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EVALUATING THE EFFECTS OF OCEAN
ACIDIFICATION ON SAND-SMELT LARVAE
THROUGH BIOCHEMICAL BIOMARKERS AND
SWIMMING ABILITY

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Resumo

A acidificação oceânica é um problema global, cujos efeitos são ainda pouco estudados. Com maior foco de investigação em organismos com exoesqueleto calcário, pouca atenção tem sido dada ao potencial impacto noutros processos e componentes do ecossistema, como o caso dos estágios iniciais do ciclo de vida de outros organismos, como os peixes. Neste sentido, o principal objetivo deste estudo foi investigar os efeitos da exposição a elevadas concentrações de $p\text{CO}_2$ no comportamento, desenvolvimento e metabolismo das fases larvares do peixe-rei, *Atherina presbyter*. As larvas em estado de desenvolvimento de flexão e pós-flexão foram capturadas no parque marinho da Arrábida, Portugal, e mantidas em condições controladas com diferentes níveis de $p\text{CO}_2$ (Controlo: $\sim 600\mu\text{atm}$; Médio: $\sim 1000\mu\text{atm}$; Elevado: $\sim 1800\mu\text{atm}$) entre 7-15 dias, antes da sua velocidade crítica de natação (U_{crit}) ser testada. Adicionalmente, foram determinados dados morfométricos e biomarcadores bioquímicos relacionados com *stress* oxidativo (SOD, CAT, LPO, danos no DNA e ROS) e metabolismo energético (ETS, LDH, IDH e Hidratos de Carbono). Os resultados sugerem que o comportamento natatório das larvas não é afectado pela exposição a elevados níveis de CO_2 ; no entanto os resultados bioquímicos apoiam a hipótese de que elevados custos energéticos poderão estar associados a alterações morfométricas, bem como à exposição a um maior *stress* ambiental. Este estudo contribui com informação relativa à sensibilidade desta espécie em condições futuras de acidificação oceânica, demonstrando que apesar da ausência de efeitos comportamentais natatórios, a resposta metabólica evidencia a existência de *stress* oxidativo em elevado $p\text{CO}_2$, cujas consequências futuras são ainda desconhecidas.

Palavras-chave: Acidificação; U_{crit} ; Biomarcadores; *Atherina presbyter*

Abstract

The ocean acidification is a critical global problem and scientific investigation of its effects is still in its infancy. Most research has been conducted on calcifying organisms and little attention has been given to the potential impact on other ecosystem processes and components, like the early life-stages of non-calcifying organisms such as fish. In this context, the main goal of this study was to investigate the effects of exposure to elevated $p\text{CO}_2$ on behavior, development, oxidative stress and metabolism of the early stages of sand-smelt, *Atherina presbyter*. Wild larvae in flexion and post-flexion stages were caught at Arrábida Marine Park, Portugal and kept in controlled conditions with different $p\text{CO}_2$ levels (Control: $\sim 600\mu\text{atm}$; Medium: $\sim 1000\mu\text{atm}$; High: $\sim 1800\mu\text{atm}$) between 7-15 days, before being tested to assess critical swimming speed (U_{crit}). Additionally, morphometric measurements and biochemical biomarkers were determined. The measured biomarkers were related with oxidative stress (SOD, CAT, LPO, DNA damage and ROS) and energy metabolism (total carbohydrate levels, ETS, LDH and IDH). Results suggest that swimming behavior of sand-smelt is unaffected by exposure to high $p\text{CO}_2$ levels; however the biochemical results suggest higher energetic costs may be associated with morphometric changes and also with the exposure to a stressful environment. This study contributes with information on this species sensitivity to future ocean acidification conditions, showing that despite the absence of swimming behavior effects, the metabolic responses demonstrate an evidence of oxidative stress at elevated $p\text{CO}_2$, whose future consequences are still unknown.

Keywords: Ocean acidification; U_{crit} ; Biomarkers; *Atherina presbyter*

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Chapter 1

Aim of the Study

The aims of this study were to evaluate the effects of high $p\text{CO}_2$ exposure on larval behavior, in particular critical swimming speed (U_{crit}), combined with the analysis of morphometric measurements and biochemical biomarkers related with oxidative stress, oxidative damage and energy metabolism of an ecological and commercial important species, the sand-smelt *Atherina presbyter* Cuvier 1829. For this purpose, larvae of two different development stages (flexion and post-flexion) were caught in the field, immediately transferred to the laboratory and randomly separated to the three $p\text{CO}_2$ treatments: Control: ~ 600 μatm , $\text{pH}=8.03$; Medium: ~ 1000 μatm , $\text{pH}=7.85$; High: ~ 1800 μatm , $\text{pH}=7.64$. After 7-15 days in treatment conditions larvae were tested in a swimming chamber for U_{crit} determination, after which fish were immediately photographed for morphometric purposes, and placed at -80°C until the following biochemical analysis were performed: 1) oxidative stress related – superoxide dismutase (SOD) and catalase (CAT) enzyme activities, levels of lipid peroxidation (LPO) and DNA damage, and levels of superoxide anion (ROS) production; 2) energy metabolism related – total carbohydrate levels, electron transport system activity (ETS) and lactate dehydrogenase (LDH) and isocitrate dehydrogenase (IDH) enzyme activities.

Chapter 2

Evaluating the effects of ocean acidification on sand-smelt larvae through biochemical biomarkers and swimming ability

Evaluating the effects of ocean acidification on sand-smelt larvae through biochemical biomarkers and swimming ability

2.1 Abstract

Although ocean acidification is a critical global problem known for decades, scientific investigation of its effects is still in its infancy. To date, most research has been conducted on calcifying organisms and little attention has been given to the potential impact on other ecosystem processes and components, like the early life-stages (larvae and juveniles) of non-calcifying organisms. Due to their vulnerable physiological state, small body size and high sensitivity to environmental variation, the effects of acidification are most likely to be detected at these early developmental stages. In this context, the main goal of this study was to investigate the effects of exposure to elevated $p\text{CO}_2$ (lower pH) on behavior, development, oxidative stress and metabolism of the early stages of sand-smelt, *Atherina presbyter*, a temperate fish species common in Atlantic European coastal waters, with economic and ecological interest. Wild larvae in flexion and post-flexion stages were caught at Arrábida Marine Park, Portugal and kept in controlled conditions with different $p\text{CO}_2$ levels (Control: ~ 600 μatm , pH=8.03; Medium: ~ 1000 μatm , pH=7.85; High: ~ 1800 μatm , pH=7.64) between 7-15 days, before being tested in a swimming chamber to access critical swimming speed (U_{crit}). Additionally, morphometric measurements and biochemical biomarkers were determined. The measured biomarkers were related with: 1) oxidative stress – superoxide dismutase (SOD) and catalase (CAT) enzyme activities, levels of lipid peroxidation (LPO) and DNA damage, and levels of superoxide anion (ROS) production; 2) energy metabolism – total carbohydrate levels, electron transport system activity (ETS) and lactate dehydrogenase (LDH) and isocitrate dehydrogenase (IDH) enzyme activities. Results suggest that swimming behavior of sand-smelt is unaffected by exposure to high $p\text{CO}_2$ levels; however at the biochemical level, results suggest higher energetic costs associated with morphometric changes and also with the exposure to a stressful environment. This study contributes with information on this species sensitivity to future ocean acidification conditions, showing that although the absence of swimming behavior effects, the metabolic responses demonstrate an evidence of oxidative stress at elevated $p\text{CO}_2$. The efficient antioxidant response capacity and the general increase in the energetic metabolism (high ETS and LDH levels) at the medium treatment, as opposed to the enzymatic inhibition detected at the high treatment may indicate that, at $p\text{CO}_2$ levels higher than the ones from the medium treatment, the capacity of larvae to restore their internal

balance could be impaired. Thus, the information obtained with this study demonstrates that ocean acidification conditions may induce different levels of stress which are not behaviorally manifested in this species, and which future consequences are still unknown.

Keywords: Ocean acidification; early life stages; U_{crit} ; Oxidative stress; sand-smelt

2.2 Introduction

Ocean acidification, as part of the global change condition, has become a strong phenomenon able to provoke direct changes on ocean biological systems and represents already a priority in the marine science research community (National Research Council, 2010). Mainly associated with rising concentration levels of CO₂, ocean acidification is an important driver of change in biological systems. The Intergovernmental Panel on Climate Change (IPCC) predicts that pCO₂ levels may reach a concentration nearly 1000 ppm by the end of 21st century (year 2100), which means a pH drop of approximately 0.3-0.4 units.

To date, most research on the impacts of ocean acidification has focused on calcifying marine organisms due to their calcium carbonate dependence (Atkinson & Cuet, 2008; Rost *et al.*, 2008; Vézina & Hoegh-Guldberg, 2008); however, the base of food webs, as well as other trophic levels and even top predators, can also be affected and at different life stages, but research on these is still scarce. The early life stages, in particular, are most likely to be affected by increasing pCO₂, due to their vulnerable physiological state, small body size and high sensitivity to environmental variation (Ishimatsu *et al.*, 2008). Recent research on acidification effects on fish larvae suggest significant disturbances across a wide range of sensory systems and neurological functions, like olfaction, vision, lateralization, hearing, learning, activity and boldness (Munday *et al.*, 2009c, 2010, 2012; Dixson *et al.*, 2010; Cripps *et al.*, 2011; Esbaugh *et al.*, 2012). Although the majority of impacts appear to be sublethal (Munday *et al.*, 2010; Ferrari *et al.*, 2012), these disturbances are presumed to have substantial impacts on predator-prey and social interactions, dispersal, habitat and settlement preference, recruitment, connectivity and population replenishment, leading to changes at higher levels of biological and ecological relevance. The pelagic larval period, although a vulnerable and susceptible stage, is ontogenetically and ecologically very important for dispersion in many species (Houde, 1997; Leis *et al.*, 2011). The replenishment and

connectivity processes are deeply correlated with recruitment potential, which is crucial for marine population's ecological functions. Changes in mortality and growth rates of larvae can easily influence the recruitment and, consequently, the population's stability (Cowen & Sponaugle, 2009). Therefore, our understanding of dispersal role on population and ecosystem-level impacts in ocean acidification conditions is fundamental. Models of dispersal, connectivity and ecological productivity are deeply dependent of behavior knowledge, particularly larval swimming abilities (Cowen, 2000; Paris & Cowen, 2004; Cowen *et al.*, 2006).

The impairment of locomotion, measured by swimming capacity, may strongly influence the ability to forage, avoid predators or unfavorable conditions, interact and survive, inducing changes in population fitness, up to the ecosystem level (Stobutzki & Bellwood, 1994; Webb, 1994; Leis, 2006; Fisher & Leis, 2010; Kolok, 1999; Plaut, 2001; Fisher *et al.*, 2005). There are several measures of swimming performance, of which critical speed (U_{crit}) is one of the most widely studied in larval fishes, providing useful information to determine and compare swimming abilities of different taxa and development stages. The U_{crit} is a measure of prolonged swimming that incorporate aerobic and anaerobic muscle activity (Brett, 1964; Reidy *et al.*, 2000) and is considered to be a useful measurement for estimating swimming ability in fishes, although it can never be achieved by fishes in nature for prolonged time and, therefore, is not directly applicable to field situations (Fisher & Leis, 2010). U_{crit} relates to the ability of the larvae to swim short distances at high speeds during short periods of time and it is a result of multiple swimming modes and metabolic support changes (Nelson *et al.*, 2012). The ability to swim is important for larvae to move between locations, habitats for settlement and also, at U_{crit} scale, to allow rapid vertical migration, which is important to achieve favorable currents to find prey (Fisher *et al.*, 2000). Swimming at U_{crit} speed is unsustainable over long periods of time (Fisher *et al.*, 2005) since for that larvae would be reallocating almost all of their aerobic scope (Post & Lee, 1996; Killen *et al.*, 2007) and therefore the oxygen delivery capacity left for biological processes associated with growth and homeostasis maintenance would most probably be insufficient (Fisher & Leis, 2010). Despite this, this measure of swimming ability is associated with other more ecologically relevant measures of swimming capacity, making the correlation of results possible (Plaut, 2001; Leis, 2006).

Organisms use most part of their energy for growth, reproduction and maintenance of their basal metabolism. The metabolic costs associated with the disruption in acid-base balance could result in a reallocation of metabolic resources to deal with the stress and, consequently, impact negatively the development and somatic growth of the animal (Matson

et al., 2012). The success of cellular defense mechanisms dealing with the stress is crucial to guarantee histological and physiological integrity as well the growth and reproductive capacity of the organisms. These responses at the sub-individual level, when indicative of a difference from the normal condition, can be considered and used as biomarkers of effect (Gestel & Brummelen, 1996). Innumerable biotic and abiotic factors can influence biomarker responses. Exposure to environmental stressors, for example, can induce dramatically the levels of Reactive Oxygen Species (ROS) leading to over accumulation and inability of the organism to reestablish balance. The failure of defense mechanisms to respond to these threats can lead to oxidative stress and, consequently, damage at cellular level including in proteins, DNA and lipids (Young & Woodside, 2001; De Jesus & Carvalho, 2008). The antioxidant enzymes are part of these defense mechanisms, playing a major role protecting or delaying the oxidative damage, peroxidation and enzymatic inactivation (Halliwell & Gutteridge, 1999; Novais *et al.*, 2014). The analysis of antioxidant activities during the defense process allows the indirect assessment of free radicals presence. Superoxide dismutase (SOD), catalase (CAT) and peroxides are examples of antioxidant enzymes able to keep superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) at non-dangerous levels in the intracellular fluid (Lesser, 2006). Other enzymes are related with energy metabolism. Some examples are the lactate dehydrogenase (LDH) that is associated with anaerobic metabolism – high levels of energy required and produced in short period, associated with stressful conditions (Ribeiro *et al.*, 1999; Diamantino *et al.*, 2001) and isocitrate dehydrogenase (IDH) related with aerobic metabolism – ATP production and oxidative stress defenses (Moreira *et al.*, 2006). The environmental variations are considered to imply high metabolic costs to guarantee the physiological homeostasis in organisms. The quantification of the energy required can be assessed by cellular energy allocation (CEA) methodology (De Coen & Janssen, 1997, 2003), providing information of energy reserves available (total carbohydrates, protein and lipids) and also the energy consumption by the measurement of the electron transport system activity (ETS) (Novais & Amorim, 2013). Thus, the energy budget of the organism could be assessed by the difference between energy available and energy consumption (De Coen & Janssen, 2003). The reallocation of metabolic resources to avoid/repair internal stress and damages may be challenging for larvae (Cunha *et al.*, 2007), costing severe consequences in particular in high energetically performances, such as the ones mentioned before. Increased metabolic rate was already correlated with size decreased in some acidification studies (Fielder *et al.*, 2005; Miller *et al.*, 2012) but also the opposite, associated with exposure of severe hypercapnia (Langenbuch & Pörtner, 2003). Survival of larval fishes may be deeply impacted

by changes in growth and development, which in turn could be affected by altered metabolic demand or reallocation of metabolic resources. The growth rate during the larval stage could also imply cascading effects to juvenile and adult phase (Searcy & Sponaugle, 2001; Hamilton, 2008). Also, changes in blood-oxygen delivery as a consequence of ocean acidification may decrease aerobic scope, which in turns reduce aerobic swimming ability (Pörtner *et al.*, 2004; Munday *et al.*, 2009c).

In this context, the aims of this study were to evaluate the effects of high CO₂ exposure on larval behavior, in particular critical swimming speed (U_{crit}), combined with morphometric measurements and analysis of biochemical biomarkers related with oxidative stress, oxidative damage and energy metabolism of an ecological and commercial important species, the sand-smelt larvae *Atherina presbyter* Cuvier 1829.

2.3 Methods

2.3.1 Seawater manipulations

Artificial seawater was used in the experiment, resulting from adding a commercial salt mixture (Tropic Marin®) to filter freshwater. CO₂ treatments were maintained by a CO₂ injection system with two pH controllers (Tunze Aquarientechnik, Germany) (medium and high treatment) or diffused with ambient air (control treatment). Each pH controller was attached to a sump of each treatment, with mechanical, biological, chemical and ultraviolet filtration. Each sump delivered equilibrated seawater into 2 35-L aquariums at ~600 ml min⁻¹.

Temperature, salinity and pH (on the National Bureau of Standards scale - NBS) of each aquarium were measured once a day by portable meter (SevenGo DuoPro, SG23) calibrated frequently with buffers (Mettler Toledo). Oxygen levels were above 90% saturation through the diffusion pumps on each sump. The resume of the parameters is represented in table 1.

Table 1 - Total alkalinity (TA), CO₂ partial pressure (pCO_2), pH, salinity (S), temperature (T) and respective standard-deviation in the three different treatments (Control, Medium and High).

	TA	pCO_2	pH	S	T
Control	2485,0 ± 58,29	690,91 ± 91,72	8,03 ± 0,06	34,5 ± 0,8	16,4 ± 0,5
Medium	2488,1 ± 63,69	1080,32 ± 49,57	7,85 ± 0,04	34,5 ± 0,8	16,4 ± 0,5
High	2654,3 ± 111,10	1854,61 ± 169,44	7,64 ± 0,05	34,5 ± 0,8	16,4 ± 0,5

2.3.2 Larvae and experimental setup

Shoals of *Atherina presbyter* larvae were collected at Portinho da Arrábida, Arrábida Marine Park (8° 28'48" N | 8° 58'59" W), Portugal, in July 2014, using a 1mm hand net. Larvae were immediately placed in a bucket with aeration and transported to the laboratory within the next 45 minutes. Organisms were placed in recirculating water aquariums, with temperature and salinity matching the field conditions, and with a 12h:12h (light: dark) photoperiod. The organisms were kept in these conditions for the first two days to recover from handling and transport stress associated. After this period, larvae were randomly assigned to the different pH treatments and reared for 7-15 days, after which they were used for behavioral and morphometric endpoints and further frozen for biochemical analyses. During the experimental period, larvae were daily fed with *Artemia* nauplii *ad libitum* with exception of the acclimation period and the U_{crit} test day to avoid some potential influence on performance.

2.3.3 U_{crit} & Morphometric Analysis

The U_{crit} swimming chamber was used following the protocols of Stobutzki & Bellwood (1994, 1997) and adapted from Faria et al. (2009). The chamber was composed by 6 parallel lanes, each with 30 mm wide 50 mm high and 180 mm long and a removable lid to allow the introduction and removal of fishes. On the top of the lid, a black strip tape functioned as visual reference in order to help the fish maintaining position. For larval retention, a mesh screen was placed upstream and downstream of each lane. The turbulence was minimized by placing a section of flow straighteners, with 40 mm long, between the chamber and the mesh screen of each lane. Previous work demonstrated that water velocity did not differ between the center of the lane and the walls (Stobutzki & Bellwood 1997; Fisher *et al.*, 2000). Linked to the swimming chamber was a submersible pump (Ecovert 510, 330 W) that collected and moved water from a tank to the chamber, which flowed out again to the tank. The velocity of this closed system water flow was controlled by a ball valve located upstream of the system. A protractor marker was added to the valve allowing marking the angles of the handle to calibrate the flow rates. The calibration was achieved by recording the time taken to fill a 5 L container and dividing it by the cross-sectional area and number of lanes. The average of the three calibrations was used as the flow speed for a determinant valve angle. A calibration curve was then made, with the angle as the predictor and the water velocity as the dependent variable, to allow the determination of the angles for the respective

velocity needed. The flow speeds of the experiment ranged from 1.5 cm s⁻¹ to approximately 18 cm s⁻¹.

For the U_{crit} measurement, water velocity was increased 1.5cm s⁻¹ every 2 minutes until larvae could no longer swim against the current, adjusting to methods from Faria & Gonçalves, 2010. Calculation of U_{crit} followed Brett (1964): $U_{crit} = U + (t/t_i * U_i)$, where U is the penultimate speed that a fish could maintain, U_i is the velocity increment, t is the time swam in the final velocity increment and t_i is the time interval for each velocity increment (2 minutes).

At the test day, larvae were randomly selected across sizes (between 6mm and 21mm total length, L_T), placed on the swimming chamber, one per lane, and acclimated at a flow speed of 1.5cm s⁻¹ for 5 minutes. Larvae from all treatments were tested under control (ambient) water conditions. Water conditions in the swim chamber have not been shown to affect swimming ability in other species (Munday *et al.*, 2009b). Individuals with symptoms of stress during this acclimation period were removed from the chamber and replaced.

After the test, individuals were immediately placed on a plastic ice cold keeper to help slow metabolism and photographed under a dissecting microscope for later measurements and classification according to developmental stage (flexion or post-flexion), using Image J software (version 1.48; <http://imagej.nih.gov/ij/>). For each larva the following morphometric measures were taken (Figure 1): Standard length (SL), Total length (TL), Anal Height (HA), Body Depth (BD), Head Length (HL), Dorsal Height (HD) and Caudal Peduncle (CP). In total, 16 individuals in flexion and 40 in post-flexion stage, per treatment, were tested. Each individual was weighted and transferred to a microtube to be stored at -80°C until biomarker analysis.

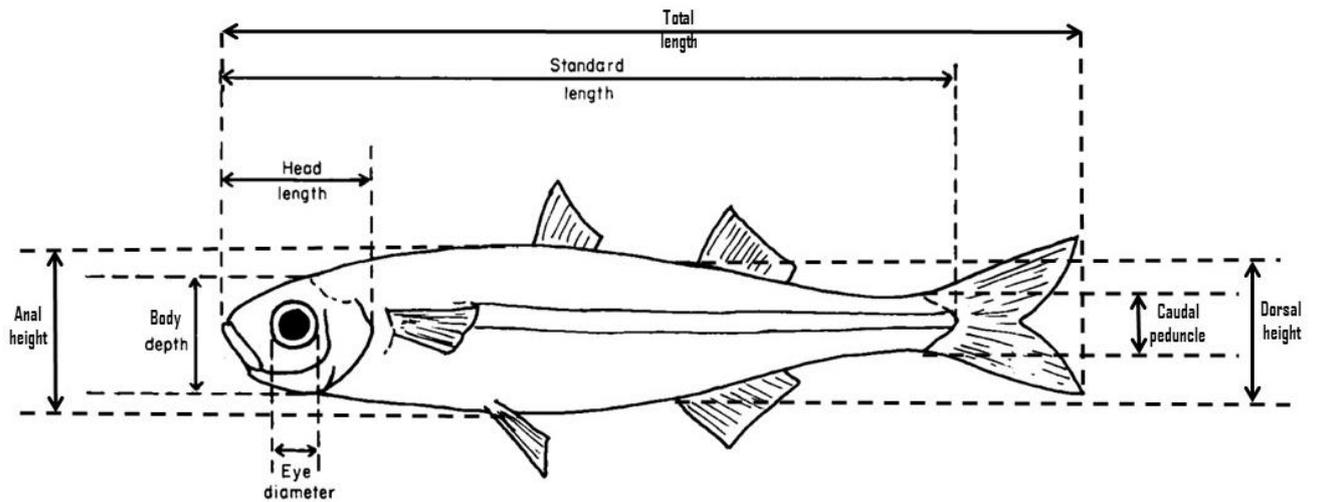


Figure 1 - Morphometric measurements of *Atherina presbyter* larvae: Total Length (TL), Standard Length (SL), Anal Height (HA), Body Depth (BD), Head Length (HL), Dorsal Height (HD), Caudal Peduncle (CP). (Adapted from Bamber & Henderson, 1985).

2.3.4 Biomarker Analysis

Each organism from the post-flexion stage, used as a single replicate, was homogenized in 1mL of potassium-phosphate buffer (0.1M, pH 7.4), using an electrical homogenizer. Part of the homogenate was separated into different microtubes to further measure: protein concentration, ETS activity and levels of lipid peroxidation (LPO), DNA damage and total carbohydrates (CBH). The rest of the homogenate was then centrifuged for 20 minutes, at 10000g (4°C), to obtain the post-mitochondrial supernatant (PMS). The PMS was used for the evaluation of SOD and CAT activities. Twenty replicates per treatment were used for these measurements. To have more concentrated samples, a different set of organisms was used for LDH and IDH activity measurements. These organisms were homogenized in 300µl of potassium-phosphate buffer (0.1M, pH 7.4) and 5 replicates per treatment were used. To quantify superoxide anion (O_2^-) levels, as a measure of ROS, 10 organisms per treatment were weighed and used for this purpose.

For flexion stage individuals, sample preparation was similar, although it was only possible to determine three biomarkers (SOD, CAT and LDH), due to the lower sizes of these organisms and therefore, low biomass available. Each replicate for this developmental stage organisms consisted in a pools of 2 individuals and a total of 8 replicates per treatment were analyzed.

All enzymatic activities and levels of mentioned parameters were determined in quadruplicate using a Synergy H1 Hybrid Multi- Mode Microplate Reader (BioTek

Instruments, Vermont, USA). In all assays, potassium -phosphate buffer (0.1M, pH 7.4) was used as blank.

For total protein quantification of each replicate homogenate and PMS fraction, bovine γ -globulin (BGG, Sigma-Aldrich, USA) was used as standard protein following the Bradford method, using 96 well flat bottom plate (Bradford, 1976). Absorbance was read at 600 nm and results expressed in mg of protein/mL.

2.3.4.1 Oxidative Stress biomarkers

SOD activity was measured following McCord and Fridovich (1969) method, adapted to microplate (Lima et al., 2007). This procedure uses the reaction between cytochrome c and superoxide radicals generated by the complex xanthine-xanthine oxidase, resulting in a reduction of cytochrome that can be measured by reading the absorbance at 550 nm for 10 minutes. SOD activity was expressed in $\text{U}\cdot\text{mg}^{-1}$ of protein using a SOD standard of $1.5 \text{ U}\cdot\text{ml}^{-1}$, where 1U represents the amount of enzyme in the sample that causes 50% inhibition of cytochrome C reduction.

CAT activity measurements were based on the consumption of substrate (H_2O_2), optimizing the protocol of Clairborne (1985). The decrease in the substrate was followed at 240 nm for 1 minute. CAT activity was expressed in $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ of protein, using a molar extinction coefficient of $40 \text{ M}^{-1}\text{cm}^{-1}$. The determination of O_2^- levels, as ROS, was conducted by following the method of Drossos *et al.*, (1995). The presence of O_2^- was determined by the capacity of the radicals present to reduce cytochrome C, which can be measured at 550 nm. Using an extinction coefficient of $19000 \text{ M}^{-1}\text{cm}^{-1}$, the amount of O_2^- produced was calculated and expressed in $\text{nmol O}_2^- \cdot \text{mg protein}^{-1}$.

The LPO levels were determined by measuring the content of thiobarbituric acid reactive substances (TBARS), following Ohkawa *et al.*, (1979) and Bird & Draper (1984). After the reaction with TBA (2-Thiobarbituric acid), absorbance was read at 535 nm and LPO levels expressed as $\text{nmol TBARS}\cdot\text{mg of protein}^{-1}$, using a molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$.

DNA damage (strand breaks) was evaluated following the DNA alkaline precipitation assay (Olive, 1988) adapted from Lafontaine *et al.*, (2000). After the precipitation of single and double stranded damaged DNA, the nucleic acids were coupled with Hoesch dye ($1 \mu\text{g}/\text{mL}$ bis-benzimide, Sigma-Aldrich) and the levels of damaged DNA were determined by

measuring fluorescence using an excitation/emission wavelength of 360/460 nm. Results were expressed as mg of DNA.mg of protein⁻¹, using calf thymus DNA as standard to extrapolate DNA concentration.

2.3.4.2 Energy metabolism related biomarkers

The determination of LDH activity was assessed using the method described and adapted by Vassault (1983) and Diamantino *et al.*, (2001), respectively. LDH activity was measured by the efficiency of this enzyme to convert pyruvate to lactate in the presence of NADH, which results in NADH oxidation and consequent decrease in absorbance. The absorbance was read at 340 nm for 5 minutes. Results were expressed as nmol.min⁻¹.mg protein⁻¹, using a molar extinction coefficient of $6.3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

The IDH activity was measured following Ellis and Goldberg (1971) with the adaptations of Lima *et al.*, (2007). The decarboxylation of isocitrate by IDH promotes the conversion of NADP⁺ to NADPH. The activity of IDH is then determined with the increase in NADPH measured at 340 nm for 3 minutes. Results were calculated according to a molar extinction coefficient of $6.22 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ and expressed as nmol.min⁻¹.mg protein⁻¹.

The total carbohydrates content was measured at 492nm according to De Coen & Janssen (1997), using a reaction of 5% phenol with H₂SO₄ (95-97%), with glucose as standard solution.

ETS activity in the mitochondria is a measurement of the cellular energy consumed (oxygen consumption rate) and can be determined following the method described by De Coen & Janssen (1997). NADPH and INT (*p* iodo-nitro-tetrazolium) solution were mixed with samples and absorbance was read at 490 nm over a 3 minutes period. The oxygen consumption was then calculated using a stoichiometrical relationship: 2 μmol of formazan formed = 1 μmol of oxygen consumed. The oxygen consumption rate was then converted into the energetic equivalent of 484kJ/mol O₂ for average carbohydrate, lipid, and protein consumption combinations (Gnaiger, 1983).

2.3.5. Statistical analysis

The influence of TL, treatment and development stage on U_{crit} swimming speed was addressed by analysis of covariance (ANCOVA), using treatment and development stage as factors and TL as a covariable. Least Significant Difference (LSD) tests for multiple

comparisons of group means were employed to determine differences between treatments and development stages (Zar, 2009).

All data were checked for normality and homoscedasticity. For morphometric and biomarkers analysis, one-way analysis of variance (ANOVA) with Holm-Sidak post-hoc test for multiple comparisons of group means was employed to determine significant differences between treatments (Zar, 2009). Kruskal-Wallis was applied when those requisites were not validated, followed by Dunn's post-hoc test for comparison between treatments. U_{crit} and morphometric parameters were evaluated using multiple linear regression analysis. The relationship between parameters was analyzed using Pearson's correlation (Zar, 2009).

When applicable, results are presented as mean \pm standard-deviation (SD). For all statistical tests, the significance level was set at $p\text{-value} \leq 0.05$. All calculations were performed using Sigma Plot software, version 11.0 and IBM SPSS Statistics, version 22.0.

2.4 Results

2.4.1. U_{crit} and morphometric analysis

The behavior endpoint analysis at different developmental stages, show the relationship between U_{crit} and total length of larvae (Figure 2). Although there were no statistically significant differences in the U_{crit} behavior after exposure to acidified conditions at any stage, some tendencies can be observed.

In the control treatment (blue line) it is possible to observe an increased swimming performance, which occurs at a faster rate during the flexion stage, after which the increase of speed tends to become stable (Figure 2).

In the medium (green line) and high $p\text{CO}_2$ treatments (red line), and at flexion stage, larvae appear to have higher critical swimming speed in comparison to larvae reared in control conditions, which is more evident for the smaller larvae in the high $p\text{CO}_2$ treatment (Figure 2). For post-flexion larvae, the U_{crit} values become closer among treatments.

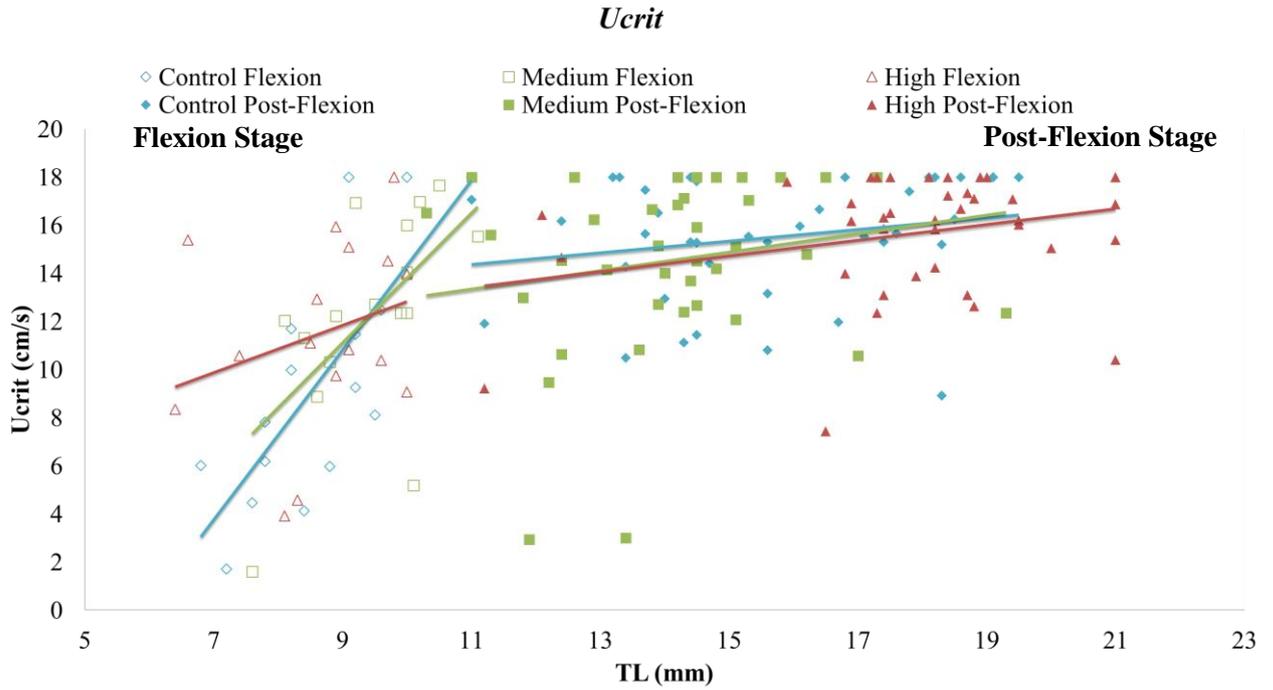


Figure 2 - U_{crit} and total length (TL) relationship at both developmental stages (Flexion and Post-Flexion) of *Atherina presbyter* larvae in different acidification treatments. Control treatment is represented in blue (\diamond and \blacklozenge) ($pH=8.03 \pm 0.06$), medium treatment is represented in green (\square and \blacksquare) ($pH=7.85 \pm 0.04$) and high treatment is represented in red (\triangle and \blacktriangle) ($pH=7.64 \pm 0.05$). No statistically significant differences were found (ANCOVA, p -value >0.05).

The morphometric analysis revealed significant differences between CO_2 treatments for some of the parameters, only in post-flexion larvae (Figure 3). Overall, it is possible to observe that the majority of the parameters present differences between treatments. Moreover, in all morphometric parameters analyzed, except for HA, the organisms from the medium treatment presented significantly lower values compared with organisms from both control and higher treatments (Figure 3).

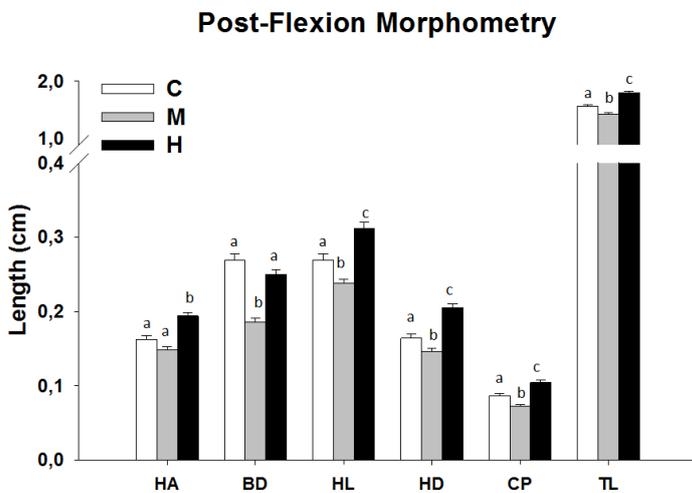


Figure 3 - Morphometric results in post-flexion stage of *Atherina presbyter* larvae in different acidification treatments. Results are expressed as mean + standard deviation. The three different treatments - Control (C, $pH=8.03 \pm 0.06$),

Medium (M, pH=7.85 ± 0.04) and High (H, pH=7.64 ± 0.05) are represented in white, gray and black bars, respectively. ^{a, b, c} Statistically significant differences between C, M and H treatments for each individual parameter (Holm-Sidak/ Dunn's, p-value<0.05). HA = Anal Height; BD = Body Depth; HL = Head Length; HD = Dorsal Height; CP = Caudal Peduncle; TL = Total Length.

The general results show that 35% of U_{crit} 's variability could be explained by TL and HL ($yU_{crit} = 3.871 + 16.686 TL - 28.104 HL$, $r=0.593$, $p<0.05$), while 65% of the U_{crit} variability is explained by other factors. For the global post-flexion morphometric results the medium treatment showed a significant relation with TL, HA, BD and CP. Thus 43% of U_{crit} 's variability can be explained by the variance of those parameters ($yU_{crit} = -6.18 + 32.461 TL + 170.289 HA - 241.510 CP - 152.158 BD$, $r=0.660$, $p<0.05$).

2.4.2 Biomarker Analysis

2.4.2.1 Flexion Stage

The results showed that no statistically significant differences were detected between acidification treatments for any of the analyzed biomarkers at the flexion stage organisms (Figure 4).

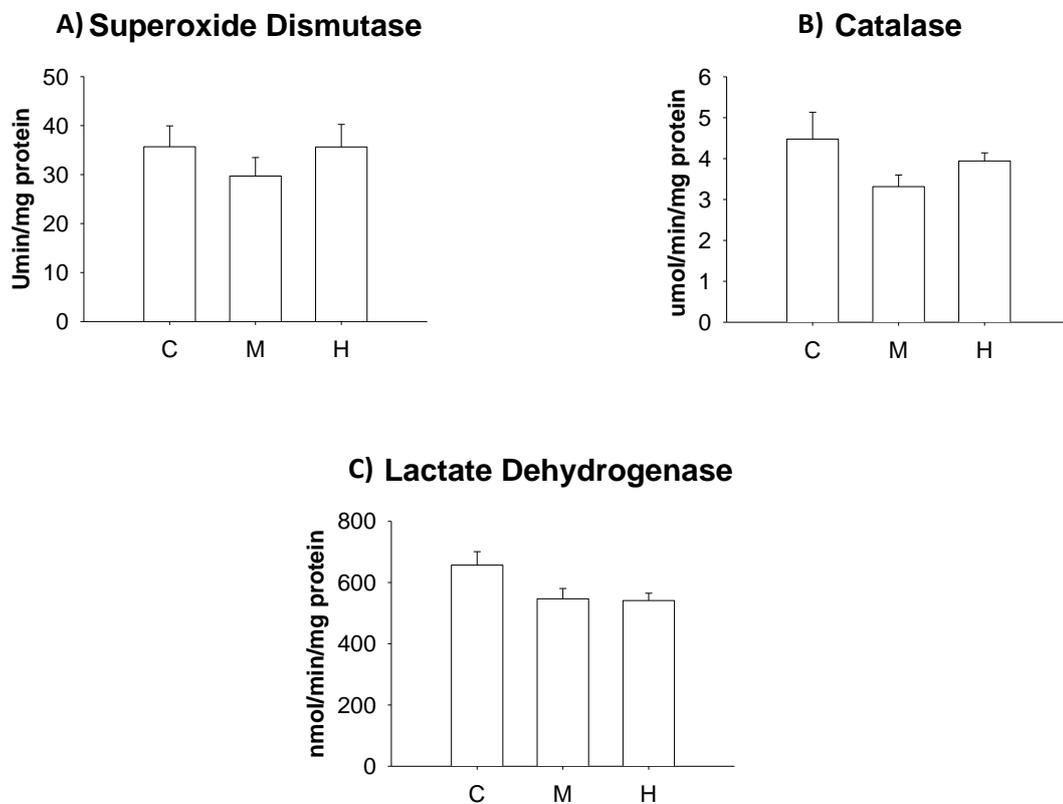


Figure 4 - Enzymatic activities of (A) superoxide dismutase, (B) catalase and (C) lactate dehydrogenase in flexion stage of *Atherina presbyter* larvae in different acidification treatments: control (C, pH=8.03 ± 0.06), medium (M, pH=7.85 ± 0.04) and high (H, pH=7.64 ± 0.05). Results express mean + standard deviation.

2.4.2.2 Post-Flexion Stage

Oxidative Stress biomarkers

The oxidative stress biomarkers measured in organisms at post-flexion stage revealed statistically significant differences between acidification treatments (Figure 5).

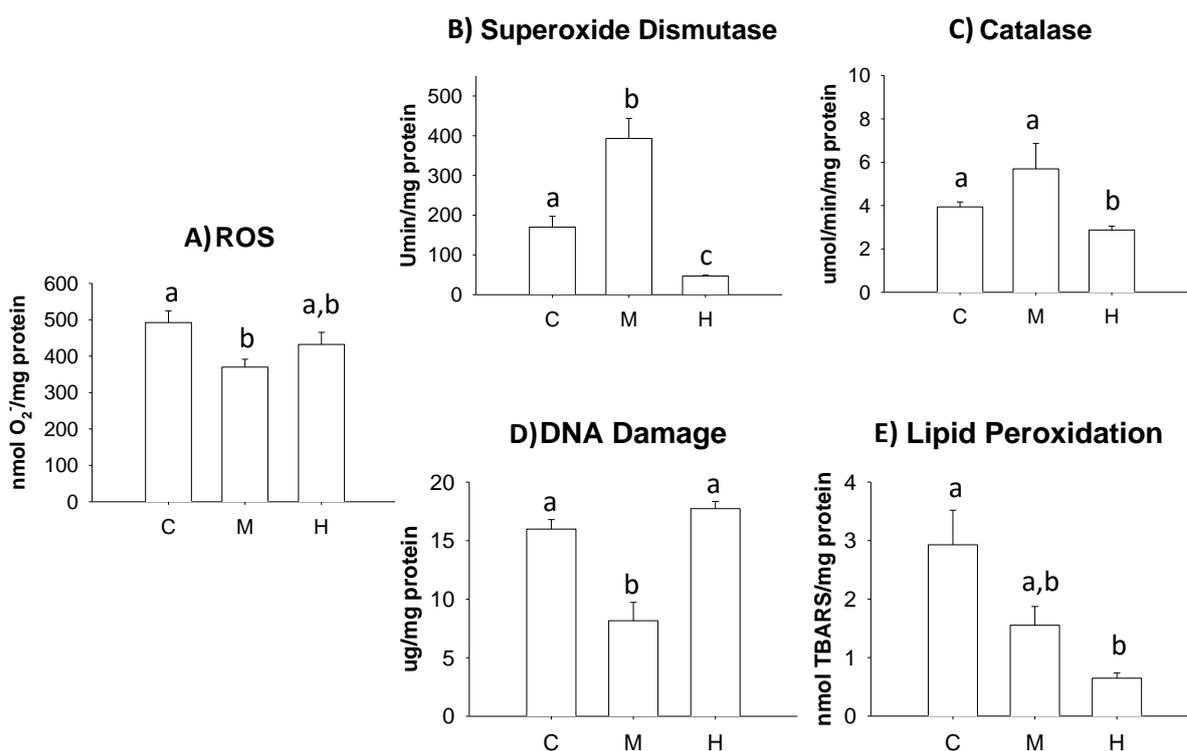


Figure 5 - Oxidative stress related parameters measured in post-flexion stage of *Atherina presbyter* larvae in different acidification treatments: control (C, pH=8.03 ± 0.06), medium (M, pH=7.85 ± 0.04) and high (H, pH=7.64 ± 0.05). (A) Superoxide anion (ROS) levels; (B) superoxide dismutase activity; (C) catalase activity; (D) DNA damage levels; (E) lipid peroxidation levels. Results are expressed as mean+standard deviation. ^{a, b, c} Statistically significant differences between C, M and H treatments for each individual parameter (Holm-Sidak/ Dunn's, p-value<0.05).

The levels of ROS, measured by means of superoxide anion production, were significantly lower in organisms exposed to the medium treatment, compared to larvae reared in control conditions (Figure 5-A). In this same medium treatment, it is possible to observe a significantly increase in SOD activity (Figure 5-B) as well as a significant decrease in the levels of DNA damage (Figure 5-D).

Regarding the high treatment, there was a significant reduction in the enzymatic activity of SOD (Figure 5-B) and CAT (Figure 5-C). Nonetheless, and although no

differences were observed in DNA damage levels compared to control conditions, the LPO values were significantly lower in the high treatment (Figure 5-E).

Energy metabolism biomarkers

The results of the measured biochemical parameters related with energy metabolism are shown in figure 6. At high $p\text{CO}_2$ levels there was a significant increase in LDH (Figure 6-A) and ETS (Figure 6-C) activity values.

No effects on IDH activity or on carbohydrates levels were observed with the acidification treatments (Figure 6-B, D).

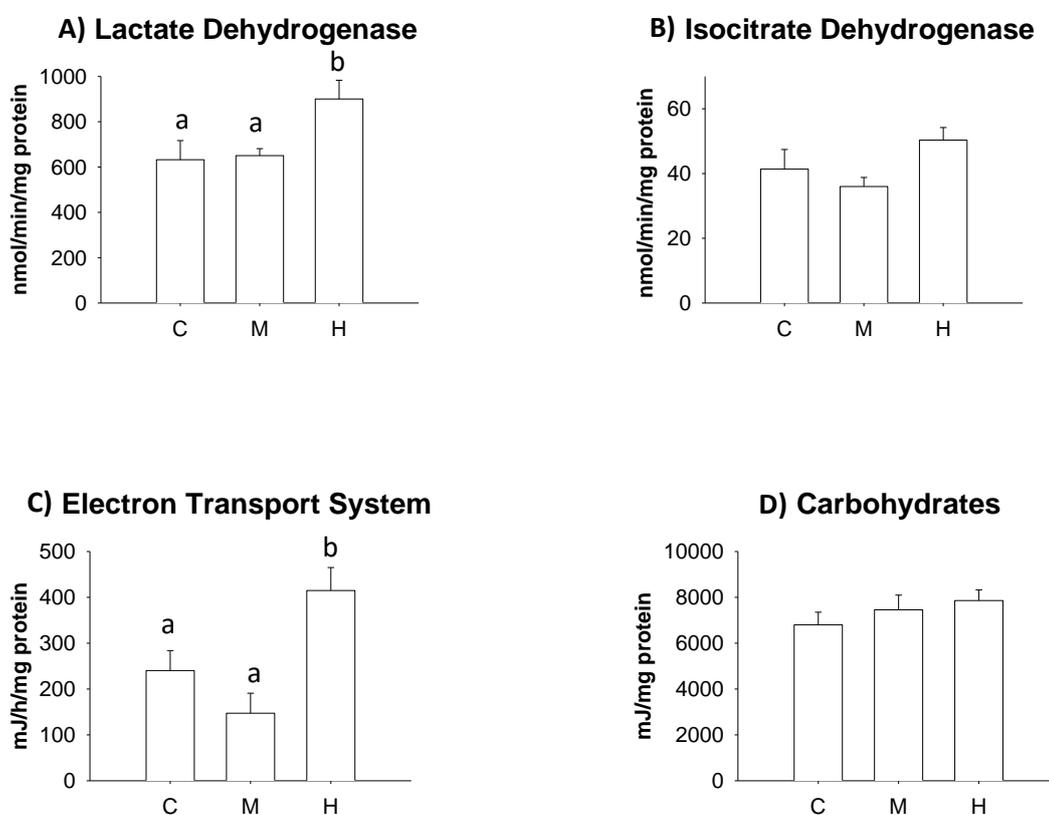


Figure 6 - Energy metabolism related parameters measured in post-flexion stage of *Atherina presbyter* larvae in different acidification treatments: control (C, $\text{pH}=8.03 \pm 0.06$), medium (M, $\text{pH}=7.85 \pm 0.04$) and high (H, $\text{pH}=7.64 \pm 0.05$). (A) Lactate dehydrogenase activity; (B) Isocitrate dehydrogenase activity; (C) Electron transport system activity; (D) Total carbohydrates content. Results are expressed as mean \pm standard deviation. ^{a, b} Statistically significant differences between C, M and H treatments for each individual parameter (Holm-Sidak, $p\text{-value}<0.05$).

2.4.3 Correlation between biochemical parameters and behavior

The following tables show the significant results after the Pearson correlation analysis performed between the U_{crit} and the biochemical biomarkers, analyzed for flexion (table 2) and post-flexion (table 3) larvae.

In flexion stage larvae there was a positive correlation between SOD and LDH ($r=0.51$, table 2), meaning that the higher the SOD activity, the higher the LDH activity and vice-versa.

Table 2 - U_{crit} and biomarkers Pearson's correlation analysis in flexion stage of *Atherina presbyter* larvae. Only the significant correlation coefficients ($p < 0.05$) are shown.

Flexion	U_{crit}	SOD	CAT	LDH
U_{crit}	1	-	-	-
SOD		1	-	0,51
CAT			1	-
LDH				1

In post-flexion larvae (table 3), the strongest correlation found between U_{crit} 's performance and biochemical biomarkers was a negative correlation with SOD ($r=-0.443$), meaning that the higher the U_{crit} , the lower the SOD and vice-versa (table 3). The other antioxidant enzyme (CAT) presented the same correlation response related with U_{crit} ($r=-0.314$, table 3). SOD had therefore a positive correlation with CAT ($r=0.36$, table 3) but a negative correlation with ROS ($r=-0.397$, table 3) and DNA damage ($r=-0.624$, table 3). From the energy metabolism related endpoints, it is possible to observe a positive correlation between ETS and LDH ($r=0.327$, table 3) and between ETS and carbohydrate levels ($r=0.669$, table 3).

Table 3 - U_{crit} and biomarkers Pearson's correlation analysis in post-flexion stage of *Atherina presbyter* larvae. Only the significant correlation coefficients are shown. * $p<0.05$; ** $p<0.01$.

Post-Flexion	U_{crit}	ROS	SOD	CAT	LPO	DNA	LDH	IDH	CBH	ETS
U_{crit}	1	-	-0,443**	-0,314*	-	-	-	-	0,288*	-
ROS		1	-0,397**	-	-	-	-	-	-	-
SOD			1	0,36**	-	-0,624**	-0,33*	-0,322*	-0,567**	-0,444**
CAT				1	0,27*	-	-	-	-0,325*	-0,429**
LPO					1	-	-	-	-	-
DNA						1	-	0,389**	0,462**	-
LDH							1	0,672**	0,408**	0,327**
IDH								1	-	-
CBH									1	0,669**
ETS										1

2.5 Discussion

The U_{crit} increased with the size of the larvae, particularly in flexion stage, ranging from an average of 1.72 to 18.0 cm s⁻¹ over a TL range from 6.6 to 21.0 mm. Comparing with results already obtained for the same species in non-acidification scenarios, it is possible to observe that within the same range of size, the maximum and minimum U_{crit} values obtained were lower than the reported (3.6 to 18.7 cm s⁻¹) (Faria *et al.*, 2014). The variation of critical speed observed at any size was also found in different no-acidification studies (Fisher *et al.*, 2005; Faria *et al.*, 2009, 2014; Faria & Gonçalves, 2010). The U_{crit} values already registered for this species, even the ones in the present study, are within the range reported for temperate and warm temperate (Faria *et al.*, 2009; Faria & Gonçalves, 2010; Leis *et al.*, 2012) perciform species which ontogeny is known, indicating that *Atherina presbyter* are among the fastest (Faria *et al.*, 2014). However, *Atherina presbyter* as a temperate species may be considered a poor swimmer if its U_{crit} values are compared with tropical reef species (6 to 100 cm s⁻¹) (Stobutzki & Bellwood 1997; Leis, 2006).

Although no statistical effects of acidification were observed in the U_{crit} , the results show some tendency for an increase in critical swimming velocity in the medium and high treatments in comparison with control at flexion stage, mainly in the smaller organisms (Figure 2). The larvae also tended to swim more and have higher velocities in these treatments at flexion than in post-flexion stage when compared to the respective controls. At a certain point, in the post-flexion stage, it is also possible to observe a graphic “plateau”, meaning a no longer exponential increasing in velocity. This could be due to the total formation of structures and complete body homeostasis that allows the organisms to achieve and stabilize higher levels of swimming velocity. As already mentioned, the increase of U_{crit} values with the ontogeny was already reported in other studies and may be associated with changes in ecology, morphology and hydrodynamics (Faria *et al.*, 2009). The early phase of rapid improvement (rapid increase of U_{crit} values) could be correlated with the pelagic - pre-settlement stage, also characterized by radical morphologic changes, compared with the later phase of slower improvement that may be associated with post-settlement stage (stabilization of U_{crit} values) (Faria *et al.*, 2009). The lack of statistical effects of ocean acidification on swimming ability was already reported in some studies, suggesting that this process may not change the swimming activity of larvae of different species, such as atlantic cod *Gadus morhua* (Melzner *et al.*, 2009; Maneja *et al.*, 2013), mahi-mahi *Coryphaena hippurus* (Bignami *et al.*, 2014), cobia *Rachycentron canadum* (Bignami *et al.*, 2013) and atlantic

herring *Clupea harengus* (Maneja *et al.*, 2015). The majority of these studies reported similar results where a decrease in pH revealed some morphologic but not swimming impacts on the larval individuals. However, other studies with different organisms revealed that ocean acidification does have an effect on their behavior (e.g. Janssen & Randall, 1975; Kikkawa *et al.*, 2003, 2004; Ishimatsu *et al.*, 2004, 2008; Munday *et al.*, 2009a; Dixson *et al.*, 2010). Species with a wide range of habitats, especially if including some with natural pH fluctuations, could have a particularly higher resistance to acidification in comparison to those exposed to fewer variations (Pörtner *et al.*, 2004; Munday *et al.*, 2008; Matson *et al.*, 2012; Bignami, 2013; Bignami & Sponaugle, 2014). Even for a single species, distinct results may be correlated with physiological and/or geographical differences, seen for example in species that can be found within and outside the intense upwelling regions (Matson *et al.*, 2012).

At flexion stage, besides the behavior, there were also no statistically significant differences detected in morphometric or biochemical endpoints (Figures 3 and 8). This can probably be partially explained by the limited number of replicates available at this development stage and the small size of the individuals, which also limited the amount of biochemical endpoints tested.

Although no differences were observed in the flexion stage, the pattern observed with the present morphometric results in the post-flexion stage reveals a tendency for smaller lengths in medium treatment and larger ones in high treatment (Figure 3). Also, the results from the multiple linear regression analysis showed that the U_{crit} 's variability couldn't be explained by morphometric parameters in flexion stage. However, almost half (43%) of U_{crit} 's variability could be explained by four morphometric parameters (TL, HA, BD and CP) in the medium treatment at post-flexion stage. Although heterogeneous growth in fish is reported to be a common phenomenon, the mechanisms associated are still not clearly understood (De Oliveira Fernandes & Volpato, 1993). The reduction in somatic growth detected at medium treatment may be associated with the high costs of internal homeostasis maintenance, functioning as an indicative of sub lethal physiological effects (Pörtner *et al.*, 2004; Fabry *et al.*, 2008; Amiard-Triquet, 2009; Timmins-Schiffmann *et al.*, 2014). Similar results are also reported in other studies with shrimps *Pandalus borealis* and mussels *Mytilus edulis* (Bechmann *et al.*, 2011), sea urchins *Strongylocentrotus droebachiensis* (Chan *et al.*, 2015) and mahi-mahi fish *Coryphaena hippurus* (Bignami *et al.*, 2014). The reduction in protein synthesis can restrict the natural performance of expensive metabolic activities such as growth and reproduction (Fabry *et al.*, 2008), which can be evaluated by RNA/DNA ratio (Bulow, 1970; Olivar *et al.*, 2009) an efficient tool that could be used, although a decrease in the ratio

may not always be correlated with a reduction in fish size (Maneja *et al.*, 2014). A smaller body size was similarly observed in other studies for larvae developed under high $p\text{CO}_2$ even with an undetectable increase of energy resources use and the authors conclude that larvae may be prioritizing energy for physiological functions associated with increased costs or that reduced body size could be associated with higher costs of growth under ocean acidification exposure (Brauner, 2008; Matson *et al.*, 2012).

However, in the high $p\text{CO}_2$ treatment, in contrast with the medium, individuals presented larger sizes, which may be possible associated with the metabolic compensation of elevated $p\text{CO}_2$ conditions, similar with the already obtained in other study with mahi-mahi fish *Coryphaena hippurus* (Bignami *et al.*, 2014). The present results are also in accordance with the study by Munday *et al.*, (2009b) where increased size of larval fish under acidified conditions was reported, suggesting that this process could be related with “increased energy intake or decreased energy expenditure”. Some authors defend that in these laboratory experiments, the costs of acid-base balance could be compensated or even over compensated by the higher amounts of prey provided in captivity – resulting in higher levels of energy intake, in comparison with the expected in natural habitat, which is something already demonstrated with mussels (Thomsen *et al.*, 2013; Stumpp *et al.*, 2011). The present results in the high treatment seem to be in agreement with the assumptions of higher energy intake since in this case there was not a decrease in energy expenditure but clearly the opposite, seen by the elevated ETS and LDH activity levels (figure 6-A, C). In fact, ETS and LDH activities were positively correlated (Table 3, Pearson’s correlation, $r=0.327$, $p<0.05$) and significantly elevated in this treatment, showing that the organisms had higher energy requirements that were probably being allocated to growth, since they presented larger sizes and no effects on behavior were observed. Therefore, given this high energy consumption (with no decreases in the carbohydrate levels – figure 6D), the larger sizes of the organisms, and the fact that all larvae from the different treatments were given the same amount of *artemia* each day, it can be hypothesized that in the high $p\text{CO}_2$ treatment the organisms are in fact feeding more than in the other treatments as a response to this stress.

The oxidative stress analyses related to high treatment may also support the hypothesis of a change in energy allocation once both enzymatic levels (SOD and CAT) presented lower values in comparison with control and medium treatments (Figure 5-B, C). Moreover, this behavior pattern is not surprising once high levels of stress are known to increase free radicals (superoxide anion and others) that can cause enzymatic inactivation. A study with flatfish larvae *Solea senegalensis*, associating hypercapnia with present (18°C) and warmer

temperatures (+4°C) reported similar results related with the apparent absence of CAT activity (Pimentel *et al.*, 2015). The authors concluded that these results possible indicate that early life stages do not possess a fully developed antioxidant defense system, which consequences in this case were translated into decreased growth, survival and an increase in skeletal deformities (Pimentel *et al.*, 2015). Although oxidative stress is widely associated with formation and accumulation of LPO (Wdziczak *et al.*, 1982; Filho *et al.*, 1993; Gutteridge, 1995; Negre-Salvayre *et al.*, 2010 Vinagre *et al.*, 2012; Cabecinhas *et al.*, 2014; Fonseca *et al.*, 2014), the obtained results show the opposite (Figure 5-E). Some authors have demonstrated that elevated CO₂ scenarios can alter the relative proportions of fatty acids in fish larvae and oysters (Díaz-Gil *et al.*, 2014; Timmins-Schiffman *et al.*, 2014). In the same acidification study, Timmins-Schiffman *et al.*, (2014) also detected the presence of lower levels of highly unsaturated fatty acids, which are very sensitive to oxidative stress damage. This lower presence may not only protect cell molecules but also cellular membranes from damage caused by ROS (Pamplona *et al.*, 2002), which may also explain the progressively lower LPO levels from control to higher pCO₂ treatment in the present study (figure 5E).

In contrast to the high treatment, in the medium treatment higher activities of SOD were observed, along with lower levels of ROS and DNA damage (Figure 5-A, B, D). SOD acts to convert and decrease superoxide anion levels within the cell, which explains the negative correlation between SOD and ROS (Table 3, Pearson's correlation, $r=-0.397$, $p<0.05$), meaning that the higher the SOD the lower the ROS levels. This elevated SOD activity may also have presented oxidative damage on DNA as can be seen by the significant lower levels of DNA damage and the negative correlation between both endpoints (Table 3, Pearson's correlation, $r=-0.624$, $p<0.05$). These energy requirements for defense mechanisms might result in less energy available for other important functions so less scope for growth. Thus, this may contribute to explain the smaller growth sizes of the organisms in medium treatment.

Changes in size induced by ocean acidification exposure may have not affected swimming behavior of the larvae in the present study but may compromise it in longer and under more realistic exposure scenarios with a wide range of complex stressors. Moreover, the sensitivity of some biomarkers to changes in environmental parameters that may be associated with ocean acidification, such as the temperature, may result in complex responses, especially when outside the optimum interval for each species (Vinagre *et al.*, 2012). Thus, in order to assess a better perception of the global effects of ocean acidification stressors on the organisms it is important to combine not only different types of stressors similar to what is

expected in the wild, but also methodologies and parameters linked with different biological organization levels.

Larval *Atherina presbyter* demonstrated some plasticity in the face of decreased pH conditions, particularly at the earlier flexion stages. The absence of statistical significant results in swimming behavior could be due to some important factors, as the stage and the period of exposure to stressful conditions, or the physiological resistance and plasticity levels to environmental variations. Nonetheless, the biochemical endpoints demonstrated that individuals are going through some changes in their metabolism to respond to the acidification stress. With these sub-cellular biomarkers results it is possible to conclude that the medium and high treatment have some negative impacts on homeostasis maintenance of larvae. Also, increased enzymatic antioxidant responses were observable only in medium treatment with significant inhibitions verified for the high treatment, which suggests that at lower pH the larvae capacity to compensate the environmental changes at internal inner may be exceed.

Chapter 3

General Conclusions

Concluding, with the prediction of more acidic waters in the near future, this work shows how resilient this species appear to be to ocean acidification phenomenon alone. It also emphasizes the importance of using different methodologies and applying endpoints from different levels of biological organization to allow a better perception and knowledge of the effects of stressors as a whole. Moreover, while the early life stages of this species demonstrated capacity to maintain the swimming performance under ocean acidification conditions, it is important to notice that, in climate change scenarios, different stressors are expected to be acting together in nature and this may have different functional impacts and ecological consequences. Also, the phenotypic and genetic plasticity necessary to cope with ocean acidification conditions may already be present in some individuals or populations nowadays, which may be the foundation for future population and community studies in different conditions.

Some ocean acidification effects may not manifest during the early life stages but can accumulate over long term impacting other endpoints like reproduction. Also, one important and rarely mentioned parameter in fish ocean acidification studies is the period limits of abrupt and short term $p\text{CO}_2$ exposure to assess a better knowledge in the adaptive capacity of the species. For future studies it is important to, not only include different methodologies and tools, but also combine different stressors, since the combined impact may overwhelm even robust and resilient species. So, although this study gives a first indication on the responses of these larvae to different acidification levels, there are some important factors that this work did not cover but it would be important to evaluate in the future like cumulative effects of long-term exposure to acidification, including transgenerational acclimation to acidified conditions, potential effects on reproduction, reversible effects/responses at changing exposures and also the combined effects of simultaneous stressors, like synergistic ones such as temperature and hypoxia, that could be experienced in nature as a result of climate change. The temperature increase is one of the ocean acidification associated phenomenon, proving in other studies to be a parameter which increases could generate greater and also contrary effects on metabolism than increases in $p\text{CO}_2$.

Some tools at the cellular, subcellular and molecular levels – including genomic, transcriptomic and proteomic processes – can provide more sensitive information that can be transposed to higher-level effects. Besides, the cellular and subcellular approaches as the ones used in the present study, along with the molecular tools, have the potential to permit more sensitive comparisons between congeneric species that possess different types of resistance to ocean acidification depending on their environment, demonstrating phenotypic adaptability or

plasticity, as well as the comparison of answers between individual and multiple stressors. These evaluations allow not only the detection of possible non-observable immediate impacts at higher levels but also the extrapolation of probable future consequences at population, community and ecosystem level, functioning as guides to predict and prevent undesirable results.

Chapter 4

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Attachments

Introduction

The ocean acidification

The anthropogenic activities have been associated with extremely high greenhouse gases emissions (especially carbon dioxide – CO₂) over the past several centuries, becoming an important component to the climate system. The resulting effects of these emissions are related with higher global mean temperatures and, of course, multiple physical and chemical changes in marine ecosystems (Figure 7). Due to the greenhouse gases trap, atmospheric and sea surface temperatures have raised 0.4-0.8°C in the past century (IPCC 2001), meaning additional abiotic pressures and complexities much higher than expected.

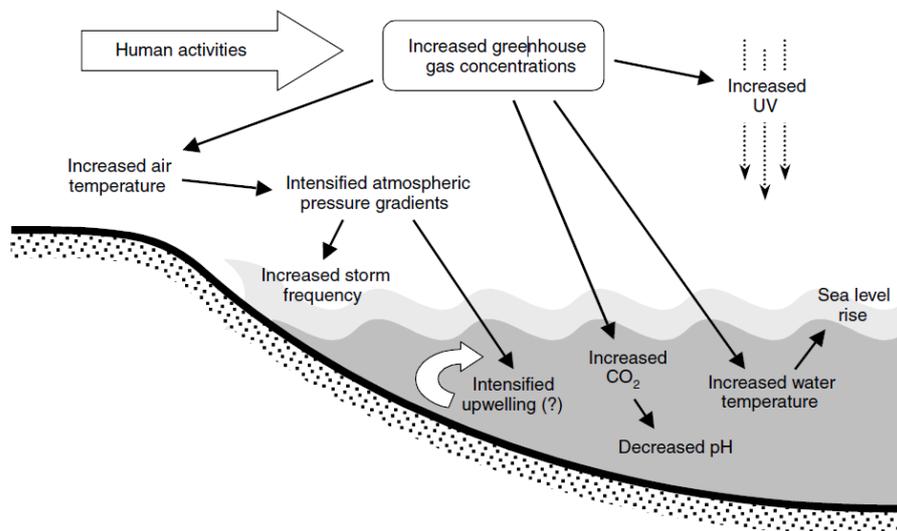


Figure 7 - Important abiotic changes related with global change. Human activities lead to higher concentrations of greenhouse gases in the atmosphere, which results in physical and chemical changes in oceans. Upwelling consequences are uncertain (adapted from Harley *et al.*, 2006).

Acidification, as one of the strongest phenomena that might cause direct changes on ocean biological processes, is contributing to the global change condition, being already a priority in the marine science research community (National Research Council, 2010). Ocean acidification is mainly associated with rising concentration levels of CO₂ and therefore as an important driver of change in biological systems. Almost half (30-40%) of all anthropogenic CO₂, with local-to-regional scale variability, is absorbed by the ocean surface and, in addition, the increase atmospheric CO₂ is also highly correlated with climatic changes such as increased global temperatures (IPCC, 2001, 2007). This leads to a multitude of cascading

reactions and effects on atmospheric, oceanic and terrestrial biodiversity (Figure 8) (Sabine *et al.*, 2004; Bernhardt & Leslie, 2013).

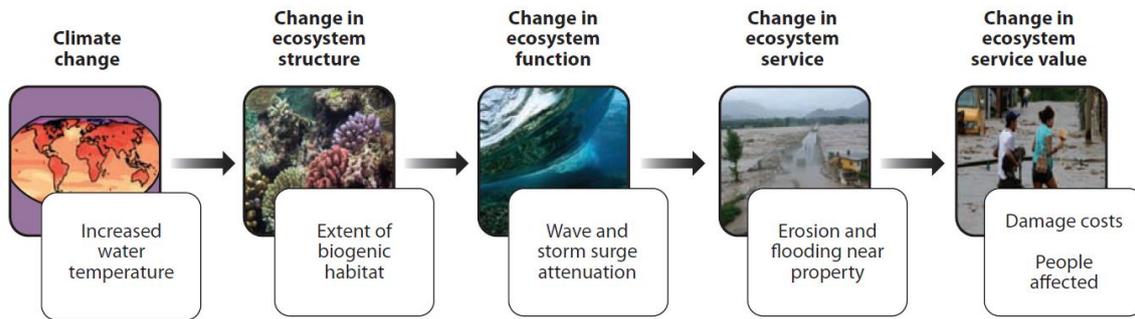


Figure 8 - Climate change effects lead to changes not only on ecosystem structure and function but also on ecosystem goods and service value (Adapted from Arkema & Samhuri, 2012 in Bernhardt & Leslie, 2013).

Ocean's acidification process starts when atmospheric CO₂ reacts with ocean surface, being absorbed by ocean water (Figure 9). This chemical reaction leads to carbonic acid formation (H₂CO₃):



Then, each molecule of carbonic acid can release one of its hydrogen ions forming bicarbonate (HCO₃⁻):



Bicarbonate can also break down, resulting in carbonate ion (CO₃²⁻):

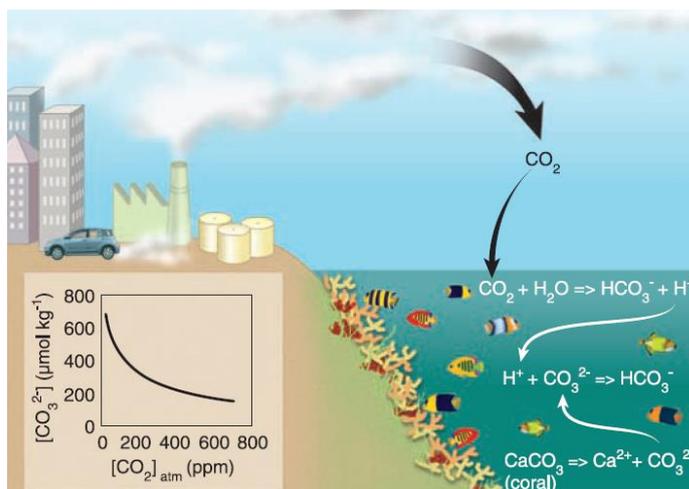
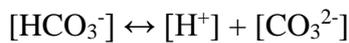
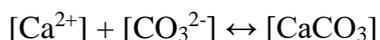


Figure 9 - Sources of atmospheric CO₂ and ocean acidification effects to marine species, especially coral communities. Decrease of available carbonate for marine calcifiers (Hoegh-Guldberg *et al.*, 2007)

For calcifying organisms, like corals, CO_3^{2-} is crucial to form calcium carbonate, along with calcium atoms (Ca^{2+}):



Nonetheless, carbonate ions can also recombine with reactive hydrogen ions forming more bicarbonate instead of calcium carbonate (Fabry *et al.*, 2008).

The loss of hydrogen ions (H^+) in the water leads to an increase in acidity measured by pH – potential to make H^+ , meaning that the more hydrogen available, the higher the acidity of the water and the lower the pH value. Thus, the higher CO_2 being produced, the more hydrogen ions will be set free into the water.

About 650 000 years prior to industrial revolution, atmospheric CO_2 concentration was 180-280 ppm (parts per million) (Raven *et al.*, 2005; Siegenthaler *et al.*, 2005; Howes *et al.*, 2015). Since the industrial era, ocean's chemistry pH dropped 0.1 units, and $p\text{CO}_2$ increased from approximately 280 ppm to nearly 390 ppm in 2010, meaning a 26% acidity increase in concentration of H^+ for global ocean (Raven *et al.*, 2005; Howes *et al.*, 2015). Today, the seawater pH mean is about 8.1 and although this represents a drop of 0.1 units since industrial era, it is important to remember that pH is measured by logarithmic scale, meaning that for each pH unit change, the concentration of hydrogen ions changes ten-fold. Under the Intergovernmental Panel on Climate Change (IPCC) scenarios, the drop of ocean's pH may reach a concentration nearly 1000 ppm by the end of 21st century (year 2100) which means a pH reduction of approximately 0.3-0.4 units (Figure 10). This value is greater than 100% increase in seawater $[\text{H}^+]$ (Houghton *et al.*, 2001; Caldeira & Wickett, 2005; IPCC, 2007).

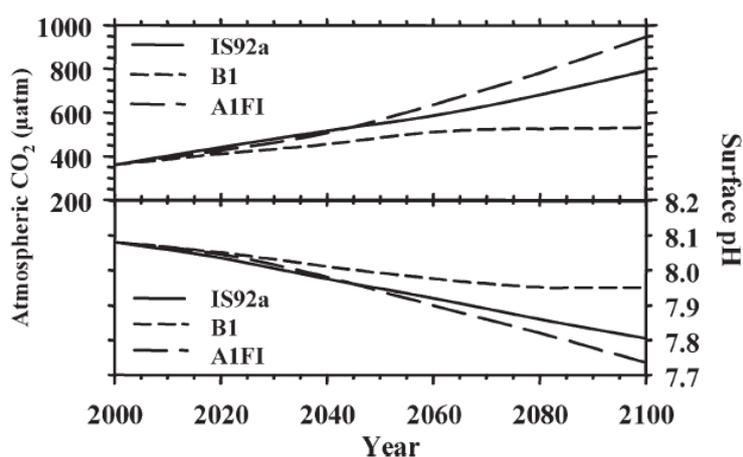


Figure 10 - Projected values of atmospheric CO_2 concentrations and seawater surface pH under three different scenarios: IS92a the "business-as-usual" CO_2 emissions, B1 the most and A1FI the least conservative scenario (Adapted from Meehl *et al.*, 2007 in Fabry *et al.*, 2008).

For the next three centuries (year 2300) the atmospheric CO₂ concentration could reach 2000 ppm, leading to an ocean pH reduction of approximately 0.77 units, marking a rate of change faster than any experienced in the past 650 000 years – 100 times faster (Caldeira & Wicket, 2005; Siegenthaler *et al.*, 2005; Forster *et al.*, 2007).

It's not surprising that higher levels of CO₂ can cause innumerable biological and ecological damages at different levels, including human societies. This basic knowledge has been the major impeller to the increasing research of ocean acidification effects among different taxa (Figure 11). The most studied taxonomic groups are calcifying cnidarian and mollusks, followed by fishes, echinoderms and crustaceans, with marine worms and sponges being the less studied (Heuer & Grosell 2014).

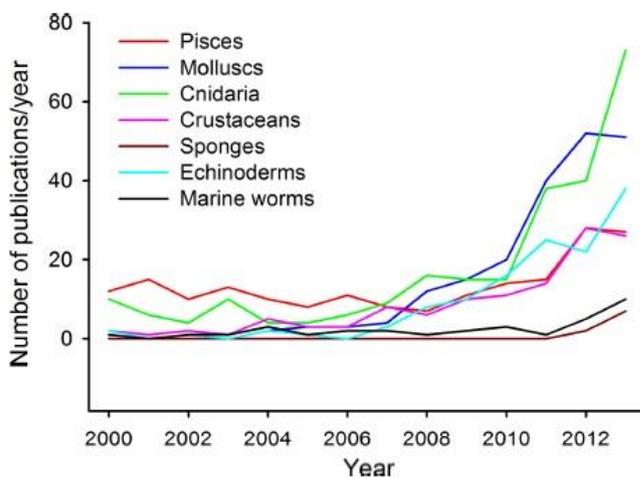


Figure 11 - The increase of ocean acidification relevant papers among different taxonomic groups since year 2000 (Heuer & Grosell, 2014).

Besides the high demonstrated interest on calcifying marine organism due to their calcium carbonate dependence, the base of food webs, as well as other trophic levels and even top predators can also be affected in different ways and at different life stages.

Research on the effects of ocean acidification on early life stages of many taxa is still scarce. Being more vulnerable to environmental changes, the probability for damage and effect detection is bigger on this early phase. Thus, it's not only essential to understand how some species can be affected by ocean acidification conditions at each developmental phase but it is also important to link and predict how changes at lower levels of biological organization will impact higher ones, like populations, community and ecosystem, so we can anticipate consequences and conservation efforts against possible extinction processes.

Impacts on marine organisms

The elevated partial pressure of CO₂ ($p\text{CO}_2$) in seawater, also known as hypercapnia, can influence marine organisms by decreasing CaCO₃ levels and causing metabolic disturbance to acid-base physiology balance (Fabry *et al.*, 2008), resulting in changes of immune system responses.

To date, besides the increased range of sensitivities of animal taxa to ocean acidification research (Figure 12), most studies have been focusing its attention on the impacts of altered seawater chemistry on calcifying marine organisms, like corals, crabs and mollusks because they're very much dependent on CaCO₃, an essential material to their exoskeleton.

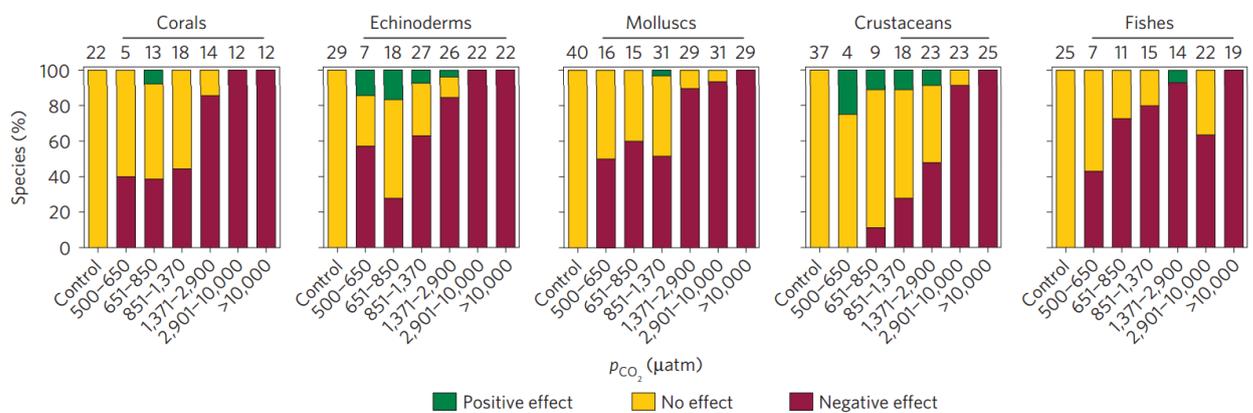


Figure 12 - Sensitivities of animal taxa to ocean acidification. Percentage (%) of coral, echinoderm, mollusc, crustacean and fish species demonstrated negative, no or positive effects on performance indicators related to $p\text{CO}_2$ ranges (μatm). Significantly count ratios are represented in bars above columns (Wittmann & Pörtner, 2013)

But, as already mentioned, the carbonate ions can also recombine with reactive hydrogen ions forming more bicarbonate instead of calcium carbonate, making it difficult for organisms to maintain, change or repair their shells and skeletons. Being more susceptible to the destruction of their external structures, they would be more exposed to predators, pathogens, infections, debility and even death, resulting in a good example of vulnerability to lower pH. Although major acidification studies use the most common calcifying organisms like the ones already cited, it is also possible to find effects close to the base of food web – foraminifera and coccolithophores (Riebesell *et al.*, 2000; Bijma *et al.*, 2002). These single cells, also covered by calcium carbonate shells, have a crucial role as phytoplankton production and ecosystem function. Therefore, with the pH decrease, the entire oceanic ecosystem might be deeply affected and most likely, the atmospheric and terrestrial ecosystems too.

Coccolithophores, for example, are responsible for producing an important contributor to the formation of clouds – dimethyl sulfide ((CH₃)₂S). Therefore threatening their existence could mean an increased planet vulnerability to harmful radiation, the reflectivity of the earth and the rate of global warming (Malin & Steinke, 2004). The quality of biological production may also be affected by this acidification phenomenon, since it has been recently demonstrated that an increase in *p*CO₂ could reduce the nutritional value of producers and their zooplanktonic predators – the primary prey for many fishes (Gao *et al.*, 2012; Rossoll *et al.*, 2012).

On the other hand, it is possible that in increased ocean acidification conditions, some species will find some advantages: “We’re pushing the oceans back to the dawn of evolution, a half-billion years ago when the oceans were ruled by jellyfish and bacteria.” – Dr. Jeremy Jackson, Scripps Institution Oceanography. These species will be capable of flourish and benefit either directly from the increased of carbon dioxide levels or indirectly from the loss of their harmed competitors. Jellyfish and sea plants are probably some examples of such organisms, giving us some notion of how future oceans may look, dominated by what we consider today as algae and invasive species (Hall-Spencer *et al.*, 2008).

Although not traditionally considered as calcifying organisms, fishes also produce CaCO₃ in the intestinal lumen and otoliths (Heuer & Grosell, 2014). Juveniles and adults are equipped with different physiological mechanisms than embryonic and larval stages, having efficient regulation process on their acid-base balance and, consequently, resistance to some acidification impacts (Brauner, 2008; Melzner *et al.*, 2009a, 2009b). Nonetheless, early life stages have potentially high vulnerability to environmental stress, which makes them an important bottleneck to population persistence (Melzner *et al.*, 2009a; Byrne, 2011). Because there is few comparable data on how early life stages cope with increased *p*CO₂, which hampers the prediction of future consequences on ecosystem level, over the last years research has focused on the early life history stages. Evidence to date suggest significant disturbances across a wide range of sensory systems and neurological functions, like olfaction, vision, lateralization, hearing, learning, activity and boldness (Munday *et al.*, 2009c, 2010, 2012; Dixson *et al.*, 2010; Cripps *et al.*, 2011; Esbaugh *et al.*, 2012). These disturbances are presumed to have substantial impacts on dispersal, habitat and settlement preference, recruitment, connectivity, population replenishment, predator-prey and social interactions, as well as biodiversity levels (Figure 13).

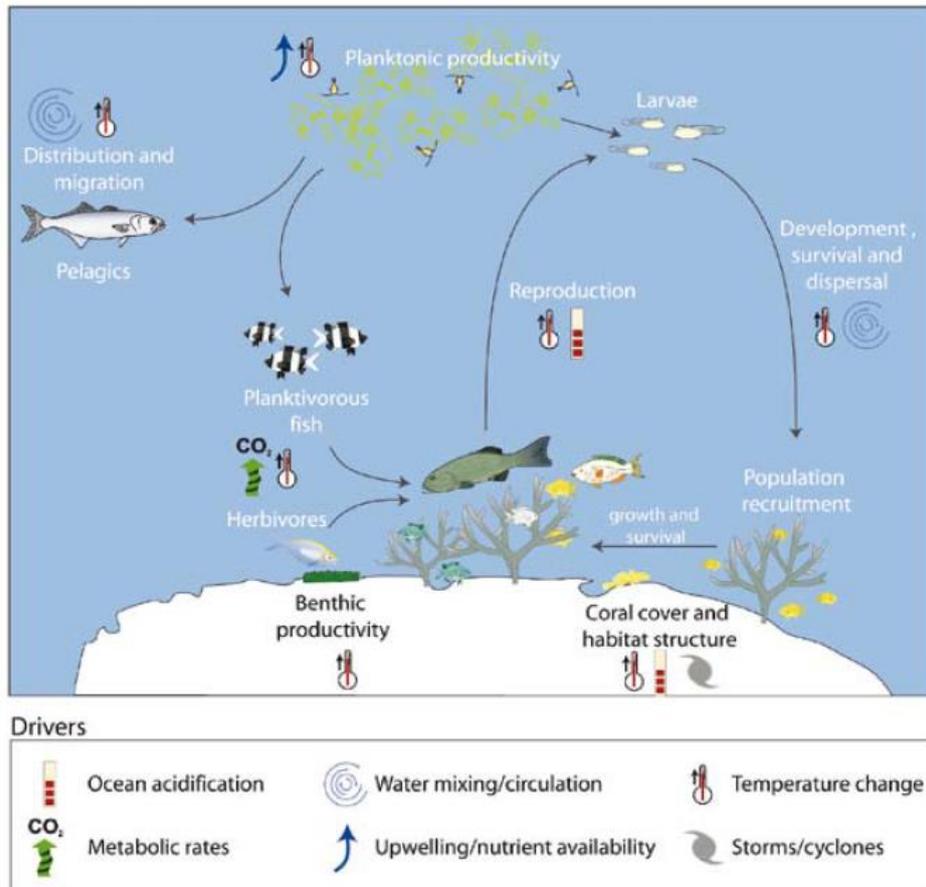


Figure 13 - Different ways ocean acidification-climate change can influence direct and indirectly fish populations and communities on different life stages (Munday *et al.*, 2007).

Pelagic larval period can last from days to months in marine fishes and is an ontogenetically and ecologically very important stage for dispersion of many species (Houde, 1997). However, it represents a very vulnerable and susceptible stage to high levels of mortality. Marine population's ecological functions are highly dependent on population replenishment and connectivity, which in turn are directly linked with recruitment potential. Recruitment is very important for populations and can be easily influenced by cumulative changes in mortality and growth rates of larvae (Cowen & Sponaugle, 2009).

The possible effects of ocean acidification on fish larvae appear to be species and population specific, also depending upon the endpoint measured. The scarce data available on this life phase show a higher susceptibility to elevated $p\text{CO}_2$ (Pörtner *et al.*, 2005) and significant reduced survival at low pH (Kikkawa *et al.*, 2003, 2004; Ishamatsu *et al.*, 2004) and behavioral disruptions at elevated $p\text{CO}_2$ (1000-1700 μatm), which include loss of ability to discriminate odors from different habitat types, predators and prey, loss of lateralization and increased "anxiety" (Munday *et al.*, 2009a; Dixson *et al.*, 2010; Cripps *et al.*, 2011).

However, other species, such as Atlantic herring (*Clupea harengus*) and Atlantic cod (*Gadus morhua*) larvae, for instance, appear to be resilient to ocean acidification (Jutfelt *et al.* 2013, Maneja *et al.*, 2013, 2015).

High levels of environmental CO₂ results in higher blood CO₂ accumulation in fishes and a decrease of blood pH, which tends to be quickly and persistently compensated by the action of base-equivalent HCO₃⁻ ions (Clairborne *et al.*, 2002). The process of acid-base balance is mainly achieved through a dynamic response at the gills and can be simplified by the reduction or reversion of pCO₂ gradient from aquatic organisms. This leads to a new steady state: the organism will try to reduce or eliminate CO₂. Teleost fishes are capable to maintain relatively constant extracellular alkaline pH (7.7-8.1) fluids. Some factors like O₂ – based ventilator drive, high CO₂ solubility in water, countercurrent exchange at the gills between water and blood flow and unidirectional water flow at respiratory surfaces make easier to excrete CO₂ of plasma for strict water breathers (Heuer & Grosell, 2014). Although the physiological adjust of HCO₃⁻ blood plasma to acidified conditions (Perry & Gilmour, 2006), fish exposed to elevated pCO₂ display continuously high levels of this element and also pCO₂ at extracellular fluids level, which may cause changes in tissues, organs, physiological processes, behavior and otolith growth. The potential to disrupt blood-oxygen deliver, also caused by acidification, can lead to effects like a decrease in aerobic scope and also changes in aerobic swimming ability (Pörtner *et al.*, 2004; Munday *et al.*, 2009c), with possible consequences on dispersal potential of larvae. Recent studies also point to an altered neurological function that lead to disruption of behaviour and sensory systems (Nilsson *et al.*, 2012; Maneja *et al.*, 2013; Munday *et al.*, 2013; Heuer & Grosell, 2014). Until some recent works, the underlying mechanisms linking high CO₂ exposure to abnormal behavior responses had been unknown. There are indications that high CO₂ interferes with neurotransmitter function, which is a determinant factor to recognize and respond to threat. In the brain of vertebrates, GABA-A is a major neurotransmitter receptor, which can be depolarized and excited in the presence of elevated sea water pCO₂ conditions and the consequently interference of acid-base relevant ions (HCO₃⁻ and Cl⁻). This results in behavioral abnormalities that can also be reversed when treated with a GABA-A antagonistic receptor (Nilsson *et al.*, 2012), which was already demonstrated in learning (Chivers *et al.*, 2014) and anxiety (Hamilton *et al.*, 2014) cases with fishes. The majority of impacts appear to be sublethal influencing the physiology (Heuer & Grosell, 2014; Timmins-Shiffmann *et al.*, 2014), growth (Parker *et al.*, 2012; Chan *et al.*, 2015), sensory function or behavior (Simpson *et al.*, 2011; Devine *et al.*, 2012; Nilsson *et al.*, 2012; Munday *et al.*, 2013), while high levels

of mortality are deeply associated with other cumulative factors like predation or temperature changing (Munday *et al.*, 2010; Ferrari *et al.*, 2012). Two studies by Munday *et al.* (2009a, 2010) illustrate well how these kind of individual effects can affect some critical factors to successful navigation, recruitment and survival of larvae in the wild although may not have a clear negative impact under experimental laboratory conditions. Besides changes in olfactory cues, predator avoidance capability, routine behavior and increase risk-taking activities, it was also possible to observe that all these changes resulted in up to a nine-fold higher *in situ* mortality of new recruits on the reef (Bignami, 2013). Nonetheless, many studies still fail to find significant effects (positive or negative) on development, growth and swimming ability (Munday *et al.*, 2009a, 2009b).

Despite the increasing number of studies on the impacts of ocean acidification on early life stages, there is still limited knowledge on physiological processes, such as osmoregulation, acid-base balance, metabolic pathways, reproduction, mitochondrial function or cardiorespiratory activity. To better understand the physiological costs and responses to ocean acidification, it is essential to understand how biochemical resources, like lipids and proteins are utilized during early life stages. Organisms use most part of their energy for growth, reproduction and maintenance of their basal metabolism. The metabolic cost associated with the disruption in acid-base balance could result in a reallocation of metabolic resources to deal with the stress and, consequently, negatively impact the development and somatic growth of the animal (Matson *et al.*, 2012). However, some organisms and especially marine invertebrates slow their metabolism in ocean acidification conditions (Melzner *et al.*, 2009a). The reduction of metabolic demand may be useful in short periods of unfavorable environmental conditions but prolonged situation could lead to damage in growth and development. Thus, metabolic activity, physiological functions and also morphologic development can be used as tools to quantify and monitor endogenous energy use during development on altered seawater chemistry and pH. A reduction in availability of energy resources during this life stage may result in slower developing or smaller larvae, which can impact predator avoidance and recruitment (Allen *et al.*, 2006). The pattern of energy costs under ocean acidification conditions may shed some information on how early life stages can deal with this water chemical changes.

Swimming behavior

For an effective monitoring or impact assessment it is essential to understand how natural and/or anthropogenic environmental changes can affect lower and higher levels of biological organization, addressing their responses to new challenges. The organisms can respond to environmental factors by changing their behavior, denouncing when the adaptation to environmental changes are occurring (Gerhardt, 2007). Depending on the species, behavior can be 10-1000 times more sensitive than the conventional LC₅₀ and it is gaining more recognition as a useful endpoint also given its higher ecological relevance in comparison with the biochemical and physiological alterations that precede it (Hellou, 2011; Sanchez-Hernandez, 2011).

Besides the small size of larval stages, their sensibility, vulnerability to environmental changes and their energetically expensive swimming capacity, they are no longer characterized as passive organisms like usual plankton components (Blaxter, 1986). In fact, some behavioral studies show that organisms in such life stages have great abilities to be strong swimmers, contributing to avoid predators, find food and control part of their dispersion (Stobutzki & Bellwood 1994; Leis & Carson-Ewart, 1997). Population replenishment and connectivity in marine populations and communities are critical for biodiversity and therefore, our understanding of dispersal role on population and ecosystem-level impacts in ocean acidification conditions is fundamental. Models of dispersal, connectivity and ecological productivity are deeply dependent of behavior knowledge, especially larval swimming abilities (Cowen, 2000, 2002; Paris & Cowen, 2004; Cowen *et al.*, 2006). Locomotion is considered to be the basis of many complex behaviors, being related to metabolic, neurological and physiological mechanisms of the animal. Thus, it is also a good indicator of anatomical condition and very important in inter-and intra-specific social interactions (Amiard, 2009). The impairment of locomotion may strongly influence the ability to forage, reproduce, avoid predators or unfavorable conditions, migrate and interact, inducing changes in population fitness, which might have consequences at the ecosystem level. For this reason, swimming velocity has been widely studied and considered to be determinant for survival (Stobutzki & Bellwood, 1994; Webb, 1994; Leis & Carson-Ewart, 1997; Kolok, 1999; Plaut, 2001; Fisher *et al.*, 2005; Faria *et al.*, 2009; Faria & Gonçalves, 2010; Fisher & Leis, 2010; Chan *et al.*, 2011; Maneja *et al.*, 2013, 2015; Faria & Gonçalves, 2014; Silva *et al.*, 2014). The larvae swimming capacities can be characterized in three different types: burst – the anaerobic muscle part; prolonged – mixing anaerobic and aerobic muscle; and sustained

– aerobic muscle part only (Webb, 1994; Kolok, 1999; Plaut, 2001). In the wild it is difficult to measure and study behavioral traits, particularly swimming ability, without disturbances. Nonetheless, there are some tools to evaluate this capability, including routine speed, *in situ* measurements, endurance performance and critical speed (U_{crit}), a useful estimate to determine the maximum swimming performance of larvae (Fisher *et al.*, 2005). U_{crit} represents a swim condition extremely rare in nature, since it's a prolonged swimming speed difficult to maintain long enough to influence dispersal. However, this measurement provides useful information to determine and compare different taxa and development stages, making possible to correlate the results with other measures of swimming capacity more ecologically relevant (Plaut, 2001; Leis, 2006). To date some studies have been contributing with knowledge on swimming abilities of many species in ocean acidification conditions. Although the obtained results particularly related with morphology, feeding behavior and physiological impacts, changed pCO_2 levels alone had no effects on swimming abilities or trajectories on the different test organisms (Faria *et al.*, 2009; Munday *et al.*, 2009c; Bignami, 2013; Maneja *et al.*, 2013, 2015; Chan *et al.*, 2015). Once larval morphologies and behaviors can affect swimming performances, some implications in swimming behavior are expected, beside the specific phenotypic plasticity that could be demonstrated in each particular species. Nonetheless, the preference of prioritizing swimming over feeding, for example, could be associated with the immediate high costs related with the inability to swim, which could be translated in failure to avoid predators or in finding food patches. Besides the limited knowledge available on this subject, this type of tradeoffs may have severe implications that could be passed over the subsequent development stages (Chan, 2012), reinforcing the necessity of more research in this area.

Biochemical biomarkers

Biomarkers can be defined as “any biological response to a stressor considered at the sub-individual level, measured in the interior of the organism or in its sub-products (blood, urine, excrements, fur, feathers, etc.), indicating a difference from the normal condition and that can not be detected from the intact organism” (Gestel & Brummelen, 1996). There are innumerable biotic and abiotic factors that can influence biomarker responses of effects/exposure and condition indices (Fonseca *et al.*, 2011). Biomarkers of effect can be characterized by a response at the biochemical or molecular level to a stress occurrence representing a cellular defense mechanism. The success of this mechanism dealing with the

stress is determinant to avoid histological and physiological changes that could result in irreversible damages, compromising the growth and reproductive capacity of the animals.

Being exposed to a wide range of environmental stressors, marine organisms face big challenges at the physiological level trying to deal for instance with oxidative stress. To obtain energy, aerobic organisms use oxygen to oxidize carbon and hydrogen-rich substrates, which is a process that forms free radicals originated by the reduction of oxygen molecules. Free radicals are chemical species that contain unpaired electrons and are capable of independent existence (Gutteridge, 1995). Highly important to cell biology, they're short-lived, highly reactive and unstable, being mostly derived by oxygen. Some examples of free radicals are the hydroperoxyl radical (HO_2^\cdot), the superoxide anion radical (O_2^\cdot), the hydrogen peroxide (H_2O_2) or the hydroxyl radical (OH^\cdot).

Superoxide anion is the precursor of most reactive oxygen species (ROS) – highly reactive oxygen radicals – and a major mediator in oxidative chain reactions (Negre-Salvayre *et al.*, 2010). ROS are a natural by-product of molecular oxygen metabolism at the membranes of mitochondria and endoplasmic reticulum and, under controlled levels, have important roles in cell signaling and redox homeostasis (Cadenas, 1989; Filho *et al.*, 1993; Vinagre *et al.*, 2012). Periods of environmental stress can induce dramatically the levels of ROS, leading to accumulation and inability of the organism to deal with these radicals. Thus, when far beyond the protective mechanisms capacity of the animal, these events can lead to oxidative stress resulting in damage in cellular macromolecules such as proteins, DNA, lipids and changes at essential minerals homeostasis (Figure 14) (Young & Woodside, 2001; De Jesus & Carvalho, 2008). Moreover, by compromising the health and viability of the cells, ROS can induce cellular responses that could lead to necrosis or apoptosis (Palmieri & Slendorio, 2007).

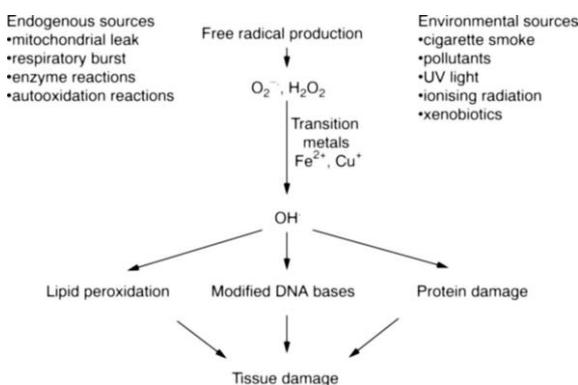


Figure 14 - Major sources of free radicals in the body and the consequences of free radical damage (Young & Woodside, 2001).

Lipid peroxidation, for instance, is considered to be one of the most complex and prevalent mechanisms related to cell damage (Cadenas, 1989; Gutteridge, 1995). It consists in free radical oxidation of polyunsaturated fatty acids (PUFAs), whose side chains of membrane lipids are particularly sensitive to this phenomenon. The process starts with hydroxyl free radical sequestering a hydrogen atom from an intact PUFA, which can result in impairment of membrane functioning – bound receptors, loss of fluidity, increased non-specific permeability to H⁺ and other ions and eventual rupture leading to release of cell and organelle contents (Gutteridge, 1995). Considering the high content of polyunsaturated fatty acids in fish, lipid peroxidation can be especially harmful but also an excellent marker related to tissue damage (Love, 1970; Halliwell & Gutteridge, 1999; Negre-Salvayre *et al.*, 2010).

ROS, as superoxide anion radical and hydroxyl radical can also cause DNA damage, which can impact the health and heritage of the organisms. These radicals can attack DNA, creating a multiple products able to be reduced or eliminated by antioxidant and repair enzymes responses (Palmieri & Sblendorio, 2007), which has been demonstrated in recent works with different taxa (Hook & Lee, 2004; Chang *et al.*, 2009) also related with pH stress factor (Choi *et al.*, 2014; Mai *et al.*, 2010; Zhou *et al.*, 2008; Wang *et al.*, 2009). The exposure to acidic conditions together with the stimulation of ROS production can lead to severe DNA damage and even apoptosis (Phillips & Ward, 2001; Antonova *et al.*, 2009). Despite the potential of marine species to be used as environmental stress models, there is an insufficiency of data related to this topic, particularly with seawater fish. There is a wide range of techniques to evaluate DNA damage, being the DNA precipitation assay one of the commonly used that allows a rapid measurement of DNA single- and double- strand breaks (Olive, 1988).

The ability to form highly reactive free radicals stimulated the evolution of defense mechanisms against these radicals in all living forms. In an organism there's a hierarchy of defense strategies to protect and deal with oxidative stress. Superimposed on different cellular compartments are gene-regulated defenses, involving the heat-shock and oxidative stress related proteins. These proteins are antioxidants (enzymatic or non-enzymatic) – chemicals with chain-breaking properties that play a major role in redox homeostasis, delaying or protecting against oxidative damage at cellular level that can also result in enzymatic inactivation (Halliwell & Gutteridge, 1999; Novais *et al.*, 2014). Nevertheless, these antioxidant defenses have a maximum capacity and, in an extreme oxidative stress situation, an antioxidant failure might occur, leading to excessive molecular and tissue injury. During the defense process, the antioxidants activities can then be used indirectly to assess free radical presence. Some examples of antioxidant enzymes are superoxide dismutase (SOD),

catalase (CAT) and peroxidases, which actions at intracellular level can keep superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) at non dangerous levels (Lesser, 2006). The SOD-CAT enzymatic mechanism is responsible for the first defense against oxygen toxicity. SOD catalyzes the dismutation of the superoxide anion radical into water and hydrogen peroxide and CAT acts to transform this hydrogen peroxide into water and oxygen (Figure 15) (Pandy *et al.*, 2003; Howcroft *et al.*, 2009).

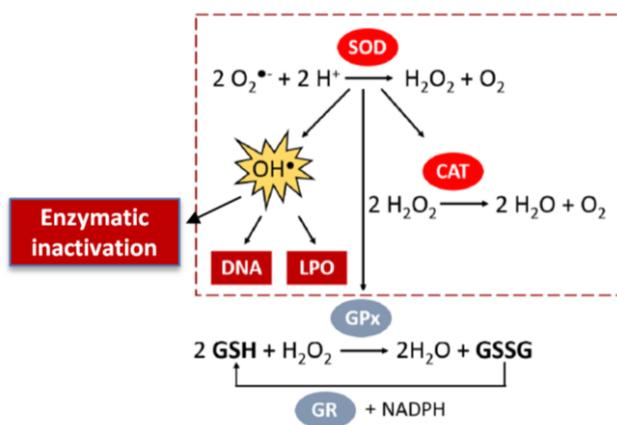


Figure 15 – SOD-CAT system in response to oxidative stress, preventing damage – lipid peroxidation (LPO), DNA damage or enzyme inactivation (adapted from Howcroft *et al.*, 2009).

Their activities have been reported in different studies of different taxa including fresh and seawater organisms (Matkovics *et al.*, 1977; Wdzieczak *et al.*, 1982; Fitzgerald, 1992), mainly regarding chemical contamination, showing that antioxidant enzymes can control and maintain a relative low rate of generation of harmful and reactive hydroxyl radical (HO), that results from the reaction of O_2^- with H_2O_2 (Chance *et al.*, 1979). Regarding acidification studies, Zhang *et al.*, (2012) demonstrated that elevated concentration of CO_2 induced the antioxidant defenses of the copepod *Centropages tenuiremis*. A same pattern result was also obtained in studies with zooxanthellae and hermatypic coral *Pocillopora capitata* (Soriano-Santiago *et al.*, 2013), in oysters (Timmins-Schiffman *et al.*, 2014) and crustacean *Artemia sinica* (Zheng *et al.*, 2015). Nonetheless a study with the spider crab *Hyas araneus* demonstrated an attenuation of oxidative stress and defense mechanisms in elevated levels of pCO_2 , possible as a limited acid-base compensation and metabolic regulation (Harms *et al.*, 2014). Some recent studies revealed that 50% of all marine crustaceans are negatively affected at pCO_2 of approximately 2,000 μatm , however more research is needed to understand better the effects and mechanisms associated.

Related to energy metabolism there are other enzymes involved in energy production that could also be used as biomarkers, providing useful information related to energy demand in

organisms exposed to stress factors, since their presence may represent the need of additional energy for physiological and biochemical functions normal maintenance (Almeida *et al.*, 2010). The isocitrate dehydrogenase (IDH) is an important enzyme of citric acid cycle, being related with ATP production and aerobic metabolism (Moreira *et al.*, 2006; Napierska & Podolska, 2008). Nonetheless, if needed, the energetic metabolism can be altered. Stressful conditions that may require an additional energy support stimulate lactate dehydrogenase (LDH) that is related with the production of energy in short periods of time and thus, anaerobic metabolism (Ribeiro *et al.*, 1999; Diamantino *et al.*, 2001). Several studies have demonstrated the successful use of the activities of these enzymes as biomarkers, using them as tools to monitor and diagnose stress exposure in fish, being mainly associated with exposure to environmental contamination (Almeida *et al.*, 2001, 2002; Rendon von Osten *et al.*, 2005; Napierska & Podolska, 2008; Vieira *et al.*, 2008, 2009; Almeida *et al.*, 2010). To my knowledge, there is only one available study associated to ocean acidification with an Antarctic krill *Euphausia superba*, reporting increased levels of LDH in high acidified conditions (Saba *et al.*, 2012).

As already mentioned, energy is required to guarantee physiological homeostasis in response to environmental variations, which is considered to have highly metabolic costs for the organisms. However the actual energy demand is rarely quantified. The cellular energy allocation (CEA) is a methodology developed by De Coen and Janssen (De Coen & Janssen, 1997, 2003) that allows the biochemical measurement of the energy reserves available (total carbohydrates, protein and lipids) and also the energy consumption that could be estimated by the measurement of the electron transport system activity (ETS). ETS confer information about oxygen consumption and cellular respiration rate at mitochondrial level (Novais & Amorim, 2013). Thus, the obtained difference between energy available and energy consumption will provide the energy budget of the organism (De Coen & Janssen, 2003). To date, CEA has been applied to a wide range of studies with different animals, including freshwater and marine ones, such as daphnia, amphipods, bivalves, gastropods and polychaetes (De Coen & Janssen, 1997, 2003; Verslycke *et al.*, 2003, 2004; Erk *et al.*, 2008; Smolders *et al.*, 2004, 2006; Moolman *et al.*, 2007; Olsen *et al.*, 2007; Stomperudhaugen *et al.*, 2009). However, with the exception of two studies; one study using sea urchins *Strongylocentrotus purpuratus* (Pan *et al.*, 2015) and the other a spider crab *Hyas araneus* (Harms *et al.*, 2014), no studies of ocean acidification effects have been reported using CEA as biomarker. According to Pan *et al.*, (2015) the sea urchins were highly impacted at cellular level but minimal on development, being reported an allocation of 84% energy available to

processes under acidification conditions. The available data suggests a dramatic compensation at the cellular level to avoid damage at other stages like development, for example. Despite the implement of the warming factor, Harms *et al.*, (2014) also reported elevated demand for metabolic substrates at ocean acidification exposure scenarios.

The effects of ocean acidification on the physiology and metabolism of marine organisms, especially fishes, are still understudied. The pressure of environmental changes towards the organisms may trigger defense strategies like antioxidant systems and repair cellular mechanisms or even abnormal behavior. The energy costs associated with this processes are mainly reallocated to guarantee physiological integrity and our capacity to use their avoidance/repair stress response mechanisms as monitoring tools could be determinant to analyze and predict higher level of changes in time.

Knowledge gaps and conservation efforts

The contact of organism's sensory structures with changed environmental chemistry conditions, as in seawater, may not create instant modifications but can alter animal's responses through a certain timescale associated with physiologic, histologic and biologic adaptation and evolution of the species to stressful parameters (Munday *et al.*, 2009a, 2010; Cripps *et al.*, 2011; Simpson *et al.*, 2011; Ferrari *et al.*, 2012; Nilsson *et al.*, 2012). In other words, a population, community or ecosystem alteration implies changes at lower biological levels, such as the molecular or cellular level (Figure 16).

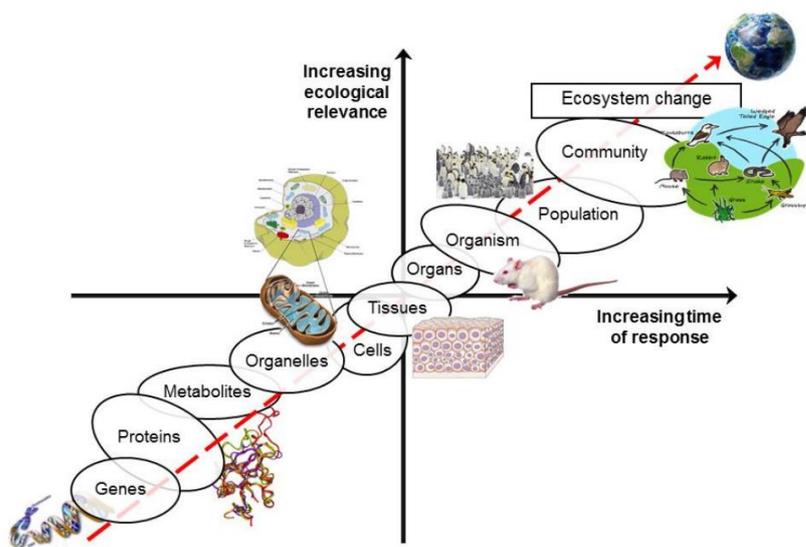


Figure 16 - The relationship between different levels of biological complexity, ecological relevance and time of response related to an environmental change or chemical exposure (Lemos *et al.*, 2010).

As early responding endpoints, the use of these lower levels as potential tools can rapidly link and anticipate knowledge to what can occur at higher levels of biological organization (population, community or even ecosystems) (Lemos *et al.*, 2010).

The expansion of research on impacts of ocean acidification is very recent which means that there are many areas in which our current understanding is limited. The impacts of ocean acidification combined with other stressful factors, on species with different life history strategies and physiological capabilities, long term effects of acidification on fish growth and reproduction, the potential for acclimatization and adaptation to acidification and the response of fish to more accurate simulations of variable ocean acidification are just some examples of research questions currently not so well understood. To better comprehend the susceptibility and adaptation of fishes to ocean acidification is crucial to have a deeply knowledge on different life history strategies and physiological characteristics. Additionally, it is also important to understand how organisms can deal with a combination of stressors associated with ocean acidification like higher levels of temperature, widespread hypoxia or altered food availability (Portner *et al.*, 2005; Fabry *et al.*, 2008). Population replenishment and connectivity are central to guarantee ecological functions and effective management of marine populations and ecosystems. On the other hand, these factors are also very susceptible to significant changes in mortality or growth rates on recruitment potential (Houde, 1997; Cowen & Sponaugle, 2009). All organism-level effects of CO₂ induced acidification may exert pressure at population-level with ecological impacts, since they can influence the dispersal, recruitment and survival – life events responsible for the replenishment of fish stocks and maintenance of biodiversity. These evaluations are important since those processes are directly correlated with fisheries productivity, economy and climate change, including ocean acidification as well (Cooley & Doney, 2009). Thus, the success of conservation and management efforts are highly dependent on focus research in this matter, enabling important evaluations of broader impacts that for now are still scarce, contributing to adapting management tools to future climate change scenarios (Doney, 2009).

Test organism

The sand-smelt *Atherina presbyter* Cuvier 1829, one of the two species of Atherinidae family, is ecological well know, with reduced dispersal capacity and strictly incidence to coastal

environments (Elliott & Dewailly, 1995). With some economic interest, this temperate fish species have a distribution from the British Isles and southern North Sea to the Canary Islands, Mauritania, western Mediterranean, Azores archipelago and Cape Verde (Quignard & Pras 1986; Santos *et al.*, 1997). In Portugal, *Atherina presbyter* can be found systematically in 3 southern sites (E, F, G in figure 17).

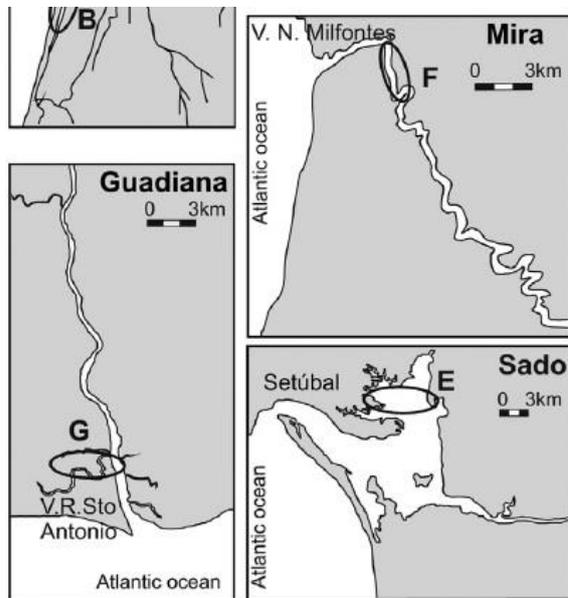


Figure 17 - Southern sites in Portugal where *Atherina presbyter* can be found (Fonseca *et al.*, 2014).

Although its adult life stage is mainly spent at sea, these animals often returned to the estuaries. The spawning of *Atherina presbyter* occurs in shallow waters, such as estuaries and coastal lagoons with some vegetation, useful as nursery grounds due to the importance of eggs attachment (Elliott & Dewailly, 1995). Hatching happens after 15-16 days of spawning at a temperature of 15°C and larvae are already well developed presenting 6.7-7.5 mm of total length and being ready to start exogenous feeding (Bamber *et al.* 1985).

This species is easy to find, collect and maintain in aquariums, with a rate of mortality near 0% (personal observations), which make it a good species to use as bioindicator. The available information about atherinids, particularly related to *Atherina presbyter* is still scarce, being the ones available mainly focused on ecological and biological aspects such as age, behavior, mortality, growth (Creech, 1992; Lorenzo & Pajuelo, 1999; Pajuelo & Lorenzo, 2000; Pombo *et al.*, 2005; Faria *et al.*, 2014) but also some on their morphological, biochemical, morphometric and genetic characteristics (Vasil'eva, 1994; Pacheco *et al.*, 2004; Fonseca *et al.*, 2014). However, there is still a paucity of information on larval behavior and

biochemical metabolism for Atherinidiformes, particularly the one of interest in this study, becoming important to understand how global change can affect this species.

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