European lobster *(Homarus gammarus)* larvae under an acidification scenario: addressing biochemical, development and behaviour responses

Lénia da Fonseca Alexandre Rato

[2016]

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A thesis submitted to the School of Tourism and Maritime Technology, Polytechnic Institute of Leiria as partial fulfilment for the requirements for the Master Degree of Science in Aquaculture, held under the scientific supervision of Professor Sérgio Miguel Martins Leandro Franco (School of Tourism and Maritime Technology, Polytechnic Institute of Leiria) and co-supervision of Professor Marco Filipe Loureiro Lemos (School of Tourism and Maritime Technology, Polytechnic Institute of Leiria) and Doctor Sara Calçada Novais (MARE, Polytechnic Institute of Leiria).

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“Onde há um safio, há um lavagante.”

from “Lavagante”, José Cardoso Pires

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RESUMO

A acidificação dos oceanos constitui uma problemática global e a realidade de que está, efetivamente, a acontecer não é uma consideração subjetiva. A Acidificação dos oceanos provocada por emissões de dióxido de carbono de origem antropogénica tem vindo a reduzir o pH das águas superficiais do Oceano e projeções preveem a continuidade deste processo. Embora muita investigação tenha sido desenvolvida no âmbito dos invertebrados que calcificam, tais como moluscos e crustáceos, poucos consideraram o estudo de efeitos ao nível sub-celular para avaliar *stress* oxidativo ou respostas funcionais do metabolismo energético em tais condições, interligando vários níveis de organização biológica.

O objetivo do presente estudo foi o de avaliar os efeitos da exposição a diferentes níveis-alvo de pCO_2 (controlo: 370 µatm; aumentado: 710 µatm) e de pH (controlo: 8.15; reduzido: 7.85) em parâmetros de crescimento bem como avaliar respostas comportamentais e bioquímicas relacionadas com *stress* oxidativo e metabolismo energético durante o desenvolvimento larvar de um crustáceo decápode. Este cenário de acidificação está de acordo com os RCPs previstos pelo Painel Intergovernamental para as Alterações Climáticas (IPCC, 2014) para o ano 2100.

Para o presente estudo, foi utilizado o crustáceo *Homarus gammarus* (L.) – sendo uma espécie com elevado valor comercial, que tem vindo a sofrer elevada pressão de pesca em águas Europeias. Fêmeas adultas provenientes da costa Atlântica Oeste Portuguesa foram obtidas de um retalhista local. Após a eclosão em ambiente laboratorial controlado, larvas provenientes da mesma progenitora foram expostas às condições acima descritas desde o momento da eclosão até à chegada ao Estádio III. A sobrevivência individual e ocorrência de ecdise foram avaliados individualmente de 12h em 12h. Réplicas de cada tratamento foram recolhidas em momentos específicos durante o Estádio I (primeiro estádio larvar) e Estádio III (último estádio larvar) para análise morfométrica e de crescimento (peso fresco e comprimento carapaça) e respostas bioquímicas. Os biomarcadores analisados incluíram parâmetros relacionados com *stress* oxidativo e danos (atividade da enzima superóxido dismutase (SOD), peroxidação lipídica (LPO) e danos no DNA) e metabolismo energético (atividade da cadeia transportadora de eletrões (ETS) e da enzima lactato desidrogenase (LDH) e quantificação de Hidratos de Carbono). Os resultados obtidos sugerem que a sobrevivência diminui e que o período inter-muda é afetado durante a exposição a cenários de acidificação. No que respeita aos parâmetros

de crescimento/morfométricos, larvas do cenário de acidificação apresentam uma tendência para crescimento diminuído, menor peso e comprimento de carapaça. As análises bioquímicas realizadas indicam a ocorrência de *stress* oxidativo sob condições de acidificação. Respostas ao nível do metabolismo energético não variaram significativamente entre tratamentos. Os resultados apontam também para que fases larvares possam possuir um sistema antioxidante ainda em desenvolvimento, tornando-as mais suscetíveis ao *stress* oxidativo.

As fases larvares são uma fase vulnerável e crucial no ciclo de vida das espécies, influenciando o recrutamento e a renovação de *stocks*. Este estudo contribui para um melhor entendimento sobre a vulnerabilidade desta espécie num cenário de alterações climáticas – Acidificação dos oceanos – ao endereçar os mecanismos envolvidos nas respostas deste crustáceo a este agente causador de *stress*.

Palavras-chave: Acidificação dos oceanos; Biomarcadores bioquímicos; Desenvolvimento Larvar; *Homarus gammarus*; Metabolismo energético*; Stress* oxidativo.

ABSTRACT

Ocean acidification is a worldwide concern and its existence is no longer a subjective consideration. Ocean acidification resulting from anthropogenic emissions of carbon dioxide has already reduced the surface ocean pH and future projections expect it will continue to do so. Although the main body of research has been developed essentially on calcifying invertebrates, such as molluscs and crustaceans, few have considered the study of sub-cellular effects to assess the occurrence of oxidative stress or alterations on the energetic metabolism under such conditions, integrating different levels of biological organization.

The focus of the present study was to evaluate the effects of exposure to different target pCO_2 (control: 370µatm; increased: 710µatm) and pH levels (control: 8.15; decreased: 7.85) on growth parameters and on behavioural and biochemical responses related with oxidative stress and energy metabolism during a decapod crustacean larval development. These exposure conditions are according with the RCPs predicted by the Intergovernmental Panel on Climate Change (IPCC, 2014) for the year 2100.

In the present study, the crustacean *Homarus gammarus* (L.) was used – as a highly valued commercial species that is facing high fishing pressure amongst European waters. Adult females were obtained from a local crustacean retailer that collected them from the Portuguese West Atlantic coast. After hatching in laboratorial controlled conditions, the larvae from the same female were exposed to the described conditions since hatching until reaching stage III. Individual survival and moulting patterns were evaluated every 12h. Replicates from each treatment were collected in specific moments at Stage I (first larval stage) and Stage III (last larval stage) for morphometric measurements (carapace length and wet weight) and biochemical biomarkers analysis. The measured biomarkers comprised endpoints related with oxidative stress and damage (superoxide dismutase activity (SOD), lipid peroxidation (LPO), and DNA damage), and with energy metabolism (Carbohydrates content and electron transport system (ETS) and lactate dehydrogenase (LDH) activities). The results suggest that survival decreases and inter-moult period is affected during the exposure to an acidification scenario. Also, concerning morphometric features, larvae from the acidified scenario tend to grow less and gain less weight. Biochemical biomarker analyses indicate the occurrence of oxidative stress under the acidified condition, whereas the energetic metabolism endpoints did not show alterations across experimental treatments. The results also indicate that early life stages may not yet

possess a fully developed antioxidant defence system, being particularly vulnerable to oxidative stress.

Larval phase is a vulnerable and crucial step in species life cycle, influencing recruitment and stock renovation. This study contributes to a better understanding of this species vulnerability under a climate change scenario – Ocean Acidification - while addressing the mechanisms involved in this crustacean response to this stressor.

Keywords: Biochemical Biomarkers; Energy metabolism; *Homarus gammarus*; Larval Development; Ocean Acidification; Oxidative stress.

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LIST OF ABBREVIATIONS

AM – After moulting

A_T – Total alkalinity

CAT – Catalase

CL- Carapace length

CO_2 – Carbon Dioxide

CO_3^{2-} - Carbonate ion

CO_2 (aq) - Dissolved CO_2

CT - Total Carbon = CO_2 (aq) + HCO_{3-} + CO_3^{2-}.

DHA - Docosahexaenoic Acid

DIC – Dissolved inorganic carbon

DO – Dissolved Oxygen

DW – Dry Weight

EPA - Eicosapentaenoic Acid

HCO_{3-} - Bicarbonate ion

H_2CO_3 - Carbonic Acid

H_2O_2 – Peroxide Hydrogen

H_2SO_4 – Sulfuric Acid

IP – Inter-moult Period

IPCC – International Panel on Climate Change

LDH – Lactate Dehydrogenase

NBS – National Bureau of Standards

OA – Ocean Acidification

OD – Optic Density

pCO_2 - Partial Pressure of Carbon Dioxide

PMS – Post-mitochondrial Supernatant

PUFA - Polyunsaturated Fatty Acids

RCP - Representative Concentration Pathways

ROS – Reactive Oxygen Species

SI – Stage I

SII – Stage II

SIII – Stage III

S.D. – Standard deviation

SOD – Superoxide dismutase

Ww – Wet weight

1. INTRODUCTION

1.1 Ocean acidification and seawater chemistry

Since the industrial revolution, anthropogenic gas emissions (namely CO_2) have shaped Earth's climate (see Figure 1.1). Higher greenhouse gases emissions are related with increasing atmospheric temperatures (IPCC, 2014). This changes also have an impact in Oceans, where Sea Surface Temperature is expected to rise 3-5°C by the end of this century (Sokolov *et al.*, 2009).

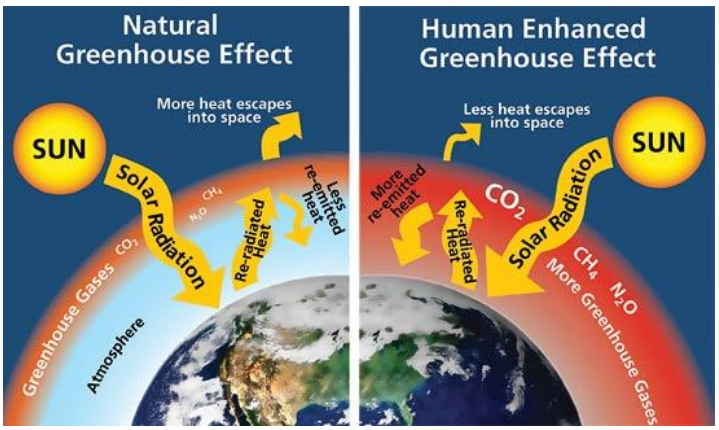

Figure 1.1 – Left: naturally occurring greenhouse gases—carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O)—normally trap some of the sun's heat, keeping the planet from freezing; Right - human activities, such as the burning of fossil fuels, are increasing greenhouse gas levels, leading to an enhanced greenhouse effect. The result is global warming and unprecedented rates of climate change. Sourced by Will Elder, NPS: https://www.nps.gov/goga/learn/nature/climate-change-causes.htm

The changes named above will also impact marine ecosystems through multiple physical and chemical changes. Earth's Oceans play a major role in the carbon cycle and are responsible by the uptake of ~48% of the atmospheric CO_2 (Raven *et al.*, 2005). It is estimated that it had absorbed approximately 155 PgC from the atmosphere over the last two and a half centuries (Sabine *et al.*, 2004; Khatiwala *et al.*, 2013). This natural process of absorption has benefited humankind by significantly reducing the greenhouse gas levels in the atmosphere and abating some of the impacts of global warming. However, the ocean's uptake of carbon dioxide is having a significant impact on the seawater chemistry, namely in the seawater carbonate system (Bolin & Eriksson, 1959; Zeebe & Wolf-Gladrow, 2001; Solomon *et al.*, 2007; Feely *et al.,* 2004; Fabry *et al.,* 2008; Zeebe, 2012). When atmospheric CO_2 exchanges across the air–sea interface, it reacts with seawater through a

series of four chemical reactions that increase the concentrations of carbon species: dissolved carbon dioxide ($CO_{2\,(aq)}$), carbonic acid (H_2CO_3) and bicarbonate (HCO_{3-})

Hydrogen ions (H^+) are produced by these reactions. This increase of the ocean's hydrogen ion concentration results in a reduction in pH, meaning an increase in acidity. Under normal seawater conditions, more than 99.99% of the hydrogen ions that are produced will combine with carbonate ion (CO_3^{2-}) to produce additional bicarbonate ions (HCO_3^-). Thus, the addition of anthropogenic CO_2 into the oceans lowers the pH and consumes carbonate ion (see Figure 1.2). According to Feely *et al. (*2004) and Fabry *et al. (*2008), H^+ ions' concentration increased 30% and carbonate ions' concentration reduced 16% since the industrial revolution.

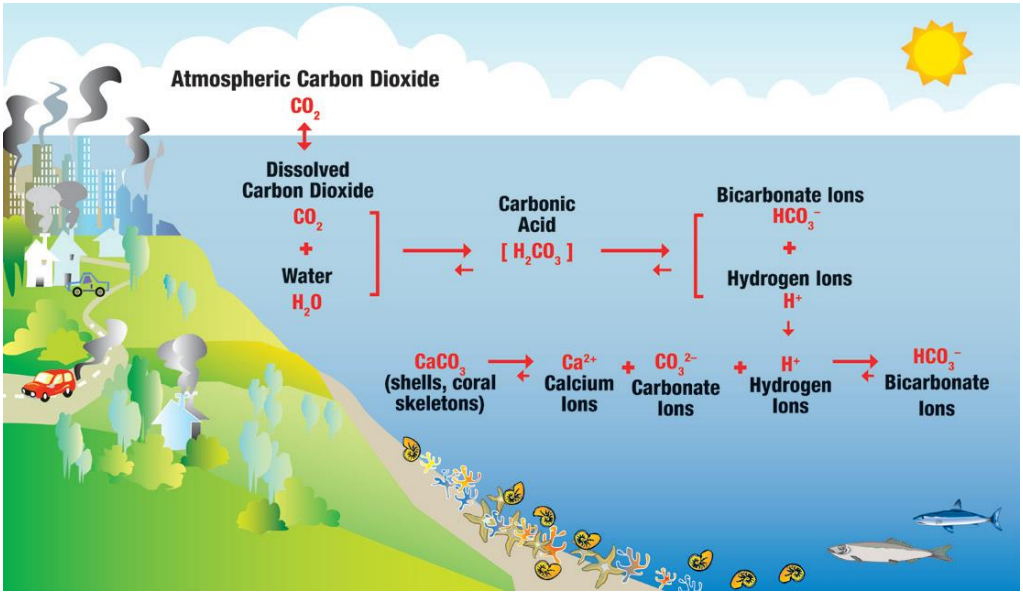

Figure 1.2 – Ocean acidification chemistry, displaying the chemical reactions that occur when CO_2 is adsorbed in the marine environment and emphasizing the fact that extra H^+ ions can react with carbonate ions and form more bicarbonate. National Research Council (2013).

The average pH of ocean surface waters has already dropped by about 0.1 units, from about 8.2 to 8.1 (average, total scale), since the beginning of the industrial revolution (Orr *et al.*, 2005a; Feely *et al.*, 2009). Estimates of future atmospheric and oceanic carbon dioxide concentrations indicate that, by the end of this century, the average surface ocean pH may be the lowest since 50 million years ago (Caldeira & Wickett, 2003). Atmospheric and oceanic CO_2 has been increasing from a 280 ppm baseline and is expect to continue the same trend. Levels of pCO_2 for 2100 were predicted to reach 1000ppm (Caldeira & Wickett, 2005; Feely *et al.,* 2010; Kroeker *et al.*, 2013). IPCC (2014) admits several

scenarios while it takes mitigation measures into account. The most extreme prediction consider a pCO_2 of ~900ppm in 2100 (see Figure 1.3).

This changes in marine environmental chemistry may affect fundamental chemical processes of the sea in coming decades (Fabry *et al.*, 2008; Doney *et al.*, 2009), impact marine biota and have socioeconomic-policy implications (Bednaršek *et al.*, 2014; Findlay *et al.*, 2014; Kelly *et al.*, 2011; Orr *et al.*, 2005; Paganini *et al.*, 2014; Schmalenbach & Buchholz., 2013; Turley & Gattuso, 2012).

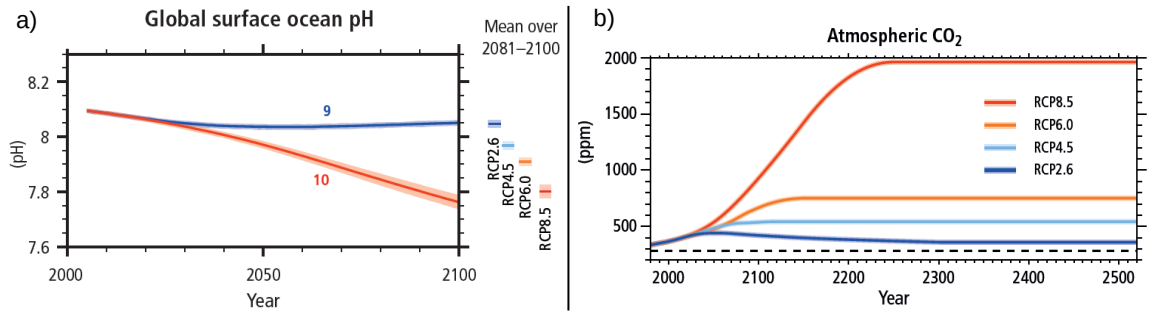

Figure 1.3 – a) Change in ocean surface pH. For all panels, time series of projections and a measure of uncertainty (shading) are shown for scenarios RCP2.6 (blue) and RCP8.5 (red). The number of CMIP5 models used to calculate the multi-model mean is indicated. The mean and associated uncertainties averaged over the 2081–2100 period are given for all RCP scenarios as coloured vertical bars on the right hand side; b) Atmospheric carbon dioxide (CO_2) for the four Representative Concentration Pathways (RCPs) up to 2300 (relative to 1986–2005) followed by a constant (year 2300 level) radiative forcing. The dashed line indicates the pre-industrial CO_2 concentration, from IPCC (2014).

Experimental manipulations of CO_2 concentrations in the field are difficult and limited to a few locals where high CO_2 naturally occurs or where large scale, costly, and labour intensive experiments have been employed (Gattuso *et al.*, 2010). Consequently, the most studies are conducted in the laboratory where CO_2 concentrations can potentially be controlled and reported accurately, and their effects isolated from those of other environmental variables. To successfully perform seawater manipulation, experimental treatments simulate future predicted CO_2 levels in the Oceans while declining pH levels, rising dissolved inorganic carbon content (DIC) and keeping total alkalinity (A_T) constant (Cornwall & Hurd, 2015).

Ideally, experimental tanks must present sufficient flow-through rates since metabolic activity of organisms can modify seawater A_T (Rost *et al.*, 2008). To determine if seawater has the desired properties, at least two components of the seawater carbonate system are measured: pH, A_T, DIC or pCO_2 (Cornwall & Hurd, 2015). This procedures have been followed by the vast majority of published studies (e.g. Small *et al.*, 2015; Small *et al.*, 2016). In order to perform ocean acidification experiments, the design systems vary across studies but usually have common features: a mixing tank (where the CO_2 in injected), a header tank (where mixed seawater passes before being in contact with the organisms) and an experimental tank (where the exposure takes place), where the word “tanks” may assume other form besides typical aquaria (Cornwall & Hurd, 2015).

1.2 Ocean acidification on marine organisms and ontogeny

The predicted levels of CO_2 for the end of the century are expected to affect estuarine and coastal areas more than the open ocean (Lachkar, 2014) due to alkalinity specific traits and the upwelling of CO_2-rich and O_2-poor seawater (Cossarini *et al.*, 2014), while they are great productivity and biodiversity areas (Swearer *et al.*, 1999) and considered the most productive in all Oceans’ extent (Denman & Powell, 1984).

A broad range of marine taxa show different responses facing ocean acidification, with some species negatively affected while other suffer no effect or are positively affected (an increase in cnidarians due to OA was presented by Attrill *et al*., 2007). The main body of research has been developed essentially on calcifying invertebrates, such as molluscs and negative effects occurred with extent gravity within that phylum (see Figure 1.4). Ocean acidification has the potential to affect the process of calcification since calcified organisms - such as planktonic coccolithophores, foraminifera and pelagic molluscs - depend on $CaCO_3$ to form their exoskeleton (Turley & Findlay, 2016). Nevertheless, ocean acidification constraints might affect other marine taxa, not just the ones who are calcification-dependent (Munday *et al.,* 2009, 2010; Pope *et al.,* 2014).

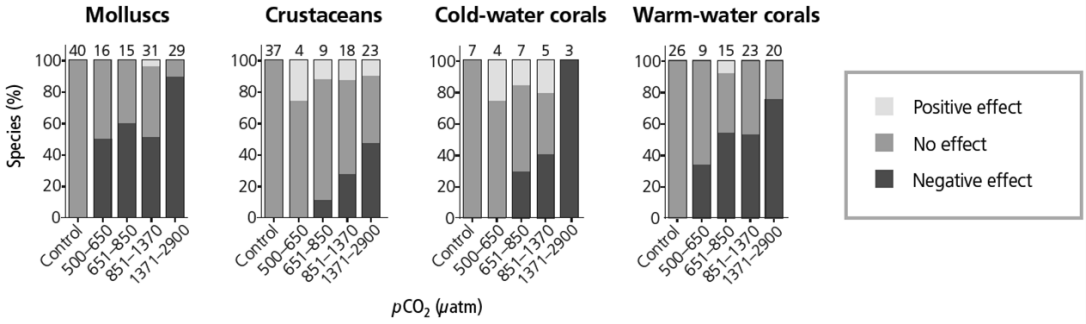

Figure 1.4 - The bottom panel compares the percentage of species sensitive to ocean acidification for corals, molluscs and crustaceans, vulnerable animal phyla with socio-economic relevance (e.g., for coastal protection and fisheries). The number of species analysed across studies is given on top of the bars for each category of elevated CO_2. For 2100, RCP scenarios falling within each pCO_2 category are as follows: RCP4.5 for 500 to 650 µatm, RCP6.0 for 651 to 850 µatm and RCP8.5 for 851 to 1370 µatm. By 2150, RCP8.5 falls within the 1371 to 2900 µatm category. The control category corresponds to 380 µatm (The unit µatm is approximately equivalent to ppm in the atmosphere), from IPCC (2014).

The early life history of calcifying marine invertebrates is assumed to be particularly sensitive to climate change drivers such as ocean acidification (OA) (Kurihara 2008; Pörtner & Farrell 2008; Melzner *et al.* 2009; Byrne, 2011).

When exposed to an elevated pCO_2 level projected to occur under the IS92a emissions scenario (740 ppmv in 2100), calcification rates in the mussel *Mytilus edulis* and the Pacific oyster *Crassostrea gigas* decrease by 25 and 10%, respectively (Gazeau *et al.,* 2007). Arthropods (including crustaceans) also shown negative effects when exposed to OA (Fabry *et al.*, 2008). The species *Hyas araneus* demonstrated less tolerance to OA reaching the Megalopa stage as well as reduced growth and calcification rates (Walther *et al*., 2010). Larvae of the shrimp *Pandalus borealis* faced increased development time (Arnberg *et al.,* 2013). If crustacean species are adversely affected by ocean acidification, then this could have far reaching ecological consequences, as crustaceans are primary and secondary consumers and an important food source for higher trophic levels. For instance, crustacean species form the bulk of the zooplankton and can be present in vast numbers, either as pelagic larvae or as adults (Whiteley, 2011).

Drifting pelagic crustacean larvae have limited swimming ability, high susceptibility to physiological stress (with causes such as pH, temperature, salinity, dissolved oxygen, pollution or mall nutrition) and struggle to find a suitable habitat for settlement and recruitment, which might explain the high mortality rates found in nature (Morgan, 1995). Upon the previous, the cumulative risk of mortality increases with the duration of its planktonic phase (Barnes & Hughes, 1999). Recruitment is an extremely important step

during species ontogeny (Caley *et al*., 1996) and might be influenced by several environmental mechanisms (Sponaugle *et al.*, 2002; Swearer *et al*., 1999). These early and highly susceptible developmental stages of marine organisms have been identified as potential life-history bottlenecks when we consider the impact of global changes on biological systems (Kurihara, 2008; Pörtner & Farrell, 2008; Byrne, 2011, 2012).

Several species of planktonic Chaetognatha, Echinodermata, Mollusca, and Vertebrata had negative responses to OA, with reduced growth and shell calcification, egg and individuals' mortality, biomass losses or even aerobic stress (Fabry et al., 2008), the overall relative sensitivity of different taxonomic groups to ocean acidification and the response of processes has been described (see figure 1.5).

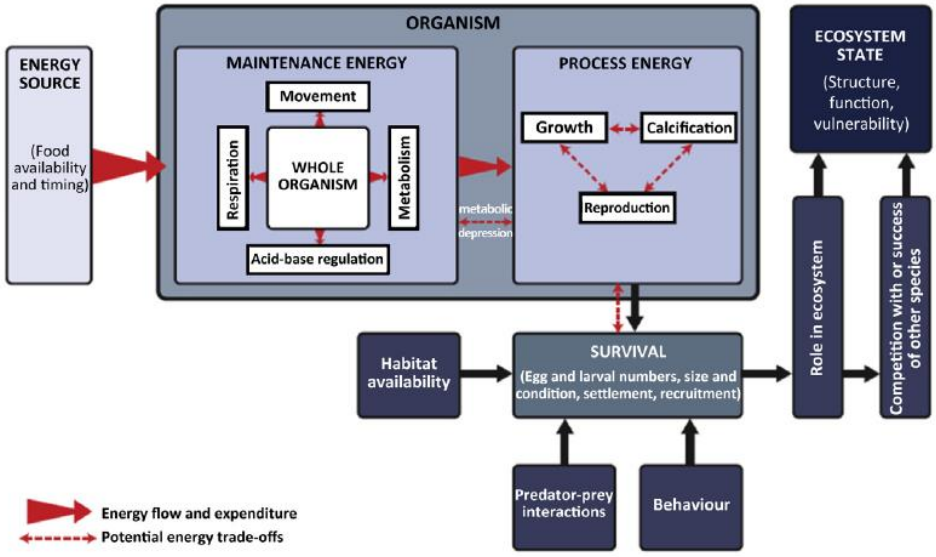

Figure 1.5 - Schematic representation of processes, energy flows and expenditures involved in organisms being able to survive, interacting with external factors that can also influence survival and the consequential impacts on the ecosystem state. Red full arrows indicate energy flows, dashed arrows represent potential energy trade-offs. Ocean acidification or other stressors can cause shifts in the energy distribution within an organism, but could also impact on the external factors, such as food availability, predator–prey interactions, and behaviour (from Turley & Findlay, 2016).

As the majority of crustaceans are committed water-breathers, they are in close contact with their external environment via the gills or equivalent structures, which are specialised for respiratory gas and ion exchange (Taylor & Taylor 1992). The effects of oceanic acidification on marine crustaceans have received increasing attention (e.g.: Wickins, 1984; Kurihara et al, 2004a,b; Kurihara & Ishimatsu, 2008; Mayor *et al.*, 2007; McDonald *et al.*, 2009; Bechmann *et al.*, 2013: Paganini *et al.*, 2014; Almén *et al.*, 2016;

Small *et al.*, 2016), however, the studies are disparate and have been conducted on widely divergent species (see Table 1.1) for varying lengths of time at different pCO_2 levels and the vast majority focused on survival and growth rates (Whiteley, 2011).

Table 1.1. Effects of elevated seawater CO_2 on indices of growth and reproductive capacity in a variety of crustacean species. pCO_2: partial pressure of CO_2 calculated from values published as ppm (mole fraction). Dashes represent absence of available data (Whiteley, 2011).

Species	pCO_2 (kPa)	pH	Time	Effect	Source
Adults					
Acartia tsuensis	0.20	7.4	27 d	No effect on survival, body size, development rate, or egg production	Kurihara & Ishimatsu (2008)
Calanus finmarchicus	0.8	6.85	72 h	No effect on adult growth, decrease in egg production	Mayor et al. (2007)
Acartia steueri	0.20–1.0	7.4–6.8	8 d	Decreased egg production at <pH 6.8	Kurihara et al. (2004a,b)
Acartia erythraea	0.51–1.0	7.0–6.8	8 d	Decreased egg production at <pH 6.8	Kurihara et al. (2004a,b)
Amphibalanus amphitrite	–	7.4	8–11 wk	No effect on growth or egg production	McDonald et al. (2009)
Semibalanus balanoides	0.09	7.7	104 d	Decreased survival	Findlay et al. (2009)
Penaeus occidentalis	–	7.6 & 7.3	56 d	Decreased growth rates	Wickins (1984)
Penaeus monodon	–	7.9–6.4	36 d	Decreased growth rates	Wickins (1984)
Palaemon pacificus	1.0	7.9	30 wk	No effect on growth	Kurihara et al. (2008)
	0.20	7.6	15 wk	Decreased growth and egg production	Kurihara et al. (2008)
Eggs/larvae					
Acartia erythraea	0.20–1.0	7.4–6.8	2d	Increase in nauplius mortality rates and hatching rate	Kurihara et al. (2004a,b)
Acartia tsuensis	0.20	7.4	27 d	No effect on development rate or hatching success	Kurihara & Ishimatsu (2008)
Calanus finmarchicus	0.81	6.95	72 h	Decreased hatching success	Mayor et al. (2007)
Euphausia superba	1.0–2.0	7.7/7.4	26 d	Decreased hatching success	Kurihara et al. (2008)
Amphibalanus amphitrite	–	7.4	8–11 wk	No effect on larval condition, cyprid size and attachment, or metamorphosis	McDonald et al. (2009)
Semibalanus balanoides	0.09	7.7	104 d	Decreased rates of embryonic development, hatching and post-larval growth	Findlay et al. (2009, 2010b)
Echinogammarus marinus	0.20	7.5	18–20 d	No effect on rates of embryonic development or hatchling number	Egilsdottir et al. (2009)
Gammarus locusta	0.10	7.6	–	No effect on growth rates to maturity	Hauton et al. (2009)
Palaemon pacificus	0.20	7.6	–	Decreased body size in settling juveniles	Kurihara et al. (2008)
Homarus gammarus	0.12	–	–	No effect on hatchling number or rate of development	Arnold et al. (2009)

Apart from survival, inter-moult period or growth, now considered “classic” fitness-related endpoints (Dupont & Pörtner, 2013) and taking into consideration the discrepancy in results across species mentioned above, a greater understanding of mechanisms of stress action is needed. This is possible through a better understanding of the biological responses at molecular and physiological levels, where calcification and respiration lead (Dupont & Pörtner, 2013). To this date, few studies have considered sub-individual physiological responses (e.g. Small *et al.*, 2015) and even fewer have considered oxidative stress and energy metabolism endpoints as key factors to assess the susceptibility of marine organisms to OA, linking it to higher levels of biological organization.

Innovative approaches such as biochemical biomarkers and omics approach in ocean acidification and climate changes research are already a reality. Huning *et al.* (2012) observed compensatory processes in shell formation of *Mytilus edulis* by assessing relevant enzymes expression, Putnam *et al.* (2012) revealed plasticity at the molecular level in the reef coral *Pocillopora damicornis*, whereas Silva *et al.* (2016) reported the occurrence of

oxidative stress and implications on energy metabolism related endpoints in the sand-smelt *Atherina presbiter* exposed to ocean acidification.

Physiological stress associated with increased environmental variability is likely to have repercussions on population distribution, growth rates, extinction risk and fitness (Boyce *et al.*, 2006). Ocean acidification promotes a decrease in haemolymph pH (hypercapnia) that can reduce the oxygen affinity of haemocyanin (Pörtner 1990), while an increase in intracellular H^+ can disrupt biological processes such as metabolism, protein synthesis, ion-regulation, and cell volume control (Grainger *et al.* 1979; Madshus, 1988; Whiteley, 1999, 2011). In *H. gammarus*, osmoregulation shifts from larval hyperosmoconforming to post-larval hyper-isoregulation over the metamorphic moult that separates the third-stage larva from the first post-larva (Thuet *et al.*, 1988), which has the adult type of osmoregulation (Charmantier, 1998). The maintenance of stable haemolymph osmolality and ionic concentrations different from those of the external medium is based on active ion transport (mainly Na^+ and Cl^-), in which different enzymes are known to play a significant function, the most well-known being the Na^+-K^+ ATPase (Conte *et al.*, 1977), which activity is still studied nowadays, concerning new field studies such as climate change (e.g. Small, 2013). Moreover, marine crustaceans, in general, possess effective extracellular buffering mechanisms to maintain haemolymph homeostasis, predominantly in the form of the physiological regulation of HCO_3^- (Cameron 1978, 1985; Truchot, 1979; Wood and Cameron, 1985; Whiteley, 1999), and have been perceived to be amongst the most 'tolerant' groups of marine invertebrates to elevated pCO_2 (Melzner *et al.* 2009, Kroeker *et al.* 2010, 2013).

Other highly mobile organisms such as fish that are capable of controlling extracellular pH through active ion transport are predicted to be more tolerant to acidification (Gutowska *et al.* 2008; Pörtner 2008; Melzner *et al.*, 2009). Consecutively, organisms unable to compensate for the reductions in extracellular pH have shown depressed metabolism, growth and fitness (Poörtner *et al.*, 2004; Michaelidis *et al.*, 2005; Siikavuopio *et al.*, 2007). Higher maintenance costs in stressful abiotic environments could cause shifts in energy allocation from reproduction and somatic growth (Kroeker *et al.*, 2010).

1.3 Biochemical biomarkers

According to Gestel & Brummmelen (1996), 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’t be detected from the intact organism” and endpoints are measured through biochemical analysis concerning enzymatic activity, stress proteins and others. Biomarkers of effect can be characterized by a response to a stressor at the biochemical or molecular level as a cellular defence mechanism, while they are unspecific for the stressor in question. This simple notion suggests that biomarkers of effect should have great potential to reflect complex exposures and should also have the ability to include aggregated and sequential exposures over time (Silins & Högberg, 2011).

An organism that is subjected to chemical (e.g. environmental pH change), physical or biological stress may experience abnormal oxidative reactions in their aerobic metabolic pathways, resulting in oxidative stress (Ranby & Rabek, 1978). Reactive oxygen species (ROS) are produced as a result of a wide range of environmental stimuli (e.g. physical parameters and xenobiotics) (Morel & Barouki, 1999) and commonly used as messenger molecules in normal cell functions. However, at increased concentrations they can disrupt normal physiological pathways (Ermak and Davies, 2002). Changes in metabolic demands during life-cycle stages can cause differential ROS production and can also potentially influence the susceptibility to oxidative stress (Fanjul-Moles & Gonsebatt, 2011).

Oxidative stress derived from the exposure of aquatic animals to different kinds of pollutants may promote a redox signal, which in turn, induces protective responses against oxidative damage (see Figure 1.6) and resets the original state of redox homeostasis after temporary exposure to ROS (Rodríguez *et al.*, 2007). If continuously generated by an aerobic metabolism, ROS can damage important biomolecules such as DNA, proteins, and lipids (Halliwell & Gutteridge, 1999). To counteract ROS-induced damage, cells have evolved defence mechanisms that act at different levels to prevent or repair such damage (Hegde *et al.*, 2008).

Under normal physiological states, antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) rapidly eliminate ROS (Yu, 1994; Abele & Puntarulo, 2004): SOD catalyses the dismutation of two superoxide radicals to hydrogen peroxide (H_2O_2), while CAT degrades H_2O_2 into water and oxygen (Holmblad & Söderhäll, 1999;

Mohankumar & Ramasamy, 2006). These mechanisms play a major role protecting or delaying the oxidative damage, peroxidation and enzymatic inactivation (Halliwell & Gutteridge, 1999; Novais *et al.*, 2014). The enzyme lactate dehydrogenase (LDH) that is related with anaerobic energetic metabolism (Ribeiro *et al.*, 1999; Diamantino *et al.*, 2001), also plays an important role in the production of the necessary energy for the maintenance of organisms' physiological homeostasis, in the absence of oxygen. Total carbohydrates and energy consumption, by the measurement of the electron transport system activity (ETS), provide information about the energetic balance (Novais & Amorim, 2013). The reallocation of metabolic resources to avoid/repair internal stress and damages may be challenging for larvae (Cunha *et al.*, 2007), with severe consequences for development, performance and survival, which will eventually impact the population and communities, and ultimately the ecosystem.

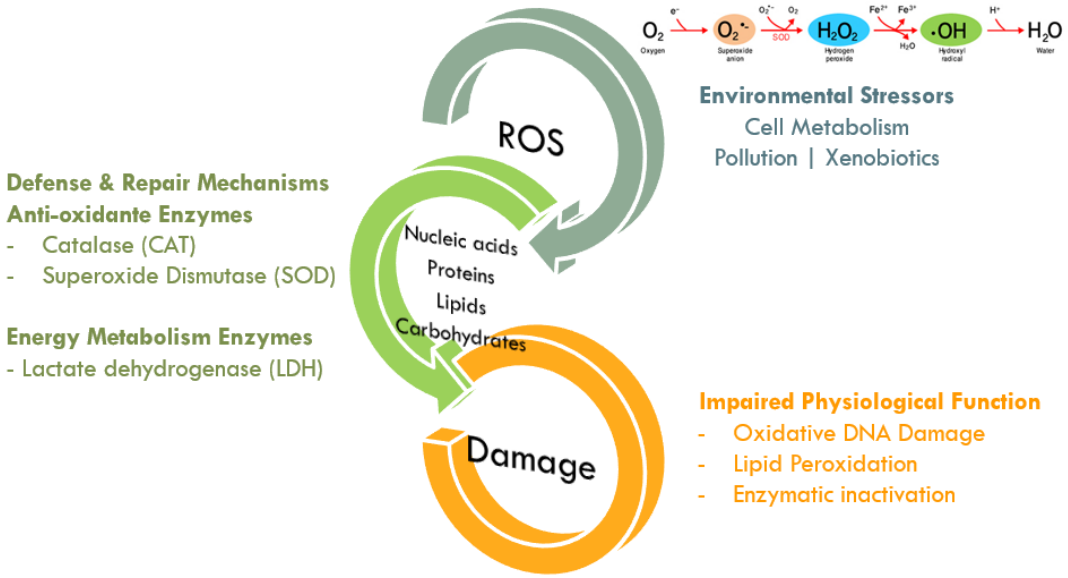

Figure 1.6 – Oxidative stress cascade. Reactive oxygen species, produced in the presence of environmental stress, activate the sub-cellular defence and repair mechanisms, which prevent the damage of nucleic acids, proteins, lipids and carbohydrates. Examples of damage caused by impaired physiological function are described.

As early responding endpoints, the use of tools addressing responses at lower levels of biological organization can rapidly link and anticipate knowledge to what can occur at higher levels of biological organization (population, community or even ecosystems, see Figure 1.7) (Lemos *et al.*, 2010). The reallocation of metabolic resources to avoid/repair internal stress and damages may be challenging for larvae (Cunha *et al.*, 2007), crustacean larvae have evolved sophisticated and redundant responses to ensure survival, some of

which may have biomass consequences compromising the individual life cycle or the population size (Fanjul-Moles & Gonsebatt, 2011).

Along their life cycle, larval, juvenile, and adult crustaceans undergo variations in respiratory and metabolic requirements that are also dependent on environmental conditions. As it may be inferred from their life cycle, crustaceans represent one of the most complex groups of invertebrates, with various aspects of their metabolism requiring regulatory processes comparable to those found in vertebrates. Studies on antioxidant defences and oxidative stress in crustacean life cycles, although very important, are relatively new and information is still limited (Fanjul-Moles & Gonsebatt, 2011).

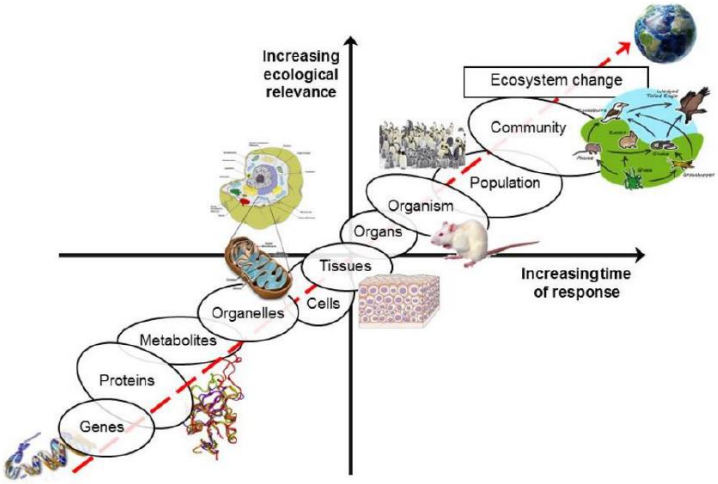

Figure 1.7 - The relationship between different levels of biological complexity, ecological relevance and time of response related to an environmental change or chemical exposure (Lemos *et al.*, 2010).

1.4 Biological model: *Homarus gammarus*

The lobster *Homarus gammarus* is included within a wide taxonomic invertebrate group, the crustaceans (Beard & McGregor, 2004); it is also an economically important species, with a higher value than its congener *Homarus americanus* (Barrento *et al.*, 2009). Ranging from Norway, along European and Mediterranean coasts with its southern limit located at Morocco (Beard & McGregor, 2004; Cobb & Wahle, 1994; Cobb & Castro, 2007; Prodöhl *et al.*, 2007, see Figure 1.8), catches as large as 5,194 ton were reported across its geographical distribution in 2014 (FAO, 2016). This species feeds on mussels, hermit crabs and polychaetes (Beard & McGregor 2004; Kristiansen *et al.*, 2004) so it has also an ecological relevance, acting as primary, secondary or tertiary consumer in the multi-

interaction, ever-changing pathways that are trophic-webs (e.g. Orensanz *et al.*, 1998; Steneck *et al.*, 2004; Worm & Myers, 2003; Frank *et al.* 2005). This species is found within the continental shelf to depths of 150 m, though is more commonly found at depths over 50 m (Holthuis, 1991). It is typically found on rocky substrates, but may also burrow into cohesive mud or form depressions in sand (Cobb & Castro, 2006).

Figure 1.8 – *Homarus gammarus'* geographical distribution (dark grey): ranging from Norway, along European and Mediterranean coasts and going until as far south as Morocco, FAO, 2016.

Large crustaceans are becoming increasingly important to coastal and continental shelf fisheries (Anderson *et al.,* 2011; Steneck *et al.,* 2011). Globally, commercial catches of crustaceans have increased ~5-fold since 1950 and are the only invertebrate group that continues to trend upward in recent years (Anderson *et al.* 2011). In Portugal, a decrease in landings has been reported in 2011 and 2012, while an increase of 30% was reported onwards (INE, 2015).

Decapod crustaceans' growth is characterized by the occurrence of a moult based growth/development/reproduction. *Homarus gammarus* reaches marketable size in the wild after 26-30 moults (5-7 years), which is strongly temperature-dependent (Beard & McGregor 2004; Charmantier & Mounet-guillaume, 1992). Sexual maturation of females does not occur before 5 to 8 years of age (Prodöhl *et al.* 2007; Browne *et al.* 2009), so even though *H. gammarus* is considered a *Least Concern Species* by IUCN (2011), higher landings (not necessarily meaning higher stocks) and long-term growth and sexual maturation are issues of concern to meet for this species future and conservation.

This species comprehend a complex life cycle (see Figure 1.9), going through three planktonic larval stages (see Figure 1.10) a metamorphic post-larvae, benthic juvenile, adult stages and the following behavioural, anatomical and physiological implications between those life stages (Cobb & Wahle, 1994; Cobb & Castro, 2007).

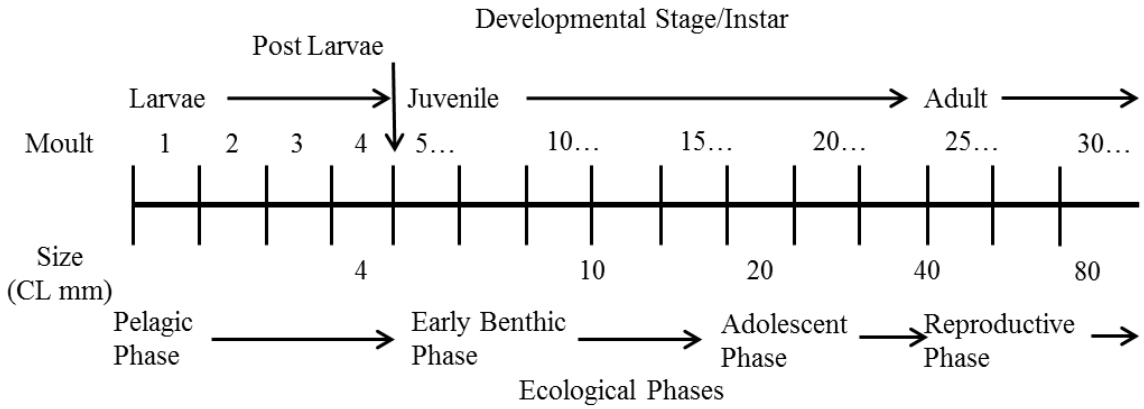

Figure 1.9 - Comparison of terminology for developmental stages and proposed ecological phases of the life history of clawed lobsters. Approximate sizes corresponding to moults are illustrated for *Homarus*. Vertical arrow indicates time of settlement, sourced from Cobb & Wahle (1994).

Stage I larvae exhibit strong positive phototaxy (Browne *et al.*, 2009; Schmalenbach & Buchholz, 2009). Stage II show diminishing positive phototaxy and increased swimming ability (Schmalenbach & Buchholz, 2009) since there is a greater development of the first pair of legs and pleopods, becoming divided. In Stage III larvae, pleopods and claws present a larger size compared to the previous stage and the separation of the first pair of uropods takes place (Browne *et al.* 2009). Stage IV represents a metamorphosed bottom seeking megalopa in transition between pelagic (larval) and benthic (juvenile) environments (Browne *et al.* 2009).

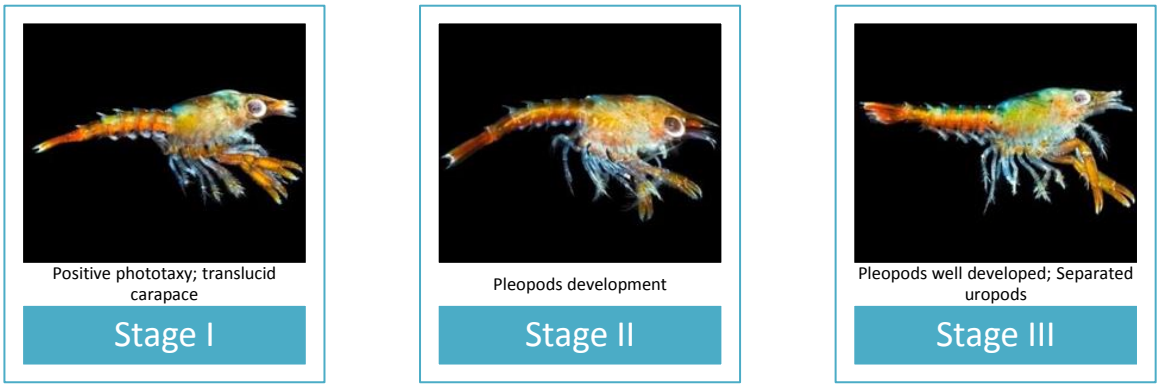

Figure 1.10 - Larval stages of the decapod crustacean *Homarus gammarus.*

Considering its economic and ecological relevance, *H. gammarus* is a broadly studied crustacean. Research topics concerning its life history traits gathered and reported knowledge on fisheries (Browne *et al.*, 2001), population dynamics and genetics (Agnalt et al, 2009; Hughes, 2000; Ulrich et al, 2001), physiology and reproduction (Free, 1998; Middlemiss *et al.*, 2016), rearing, aquaculture and stock enhancements (Beard & Wickins, 1992; Benavente *et al.*, 2010; Branford, 1978; Evjemo *et al.*, 2009; Jørstad *et al.*, 2009, Schoo *et al.*, 2014) and climate change as well (Arnold *et al.*, 2009; Schmalenbach & Franke, 2010; Small *et al.*, 2015; Small *et al.*, 2016;). This species, target of comprehensive studies, is a worthy study model where major inferences may be obtained considering their physiology and conservation measures taking the prospects of current/future global changes in an important economic and ecological resource.

1.5 Larval rearing of *Homarus gammarus*

For many commercially important species, the interest in aquaculture production for stock enhancement or food is often inversely proportional to fishery landings (Tlusty *et al.*, 2005). Stock enhancement experiments in Europe have been evaluated by Bannister & Addison (1998) and, in an attempt to develop new methods, a large-scale lobster enhancement experiment was initiated in 1990 in Norway to assess if releases of hatchery-produced juveniles can stabilise recruitment and hence increase stock on a long-term basis (Agnalt *et al.* 1999). Restocking programs have been implemented in several European countries (Beard & Wickins 1992; Van Der Meeren 2005; Benavente *et al.* 2010; Schmalenbach *et al.* 2011), especially Norway, Ireland, United Kingdom and France. These

conservation efforts not only reinforced natural stocks of *Homarus gammarus* but gave great insights on how the larval rearing of this species is performed, through years and years of research, protocol optimization and know-how (e.g. National Lobster Hatchery).

Intensive rearing for closed-cycle aquaculture purposes is already being done in Norway, where the Norwegian Lobster Farm produces plate size *Homarus gammarus*. Nevertheless, achieving economic feasibility isn't easy as this species' rearing presents some constraints, namely cannibalism (Kristiansen *et al.* 2004; Prodöhl *et al.* 2007; Browne *et al.* 2009). High densities intensify this behaviour so the captive rearing requires reduced stocking densities or even individual rearing, resulting in high initial investments and intensive maintenance procedures (Browne *et al.* 2009; Schmalenbach *et al.* 2009; Benavente *et al.* 2010). The individual rearing of lobster larvae may also be applied in experimental research studies since outcomes may be influenced due to cannibalism instead of the experimental treatment itself, being survivorship an example.

One of the major constraints in marine aquaculture is efficient larval culture (Rainuzzo *et al.*, 1997). Most marine larvae require live prey. Prey items used in aquaculture must have an adequate size, a high nutritional profile, swimming ability and capacity to survive in the target species culture environment (Kumlu & Jones, 1995a,b). The most common preys used in the larval rearing of *Homarus gammarus* larvae are enriched *Artemia* nauplii and frozen Mysis (e.g. Beard & Wickins, 1992; Kristiansen et al., 2004; Schmalenbach & Franke, 2010; Scolding et al., 2012). Artemia nauplii swim slowly and can be reliably cultured at high densities (Conklin, 1995; Narciso, 2000; Beck & Turingan, 2007). However, these organisms are not natural preys in the wild (Figueiredo *et al.*, 2009) and have an unsatisfactory nutritional profile for most marine larvae since they lack certain essential polyunsaturated fatty acids (Léger *et al.*, 1987; Narciso & Morais, 2001). Long-chain polyunsaturated fatty acids (polyunsaturated fatty acid with ≥20 C atoms, PUFA) such as eicosapentaenoic (EPA, 20:5n−3) and docosahexaenoic (DHA, 22:6n−3) acid are particularly important for larval growth but cannot be synthesized *de novo*, and therefore, need to be taken up from food (Bergé & Barnathan, 2005). A low level of essential PUFA in the diet reduces larvae physiological condition, and therefore reduces their chances of survival and growth (Anger, 1998).

As passive filter feeders, *Artemia* nauplii may be enriched with microalgae, yeast and probiotics. In the case of microalgae enrichment, the selected microalgae species is a crucial issue for the improvement of *Artemia* growth, modifying both its growth rate and its

biochemical composition (Seixas *et al.*, 2009). The cryptophyte flagellate *Rhodomonas* fatty acid composition was studied by several authors. Although Trembley *et al.* (2007) reported low levels of PUFAs (namely EPA and DHA), Seixas *et al.* (2009) observed that *Rhodomonas lens* enriched *Artemia* contained higher levels of eicosapentaenoic acid (EPA, 6.2%) than artemia juveniles fed with *Tetraselmis suecica* (4.1%, P≤

0.01), whereas docosahexaenoic acid (DHA) was only found in juveniles fed with Rhodomonas lens (1.1%). This microalgae has a high content in protein which also promoted faster Artemia growth (Seixas et al., 2009). Nevertheless, the enrichment of Artemia nauplii in the rearing of *Homarus gammaus* larvae included several other microalgae such as *Isochrysis galbana* (Uglem *et al.,* 2006), *Chaetoceros spp*. (Browne *et al.,* 2009) or the Self-Emulsifying Lipid Concentrate – SELCO (Scolding *et al.*, 2012), which is rich in Highly Unsaturated Fatty Acids (HUFA's) and contains particularly high levels of the essential Omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

1.6 Thesis aim and objectives

The knowledge on the sensitivity of several marine taxa to climate change, namely to Ocean Acidification scenarios, is increasing. Some information is now available on how plankton, molluscs, fish or crustaceans cope with this stressor. The study of responses through species' ontogeny allows a further comprehensive understanding on how ocean acidification might affect this ecosystem in the future. Nevertheless, the vast majority of studies have focused on specific life-history stages.

While the assessment of crustaceans' susceptibility to Ocean Acidification has been evaluated through developmental and behavioural responses, few considered the importance of insights at the physiological functioning.

Consequently, the aim of this thesis is:

To investigate the developmental and physiological sensitivities of *Homarus gammarus* larvae – an ecologically and economically relevant decapod crustacean - when exposed to elevated levels of pCO_2 in line with the IPCC predictions for 2100.

The detailed objectives of this study are three-fold:

1 – To investigate the developmental and behavioural responses of lobster larvae through the entire larval development (survival, growth, inter-moult period) when exposed to ocean acidification levels predicted by 2100.

2- To give the first insight on the biochemical responses of *Homarus gammarus* larvae under an Ocean Acidification scenario (oxidative stress and energy metabolism related endpoints) and discuss how physiological sensitivities may be linked to changes in life history traits.

3- To understand the proper rearing conditions and survival of the individuals through the larval development and performing quality aquaculture techniques, while manipulating chemical properties of seawater and maintaining the desired treatment conditions at all times.

2. MATERIALS AND METHODS

2.1 Broodstock husbandry

Newly hatched larvae of *Homarus gammarus* were obtained from a single berried female at CETEMARES Building aquaculture facilities (Peniche, Portugal). The female was provided by a local crustacean retailer (Frutos do Mar, Portugal), the same week it was caught in Portuguese West Atlantic coastal waters.

After the acclimatization due to the 30-minute transport to CETEMARES facilities' , the female was kept in a tank (V=500L) connected to a recirculation water system (TMC System 2500/5000 Filtration Unit), kept at a salinity of 35 PSU, temperature of 19°C and dissolved oxygen values close to saturation (DO ≥ 8mg.L^{-1}). Natural light/dark cycle was used throughout the rearing and experimental period (11h:13h light/dark). The amount of nitrites, nitrates and ammonia were always kept under 0.25mg/L.

The female was fed once a day at noon with whole mussels (*Mytilus edulis*) *ad libitum* and provided with refuge to promote wellbeing and minimize stress. It was considered successfully acclimatized since in less than 24h after the arrival it was already feeding.

To confirm the maturation stage of females, the Embryonic Eye Index (Browne *et al.* 2009 and Beard *et al.* 1985) was calculated from measurements in a small batch of eggs. This procedure was important for the assessment and calculation of the hatching time in function of temperature. Egg maturation and hatching was allowed to occur naturally, without any thermal, osmotic or mechanic stress.

2.2 Experimental design

2.2.1 – Experimental system set-up and seawater manipulation

The experimental system consisted in two independent recirculating aquaculture systems filled with previously filtered natural seawater and equipped with aerobic biological filtration (BioBalls and Fluidized Sand Filter – ReefSet)), mechanic filtration (protein skimmer – AquaMedic Blue 1000), chemical sterilization (UV Sterilizer – TMC Vecton V2) and water temperature control (chiller HAILEA, HC-150A; Aquarium heater EHEIM).

Each sump (V=60 L) delivered equilibrated seawater to a 60L transparent tank at a flow rate of 360L.h^{-1}. Each tank held 47 individual rearing experimental units (V= 0,2L) with a cylinder-spherical shape (see Figure 2.1). A gentle flow of ambient air was provided at the surface of each individual rearing units to promote water flux inside the units. The rearing system was allowed to mature for 6 weeks before the experiment took place.

The experimental treatments targeted the exposure to two different pCO_2 (control: 370 µatm; increased: 710 µatm) and pH (control: 8.15; decreased: 7.85) scenarios. These scenarios are in line with the present conditions in Portuguese West Atlantic coastal waters and with IPCC RCPs for year 2100, where pH is expected to decrease 0.15-0.35 pH units, depending on the scenario family.

Larvae were exposed to a nominal seawater temperature of around 19ºC (see table 2.1). This temperature resembles the Mean Sea Surface Temperature (SST) from 1981-2010 of Portuguese central waters in the month the experiment took place (NOAA Optimum Interpolation (OI) SST V2) and was coincident with current field measurements for that month.

Seawater manipulation was performed in each treatment's sump through the injection of pure CO_2 (SERA CO_2 gas bottle) and controlled by a pH controller (Flora Fertilization System and Ceramic, SERA) in order to achieve the desirable pH values in every experimental units. The CO_2 injection was regulated to a hysteresis of 0,001 to prevent large pH oscillations and therefore the undesired pulse effect. The experimental system with CO_2 injection was allowed to equilibrate before the essay took place.

Water chemistry was monitored daily. The pH measurements following the National Bureau of Standards Scale (NBS Scale) took place two times a day in 3 reference experimental units of each treatment using a pH electrode connected to a hand-held pH meter which was calibrated every two days with pH buffer standards. Temperature (kept at 19 ºC) and salinity (kept at 35 PSU) were measured twice a day in the same reference experimental units of each treatment with a handheld multiparameter meter (YSI, Professional Plus) and a refractometer (V^2, TMC), respectively. Also DO (mg.L^{-1}) was measured using the same multiparameter meter.

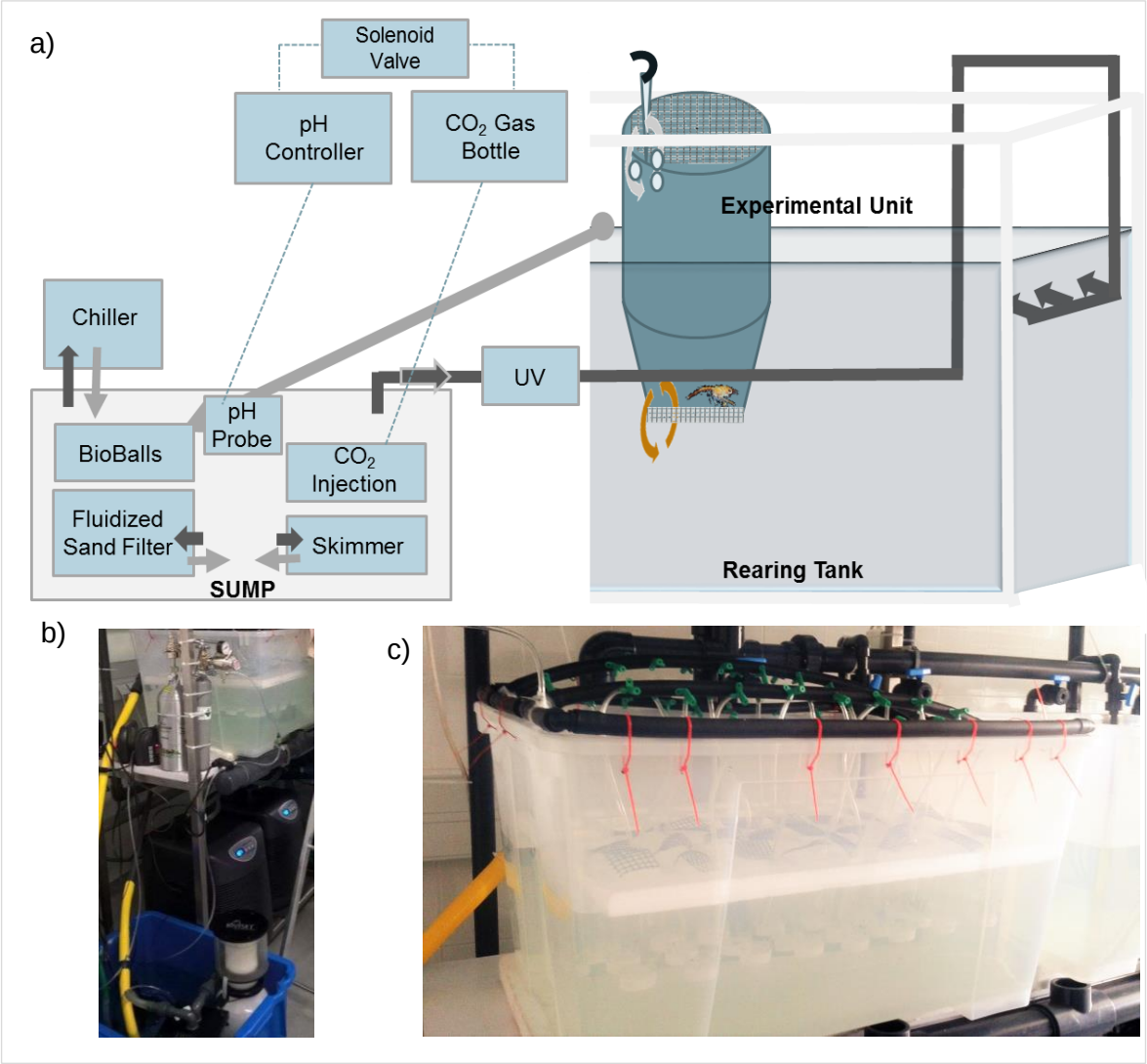

Figure 2.1 – Experimental setup: a) Scheme of the designed RAS, ➡ represent seawater inflow, ➡ represent seawater outflow and ----- the CO_2 injection set-up; b) Real image of the system set-up; c) close-up on one of the rearing tank and individual rearing experimental units.

Total Alkalinity (A^T) was measured through manual potentiometric titration to a pH endpoint of 4.5 using standardized sulfuric acid (H_2SO_4; 0,019N) on a closed vessel. pCO_2 (µatm) was derived from TA, pH, temperature and salinity with the CO2SYST program (Lewis & Wallace, 1998), with constants provided by Mehrbach et al. (1973) and refitted by Dickson and Millero (1987) and KSO_4 constants from Dickson (1990). Sampling occurred every 5 days in triplicate from experimental units, kept in Borosilicate flasks without any air between the water mass until reaching the temperature in which the titration would take place (room temperature, 25 ºC) and then analysed. The used benchtop pH meter (WTW, inolab® pH 7110) was always calibrated before titration took place. Water-chemistry parameters for both treatments during the exposure period are presented in table 2.1. For

the equilibration of the target conditions, and since the response time of chillers and gas exchange rates are directly related with temperature, room temperature was controlled with the support of air conditioning.

Table 2.1. Water chemistry parameters over the course of the experimental period (Mean ± SD): temperature (°C), salinity, pH (NBS Scale) – measured in the experimental units, total alkalinity (A_T, mg.L-1 $CaCO_3$), carbon dioxide partial pressure (pCO_2, µatm), pH (NBS Scale), bicarbonate concentration ([HCO3-], µmol.kg-1), carbonate concentration ([CO_3^{2-}], µmol.kg-1). Parameters calculated using CO_2SYS program (Lewis & Wallace 1998) with constants provided by Mehrbach *et al.* (1973) refitted by Dickson & Millero (1987) and KSO_4 constants from Dickson (1990).

	370 µatm | Control	710 µatm | Ocean Acidification
T (ºC)	18,90 ±0,23	19,10 ±0,25
Salinity	35,00 ± 0,74	35,00 ± 0,74
pH (NBS Scale)	8,14 ±0,03	7,84 ±0,05
TA (mg.L^{-1} $CaCO_3$)	175,32 ±4,39	155.47 ±1,88
pCO_2 (µatm)	322,80 ±15,61	733,80 ±72,85
[HCO_3^-]	1403,40 ± 24,22	1391,50 ± 16,81
[CO_3^{2-}]	127,30 ± 9,03	55,3 ± 4,78
DO (mg.L^{-1})	6,70 ± 0,37	6,70 ± 0,34

2.2.2 – Experimental Procedure

The chosen, acclimatized, female had a large, healthy and very close to complete maturation egg mass. In the first hatching day, newly hatched larvae (Basal; N=24) were immediately and gently collected with a sieve, measured, weighed and preserved for further biochemical biomarkers analysis. The remaining larvae were collected with a sieve right after hatching to a 40 L bucket with low flux aeration supply to promote locomotion and prevent cannibalism. After, larvae were separated to two buckets, acclimatized for 30 minutes with increasing proportions of the corresponding treatment seawater and then individually transferred to each experimental unit, using a disposable Pasteur pipette with trimmed tip. Once the assay begun, survival and moulting events were assessed every 12h during the entire experiment (see Figure 2.2), through non-invasive methods (visual inspections).

The first sampling moment took place 24h after the exposure (Stage I; N=24). Right after, the sampled larvae were replaced with newly hatched larvae from the same female which were also individually collected, measured and preserved (Basal; N=24) and acclimatized and exposed to the target scenarios (N=24). Once larvae had reached the Stage III (SIII - 48h after moulting [AM]), they were randomly selected (N=18), collected and preserved as mentioned above.

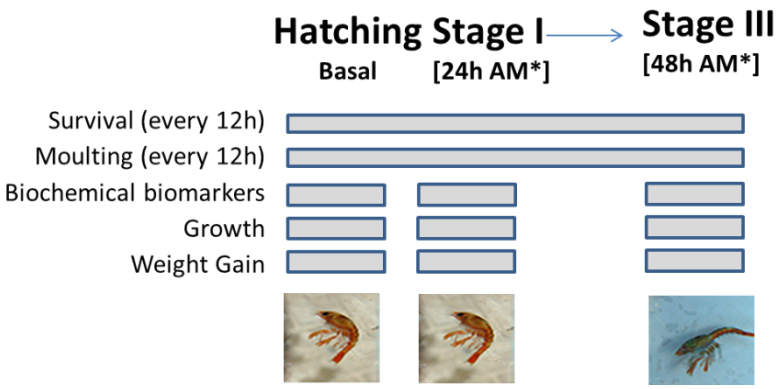

Figure 2.2 – Scheme of the sampling design, addressed endpoints and used life stage in the experiments.

2.2.3 –Live feeds: Artemia franciscana nauplii enriched with Rhodomonas lens

Homarus gammarus larvae were fed 48h-old enriched *Artemia franciscana* nauplii at a concentration of 1nauplii.mL^{-1} (200 nauplii per experimental rearing unit), with a daily 100% feed renewal.

Commercial artemia cysts (Ocean Nutrition®) were decapsulated and put to hatch at 25 ºC. 24h after the hatching peek and quantification, the needed amount of artemia nauplii was enriched for 24h with *Rhodomonas lens* at a density of 3x10^{5} cel.mL^{-1} (dry weight [DW] of 0,68g.day^{-1}) in 2 L cylinder-conical vessels (Aquabreed, Aqua Medic).

Rhodomonas lens was aseptically cultured from a previously owned highly concentrated batch. Approximately 1L was used to a final volume of 5 L in 6 L containers. F/2 medium was added twice a week at a proportion of 3.5 mL.L^{-1}).

A growth curve was determined for this microalgae taking into consideration Cetemares' specific culture conditions. In order to estimate culture concentration and

quantify the needed daily amount to enrich artémia nauplii, a relation between OD and concentration was obtained (see Figure 2.3).

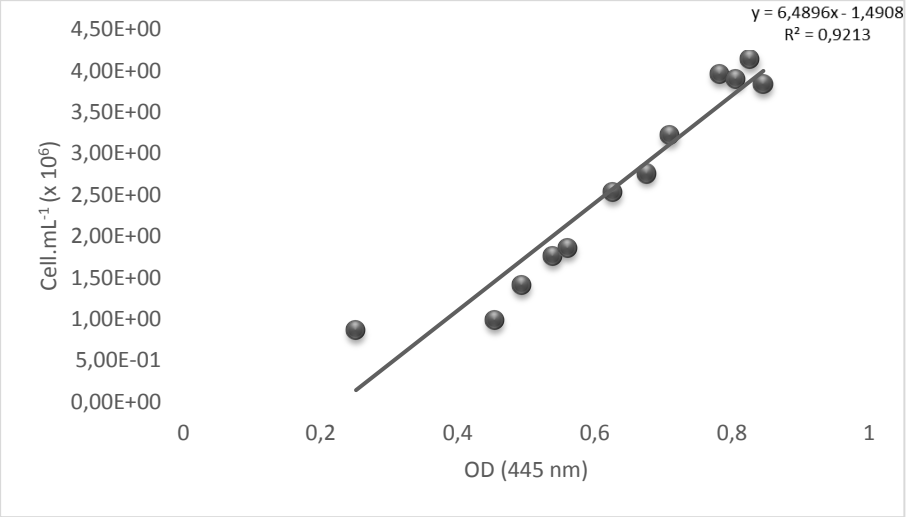

Fig. 2.3 - Relation between OD and concentration for the cryptophyte *Rhodomonas lens* in Cetemares building considering an absorbance at 445nm. $\mu.day^{-1}$ = 0,364, duplication time = 1.9 days.

2.3 Determination of Behaviour and Development Responses: Survival, Inter-moult Period, Morphometric Analysis, and Growth

2.3.1 – Organism Survival

The number of live individuals was counted every 12h in each rearing unit and cumulative survival was expressed as the percentage of the number of individuals introduced at day 0. This also took into account individuals removed for measurements: 24h after exposure that were replaced and the ones 48h after reaching Stage III that were not. Survival was also expressed as the percentage of individuals present in each scenario from the previous stage, to capture stage-specific changes in survival.

2.3.2 – Inter-moult Period Assessment

Organisms were assessed individually every 12h until the end of exposure, moulting events were registered and inter-moult period (IP) was expressed as the mean (days) of the duration of each larval stage.

2.3.3 – Morphometric Analysis

While in a Petri dish with the respective scenario seawater, each larvae was individually laid flat on their right-hand side, with the help of a needle, to be photographed with a camera attached to a stereomicroscope (ZEISS, Stemi 305 EDU Mikroskop-Se). Photographs were later analysed with an Image Software (ZEN 2, ZEISS) to measure Carapace length (CL: rear of eye socket to rear of carapace) and therefore extrapolate growth. Individuals were then rinsed with distilled water, excess water was removed by gently blotting with fine tissue paper and wet weight (mg) was measured with an Analytical scale. This process, repeated to each individual, took no more than one minute per organism.

The organisms were individually placed in a previously identified microtube on ice and when all were sampled, placed immediately at -80ºC until biochemical biomarkers analysis.

2.4 Determination of Biochemical Responses: Biochemical Biomarkers Analysis

Due to the small size of the individuals – and therefore low available biomass –, Stage I larvae were arranged in pools of 3 and each pool was assumed as one replicate (N=8) whereas in Stage III, with bigger biomass available, only 2 individuals were needed per pool (N=9). This methodology was applied to each treatment.

Each sample was then homogenised in 750 µL of potassium-phosphate buffer (0.1M; pH 7.4), using a mechanical homogenizer. Several aliquots of the homogenate were separated into different microtubes for later DNA damage, LPO and carbohydrates content measurements. A part of the homogenate was also used to quantify ETS activity after being centrifuged at 1000g for 10 minutes at 4ºC. The rest of the homogenate was centrifuged at 10,000g for 20minutes at 4ºC to obtain post-mitochondrial supernatant (PMS) that was used to assess soluble protein content and SOD, CAT and LDH enzymatic activities. All samples were preserved at -80 ºC until further analysis.

The parameters above were determined in triplicate technical replicates 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.

2.4.1 - Oxidative stress *related biomarkers*

SOD activity was measured following McCord & 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 c that can be measured following the increase in absorbance at 550 nm for 10 minutes. SOD activity was expressed in $U.mg^{-1}$ of protein using a SOD standard of 1.5 $U.ml^{-1}$, where 1 U 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 $\mu mol.min^{-1}.mg^{-1}$ of protein, using a molar extinction coefficient of 40 $M^{-1}.cm^{-1}$.

DNA damage (strand breaks) was evaluated following the DNA alkaline precipitation assay (Olive, 1988), adapted by Lafontaine *et al.*, (2000). After the precipitation of intact single and double stranded DNA, the damaged nucleic acids remaining in the supernatant were coupled with Hoesch dye (1 µg/mL bis-benzimide, Sigma-Aldrich) and its levels were determined by measuring fluorescence using an excitation/emission wavelength of 360/460 nm. Results were expressed as mg of DNA.mg Ww^{-1}, using calf thymus DNA as standard to extrapolate DNA concentration.

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 nmol TBARS.mg Ww^{-1}, using a molar extinction coefficient of $1.56x10^5$ $M.cm^{-1}$.

2.4.2 - 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). LDH activity was measured by the efficiency of this enzyme to convert pyruvate to lactase 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 $6.3x10^3$ $M^{-1}.cm^{-1}$.

The total carbohydrates content was measured at 492nm according to De Coen & Janssen (1997), using a reaction of 5% phenol with sulfuric acid (H_2SO_4, 95-97%), with glucose as standard solution. Results were expressed as mJ. mg Ww^{-1}.

ETS activity in the mitochondria is a measurement of the cellular energy consumption (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 stochiometrical relationship: 2 µmol of formazan formed = 1 µmol of oxygen consumed. The oxygen consumption rate was then converted into the energetic equivalent of 484 kJ. mol O_2^{-1} for average carbohydrate, lipid, and protein consumption combinations (Gnaiger, 1983).

2.5 Statistical analysis

For the analysis of survival, a Kaplan-Meier analysis was performed to determine cumulative survival and a Breslow (Generalized Wilcoxon) test was employed to test significant differences between Control and Ocean Acidification scenarios. For each larval stage, an Independent Samples T-test was employed to determine significant differences between scenarios on IP, growth, Weight Gain and Biochemical responses.

For all statistical tests, the significance level was set at p-value ≤ 0.05. All calculations were performed with IBM SPSS Statistics 22. Where applicable, results are presented as Mean ± Standard Deviation (S.D.).

3. RESULTS

3.1 Behaviour and development responses: survival, inter-moult period, morphometric analysis and growth

3.1.1 – Survival

The overall cumulative survival of *Homarus gammarus* larvae throughout the exposure time to Control and Ocean Acidification scenarios presented a tendency to be lower at the OA scenario (see Figure 3.1), where an accentuated declining on survival at day 4 was observed during the first moulting event, from Stage I to Stage II. From this day onwards, survival showed a strong decreasing tendency, with the last exposure day showing a cumulative survival of 76.6% in Control and 63.8% in the OA scenario (see Table 3.1). The survival of *H. gammarus* in terms of % survival from previous stage emphasizes the declining in survival after moulting into Stage II (see Figure 3.2), around 20%. Stage-specific cumulative survival states the survival (%) at each stage and in the end of the exposure (Table 3.1).

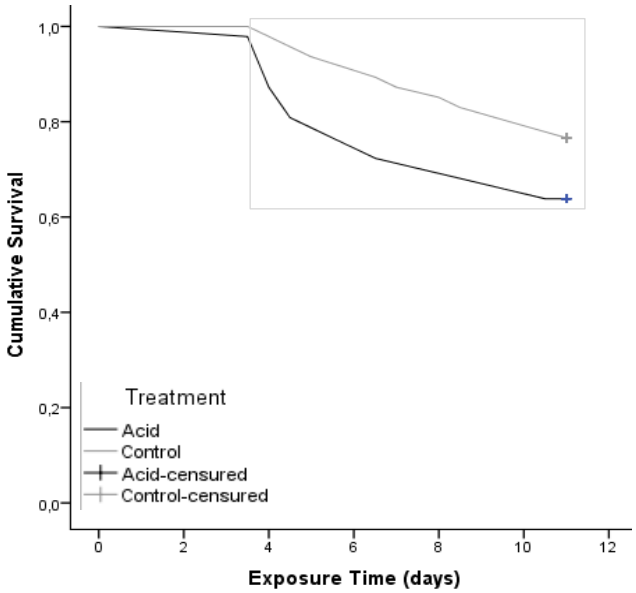

Figure 3.1 – Cumulative survival of larvae of *Homarus gammarus* reared under control and ocean acidification scenarios (Mean ± S.D., N=46) during 11 days until 48h after reaching larval stage III. Grey line indicates Control scenario and black line indicates Acidification scenario. "+" in the end of each line represents censured data, where the remaining larvae ceased to be monitored.

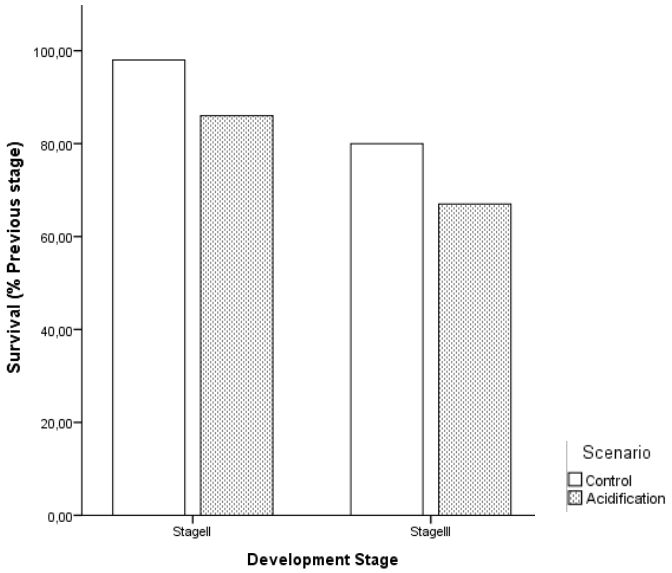

Figure 3.2 – Survival in % from previous stage of larvae of *Homarus gammarus* reared under control and ocean acidification scenarios. White bars indicate Control scenario and dotted bars indicate Acidification scenario.

3.1.2 - Inter-moult Period

Inter-moult Period (IP), in terms of time (days) duration of each stage (see Figure 3.3), of *H. gammarus* throughout larval development under control and acidification scenarios showed that SI Larvae from Acidification scenario took significantly less time to moult into SII than the individuals from the Control scenario (p-value = 0.003). The opposite pattern was observed when considered the IP from SII-SIII, where larvae from Acidification scenario took more time to moult into Stage III than larvae from the Control (p-value = 0.019). The IP – in terms of time (days) duration of each stage - can be found in Table 3.1.

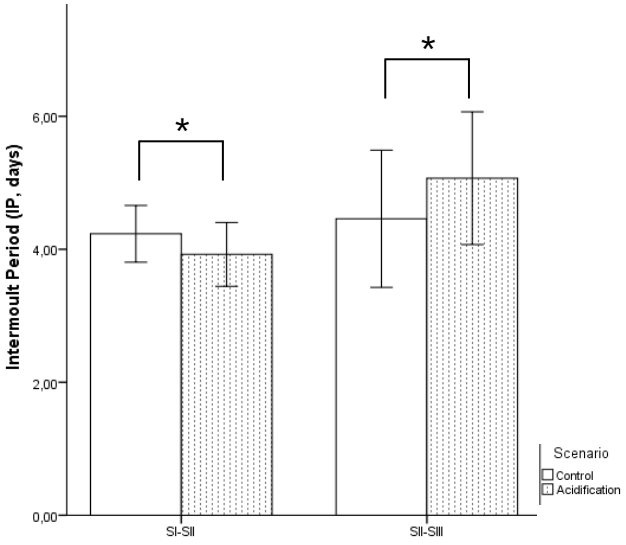

Figure 3.3 – Inter-moult Period of larvae of *Homarus gammarus* reared under control and ocean acidification scenarios (Mean ± S.D.): SI-SII ($N_{control}$= 43; N_{acid} = 39) and SII-SIII ($N_{control}$= 36; N_{acid} = 29). Striped bar indicate Control scenario and dotted bars indicate Acidification scenario. * Indicate significant differences between scenarios in each inter-moult Period (Independent Samples T-test, p-value ≤ 0.05).

Table 3.1. – Life history traits of *Homarus gammarus* throughout larval development under Control and Ocean Acidification scenarios (Means ± S.D.): Survival (cumulative % of initial number), inter-moult period (duration of the stage until moulting, days).

	370 µatm | Control	710 µatm | Ocean Acidification
Survival (%)		
Stage II	97.6	80.47
Stage III	80.48	69.7
Final	76.6	63.8
Inter-moult Period (d)		
Stage I – Stage II	4.2 ± 0.3	3.9 ± 0.47
Stage II – Stage III	4.6 ± 1	5.1 ± 1
Total	8.8	9.0

3.1.3 - Morphometric Analysis and Growth

Weight gain (mg) of *Homarus gammarus* throughout larval development under control scenario and acidification scenario is displayed in Figure 3.4. Significant differences were not found between treatments in SI or SIII larvae at the sampling moment. Nevertheless, in the Acidification scenario, a trend to less weight gain can be observed for both stages.

Overall, 24h after the exposure, SI larvae from Control and Acidification weighted in average 8.4% and 4.9% more, respectively, when compared with their newborn values. SIII larvae, 48h after moulting and with the same exposure time, gained an average 120.6% and 138.1% body weight, respectively.

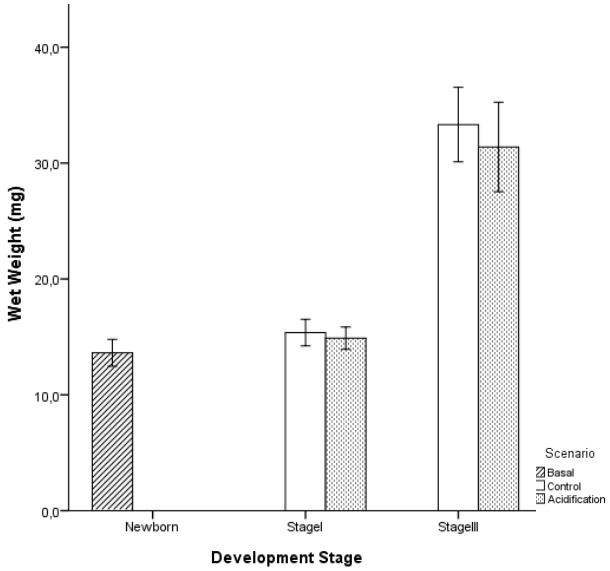

Figure 3.4 – Wet weight of larvae of *Homarus gammarus* reared under control and ocean acidification scenarios (Mean ± S.D.): Newborn (N=48), Stage I (N=24) - 24h after exposure - and Stage III (N=18), 48h after moulting and with the same exposure time. Striped bar indicates Basal scenario, white bars indicate Control scenario and dotted bars indicate Acidification scenario.

Carapace length (µm) of *H. gammarus* throughout larval development under control scenario and acidification scenario is displayed in Figure 3.5. Significant differences were not found between treatments in SI but there were differences in SIII larvae at the sampling moment, where larvae from the Acidification scenario were significantly smaller (p-value = 0.000). Overall, when compared with newborn carapace length, SI larvae - 24h after the exposure - from Control and Acidification grew in average 0.7% and 0.4%, respectively, whereas SIII larvae - 48h after moulting and with the same exposure time - grew in average 47.1% and 35.5%, respectively.

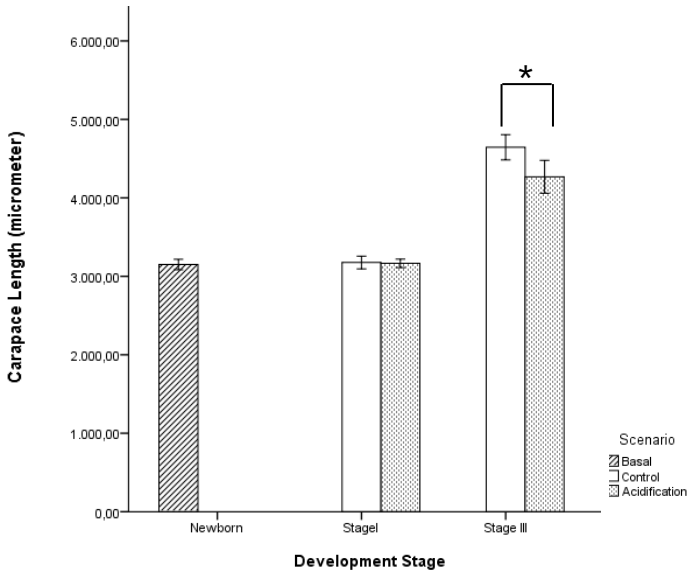

Figure 3.5 – Carapace length of larvae of *Homarus gammarus* reared under control and ocean acidification scenarios (Mean ± S.D.): Newborn (N=48), Stage I (N=24) - 24h after exposure - and Stage III (N=18) after moulting and with the same exposure time. Striped bar indicates Basal scenario, white bars indicate Control scenario and dotted bars indicate Acidification scenario. * Indicate significant differences between scenarios in each inter-moult Period (Independent Samples T-test, p-value ≤ 0.05).

3.2 Biochemical biomarkers responses: oxidative stress & energy metabolism

3.2.1 - Oxidative stress *related biomarkers*

Oxidative stress related responses of *H. gammarus* throughout larval development under the control and acidification scenario are displayed in Figure 3.6. When compared with Basal and Control scenarios, SOD activity in acidification showed a tendency to decrease in SI larvae, and was significantly lower in SIII (p-value = 0.033; Fig. 3.6a). Catalase activity was not detected on the larval stages of *H. gammarus* larvae, for both scenarios.

No significant differences were found in LPO quantification between scenarios for both development stages but a tendency to decreased LPO in acidification can be observed (Fig. 3.6b). DNA damage was very low at newborn larvae and increased till SI, where no differences were found between treatments (Fig. 3.6c). However, in SIII, larvae had significantly higher DNA damage levels in the acidification scenario than in control (p-value = 0.031).

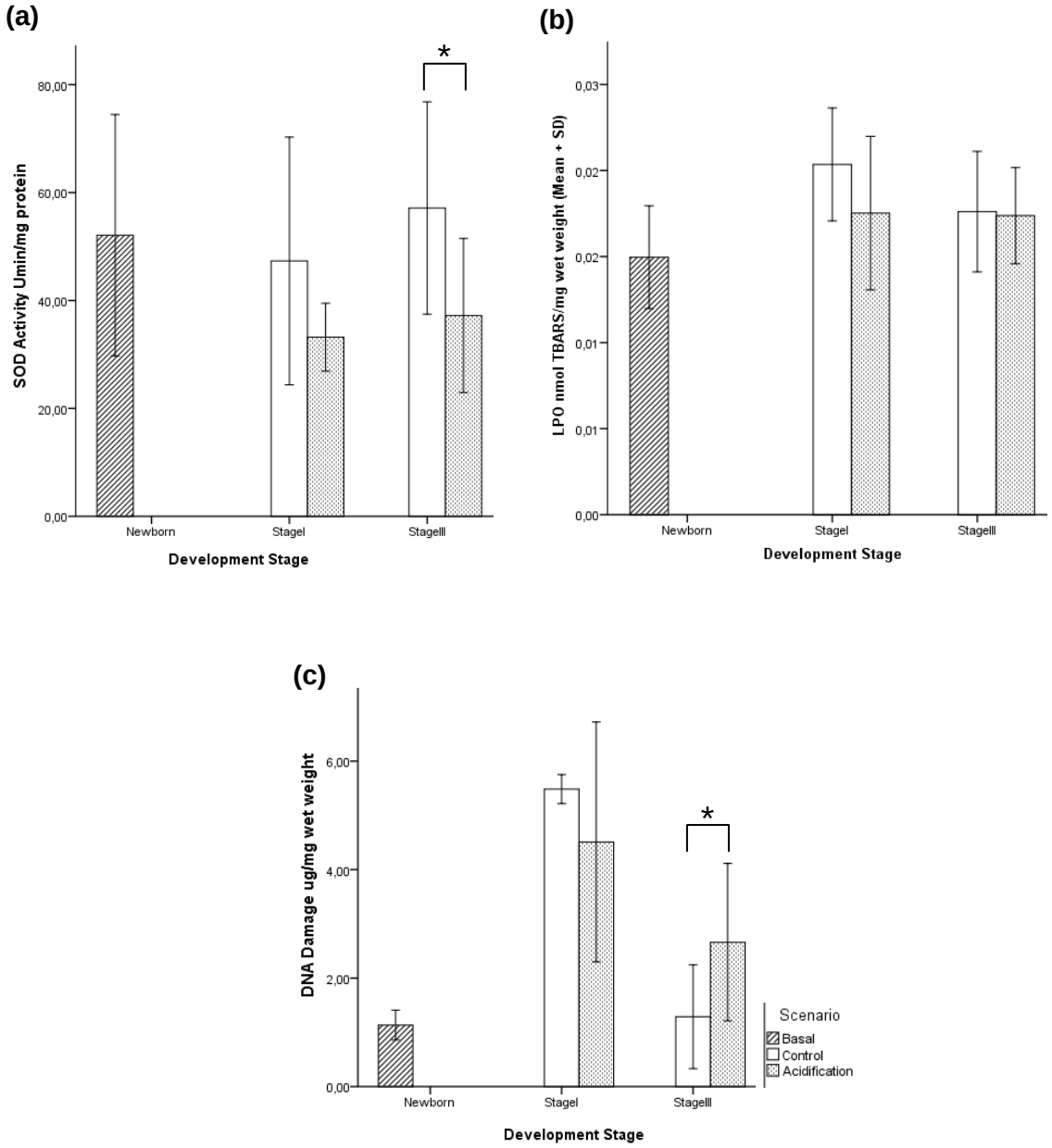

Figure 3.6 – Oxidative stress responses of *Homarus gammarus* larvae reared under control and ocean acidification scenarios (Mean ± S.D.): Newborn (N=16), Stage I (N=8) - 24h after exposure - and Stage III (N=9), 48h after moulting and with the same exposure time, (a) Superoxide dismutase (SOD) activity, (b) Lipid peroxidation (LPO) and (c) DNA damage levels. Striped bar indicates Basal scenario, white bars indicate Control scenario and dotted bars indicate Acidification scenario. * Indicate significant differences between scenarios in each inter-moult Period (Independent Samples T-test, $p \leq 0.05$).

3.2.2 - Energy Metabolism related biomarkers

Energy Metabolism related responses of *H. gammarus* throughout larval development under control and acidification scenario are displayed in Figure 3.7. No significant differences on acidification scenario were observed at any larval development stage in any of the energy metabolism related endpoints analysed.

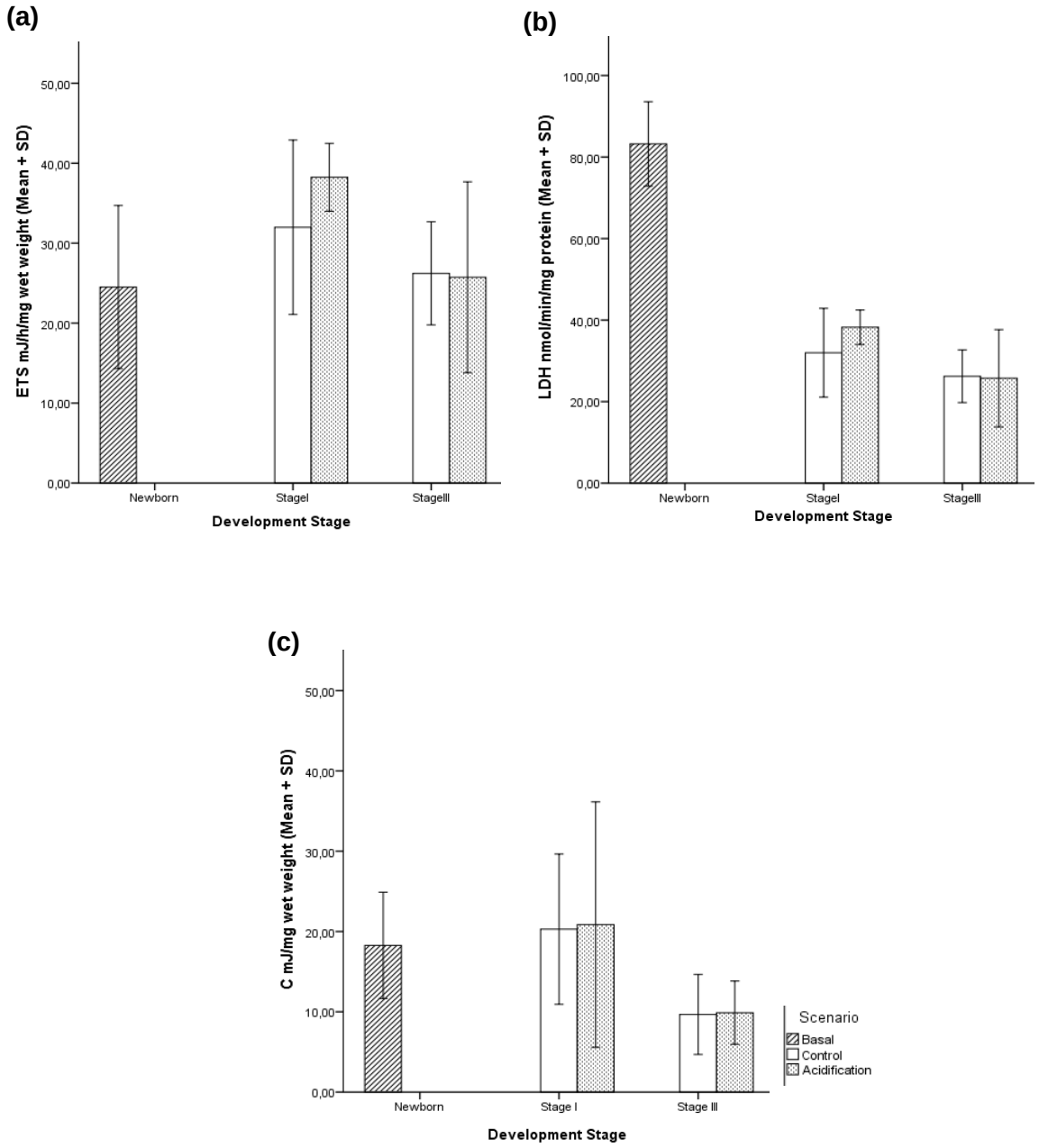

Figure 3.7 – Energy metabolism related responses of *Homarus gammarus* larvae reared under control and ocean acidification scenarios (Mean ± S.D.): Newborn (N=16), Stage I (N=8) - 24h after exposure - and Stage III (N=9), 48h after moulting and with the same exposure time, (a) Electron Transport System (ETS) activity, (b) Lactate dehydrogenase (LDH) activity and (c) Carbohydrates content (C) levels' quantification. Striped bar indicates Basal scenario, white bars indicate Control scenario and dotted bars indicate Acidification scenario.

4. DISCUSSION

4.1 Overview

Invertebrates are affected by increased levels of pCO_2 (Gonzalez-Bernatetal, 2012) and, as Whiteley (2011) referred, crustaceans are particularly tolerant to climate change scenarios predicted to 2100 - and even 2300. Nevertheless, their tolerance is short over time and starts to decrease with long-term exposures. Also, Wittmann & Pörtner (2013) studied the responses of several taxa to ocean acidification and only 10% of the analysed crustacean species were negatively affected by the pCO_2 range in this study (651-850 µatm), whereas 80% revealed no effects and 10% were positively affected by these scenarios.

Below the findings on the biochemical, development and behaviour responses of larvae of *H. gammarus* exposed to future ocean acidification triggered by elevated pCO_2 levels will be discussed. Finally, conclusions and considerations about possible future implications for this species and the ecosystem are addressed.

4.2 Behaviour and development responses: survival, inter-moult period, morphometric analysis and growth

At the end of the exposure time, larvae survival was lower, yet not statistically different, in the ocean acidification scenario (63.8%) than in the control scenario (76.6%). While tendencies to lower survival were also observed in other climate change studies that included crustacean larvae and namely *H. gammarus* exposure to OA (e.g. Arnold *et al.*, 2009; Long *et al.*, 2013; Small *et al.*, 2015; Small *et al.*, 2016; Spice *et al.*, 2007; Walther *et al.*, 2010;), stage-specific moulting patterns obtained in this study were not similar to the ones named above.

Moulting of decapod crustaceans is affected by extrinsic factors such as temperature, salinity, light intensity, pollutants and also by intrinsic factors such as nutritional state and hormones (Kleinholz, 1985). Moulting into SII occurred earlier in larvae exposed to OA - along with a mortality peak. This pattern was observed by Díaz *et al.* (2003) in the decapod crustacean *Palaemonetes argentinus* showing increased moulting frequency when facing environmental stress. An opposite pattern was observed in SII-SIII moulting event, where exposure to OA for a longer period, reflected on longer moulting

period. Medium-term exposure to high pCO_2 levels, leading to hypercapnia, has the potential to adversely affect growth – and therefore moulting - by diverting energy towards the maintenance of effective compensatory responses (Whiteley, 2011). Thus, delayed moultings – when not correlated with temperature variations - might be due to eco-physiological stress, oxidative stress and damage, as larvae allocate resources into survival instead of growth and development. Chen & Chen (2003) reported lower moulting frequency in the prawn *Macrobrachium rosenbergii* when exposed to acidified water.

Although stage-specific moulting patterns were different between treatments, the overall larval development time of *H. gammarus* (until stage III) when exposed to Ocean Acidification wasn't significantly different from the Control scenario (~9days), as previously observed by Arnold (2009) and Small *et al.*, (2015). In a great extent, temperature is a major key-driver of embryonic, larval and post-larval development time and growth in crustaceans (as seen in Charmantier & Mounet-Guillaume, 1992; Nicosia & Lavalli, 1999; Schmalenbach & Franke, 2010 and Styf *et al.*, 2013) and larval development might not be affected by the 2100 predicted levels for Ocean Acidification *per se* (e.g. Small *et al.*, 2015), although different responses are shown by different crustacean species (Whiteley, 2011): *Hyas araneus* larval development was delayed only when exposed to extreme OA levels (Walther *et al.*, 2010), whereas *H. americanus* has shown development differences when exposed to 2100 pCO_2 predictions (in Keppel *et al.*, 2012). Species with a wide range of habitats, especially if including natural pH fluctuations, may have a particularly higher resistance to acidification in comparison to those exposed to fewer fluctuations.

Another explanation for a non-affected overall moulting performance is that exoskeletons of planktonic decapod larvae are unmineralised, while those of megalopa and benthic juveniles are only partially calcified (Anger, 2001). This is likely to reduce the potential negative effects of ocean acidification during larval moults as calcification effects tend to be the most critical for these species (Whiteley, 2011). Also crustaceans are believed to use calcium bicarbonate as primary source for the production of $CaCO_3$ instead of the carbonate ions that lacks in ocean acidification scenarios (Cameron, 1989) which gives them tolerance concerning moulting and calcification (Kroeker *et al.*, 2010; Kroeker *et al.*, 2011b).

Negative morphometric responses to ocean acidification have been commonly seen in benthic and pelagic marine invertebrates. Shirayama & Thorton (2004) reported reduced larval development and size in the sea urchins *Hemicentrotus pulcherrimum* and

Echinodetra mathei, whereas Bechmann *et al.* (2010) reported the same in early life stages of shrimp (*Pandalus borealis*) and Mussel (*Mytilus edulis*). In this study, *H. gammarus* larvae exposed to ocean acidification exhibited a trend to reduced weight gain (Ww) and presented reduced growth (carapace length). Keppel *et al.* (2012) observed reduced rates of growth in *H. americanus*, and Fabry *et al.* (2008) and Whiteley (2011) have reported these same responses in several marine taxa when exposed to ocean acidification.

With the increasing of exposure time, SII-SIII inter-moult period was longer in acidification treatment but larvae were significantly smaller than the larvae from Control. This reduction in growth may be associated with a growth trade-off towards the high costs of internal homeostasis maintenance (Amiard-Triquet, 2009; Fabry *et al.*, 2008; Pörtner *et al.*, 2004).

The developmental responses obtained in the present study and in previously published (e.g. Keppel, 2012; Small *et al.*, 2015), where significant differences were not found on survival but were found on growth, support that effects on growth are a more sensitive indicator of environmental stress at an earlier stage than effects on survival. While the previous is well recognized, the present research confirms growth as a more sensitive indicator of stress than moulting frequency (seen also in Cowgill, 1989), besides the advantage of having higher ecological relevance.

In 2009, Arnold and co-workers stated that *H. gammarus* carapace length remained unaffected between stages I-IV and carapace mass was affected in the end of an exposure to OA of 1200 ppm. These results discrepancy suggest an emerging pattern of differing responses within taxonomic groups (Ridgwell *et al.*, 2009; Pistevos *et al.*, 2011) but may also convey the hypothesis of different responses across geographically disconnected but concomitant populations. This was already found to happen with crustaceans when considering other environmental stressors such as temperature (Leandro *et al*., 2006b).

4.3 Biochemical biomarkers responses: oxidative stress & energy metabolism

ROS are commonly used as messenger molecules in normal cell functions; however, at increased concentrations they may disrupt normal physiological pathways and cause cell damage or death (Ermak & Davies, 2002). Biochemical biomarkers are generally considered to be more sensitive than whole-organism responses (Jemec *et al.*, 2009) since

responses are obtained earlier at the sub-individual level and can give a better overview on the mechanisms of effect of a given stressor.

The overall sensitiveness on survival, moulting, and growth during ocean acidification obtained in this study was in part in concordance with research that included *H. gammarus* larval development as well as other crustacean species. However, the innovative approach to biochemical biomarkers allowed a more detailed assessment of sub-individuals responses and physiological fitness underlying higher level of organization effects.

H. gammarus SIII larvae exposed to high levels of pCO_2 demonstrated to have significantly less SOD activity, which had already been observed in the crustacean copepod *Calanus sinicus* (Dajuan *et al.*, 2016) and the pelagic fish *Atherina presbiter* (Silva *et al.*, 2016). This result indicate that the organisms are probably not investing their energy for antioxidant defences which, in case of increasing ROS formed in the OA scenario, can result in damage in macromolecules, as actually verified with DNA damage results. DNA damage was superior in acidification scenario in SIII larvae, which was also found in the decapod *Litopenaeus vannamei* when exposed to pH stress (Wang *et al.*, 2009). Cells exhibit a broad spectrum of responses to oxidative stress, depending on the stress type and level encountered. Oxidative stress exceeding the antioxidant capacity level may induce oxidative damage, but low-level stress may enhance the defence capacity (Niki *et al.*, 2005). On an immature or inhibited antioxidant system, as suggested in the present study, physiological burdens may occur. In this case, at the sub-individual level the SOD activity reduction might have led to the higher DNA damage observed, with further consequences at the individual level with reduced growth. The latter was also observed in larvae of the flatfish *Solea senegalensis* exposed to hypercapnia by Pimentel *et al.* (2015). Ultimately, the breakdown of antioxidant defences may contribute to oxidative stress, that will lead to damage (Howcroft *et al.*, 2009) that, in its turn, may compromise survival (Borgeraas & Hessen, 2002).

Although oxidative stress is widely associated with formation and accumulation of lipid peroxidation (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 results obtained in this study showed an opposite tendency in the last Stage (III). Some authors have demonstrated that elevated pCO_2 levels can alter the content of fatty acids in fish larvae, oysters, gastropods and crustaceans (Díaz-Gil *et al.*, 2014; Garzke et al, 2016;

Leu et al, 2013; Regino et al, 2015; 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 slightly lower LPO levels from control to acidification scenario in the present study.

The developmental sensitivity to elevated pCO_2 seems to be in line with the increasing time of exposure to OA, since significant differences were found mostly in Stage III larvae. Nevertheless, these very same larvae also went through two moulting events in less than 10 days, possibly decreasing their physiological resistance when facing an ocean acidification scenario. Moulting might have detrimental effects on the organisms since it is a stressful and energy-consuming event, where even starvation occurs (Sugumar *et al.*, 2013). The continued exposure to OA in *Necora puber* also had a detrimental effect at day 16 even though the organisms were able to cope with OA-related stress for the first 24 hours (Spicer *et al.* 2007).

No differences were found in *H. gammarus* larvae exposed to acidification scenarios, concerning energy metabolism related biomarkers. Smaller body size was similarly observed in other studies for larvae developed under high pCO_2 even with an undetectable increase of energy resources. The authors concluded 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). In fact, the increase in energetic costs associated with ion-regulation has recently been used to explain differences in protein synthesis rates in the tropical prawn *Macrobrachium rosenbergii* (Intanai *et al.* 2009), as protein synthesis rates are a major determinant of growth (Whiteley, 2011) - the growth differences obtained in this study support this concept. OA scenarios were also shown to cause immune suppression and protein damage in the crustacean *Nephrops norvegicus* (Hernroth *et al.*, 2012).

Another interesting outcome of this study was that during protocol optimization and after several changes in reagent proportions, sample dilutions or total aliquot samples' volume, the antioxidant activity of CAT could not be detected at any larval stages for both scenarios, leading to the hypothesis that this important antioxidant enzyme was not present in these larval stages. This was also observed in a study where *Solea senegalensis* larvae

showed apparent absence of CAT activity and where the authors concluded that these results possible indicate that early life stages do not possess a fully developed antioxidant defence system (Pimentel *et al.*, 2015), making them potentially more vulnerable to oxidative stress and relying on other enzymes to tackle this issue. However, when testing CAT in *H. gammarus* reared under Control conditions until reaching the early benthic phase (juveniles; 2.5 month-old), this enzyme activity could already be found (2.66 ± 0.49 µmol/min/mg protein). These observations support the idea that species' physiological functioning is different in close life events such as larval and juvenile stages and also across their ontogeny, where in this case juvenile lobsters already have a more mature antioxidant system than larvae. This also means that a species susceptibility to a stressor might not be the same across different life stages and highlights the importance of long-term, multi-stage exposures to stressors.

Homarus gammarus larval development seems to be influenced by the elevated pCO_2 levels predicted to occur by 2100 (IPCC, 2014), since growth was different across scenarios. Oxidative stress mechanisms' reduction (SOD) and higher levels of DNA damage indicate that ocean acidification may have adverse effects on the physiology of lobsters, which previously had been overlooked in studies of basic parameters such as lobster survival or calcification. In the end, Stage III sensitiveness was most probably not due to lack of Ca_3^{2-} ions – a direct consequence of changing ocean chemistry - but due to physiological compensation during stressful environmental conditions. These findings support the idea that life history dynamics can ultimately be linked to the environment via physiological processes (Calow & Forbes 1998; Ricklefs & Wikelski 2002; Young *et al.* 2006).

5. CONCLUSIONS AND FUTURE PERSPECTIVES

Due to their economic and ecological importance, nature, life-cycle and moulting behaviour, crustaceans are excellent organisms to understand how environmental and endogenous factors shape complex life cycles. *Homarus gammarus* was considered an effective model species since it has a rather short larval development time and larval stages are relatively large when compared with other crustacean larvae (e.g. *Necora puber*, *Maja squinado, Cancer pagurus)*. For both life phases – larvae and adults – studies and rearing practices have been optimized to this species which turned their maintenance and rearing in the laboratory a less demanding but still challenging task.

Homarus gammarus larvae demonstrated some susceptibility to the IPCC's predicted pCO_2 levels for 2100, although the analysed scenario was the most conservative from the forecasted. Higher levels of pCO_2 might occur within the nearby future so it is important to study this organisms'/population responses at different pCO_2/pH scenarios, including the ones predicted for 2300. Besides acidification, the ocean faces several other global factors related to climate change, in the form of physical or chemical forcing. Phenomena such as global warming and respective SST rising, hypoxia, salinity shifts and the OA itself may act alone or combined and have implications in the future of marine populations.

Despite the non-lethal effects of this acidification scenario, a deeper analysis at the biochemical and physiological level, allowed to conclude that these conditions may affect the organisms functioning with probable implications in their future fitness. The indication of the occurrence of oxidative stress in this OA scenario (SOD reduction and higher DNA damage) was followed by reduced growth and increased inter-moult period from SII to SIII. In nature, slowed progress through moults could result in greater time in the water column as pelagic drifting plankton, where this larvae are vulnerable to pelagic predators and environmental shifts, potentially leading to reduced benthic recruitment. Given the results mentioned above, it would be interesting to study how longer exposure times would affect this species fitness, namely during settlement which is a very demanding physiological process and since larval phase in marine organisms is of the uttermost sensitiveness.

Further comprehensive and robust analysis, besides the now trivial parameters such as survival, growth or calcification, should be performed in the future to assess the susceptibility or plasticity of marine organisms when facing ocean acidification or other

related global change scenarios. These innovative analyses may comprehend the “omics” family where several endpoints might be analysed. These tools would allow a better understanding of sub-cellular mechanisms underlying the effects and comprehend its implications not only on the individual but also at higher levels of biological organization.

Several reports have alleged that moulting in crustaceans might be delayed by endocrine disruption when exposed to environmental stressors, even though these stressors have been reported as chemical xenobiotics (e.g. Zou, 2005). Further investigations on how OA and other climate change stressors might affect crustaceans’ endocrine regulation would be a great approach, namely taking into consideration Ecdysteroids (moulting hormones).

This recent field of research – climate change in marine environment – and the different responses amongst and within taxonomic taxa upsurge the importance of studies of this nature, whereas not just a species should be studied but its responses across several geographic locations should also be considered.

It is important to keep in mind that adaptation and evolution (in any form) are intrinsic features of all living organisms and this kind of studies do not take those into consideration since they are exposed to severe environmental conditions with no gap for gentle increasing. Nevertheless, is generally accepted that the future rates of marine environmental change are sufficiently high to diminish the potential for adaptation despite organisms endeavour (e.g. coral bleaching).

The individual recirculating rearing system designed specifically for this species’ larval study showed remarkable results not only by allowing to perceive individual responses while maintaining the targeted scenario conditions but also by eliminating a key constraint: cannibalism. Nevertheless, progress is always required and an optimized RAS that will allow studying diverse species is currently being developed to increasingly approximate this to the most realistic scenarios.

In aquaculture, the elevated densities used relatively to the natural environment, cause the natural processes to happen faster than they would in the wild. Farmed organisms and filter bacteria breathe, releasing carbon dioxide into the water, which forms carbonic acid. Also, during the aerobic breakdown of biological wastes, such as the conversion of ammonia to nitrate in a biofilter, hydrogen ions are released and

accumulate. Both of these processes cause the pH of the water to decrease leading to hypercapnia in reared organisms. In this study, larval growth of *H. gammarus* was affected by lower pH levels. These results show the importance in maintaining acceptable pH levels during the rearing of this economically important crustacean, whether for repopulation of stocks – where bigger individuals have more chances to survive in the wild - or for closed-cycle aquaculture. Either way, the aquaculture industry is a major key-player to this species future sustainability.

In the end, understanding physiological responses behind life history traits is a critical process in linking organism and population changes to environmental changes and formulating successful stock management and species conservation strategies in an increasingly more certain global change scenario.

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