Germany). After 5 minutes of microplate incubation at room temperature, the absorbance was read at 415 nm using a microplate reader (BIO-RAD, Benchmark, USA). TAC was calculated from a calibration curve, based on a series of trolox standards, ranging from 0 to 0.33 mM. The results were expressed according to the total protein content as mM/mg total protein.[72]

3.6.7 Neurotoxicity: Acetylcholinesterase Activity (AChE)

Acetylcholinesterase (AChE) activity (EC 3.1.1.7) was determined using an adaptation of the method previously described by Ellman[71] and adapted to a 96-well microplate. In this assay,

50 µL of each sample (muscle and brain) and 250 µL of a reagent mix was added to the microplate wells (Greiner Bio-one, Austria) and the absorbance was read each minute for 10 minutes at 415 nm, using a microplate reader (BIO-RAD, Benchmark, USA). The negative controls were also included (addition of 50 µL of buffer was added instead of sample). The reagent mix was composed by 50 mM, sodium phosphate buffer (pH 8.0; Sigma-Aldrich, Germany), 10 mM DTNB (5,5'-dithio-bis-2-nitrobenzoic acid; Sigma, Germany) and 75 mM ACTI (acetylthiocholine, Sigma, Germany). The AChE activity was determined using the Equation 12, considering the extinction coefficient factor of 0.00781 μM^{-1} cm^{-1} of DTNB. The results were normalized to the total protein content and expressed in nmol/min/mg total protein.

$$AChE\ (nmol/min/mL) = \frac{Abs_{415}/min \times V(mL)}{0.00781 \times V_{sample}\ (mL)} \quad (15)$$

3.6.8 Cellular Damage: Lipid Peroxidation (LPO)

Lipid peroxidation (LPO) was determined following the thiobarbituric acid reactive substances (TBARS) method described by Ohkawa *et al.*[58] and adapted by Correia *et al.*[72] LPO was determined through the quantification of specific end-product of the lipid oxidative degradation process, i.e. malondialdehyde (MDA).[58,72] Briefly, 5 µL of sample or standard and 45 µL of 50 mM monobasic sodium phosphate buffer (Sigma-Aldrich, Germany) were added to microtubes. Afterwards, 12.5 µL of sodium dodecyl sulphate (SDS 8.1%, Merck, Germany), 93.5 µL of trichloroacetic acid (TCA 20%, pH 3.5; Panreac, Spain), 93.5 µL of thiobarbituric acid (TBA 1%, Sigma-Aldrich, Germany) and 50.5 µL of Milli-Q grade ultrapure water were added to each microtube. Then, this mixture was centrifuged at 2,000 *g* for 1 minute and the lids of the microtubes were punctured before incubation in boiling water for 10 minutes. Subsequently the microtubes were immediately placed on ice for a few minutes to cool to room temperature, and 62.5 µL of Milli-Q grade ultrapure water were added and then centrifuged at 2,000 *g* for 1 minute. Duplicates of 150 µL of the supernatant of each sample were added to 96-well microplate (Greiner Bio-one, Austria). Absorbance was read at 530 nm, using a microplate reader (BIO-RAD, Benchmark, USA). MDA concentrations were quantified based on a calibration curve ranging from 0 to 0.1 µM TBARS, performed with MDA (Sigma-Aldrich, Germany), in order to calculate the lipid peroxides formed in the samples. The results were expressed in relation to total protein content, in µM/mg total protein.

3.6.9 Heat Shock Response (HSP70/HSC70 content)

The heat shock response (HSR) was assessed from heat shock protein 70 (HSP70/HSC70) production which was quantified through Enzyme-linked Immunosorbent Assay (ELISA) based on a protocol from Njemini *et al.*[96] Briefly, 50 µL of each sample or standard were added to a

96-well microplate (Greiner Bio-one, Austria), and left to incubate overnight at 4 ºC. Afterwards, the microplates were washed three times with a 0.05% PBS-Tween-20 solution (Sigma-Aldrich, Germany) and 200 µL of blocking solution (composed by 1% BSA (Nzytech, Portugal) in PBS) were added to each well and left to incubate at 37 ºC for 90 minutes in the incubator (Labnet, USA). Subsequently, the microplates were washed again (3X with PBS and 0.05% of Tween-20 solution) and 50 µL of primary antibody solution (anti-HSP70/HSC70, Santa Cruz Biotechnology; diluted to 1 µg/mL in 1% BSA solution) was added to each well and incubated overnight at 4 ºC. After another washing step, 50 µL of secondary antibody solution (anti-mouse IgG Fc specific - alkaline phosphatase, Sigma-Aldrich, Germany; diluted to 1 µg/mL in 1% BSA solution) were added to each microplate well and incubated for 90 minutes at 37 ºC. After another wash, 100 µL of alkaline-phosphatase substrate [composed by 100 mM NaCl (Panreac, Spain), 100 mM Tris-HCl (Sigma-Aldrich), 50 mM $MgCl_2$ (Sigma-Aldrich, Germany) and 27 mM PnPP (4-nitrophenyl phosphate disodium salt hexahydrate, pH 8.5 (Sigma, Germany)], were added to each microplate well and incubated for 30 minutes at room temperature. Finally, 50 µL of stop solution (3M NaOH, Panreac, Spain) were added to each microplate well and the absorbance measured at 405 nm, using a microplate reader (BIO-RAD, Benchmark, USA). HSP70/HSC70 contents in the samples were calculated using the standard curve of purified HSP70 active protein (OriGene Technology, USA) with serial dilutions ranging from 0 to 2 µg/mL. The results were expressed in relation to the total protein content of the samples as µg/mg total protein.

3.6.10 Ubiquitin Content (Ub)

Ubiquitin was quantified through an indirect ELISA described by Njemini *et al.*[94], in an adaptation to a 96-well microplate as previously described by Rosa *et al.*[95] Briefly, 50 µL of sample or standard were added to a 96-well microplate (Greiner Bio-one, Austria), and left to incubate overnight at 4 ºC. Afterwards, the microplates were washed three times with a PBS solution containing Tween-20 and 200 µL of blocking solution (composed by 1% bovine serum albumin (BSA, Sigma-Aldrich, USA) in PBS), were added to each well and left to incubate at 37 ºC for 90 minutes in the incubator (Labnet, USA). Subsequently, the microplates were washed again (3X with PBS and 0.05% Tween-20 solution) and 50 µL of primary antibody solution (Ub (P4D1) Sc-8017, mouse monoclonal IgG, Santa Cruz Biotechnology, Portugal; diluted to 1 µg/mL in 1% BSA in PBS solution), were added to each microplate well and incubated overnight at 4 ºC. After another washing, 50 µL of secondary antibody solution (anti-mouse IgG Fc specific-alkaline phosphatase, Sigma-Aldrich, Germany; diluted to 1 µg/mL in 1% BSA in PBS solution) were added to each microplate well and incubated for 90 minutes at 37 ºC. After another wash, 100 µL of alkaline-phosphatase substrate [composed by 100 mM NaCl (Panreac, Spain), 100 mM Tris-HCl (Sigma-Aldrich), 50 mM $MgCl_2$ (Sigma-Aldrich, Germany) and 27 mM PnPP (pH 8.5, Sigma, Germany)] were added to each microplate well and incubated for 30 minutes at

room temperature. Finally, 50 μL of stop solution (3 M NaOH, Panreac, Spain) were added to each microplate well and the absorbance measured at 405 nm, using a microplate reader (BIO-RAD, Benchmark, USA). Ubiquitin content was calculated using the standard curve prepared by sequential dilutions of purified ubiquitin active protein (Santa Cruz Biotechnology, Portugal), ranging from 0.8 to 0.0125 μg/μL. The results were expressed in relation to the total protein content of the samples as μg/mg total protein.

3.7 Statistical Analysis

All data were first tested for assumptions of normality of distribution and homogeneity of variances using Kolmogorov-Smirnov's and Levene's test, respectively. Data were log-transformed whenever these assumptions of analysis of variance (ANOVA) were not verified. One-way ANOVA analysis, followed by post-hoc tests (Tukey HSD and unequal N HSD) were performed to identify significant differences in fish haematological parameters (percentage of erythrocytes and leukocytes in relation to total blood cells), morphometric data (TW and TL), muscle AChE activity and other biochemical biomarkers (SOD, CAT, GST, GPx, TAC, LPO, HSP70/HSC70 and Ub). These differences were detected between the four treatments (Control, Acidification, Warming and Acidification + Warming) in each tissue (brain, liver, muscle and gills). Whenever log-transformed data did not verify the assumptions required of ANOVA, then non-parametrics tests were performed, using two independent comparison tests, followed by the Mann-Whitney U test. Statistical analyses were performed using significance levels of 0.05, using STATISTICA™ software (Version 8.0, StatSoft Inc., USA). All data were expressed as mean±standard deviation (mean±SD).

4. RESULTS

4.1 Animal Fitness

During the 60 days of exposure to the different climate change scenarios, it was found that prolonged exposure to warming (23 ºC) and acidification (pH=7.7, pCO_2~1000 µatm), either acting in isolation or in combination, were not lethal for juvenile *D. cervinus*.

After the acclimation exposure period significant differences in total weight (TW) were detected among the four exposure treatments (Figure 4.1). It was also observed that warming acting alone or combined with acidification induce a significant higher growth in TW when comparing to the control and acidification alone (p<0.001). No significant changes were found in fish's length (TL) under the different treatments.

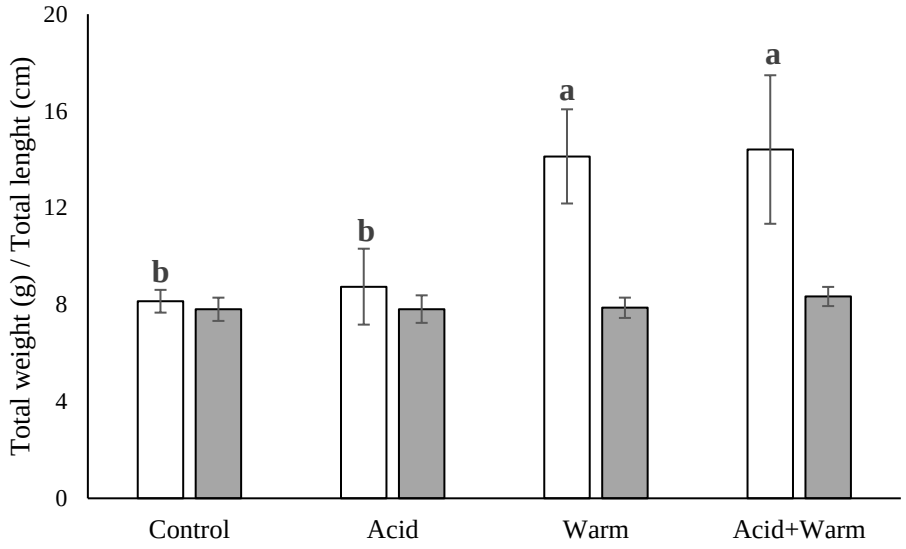

Figure 4.1- Total weight (g, white) and total length (cm, grey) of juvenile *Diplodus cervinus* in the four treatments after 60 days of exposure (mean±SD; n=8). Different letters (a-b) indicate significant differences (p<0.05) between treatments. Abbreviations: Control – reference temperature and pH conditions (T=19 ºC and pH=8.0); Acid – simulated acidification (T=19 ºC and pH= 7.7); Warm – simulated warming (T=23 ºC and pH=8.0); Acid+Warm - simulated acidification and warming (T=23 ºC and pH=7.7).

Fish fitness indexes (HSI, VSI and K) after 60 days of exposure to climate change scenarios are presented in Figure 4.2. While viscerosomatic index (VSI) was not significantly affected by warming and acidification whether alone or combined (p>0.05), a significant decrease in the hepatosomatic index (HSI) was found when the fish were exposed to warming scenarios (alone or in combination with acidification; p<0.05). This decrease was more significant when the fish were exposed to warming alone rather than when both stressors were induced (0.54±0.05 and 0.76±0.11, respectively). Also, a significantly higher Fulton's condition index (K) was found in fish exposed to acidification and warming combined, compared to the fish in control and exposed

to warming alone (1.50±0.08, 1.59±0.07, 1.56±0.06 and 1.70±0.08, for Control, Acid, Warm and Acid+Warm treatments, respectively; $p<0.05$).

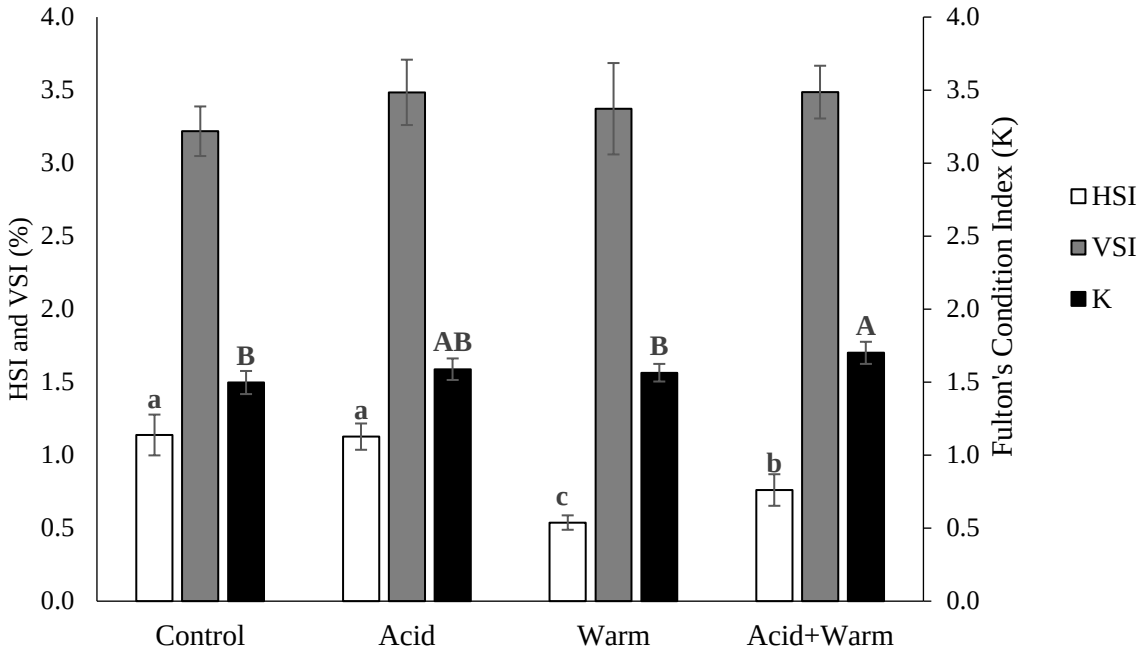

Figure 4.2- Hepatosomatic index (HSI, white), viscerosomatic index (VSI, grey), and Fulton's condition index (K, black), in juvenile *Diplodus cervinus* after 60 days of exposure (mean±SD; n=8). Different letters (a-c for HSI; A-B for K) indicate significant differences ($p<0.05$) between treatments. Abbreviations: Control – reference temperature and pH conditions (T=19 ºC and pH 8.0), Acid – simulated acidification (T=19 ºC and pH 7.7), Warm – simulated warming (T=23 ºC and pH 8.0) and Acid+Warm – simulated acidification and warming (T=23 ºC and pH 7.7).

4.2 Haematological Parameters

The erythrocytes and leucocytes (lymphocytes and macrophages) cells were identified and their percentage determined in peripheral blood cell of juvenile *Diplodus cervinus* in the four treatments tested (shown in Figure 4.3).[91]

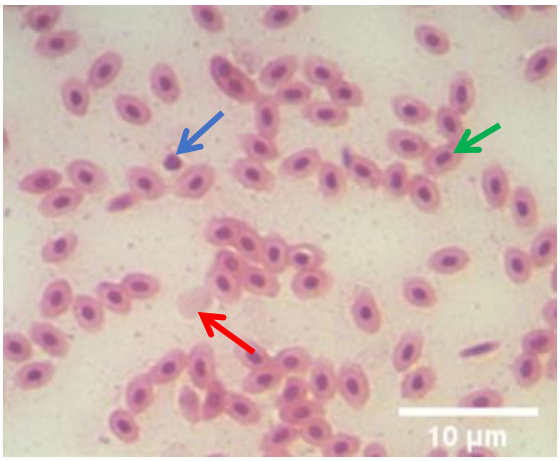

Figure 4.3- Representative image of peripheral blood cells of juvenile *Diplodus cervinus* where is possible to notice erythrocytes (green arrow), lymphocytes (blue arrow) and macrophages (red arrow).

In order to determine whether alterations in red and white blood cells counts occurred following fish exposure to the tested climate change scenarios, it was assessed the percentage of erythrocytes and leucocytes, respectively (Table 1.1). The leucocytes were considered as the sum of lymphocytes and macrophages levels.

Table 1.1 - Erythrocytes and leucocytes counts (in percentage; mean±SD; n=32). Different letters (a-b) indicate significant differences (p<0.05) between treatments (columns). Abbreviations: Control – reference temperature and pH conditions (T=19 ºC and pH 8.0), Acid – simulated acidification (T=19 ºC and pH 7.7), Warm – simulated warming (T=23 ºC and pH 8.0) and Acid+Warm – simulated acidification and warming (T=23 ºC and pH 7.7).

	Erythrocytes (%)	**Leucocytes (%)**
Control	96.48 ± 3.69 ^{b}	2.57 ± 0.34 ^{a}
Acidification	97.56 ± 2.23 ^{a}	2.00 ± 0.25 ^{ab}
Warming	97.57 ± 1.43 ^{a}	1.82 ± 0.16 ^{b}
Acidification + Warming	97.65 ± 1.70 ^{a}	1.97 ± 0.22 ^{ab}

As expected, erythrocytes were the main component of blood cells, being the most abundant cells present in fish blood smears in all treatments (98-99%).[98] The erythrocytes percentage significantly increased when the fish were exposed to the three scenarios of climate change in comparison with control (p<0.05). In contrast, the percentage of leucocytes was significantly higher in control (2.57%) and only significantly decreased when fish were exposed to warming acting alone (1.82%; p<0.005).

4.3 Biochemical Analyses

4.3.1 Superoxide Dismutase (SOD)

The activity of SOD was measured in the different tissues studied from juvenile *Diplodus cervinus* and is presented in terms of percentage of inhibition of NBT-diformazan per mg of total protein as shown in Figure 4.4. SOD activity is directly proportional to the inhibition of NBT-diformazan complex formation, which means that when the percentage of inhibition is higher, there is also a higher SOD activity.

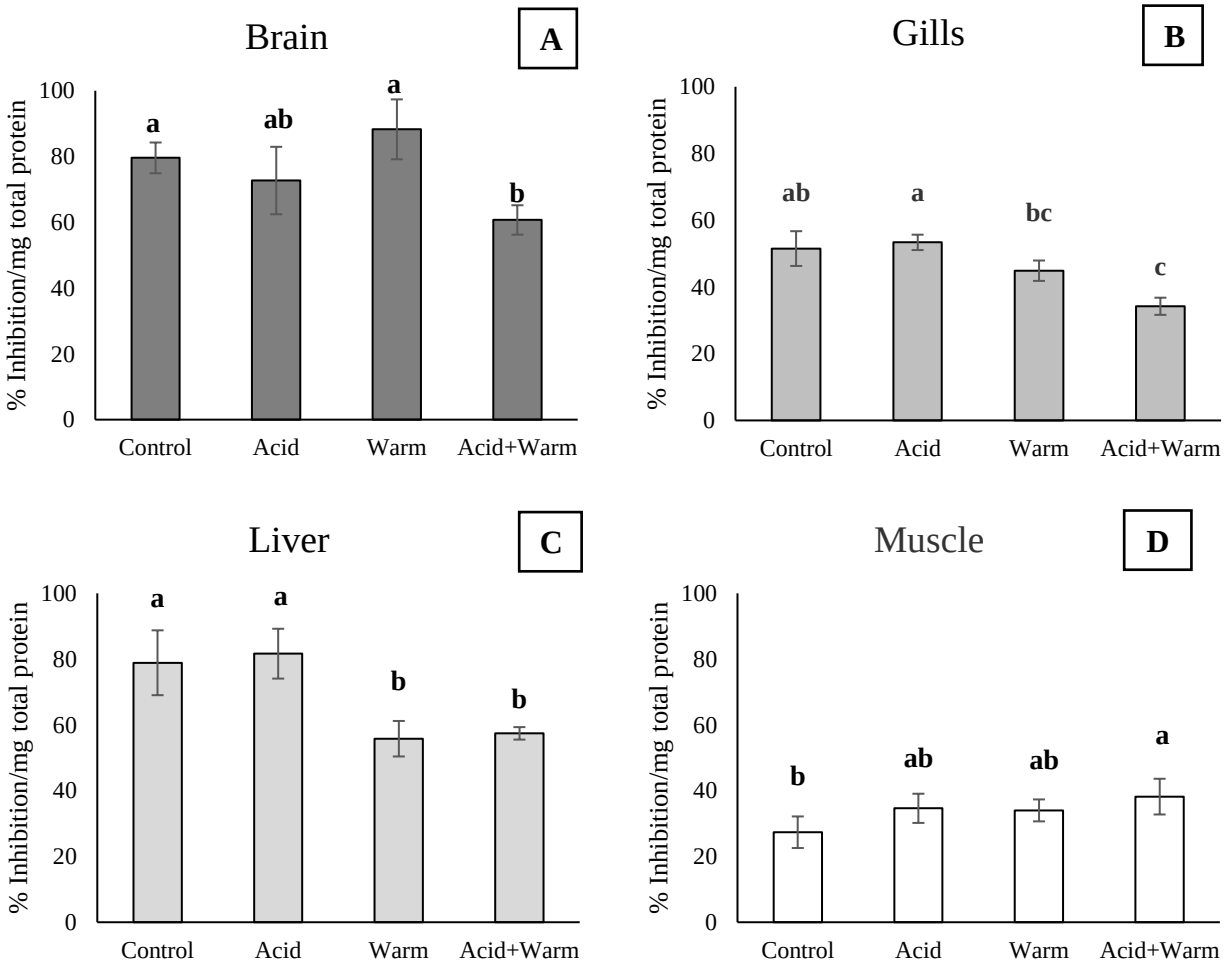

Figure 4.4- Superoxide dismutase activity (SOD) in A) brain, B) gills, C) liver and D) muscle of juvenile *Diplodus cervinus* after 60 days of exposure to the different treatments (mean±SD; n=8). Different letters (a-c) indicate significant differences (p<0.05) between treatments. Abbreviations: Control – reference temperature and pH conditions (T=19 ºC and pH=8.0); Acid – simulated acidification (T=19 ºC and pH=7.7); Warm – simulated warming (T=23 ºC and pH=8.0); Acid+Warm - simulated acidification and warming (T=23 ºC and pH=7.7).

Among the different tissues, the brain and liver presented significantly higher percentage of SOD activity (reaching 88.3% and 81.6%, respectively) when comparing to the other tissues (attaining 53.4% and 38.2% in gills and muscle, respectively; p<0.0002).

In fish brain no significant differences where observed when exposed to acidification and warming alone when comparing to the control (Figure 4.4A), but when these two stressors were combined, there was found a significant decrease in SODs activity (p=0.038).

The gills and the liver revealed a similar behaviour in SODs activity (Figure 4.4B-C). In both tissues, no significant changes were observed between the control and the acidification treatment (p>0.05). Exposure to warming alone or combined with acidification induced a significant decrease in SODs activity compared to control and acidification alone (p<0.0005). While in the liver no significant changes were observed between the Warm and Acid+Warm treatments, in the gills it was observed a significant decrease in SODs activity when the fish were exposed to both stressors when comparing to exposure to warming alone (p<0.05).

SODs activity in fish muscle tended to increase when exposed to climate change scenarios, but this increase was only significant when the fish were exposed to warming and acidification acting simultaneously when comparing to control (p=0.009; Figure 4.4D).

4.3.2 Catalase (CAT) Activity

CAT activity was measured in the different tissues studied from juvenile *Diplodus cervinus* and is presented in Figure 4.5.

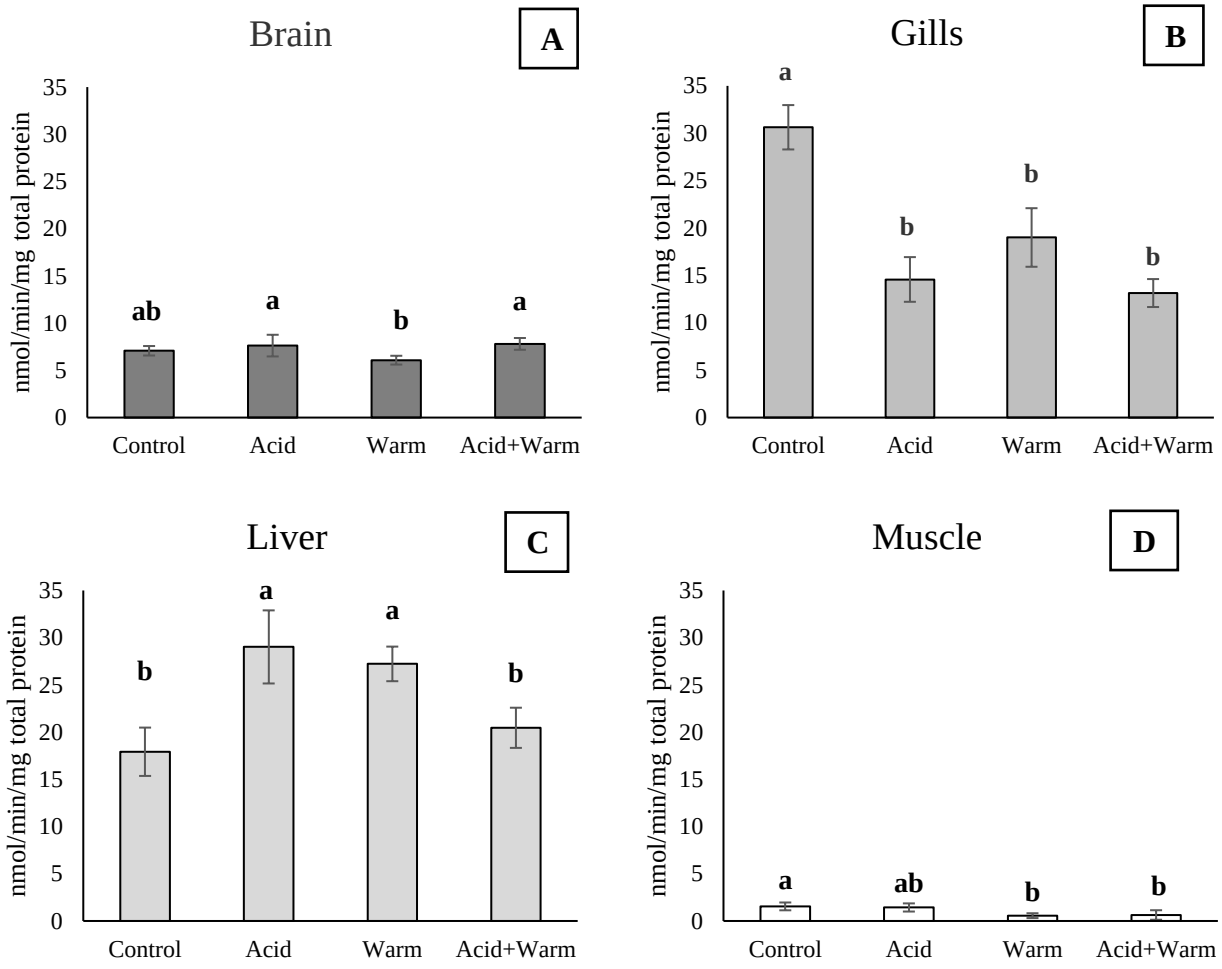

Figure 4.5- Catalase activity (CAT) in A) brain, B) gills, C) liver and D) muscle of juvenile *Diplodus cervinus* after 60 days of exposure to the different treatments (mean±SD; n=8). Different letters (a-b) indicate significant differences (p<0.05) between treatments. Abbreviations: Control – reference temperature and pH conditions (T=19 ºC and pH=8.0); Acid – simulated acidification (T=19 ºC and pH=7.7); Warm – simulated warming (T=23 ºC and pH=8.0); Acid+Warm - simulated acidification and warming (T=23 ºC and pH=7.7).

The measured CAT activity was significantly higher in the liver and gills (29.0 and 30.6 nmol/min/mg total protein, respectively) comparatively to the other tissues (7.8 and 1.6 nmol/min/mg total protein in the brain and muscle, respectively) (p<0.002).

In fish brain, although values in Control treatment were not significantly different from those in the remaining treatments, the exposure to acidification, alone or combined with warming resulted in significantly higher CAT activity (p=0.030 and p=0.015, respectively; Figure 4.5A) that when warming acting alone.

In fish gills and muscle, a significantly decrease was found in CAT activity when the fish were exposed to the three climate change scenarios (i.e. stressors acting individually or in

interaction) in comparison with control ($p<0.05$; Figure 4.5B and D), except for Acid treatment in muscle.

In fish liver, while a significant increase in CAT activity was found when the fish were exposed to acidification and warming alone (p=0.0004 and p=0.0017, respectively), when the stressors acted simultaneously no significant changes where observed compared to control (Figure 4.5C). This increase in CAT activity for exposure to acidification and warming alone were also significantly higher when comparing to the co-exposure of the two stressors (p=0.0014 and p=0.0138, respectively).

4.3.3 Glutathione-S-Transferase (GST) Activity

The activity of GST measured for the different tissues in juvenile *Diplodus cervinus* is shown in Figure 4.6.

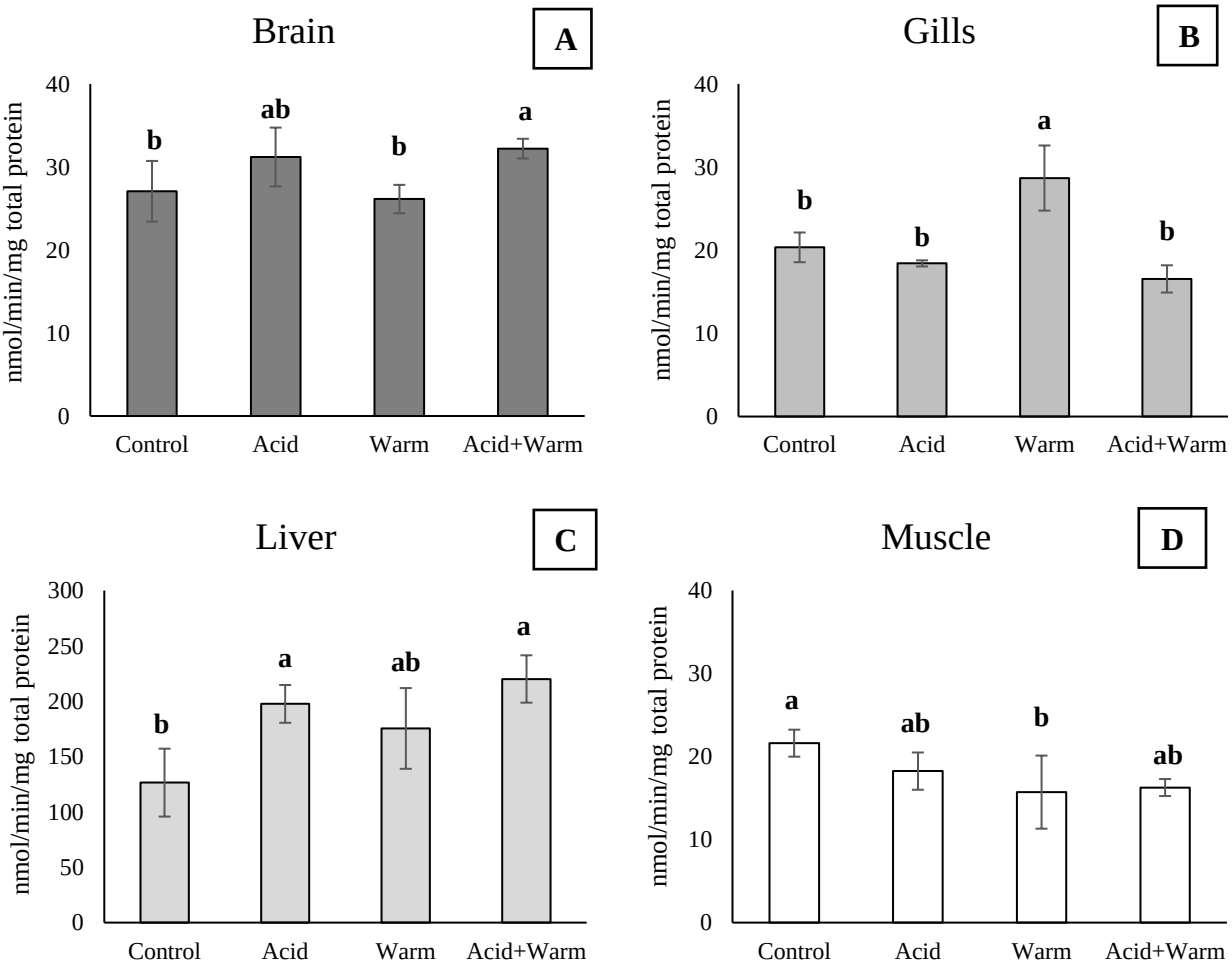

Figure 4.6- Glutathione S-transferase activity (GST) in A) brain, B) gills, C) liver and D) muscle of juvenile *Diplodus cervinus* after 60 days of exposure to the different treatments (mean±SD; n=8). Different letters (a-b) indicate significant differences ($p<0.05$) between treatments. Abbreviations: Control – reference temperature and pH conditions (T=19 ºC and pH=8.0); Acid – simulated acidification (T=19 ºC and pH=7.7); Warm – simulated warming (T=23 ºC and pH=8.0); Acid+Warm - simulated acidification and warming (T=23 ºC and pH=7.7).

The liver presented significantly higher GST activity (reaching 183.1 nmol/min/mg total protein) when comparing to the other tissues (attaining 7.2, 18.0 and 1.1 nmol/min/mg total protein in brain, gills and muscle, respectively; $p<0.0002$).

While no significant changes were found when the fish brain were exposed to acidification and warming alone, the co-exposure to these stressors significantly induce an increase in GST activity, when comparing to the control ($p=0.047$; Figure 4.6A). There was also observed a significant increase in the activity for Acid+Warm treatment when comparing to the exposure to warming alone ($p=0.012$).

Although no significant changes were found in fish gills between the control and acidification, alone or combined with warming, significant lower GST activities were observed in these three treatments when the fish were exposed to warming alone ($p=0.0010$, $p=0.0004$ and $p=0.0003$ in Control, Acid and Acid+Warm treatments, respectively; Figure 4.6B).

The exposure to climate change scenarios induced positively GST activity in the liver, being this increase significant in the Acid and Acid+Warm treatments when comparing to the Control treatment ($p=0.0064$ and $p=0.0008$, respectively; Figure 4.6C).

In fish muscle, GST activity only significantly decreased when the fish were exposed to the warming treatment alone, compared to control ($p=0.037$; Figure 4.6D).

4.3.4 Glutathione Peroxidase (GPx) Activity

GPx activity determined for the different tissues studied in juvenile *Diplodus cervinus* is presented in Figure 4.7.

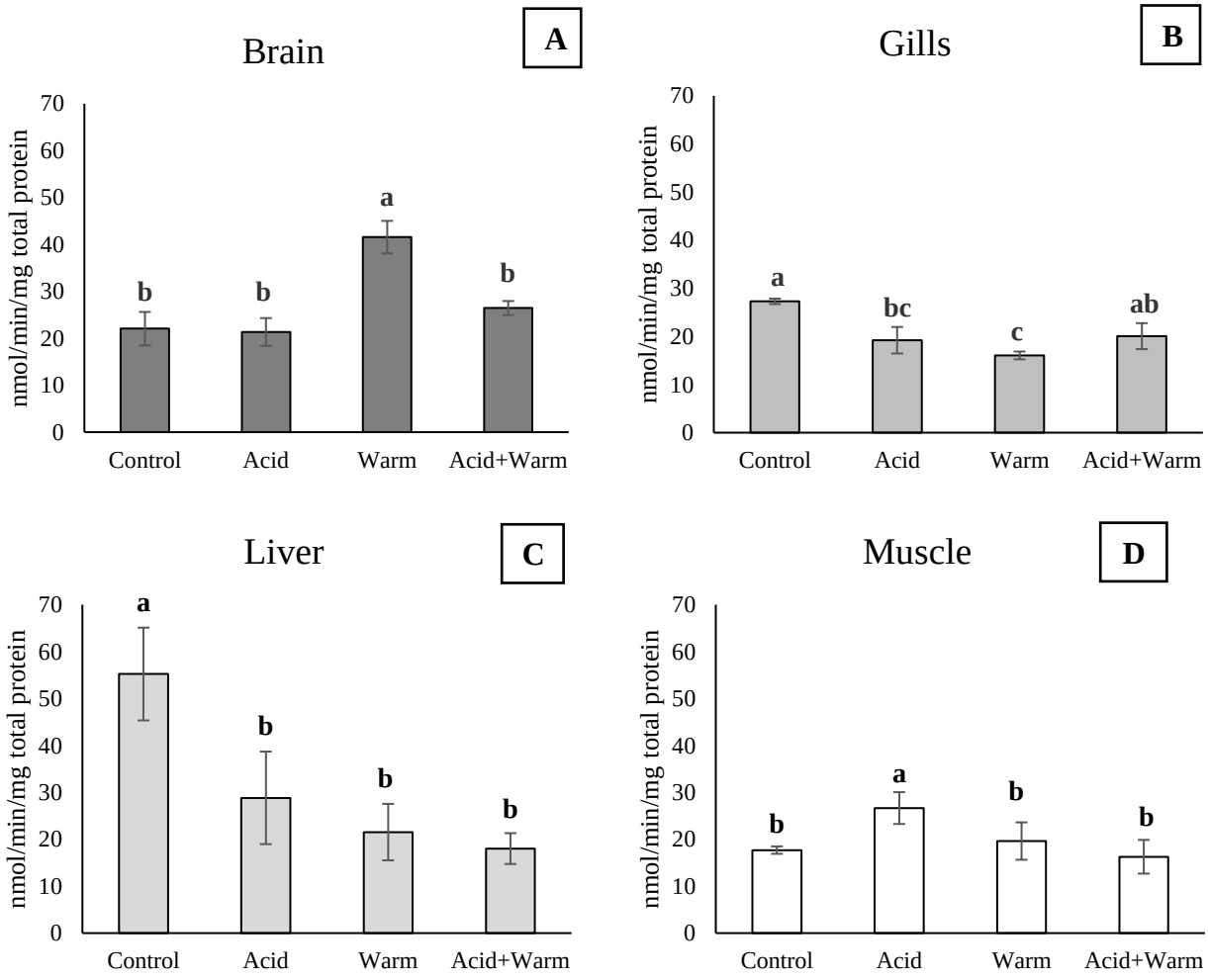

Figure 4.7- Glutathione peroxidase activity (GPx) in A) brain, B) gills, C) liver and D) muscle of juvenile *Diplodus cervinus* after 60 days of exposure to the different treatments (mean±SD; n=8). Different letters (a-c) indicate significant differences (p<0.05) between treatments. Abbreviations: Control – reference temperature and pH conditions (T=19 ºC and pH=8.0); Acid – simulated acidification (T=19 ºC and pH=7.7); Warm – simulated warming (T=23 ºC and pH=8.0); Acid+Warm - simulated acidification and warming (T=23 ºC and pH=7.7).

Among the different tissues, the brain and liver (27.1 and 30.9 nmol/min/mg total protein, respectively) were the tissues that exhibited significantly higher GPx activity when comparing to the other tissues (21.2 and 20.5 nmol/min/mg total protein in gills and muscle, respectively) (p<0.05).

In fish brain, there was only observed a significantly higher GPx activity in warming acting alone in comparison with the other remaining treatments (p=0.0002; Figure 4.7A).

GPx activity revealed a similar behaviour in the fish gills and liver. When the fish were exposed to climate change scenarios, a significant decrease was observed when comparing to control (p<0.05; Figure 4.7B-C), being more pronounced in the liver. Moreover, while in the liver no significant changes were observed between the three treatments of exposure to the abiotic

stressors, acting alone or combined, in the gills a significant decrease was observed when exposed to warming alone when comparing to exposure to both stressors (*p*=0.032).

Finally, in the fish muscle, a significant increase in GPx activity was only found when exposed to acidification acting alone, when comparing to the other treatments (*p*=0.003, *p*=0.015 and *p*=0.0025 in Control, Warm and Acid+Warm, respectively; Figure 4.7D).

4.3.5 Total Antioxidant Capacity

Total antioxidant capacity (TAC) measured for the different tissues in juvenile *Diplodus cervinus* is shown in Figure 4.8 and is presented in terms of trolox equivalent antioxidant capacity, an analogous to the natural antioxidant, vitamin E.

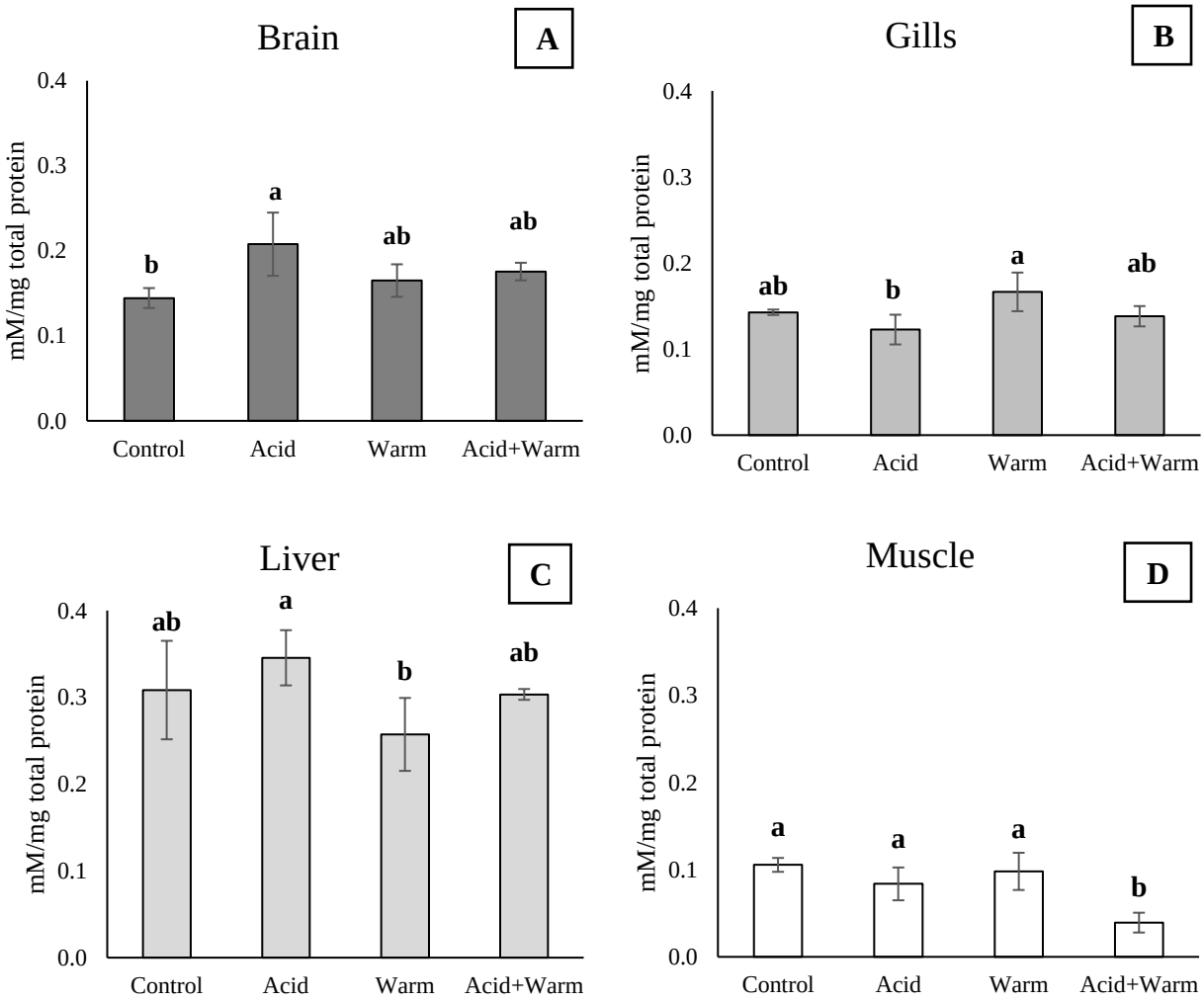

Figure 4.8- Total antioxidant capacity (TAC) in A) brain, B) gills, C) liver and D) muscle of juvenile *Diplodus cervinus* after 60 days of exposure to the different treatments (mean±SD; n=8). Different letters (a-b) indicate significant differences ($p<0.05$) between treatments. Abbreviations: Control – reference temperature and pH conditions (T=19 ºC and pH=8.0); Acid – simulated acidification (T=19 ºC and pH=7.7); Warm – simulated warming (T=23 ºC and pH=8.0); Acid+Warm - simulated acidification and warming (T=23 ºC and pH=7.7).

The liver was the tissue that exhibited significantly higher TAC (0.31 mM/mg total protein) when comparing the other tissues (0.17, 0.14 and 0.08 mM/mg total protein in brain, gills and muscle, respectively; $p<0.0002$).

In the fish brain, no significant changes were found for TAC between the three scenarios of climate change exposure ($p>0.05$; Figure 4.8A). However, it was observed a significantly increase for TAC when the fish were exposed to the acidification alone comparing with control ($p=0.004$).

While TAC significantly increased in the gills when the fish were exposed to warming alone when comparing to the acidification treatment ($p=0.012$; Figure 4.8B), the inverse effect was found for the liver ($p=0.019$; Figure 4.8C) and a significant decrease in TAC was observed. No significant changes were found between control and the other three climate change scenarios in both tissues ($p>0.05$).

Although no significant changes were found in TAC for fish muscle between the control treatment and warming or acidification acting alone, the co-exposure to these two abiotic stressors (i.e. Acid+Warm) significantly decreased TAC when comparing to the other three treatments ($p=0.0003$, $p=0.0046$ and $p=0.0005$ for Control, Acid and Warm treatments, respectively; Figure 4.8D).

4.3.6 Acetylcholinesterase (AChE) Activity

The AChE activity measured in the muscle of juvenile *Diplodus cervinus* is presented in Figure 4.9 in terms of acetylthiocholine, an analogous of the neurotransmitter acetylcholine.

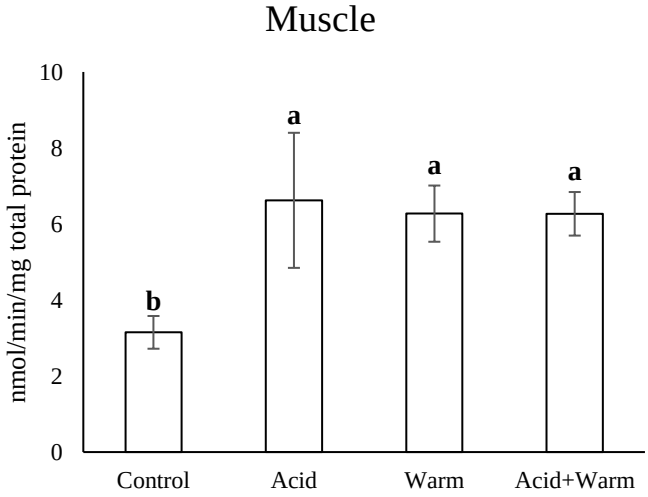

Figure 4.9- Acetylcholinesterase activity (AChE) in muscle of juvenile *Diplodus cervinus* after 60 days of exposure to the different treatments (mean±SD; n=8). Different letters (a-b) indicate significant differences ($p<0.05$) between treatments. Abbreviations: Control – reference temperature and pH conditions (T=19 ºC and pH=8.0); Acid – simulated acidification (T=19 ºC and pH=7.7); Warm – simulated warming (T=23 ºC and pH=8.0); Acid+Warm - simulated acidification and warming (T=23 ºC and pH=7.7).

When the fish were exposed to the three climate change scenarios, it was found a significant increase in AChE activity when comparing to control (p=0.0013, p=0.0031 and p=0.0009 for Acid, Warm and Acid+Warm treatments, respectively). Additionally, there were not observed significant changes between the three treatments of exposure to the abiotic stressors (p>0.05).

Furthermore, it should also be noted that was not possible to determine AChE activity in fish brain samples, due to some experimental errors that led to the loss of method sensibility and consequently the obtained values of AChE activity were very low.

4.3.7 Lipid Peroxidation (MDA content)

Lipid peroxidation determined in the different tissues is presented in Figure 4.10 and expressed in terms of MDA concentration, a specific end product of this lipid degradation process.

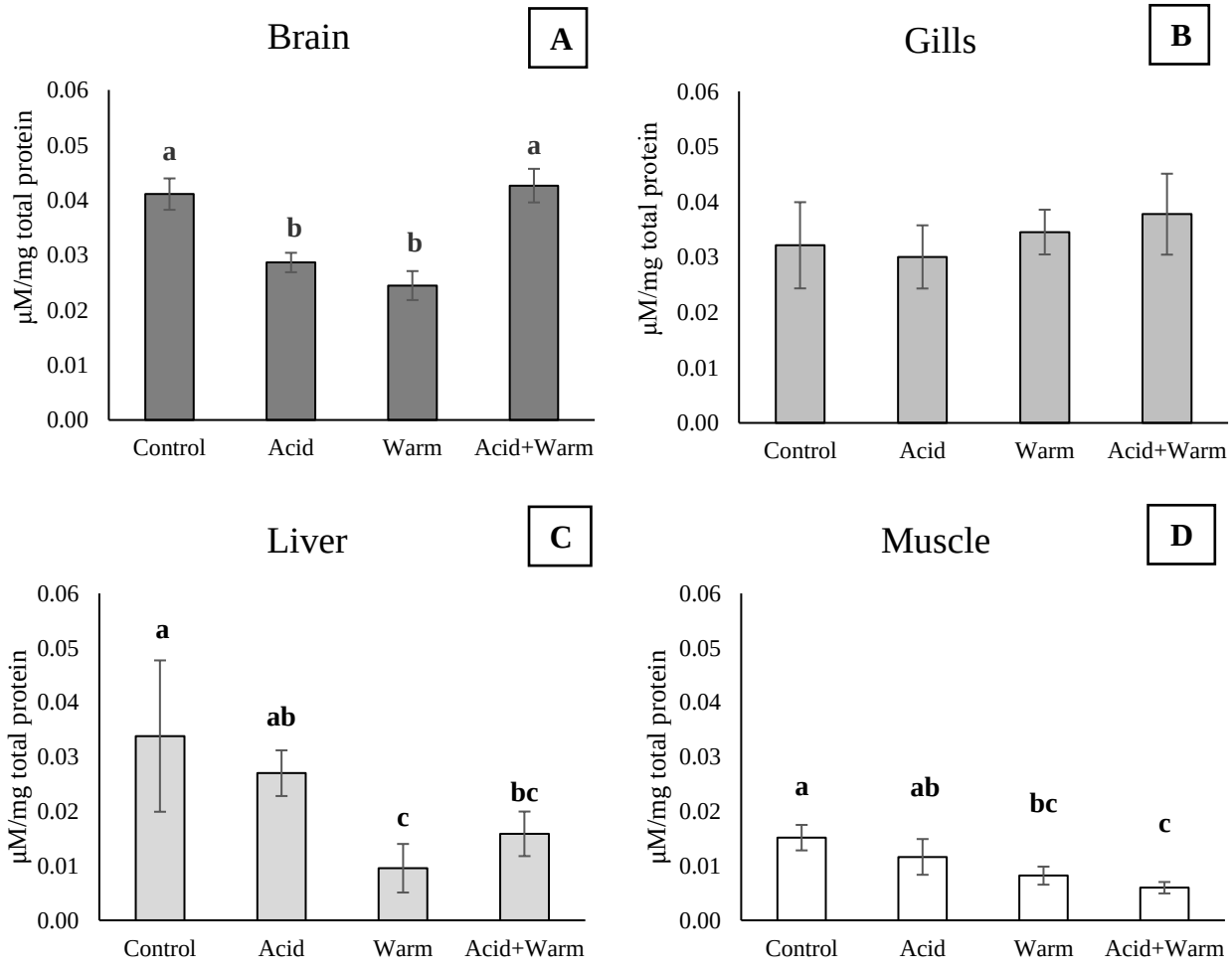

Figure 4.10- Lipid peroxidation (MDA content) in A) brain, B) gills, C) liver and D) muscle of juvenile *Diplodus cervinus* after 60 days of exposure to the different treatments (mean±SD; n=8). Different letters (a-c) indicate significant differences (p<0.05) between treatments. Abbreviations: Control – reference temperature and pH conditions (T=19 ºC and pH=8.0); Acid – simulated acidification (T=19 ºC and pH=7.7); Warm – simulated warming (T=23 ºC and pH=8.0); Acid+Warm - simulated acidification and warming (T=23 ºC and pH=7.7).

The MDA concentration measured was significantly lower in the muscle (reaching 0.01 μM/mg total protein), when comparing to the other tissues (attaining 0.033, 0.033 and 0.022 μM/mg total protein in the brain, gills and muscle respectively; $p<0.001$).

In the fish brain, a significant decrease in the LPO was observed when the fish were exposed to acidification and warming acting alone, comparing to control ($p=0.0008$ and $p=0.0002$, respectively) and to acidification combined to warming ($p=0.0003$ and $p=0.0002$, respectively; Figure 4.10A).

Although no significant changes were found in fish gills between the different treatments ($p>0.05$), there was observed a trend to increase in LPO when the fish were exposed to warming, whether alone or combined with acidification (Figure 4.10B).

In the fish liver and muscle, a significant decreased was found for LPO when exposed to warming, acting alone or combined with acidification comparing with control ($p=0.0005$ and $p=0.023$ in the liver, respectively and $p=0.0014$ and $p=0.0002$ in the muscle, respectively; Figure 4.10C-D). Additionally, the fish liver revealed significantly lower LPO content when exposed to warming alone in comparison to acidification alone ($p=0.001$; Figure 4.10C). Also, fish muscle presented significantly lower LPO content when the fish were co-exposed to the both stressors when comparing to acidification acting alone ($p=0.001$; Figure 4.10D).

4.3.8 Heat Shock Proteins (HSP70/HSC70)

Heat shock protein HSP70/HSC70 concentration determined for the different tissues in juvenile *Diplodus cervinus* is presented in Figure 4.11.

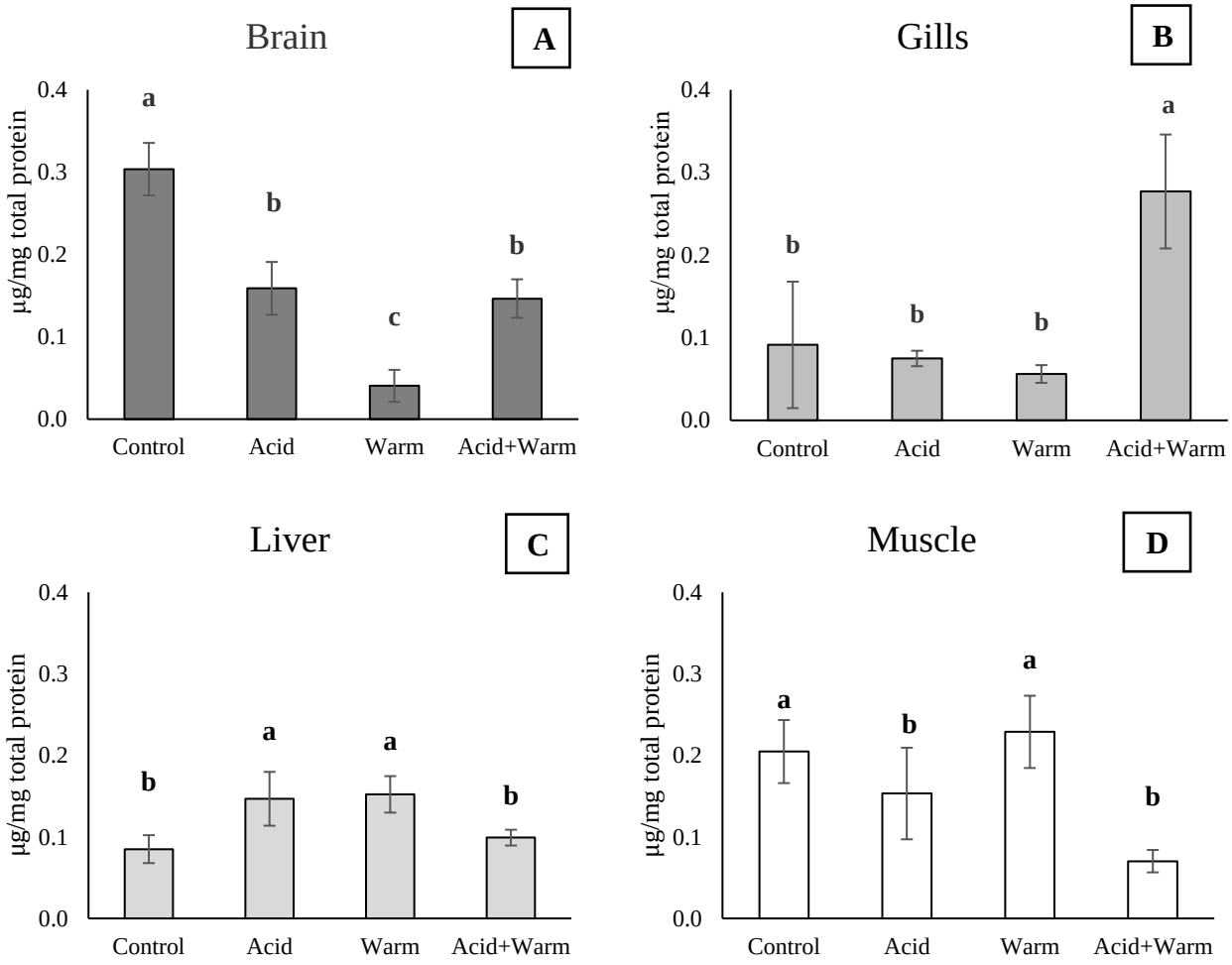

Figure 4.11 - Heat shock protein concentration (HSP70/HSC70) in A) brain, B) gills, C) liver and D) muscle of juvenile *Diplodus cervinus* after 60 days of exposure to the different treatments (mean±SD; n=8). Different letters (a-c) indicate significant differences (p<0.05) between treatments. Abbreviations: Control – reference temperature and pH conditions (T=19 ºC and pH=8.0); Acid – simulated acidification (T=19 ºC and pH=7.7); Warm – simulated warming (T=23 ºC and pH=8.0); Acid+Warm - simulated acidification and warming (T=23 ºC and pH=7.7).

No significant changes were found in HSP70/HSC70 expression between the different tissues (p>0.05).

In fish brain, when exposed to climate change scenarios there was observed a significant decrease in HSP70/HSC70 levels comparing with control (p=0.0002, Figure 4.11A), particularly in warming acting alone. Also, significantly lower concentrations were found when the fish were exposed to warming alone in comparison with acidification acting alone or combined with warming (p=0.0006 and p=0.0005, respectively).

Even though the exposure to acidification and warming alone, in the fish gills, did not induce significant changes when comparing to the control, the co-exposure to both stressors combined (i.e. Acid+Warm) significantly increased HSP70/HSC70 concentration when comparing to the

other treatments (p=0.036, p=0.011 and p=0.005 for control, acidification and warming, respectively; Figure 4.11B).

In the fish liver, there was observed a significant increase in HSP70/HSC70 expression when the fish were exposed to acidification and warming acting alone comparing to control (p=0.009 and 0.012, respectively; Figure 4.11C). Furthermore, the co-exposure to both stressors did not induce significant changes compared to control ($p>0.05$).

HSP70/HSC70 concentration in fish muscle significantly decreased when the fish were exposed to acidification, whether alone or combined with warming, compared to control (p=0.044 and 0.0004, respectively; Figure 4.11D). Nevertheless, warming alone did not induce significant changes comparing to control ($p>0.05$).

4.3.9 Ubiquitin Concentration (Ub)

Ubiquitin concentration determined for the different tissues in juvenile *Diplodus cervinus* is presented in Figure 4.12.

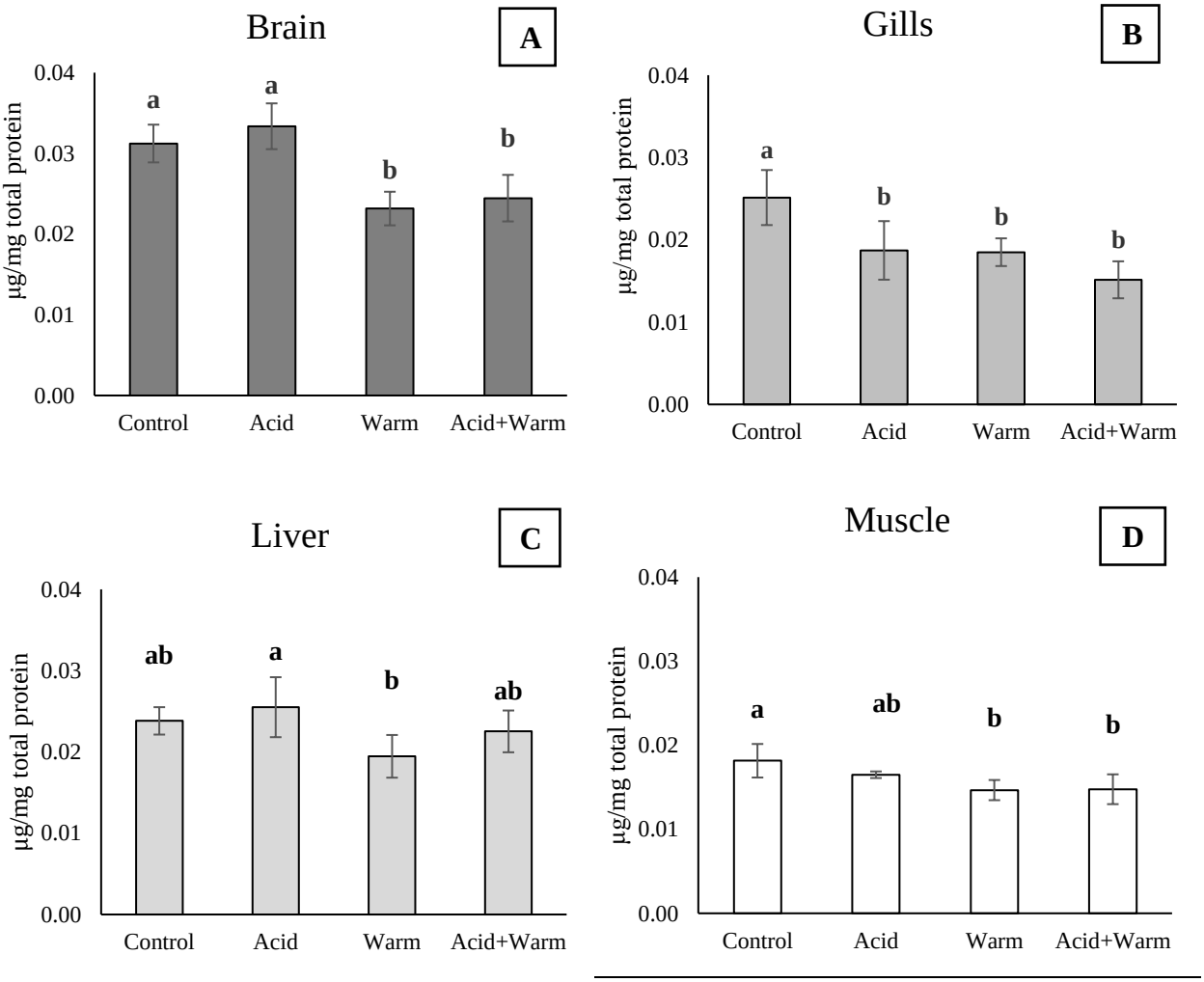

Figure 4.12- Ubiquitin concentration (Ub) in A) brain, B) gills, C) liver and D) muscle of juvenile *Diplodus cervinus* after 60 days of exposure to the different treatments (mean±SD; n=8). Different letters (a-b) indicate significant differences (p<0.05) between treatments. Abbreviations: Control – reference temperature and pH conditions (T=19 ºC and pH=8.0); Acid – simulated acidification (T=19 ºC and pH=7.7); Warm – simulated warming (T=23 ºC and pH=8.0); Acid+Warm - simulated acidification and warming (T=23 ºC and pH=7.7).

The brain was the tissue that exhibited significantly higher Ub concentration (0.028 µg/mg total protein) when comparing the other tissues (0.023, 0.019 and 0.016 µg/mg total protein in liver, gills and muscle, respectively; p<0.05).

While in the fish brain and muscle, warming acting alone or combined with acidification induced a significant decrease in Ub content compared to the control (p=0.012 and p=0.011 in the brain and p=0.011 and p=0.013 in the muscle, respectively; Figure 4.12A,D), the exposure to acidification alone did not show significantly alterations (p>0.05).

In the fish gills, there was observed a significant decrease in Ub concentration when exposed to acidification and warming, whether acting alone or combined, compared to control (p= 0.0115, p=0.0091 and p=0.0004, respectively; Figure 4.12B).

Although no significant changes were found in fish liver between the three climate change scenarios and control ($p>0.05$), the exposure to warming alone significantly reduced Ub concentration in the relation to acidification acting alone (Figure 4.12C; p=0.014).

5. DISCUSSION

Even though climate change is considered one of the main global environmental threats to the marine biosphere, the impacts of these abiotic stressors on the physiological responses of marine organisms are poorly known and require further understanding. This study assesses, for the first time, the impacts on the defence mechanisms against oxidative stress of the juvenile zebra seabream (*Diplodus cervinus*) under ocean warming and acidification scenarios.

During the 60 days period of exposure to the different climate change scenarios, it was found that warming (T=23 ºC) and acidification (pH=7.7, pCO_2~1000 µatm), either acting in isolation or in combination, were not lethal for juvenile *D. cervinus*.

The fish exposure to environmental stressors showed a significant increase in body weight after the exposure to warming conditions, whether alone or combined with acidification (Figure 4.1). These results are in agreement with previous findings where the exposure to warming temperatures induces a higher metabolism rate and enhances animal growth in ectotherms.[99–101] It was also found a significant increase in index K when both stressors were combined (Figure 4.2).

Although an increase was observed in growth, a significant decrease in HSI was found in Warm and Acid+Warm treatments (Figure 4.2), like it was previously described by Maulvault *et al.*[101] when *D. sargus* were under severe stress conditions (warming, acidification and triclosan). These results suggest that warming conditions can induce severe metabolism changes that can interfere with glycogen deposition in liver, reducing their structural components, like lipids and proteins, thus affecting liver weight.[102,103]

Haematological parameters revealed that when fish were exposed to acidification and warming, alone or combined, a significant increase in the percentage of erythrocytes was observed in comparison with the control treatment (Table 1.1). On the other hand, a significant decrease in the percentage of leucocytes was only found when the fish were exposed to warming acting alone. Although our results are in agreement with Magnadóttir[104], which concluded that temperature changes reveal a suppressive effect on fish immune system, few studies focus on the impact that these environmental stressors have on blood cells and further research is still needed.

Overall, marine organisms have developed efficient antioxidant mechanisms against oxidative stress induced by climate change-related stressors, as ocean warming and acidification.[50] The antioxidant enzymes SOD, CAT and GST play a major role in this detoxifying process: SOD is the first line of defence and converts superoxide radicals in H_2O_2 and O_2 and then, CAT converts H_2O_2 into water and oxygen, while GST transforms reactive species that escape the first line of defence, preventing in this way the propagation of the lipid peroxidation chain reaction.[51] GPx also detoxifies hydrogen peroxide, completing in this way the CAT function. TAC gives a measurement of the capacity to inhibit oxidation, LPO the extension of the

lipid peroxidation damage and AChE the level of neurotoxicity. Hence, this work presents an important multiple biomarker approach to evaluate climate change-related oxidative stress.

Levels of antioxidant enzymes (SOD, CAT, GPx, and GST) and other mechanisms (TAC, LPO and AChE) in brain, gills, liver and muscle of juvenile *D. cervinus* suggest that exposure to ocean warming and acidification leads to cellular adjustments that can lead to physiological impairments. Environmental stressors, like warming and acidification, can generate oxidative stress and ROS production, so in response, compensatory mechanisms (regardless of the tissue) are usually followed by a significant increase on the antioxidant enzymes activities in order to adapt and overcome the stress induced, but the opposite (inhibition) can occur when the stressors severity and duration overwhelm the cell's capability to produce compensatory responses, leading to exhaustion and making the organisms more susceptibility to environmental stress and potential adverse effects.[51,54,105–107]

Our results are in agreement with previous findings (e.g. [99,106]), which demonstrated that the exposure to environmental stressors result in an increase or decrease of the activities of antioxidant enzymes, according to fish tissue and biomarker. Different tissues can show distinct responses (e.g. higher GST activity in liver than in brain, gills and muscle; and GST liver activity increased under acidification combined with warming, whereas CAT muscle activity was inhibited by both stressors) which can be related with the different cells' composition of the tissues.[101] Therefore, the importance of evaluate the differential responses of fish tissues to warming and/or acidification are highlighted by the present findings in this thesis and the impacts of these environmental stressors on a whole organism context are provided.

Since SOD is the first enzyme to interact with the ROS, it can be used as an early signal of exposure to oxidative stress and several studies showed that an increase in SOD activity is a marker for antioxidant defence mechanisms in different marine organisms.[108–111] In this study, the muscle was the only tissue that revealed a trend to increase in SOD activity, when exposed to climate change scenarios, but this increase was only significant when the fish were co-exposed to warming and acidification (Figure 4.4D). Thus, the muscle was the only tissue still able to develop an adaptive response to oxidative stress.[112] On the other hand, SOD activity significantly decreased in fish brain when exposed to warming and acidification combined. Also, a significant decrease in SOD activity was observed in gills and liver when fish were exposed to warming alone or in co-exposure with acidification. This reduction in SOD activity in the three tissues can be related to their inability to eliminate the free radicals and ROS mediated denaturation, that lead to the inactivation of this enzyme, which has been previously described.[99,105,113,114]

CAT, as it was previously mentioned, complements SOD activity in the first line of defence against oxidative stress. The present results obtained in the brain showed a significant decrease in CAT activity when fish were exposed to warming alone in comparison with acidification treatments, acting alone or combined with warming (Figure 4.5A). These results are in agreement with the trend observed by Maulvault *et al.*[112] in *Dicenthrachus labrax*. While for the gills, there

was observed a significant decrease in CAT activity when fish were exposed to warming and acidification, acting alone or in combination, comparing with the control treatment (Figure 4.5B), for the muscle the significant decrease was only found under warming, acting alone or combined with acidification (Figure 4.5D). The decrease in CAT activity have been reported as a result of hydrogen peroxide and other oxyradicals accumulation that exceed the enzymes reduction capability, demonstrating that these tissues cannot produce compensatory mechanisms and consequently oxidative damage occurs.[60] In contrast, there was found in the fish liver a significant increase of CAT activity under warming and acidification, acting alone (Figure 4.5C), indicating that this tissue is the only one still able to respond positively to oxidative stress and eliminate H_2O_2, allowing the production of compensatory mechanisms against these abiotic stressors. [99,112,115]

GST, as it was mentioned above, is an enzyme that complements the first line of defence, having an important role in protection against oxidative stress.[63,64] It has been previously reported that marine animals in response to warming and acidification, increase GST levels as an antioxidant mechanism, such as in studies performed with sole post-metamorphic larvae (*Solea senegalensis*)[45], tropical and temperate shrimp (*Lysmata amboinensis* and *Lysmata seticaudata)*[116] and juvenile sea bream *(Sparus aurata)*[117]. Therefore, it was expected, that juvenile *D. cervinus* GST levels increased under exposure to warming and acidification, preventing the damage from oxidative stress. Our results obtained in fish liver are in agreement with the previous findings, where there was observed a trend to increase in GST activity when exposed to climate change scenarios. These results were only significantly higher in liver when fish were exposed to acidification treatments, whether alone or combined with warming in comparison with the control treatment (Figure 4.6C), suggesting that the antioxidant defence mechanisms are active in an attempt to protect cells against the oxidative stress[118] and the liver is the only tissue that can still respond and produce compensatory mechanisms in the different treatments. Additionally, it was found that the average values for GST activity were higher in the liver when compared to the other studied tissues, as expected since the liver is the main organ for detoxification and play a major role in transformation and excretion of ROS that escaped the first line of defence.[119] There was also found a significant increase in GST levels for the brain when fish were exposed to both acidification and warming combined and for the gills under warming acting alone, comparing to the control treatment (Figure 4.6A-B). Concerning the fish muscle, it was observed a trend to decrease in GST activity when exposed to climate change scenarios, being only significant when the fish were exposed to warming alone in comparison to the control treatment (Figure 4.6D). This decrease in the fish muscle can be due to the exhaustion of GSH, resulting in the reduced amount of substrate for GST to act on, as it was previously reported in several studies[112,118,119]. These differences obtained between tissues (increase in GST activity for liver, brain and gills and decrease for muscle), demonstrate that the different tissues respond differently to the exposure of the same abiotic stressors.

GPx complements CAT function, reducing also the hydrogen peroxide induced by the oxidative stress.[51] While in the fish brain the results showed a significant increase in GPx activity when fish were exposed to warming alone (Figure 4.7A), in the fish muscle, a significant increase was observed under acidification alone (Figure 4.7D), comparing to the other tissues. This upregulation of GPx activity was previously described in a study performed with *Chiloscyllium plagiosum*[120] and also in *Gammarus locusta*[121]. In the fish gills and liver, the opposite trend was detected, and a significant decrease was observed when the fish were exposed to warming and acidification, alone or combined, comparing to control (Figure 4.7B-C). The results observed for GPx activity are in contrast with what was found for CAT activity, showing that when CATs ability to reduce hydrogen peroxide is compromised, GPx can in some extent compensate CATs function and prevent oxidative damage.

Total antioxidant capacity (TAC) is a measurement of a substance capacity to inhibit oxidation and can give a general idea of the response against oxidative stress (enzymatic and non-enzymatic). Results found for the brain showed a significant increase when exposed to acidification alone comparing to control (Figure 4.8A). In the fish gills and liver, there was not observed significant differences between the control and climate change scenarios (Figure 4.8B-C). While in the gills a significant increase was found for the warming treatment in comparison with the acidification treatment, in the liver, the opposite trend was observed. Concerning the fish muscle, there was only detected a significant decrease when the fish were exposed to acidification and warming combined in comparison with the remaining treatments (Figure 4.8D). The increase detected for TAC, in the brain, are in agreement with the ones reported by Lopes *et al*.[121] , although their study was performed with amphipod species *Gammarus locusta*.

Although AChE has been well reported as an important biomarker for neurotoxicity in studies performed with organisms exposed to contaminants[70,122], other studies reported that it can also be used as an indicator of environmental stress like the exposure to warming and acidification[123,124]. The results found for fish muscle showed that exposure to warming and acidification, alone or combined, induced a significant increase in AChE activity (Figure 4.9). As it was previously mentioned, warming and acidification can generate oxidative stress, so the present findings are in agreement with Melo *et al.*[123] and Duriex *et al.* [124], which indicate that oxidative and thermal stress are known to increase AChE activity in vertebrate models, leading to behavioural changes.[127,128] Our results are also in agreement with Rosa *et al.*[115], that found an increase in AChE activity of *Chiloscyllium punctatum* under exposure to warming and acidification, alone or combined. The increased AChE levels may be occurred probably as a response to reduce the neurotransmitter excess in the synaptic clefts.[115]

ROS have the ability to generate lipid peroxidation (LPO), causing cellular membrane damage and could lead to death.[51] The MDA content is usually used as an indicator of lipid peroxidation when exposed to oxidative stress in cells.[39,58] The results obtained in the fish liver, muscle and brain of *D. cervinus* revealed a trend to decrease the MDA concentration when

exposed to climate change scenarios (Figure 4.10A, C and D). In the fish brain, this decrease was only significant when the fish were exposed to warming and acidification alone, comparing to the control treatment. Additionally, in the fish liver and muscle, warming (alone or combined with acidification) promoted a significant decrease in LPO in comparison with the control treatment. These results found for brain, liver and muscle (decrease in LPO) are in contrast with what usually was reported for other species under similar climate change scenarios (e.g. [45,97,115]). On the other hand, a LPO decrease was also observed by Jesus *et al.*[129] in *Squalius carolitertii* and *Squalius torgalensis*. Nevertheless, these authors considered that the tested scenarios were not enough to induce lipid peroxidation or antioxidant mechanisms when fish were exposed to higher temperatures or pH variations throughout the year (especially in the summer season). In contradiction with these facts, the obtained results in the present thesis demonstrated that the exposure to +4 ºC and to -0.3 pH units indeed activated the some antioxidant mechanisms (e.g. increased GST and CAT levels in the liver) and can be able to respond to the oxidative stress and probably can be enough to remove the final products of lipid peroxidation. On the other hand, the gills were the only tissue that, although not significant, showed an increase in lipid peroxidation when exposed to climate change scenarios (Figure 4.10B). These results are in agreement with the fact that the antioxidant response only prevented ROS production and oxidative stress to some extent, leading to the consequent cell damage. Similar results were also reported in *D. labrax* exposed to warming conditions.[112]

Heat shock proteins are molecular chaperones that under conditions of oxidative stress, especially to elevated temperatures, show a tendency to increase their expression.[130,131] Results from HSP70 concentration in the fish brain showed a significant decrease when exposed to warming and acidification, whether alone or combined, in comparison with the control treatment (Figure 4.11A). This decrease was also significantly lower under warming conditions compared to acidification, acting in isolation or in combination with warming. Concerning the fish muscle, there was also found a significant decrease in HSP70/HSC70 content when the fish were exposed to acidification, alone or combined with warming, in comparison with the control and warming alone (Figure 4.11D). In fact, the HSP70/HSC70 down-regulation can be due to severe stress exposure and metabolic depression, showing that HSP70/HSC70 is compromised.[106] In relation to the fish gills and liver, the opposite behaviour was observed, and an increase in HSP70/HSC70 was observed (Figure 4.11B-C). While for the gills there was found a significant increase when the fish were co-exposed to acidification and warming, in the liver the significant increase was only detected when the stressors acted individually, comparing to the control. These results found for the gills and liver showed that these tissues can respond to the stress induced by warming and acidification in order to keep their native protein structure intact, and HSP70/HSC70 concentration increases as expected, like it was previously described[106,132,133].

Although under oxidative stress it is expected an increase in ubiquitin concentration[79], the present findings showed a tendency to significantly decrease the Ub content of fish exposed to

climate change scenarios in brain, gills and muscle, except under acidification acting alone in brain and muscle (Figure 4.12A,B,D). Moreover, it was observed a significant decrease in Ub concentration of fish liver under warming conditions in comparison with acidification alone (Figure 4.12C). This decrease in Ub content has been previously reported as a result of prolonged exposure to warming and acidification, where protein synthesis is compromised since it is an energy demanding process that requires high energetic costs.[106,112]

In conclusion, the distinct fish tissues responses elicited by the different scenarios of climate change-related stressors are then described in detail. In the brain, the results revealed that this tissue can respond to oxidative stress to some extent since a tendency to increase in GST, GPx and TAC were observed when fish were exposed to climate change scenarios. These increased activities were only significant when the fish were exposed to acidification for TAC, to warming for GPx and under co-exposure to both stressors for GST, all in comparison with the control. In contrast, the extreme exposure to ROS and the compromise in the efficiency of antioxidant mechanisms was shown by the significant decrease found in LPO (Acid and Warm alone), HSP70/HSC70 (all treatments), and Ub content (Warm and Acid+Warm) as well as in SOD activity (Acid+Warm).

Concerning the gills, the antioxidant machinery is severely compromised, and a decrease in the activities of the different biomarkers were observed when fish were exposed to the different scenarios (e.g. SOD activity in Acid+Warm; CAT activity and Ub content in all treatments of climate change; GPx activity in Acid and Warm alone, comparing to control). For this tissue, a significant increase in activity were only found for GST under exposure to warming alone and for HSP70/HSC70 content under co-exposure to warming and acidification combined. The reduced ability of this tissue to respond to oxidative stress may be due to the fact that the gills have diminished levels of antioxidant enzymes, they are in close contact with the media and quick absorption.[134]

The present findings showed that the highest values for the antioxidant enzyme activities (for SOD, CAT, GST, GPx and TAC) were generally observed for the liver in comparison to the other tissues as expected, since the liver is the main tissue responsible for ROS detoxification produced by oxidative stress.[119] The results found in the liver, suggest that oxidative stress is occurring, and the several defence mechanisms are working together in order to develop a response to the stress that is imposed by warming and acidification. The treatments where the fish were exposed to warming and acidification induced a significant increase in GST (Acid and Acid+Warm) and CAT activities as well as in HSP70/HSC70 content (both in Acid and Warm alone) in order to remove ROS. Moreover, the increase found in CAT activity was also accompanied by a decrease in GPx activity (in all treatments of climate change), since both enzymes have the same substrate, and therefore they will compete for it. Also, the significant decrease found in SOD activity and LPO (Warm and Acid+Warm), suggests that other alternative defence mechanisms are activated

to remove ROS, explaining in this way the increase in GST activity and in HSP70/HSC70 concentration.

Finally, in relation to the muscle, it was observed that this tissue can respond to oxidative stress in some extent, since a decrease in LPO was detected when fish were exposed to warming alone or in combination with acidification. Additionally, the significant decrease found in CAT (Warm and Acid+Warm), GST (Warm) and TAC activities (Acid+Warm) as well as in HSP70/HSC70 (Acid and Acid+Warm) and Ub levels (Warm and Acid+Warm) indicate that antioxidant machinery is overwhelmed by the oxidative stress under exposure to climate-change related stressors. In contrast, there was also observed a significant increase in SOD activity under co-exposure to both stressors combined. Moreover, AChE activity significantly increase when exposed to warming and acidification, whether alone or combined, while GPx activity increase when fish were exposed to acidification alone.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

The present work provided an multi-biomarker response approach to assess the interactive effects of ocean warming and acidification on juvenile zebra seabream. Despite the different physiological responses of distinct organism´s tissues to the environmental stressors, the results showed that the juvenile fish stress responses (animal fitness, fish immunity, antioxidant mechanisms, neurotoxicity and molecular chaperones) were strongly affected by increased temperature (+4 ºC) and pCO_2 (+1000 µatm), acting alone or in combination.

In the present study, the exposure to ocean warming and acidification was not lethal for juvenile *Diplodus cervinus*, but the cellular defence mechanisms were compromised. Additionally, the four fish tissues studied (brain, gills, liver and muscle) responded differently to the stress imposed by climate change scenarios.

Warming induced a significant increase in weight, but a significant decrease was found in HSI when fish were exposed to warming, alone or combined with acidification, which indicate that warming conditions can lead to severe metabolism changes that can interfere with glycogen deposition in liver, reducing their structural components, like lipids and proteins, thus affecting liver weight.

Haematological parameters revealed that warming alone induced a significant decrease in leucocytes percentage in blood in comparison to the control, while the erythrocytes percentage significantly increased when exposed to all climate change scenarios.

The brain is capable to respond to environmental stressors by increasing the expression of important antioxidant enzymes, like GST and GPx activities. Although oxidative stress compromised some fundamental defence enzymes, the activities of GST and GPx, along with other antioxidant mechanisms (e.g. increase in TAC) may be enough to fight oxidative stress in this tissue since a significant decrease in SOD activity, LPO, HSP70/HSC70 and Ub content was observed.

The gills showed a lower ability to respond to oxidative stress (e.g significant decrease in SOD, CAT and GPx activities as well as Ub content), possibly because gills are in close contact with the media as well as gills' cells generally have reduced amounts of antioxidant enzymes.

Liver is the tissue that generally revealed higher levels for the different biomarkers analysed. Although SOD and GPx activities showed a decrease after exposure to abiotic stressors, the other antioxidant enzymes increased to remove ROS and to avoid LPO, as shown by the decrease in MDA concentration. Overall, the results showed a significant increase in CAT and GST activities, TAC and HSP70/HSC70 concentration.

The muscle revealed that it was still capable to produce some compensatory mechanisms against oxidative stress by the significant increase in SOD, GPx and AChE activities. These increase in the enzymes' activities may reduce the damage caused by ROS, since a significant decrease was found in LPO when exposed to warming alone or in combination with acidification.

In contrast, a significant decrease was found for CAT and GST activities, TAC, HSP70/HSC70 and Ub content.

Hence, the present findings indicate that prolonged exposure to ocean warming and acidification can compromise the cellular adjustments and antioxidant responses of marine organisms, and thus lead to physiological impairments in the different fish tissues.

Considering the expected scenarios of climate change by the end of the 21st century, it is of paramount importance to evaluate the effects of other environmental stressors that may act in synergy with temperature and pH, such as hypoxia, salinity or pollution, which also influence the response of marine ecosystems. It would also be interesting to study the effects of these stressors on the physiological responses over time, collecting samples with different periods of exposure.

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8. ANNEXES

Table A1- Seawater carbonate chemistry data for juvenile *Diplodus cervinus* under different climate change scenarios: Control – 19 °C and pH 8.0; Acidification (T=19 ºC, pH=7.7); Warming (T=23 ºC, pH=8.0); Acid+Warm - Acidification + warming (T=23 ºC, pH=7.7). Total carbon (CT), carbon dioxide partial pressure (pCO_2), bicarbonate concentration (HCO_3^-) and saturation states of aragonite (ΩAra) and calcite (ΩCal) were calculated with CO_2SYS using temperature, salinity, pH total scale (pHT) and total alkalinity (AT). Values are represented as means±SD.

	Control	**Acidification**	**Warming**	**Acidification + Warming**
measured				
Temperature (°C)	19.1 ± 0.3	19.0 ± 0.21	23.1 ± 0.2	22.9 ± 0.2
Salinity (‰)	35.5 ± 0.5	35.5 ± 0.6	35.5 ± 0.4	35.4 ± 0.5
pH_T (units)	7.99 ± 0.02	7.65 ± 0.18	8.01 ± 0.03	7.69 ± 0.06
AT (µmol/kg)	2250.2 ± 205.1	2325.6 ± 217.0	2242.1 ± 212.8	2291.8 ± 187.8
calculated				
CT (µmol/kg)	2019.2 ± 189.9	2224.2 ± 210.4	1970.2 ± 192.5	2171.8 ± 181.2
pCO_2 (µatm)	437.3 ± 47.1	1014.0 ± 106.8	416.2 ± 46.1	1020.9 ± 114.3
HCO_3^- (µmol/kg)	1839.6 ± 172.7	2098.5 ± 198.1	1766.3 ± 171.4	2038.8 ± 170.0
ΩAra	2.55 ± 0.28	1.42 ± 0.16	2.99 ± 0.35	1.60 ± 0.16
ΩCal	3.92 ± 0.44	2.19 ± 0.25	4.55 ± 0.53	2.44 ± 0.25

Table A2- Formulation, ingredients and proximate chemical composition (% dry weight, dw) of the experimental diet used for juvenile zebra seabream (mean ± standard deviation).

Ingredients (%)	Diet
Fishmeal LT70[1]	40.0
Fish protein concentrate[2]	7.8
Squid meal[3]	10.0
Chlorella[4]	2.0
Soy protein concentrate[5]	4.0
Soybean meal 48[6]	4.0
Wheat meal[7]	6.0
Fish oil[8]	12.0
Soy lecithin[9]	2.0
Guar gum[10]	1.2
Macroalgae[11]	5.0
Antioxidant[12]	0.5
Monocalcium phosphate[13]	1.0
Glycerol[14]	3.5
Vitamin and mineral premix[15]	1.0
Proximate chemical composition (dw)	**Control**
Moisture (%)	4.55 ± 0.03
Protein (%)	57.71 ± 0.82
Fat (%)	16.89 ± 0.31
Total carbohydrates[16]	16.17 ± 1.07
Ash (%)	9.24 ± 0.02
Gross energy (kJ/g)	21.86 ± 0.15

[1] Peruvian fishmeal LT: 67% crude protein (CP), 9% crude fat (CF), EXALMAR, Peru.
[2] CPSP 90: 84% CP, 12% CF, Sopropêche, France.
[3] Super prime without guts: 82% CP, 3.5% CF, Sopropêche, France;
[4] Chlorella powder: 62.5% CP, 9.2% CF, ALLMICROALGAE, Portugal.
[5] Soycomil P: 65% CP, 0.8% CF, ADM, The Netherlands.
[6] Dehulled solvent extracted soybean meal: 47% CP, 2.6% CF, Cargill, Spain;
[7] Whole wheat: 10.2% CP, 1.2% CF, Casa Lanchinha, Portugal.
[8] SAVINOR UTS, Portugal
[9] LECICO P 700IPM, LECICO GmbH, Germany.
[10] Guar gum HV109, SEAH International, France.
[11] OceanFeed: 10.2% CP, Ocena Harvest Technology, Ireland.
[12] Paramega PX, Kemin Europe NV, Belgium
[13] Monocalcium phosphate: 22% phosphorus, 16% calcium, Fosfitalia, Italy.
[14] Rapeseed-derived crude glycerol, IBEROL, Portugal.
[15] PREMIX Lda, Portugal: Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 500 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate,7.5 mg; sodium chloride, 400 mg; excipient wheat middlings.
[16] Calculated as, total carbohydrates (starch, free sugars, cellulose) = 100-(protein+fat+ash).

Table A3- Total length (TL; cm) and total weight (TW; g), Fulton's condition index (K), hepatosomatic index and visceral indices (HSI and VIS, respectively) in juvenile *Diplodus cervinus* in the four treatments after 60 days of exposure (mean ± SD; n=8). In each column, different letters indicate significant differences ($p < 0.05$) between treatments. Abbreviations: Control – reference temperature and pH conditions (i.e. T=19 ºC and pH=8.0); Acidification – simulated acidification (T=19 ºC and pH= 7.7); Warming – simulated warming (T=23 ºC and pH= 8.0); Acidification + Warming - simulated acidification and warming (T=23 ºC and pH=7.7).

	TL	**TW**	**K**	**HSI**	**VSI**
Control	7.66 ±1.73	7.81 ± 0.48	1.59 ± 0.21	0.98 ± 0.24	3.37 ± 0.38
Acidification	7.95 ± 2.00	7.82 ± 0.57	1.63 ± 0.20	1.05 ± 0.24	3.63 ± 0.46
Warming	10.38 ± 3.86	8.40 ± 0.81	1.68 ± 0.21	0.85 ± 0.37	3.34 ± 0.64
Acidification + Warming	11.86 ± 3.93	8.79 ± 0.77	1.69 ± 0.17	0.82 ± 0.24	3.42 ± 0.54

Table A4- Tissue molecular biomarker levels in juvenile *Diplodus cervinus* in four treatments after 60 days of exposure (mean ± SD; n=8). Abbreviations: CAT – catalase; SOD – superoxide dismutase; GST – glutathione S-transferase; GPx – glutathione peroxidase; TAC –total antioxidant capacity; AChE – acetylcholinesterase; LPO – lipid peroxidation; HSP70/HSC70 – heat shock proteins; Ub – total ubiquitin.

		CAT activity (nmol/min/mg protein)	**SOD activity (% inhibition/ mg total protein)**	**GST activity (µmol/min/mg protein)**	**GPx activity (nmol/min/mg protein)**	**TAC (mM/mg protein)**	**AChE activity (nmol/min/mg protein)**	**LPO (µM/mg protein)**	**HSP70/HSC70 concentration (µg/mg protein)**	**Ub concentration (µg/mg protein)**
Control	**Brain**	7.08 ± 0.50	79.59 ± 4.68	27.07 ± 3.66	22.05 ± 3.57	0.15 ± 0.02		0.041 ± 0.003	0.30 ± 0.03	0.031 ± 0.002
	Gills	30.63 ± 2.34	51.50 ± 5.21	20.35 ± 1.79	27.29 ± 0.57	0.14 ± 0.003		0.032 ± 0.008	0.09 ± 0.08	0.025 ± 0.003
	Liver	17.92 ± 2.56	78.89 ± 9.86	126.45 ± 30.69	55.22 ± 9.89	0.31 ± 0.06		0.034 ± 0.014	0.08 ± 0.02	0.024 ± 0.002
	Muscle	1.55 ± 0.41	27.37 ± 4.80	21.61 ± 1.62	17.69 ± 0.79	0.11 ± 0.008	3.15 ± 0.43	0.015 ± 0.002	0.20 ± 0.04	0.018 ± 0.002
Acidification	**Brain**	7.62 ± 1.15	72.68 ± 10.25	31.20 ± 3.54	21.35 ± 2.96	0.21 ± 0.04		0.028 ± 0.002	0.16 ± 0.03	0.033 ± 0.003
	Gills	14.57 ± 2.36	53.36 ± 2.30	18.42 ± 0.37	19.20 ± 2.75	0.12 ± 0.02		0.030 ± 0.006	0.07 ± 0.009	0.019 ± 0.004
	Liver	29.02 ± 3.87	81.66 ± 7.58	197.57 ± 17.07	28.83 ± 9.86	0.35 ± 0.03		0.027 ± 0.004	0.15 ± 0.03	0.026 ± 0.004
	Muscle	1.43 ± 0.43	34.64 ± 4.44	18.25 ± 2.24	26.65 ± 3.39	0.08 ± 0.019	6.62 ± 1.78	0.011 ± 0.003	0.15 ± 0.06	0.016 ± 0.0004
Warming	**Brain**	6.08 ± 0.47	88.26 ± 9.11	26.13 ± 1.71	41.56 ± 3.47	0.16 ± 0.02		0.024 ± 0.003	0.04 ± 0.02	0.023 ± 0.002
	Gills	19.00 ± 3.09	44.87 ± 3.06	28.68 ± 3.92	16.05 ± 0.81	0.16 ± 0.02		0.035 ± 0.004	0.06 ± 0.01	0.018 ± 0.002
	Liver	27.23 ± 1.83	55.80 ± 5.40	175.40 ± 36.47	21.53 ± 6.02	0.26 ± 0.04		0.010 ± 0.04	0.15 ± 0.02	0.019 ± 0.003
	Muscle	0.56 ± 0.27	34.00 ± 3.34	15.71 ± 4.40	19.62 ± 3.98	0.10 ± 0.021	6.27 ± 0.74	0.008 ± 0.002	0.23 ± 0.04	0.015 ± 0.001
Acidification + Warming	**Brain**	7.81 ± 0.62	60.71 ± 4.48	32.20 ± 1.19	26.45 ± 1.50	0.18 ± 0.01		0.043 ± 0.003	0.15 ± 0.02	0.024 ± 0.003
	Gills	13.14 ± 1.47	34.21 ± 2.57	16.55 ± 3.91	20.06 ± 2.70	0.14 ± 0.01		0.038 ± 0.007	0.28 ± 0.07	0.015 ± 0.002
	Liver	20.46 ± 2.13	57.42 ± 1.90	220.08 ± 21.39	18.02 ± 3.28	0.30 ± 0.006		0.016 ± 0.004	0.10 ± 0.01	0.022 ± 0.003
	Muscle	0.63 ± 0.51	33.88 ± 1.00	16.27 ± 1.03	16.28 ± 3.60	0.04 ± 0.011	6.77 ± 1.22	0.006 ± 0.001	0.07 ± 0.01	0.015 ± 0.002