MESTRADO CIÊNCIAS DO MAR - RECURSOS MARINHOS

BIOLOGIA E ECOLOGIAS MARINHAS

Future ocean warming and acidification: Simultaneous impacts on the green crab *Carcinus maenas*

Sónia Ferreira

M

2021

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M.ICBAS **2021**

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Future Ocean Warming and Acidification: Simultaneous Impacts on the green crab *Carcinus maenas*

Dissertation for the Master Degree in Marine Sciences – Marine Biology submitted to the Abel Salazar Institute of Biomedical Sciences from de University of Porto

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AGRADECIMENTOS

Gostava de agradecer em primeiro lugar às instituições que me acolheram, Instituto de Ciências Biomédicas Abel Salazar (ICBAS) e ao Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR), e também à Faculdade de Ciências da Universidade do Porto (FCUP) onde iniciei o meu percurso académico, responsável pelas minhas bases científicas. Este trabalho foi parcialmente financiado pelo Projeto SEEINGSHORE (referência SEEINGHSHORE/IA/2018-089), cofinanciado pelo POCI (Programa Operacional Competitividade e Internacionalização), COMPETE 2020, Portugal 2020 e União Europeia através do FEDER, e pela FCT através do OE e pelo projeto ATLANTIDA (NORTE-01-0145-FEDER-000040), cofinanciado pelo Programa Operacional Regional do Norte (NORTE 2020), através do Portugal 2020 e do FEDER - Fundo Europeu de Desenvolvimento Regional.

Ao Doutor Francisco Arenas e à Doutora Laura Guimarães os meus agradecimentos pela orientação, apoio e disponibilidade durante o percurso prático e teórico. Ficamos muito agradecidos à empresa MARE EST NOSTER (Matosinhos, Portugal) por fornecer os animais para a realização deste trabalho.

Agradeço a todos os meus colegas de Mestrado, em especial às minhas amigas Gisela Costa e Leonor Almeida pela amizade ao longo destes dois anos. Agradeço ainda a todos os colegas do laboratório e grupo BET (Benthic Ecology Team) do CIIMAR, em particular ao meu colega Harold Casalis e ao Luís Pereira pelo apoio e disponibilidade constante ao longo deste percurso e ainda à Julia e à Rithielli, pelo companheirismo e amizade. Agradeço também aos colegas de laboratório do grupo METOX e EDEC pelo acompanhamento e ajuda durante o meu trabalho laboratorial no CIIMAR.

Ao Nélson Alves, um enorme obrigada pela amizade e pelo apoio persistente em todos os momentos, bons ou menos bons, ao longo de todo o meu percurso académico. Obrigada também a todos os meus "lamas" de Biologia, vocês são incríveis.

A todos os meus amigos mais queridos, e em especial ao Francisco, à Sofia, ao Carlos e ao Tiago nestes últimos anos, agradeço pela presença constante na minha vida e pelo apoio fulcral e incessante este percurso. Não posso esquecer também de agradecer a

todas as pessoas e profissionais que me prepararam, de alguma maneira, para chegar até aqui. Obrigada Professor Arlindo, por me fazer gostar ainda mais e me ter incentivado a percorrer o caminho da Biologia.

E por fim, e não menos importante, um obrigada gigante à minha família, especialmente aos meus pais e irmão pela compreensão e apoio incansável, e ainda à Drª Cathia Chumbo pelo acompanhamento profissional e suporte emocional numa fase menos boa.

Á minha mãe, um obrigada não chega para agradecer a companhia e amparo nos momentos menos bons, e pelo total suporte das minhas escolhas. És e serás sempre aquele porto seguro a quem posso sempre voltar.

Declaração de Honra

Declaro que a presente dissertação é de minha autoria e não foi utilizada previamente noutro curso ou unidade curricular, desta ou de outra instituição. As referências a outros autores (afirmações, ideias, pensamentos) respeitam escrupulosamente as regras da atribuição, e encontram-se devidamente indicadas no texto e nas referências bibliográficas, de acordo com as normas de referenciação. Tenho consciência de que a prática de plágio e auto-plágio constitui um ilícito académico.

29 de outubro de 2021

O estudante,

Sónia Isabel Ribeiro Ferreira

Abstract

Anthropogenic climate change is gradually provoking ocean warming and acidification, noticeable even at a small temporal scale. The available literature indicates varying responses of different species to the exposure levels indicated by climate predictions. Thus, perceiving the responses of key species to such climate changes is critical to determining how the marine environment may be affected in the future and develop measures to increase their resilience. This study investigated combined effects of predicted levels of ocean warming and acidification on some fitness-linked species traits and responses such as the mechanical properties of *Carcinus maenas* shell, crabs' predatory behaviour, metabolic rates (O_2 consumption) and activity of neurotransmission (acetylcholinesterase, AChE) and energy production (lactate dehydrogenase, LDH, and isocitrate dehydrogenase, IDH) enzymes. The crabs were exposed for 30 days to six treatments resulting from the combination of two pH levels (ambient: 8.02; low: 7.88) and three temperatures (18 ºC. 22 ºC and 26 ºC). The experimental pH and temperatures were selected according to the recent predictions of the International Panel on Climate Change (IPCC). During exposure, the mortality, predatory behaviour and metabolic rates were assessed. Metabolic rate measurements focused on two conditions of O_2 consumption: when animals were fed and when they were deprived from eating by 48h prior to the measurements. Predatory behaviour trials consisted in investigating the number of feeding attempts of *C. maenas* on *Mytillus* sp*,* from collected nocturnal videos using animals from each treatment, after 12, 18 and 24 days after exposure on the mesocosms. After exposure biological sampling was done: carapace was used to assess possible effects on the calcifying structure and organs and tissues were removed to determine the activities of AChE (ganglion and muscle), LDH (muscle) and IDH (muscle). Different interactions between pH and temperature were observed across measurements. Whereas significant effects of pH were observed in carapace hardness and width, temperature seems to have a preponderant effect on IDH activity and crab survival despite pH treatments. This study suggested synergistic negative effects of these climate change drivers on some of the responses, although on overall the resilience of this species is high probably enough to cope with the stress resulting from near future warming and acidification trends.

Key-words: Climate change, pH, Temperature, CO_2, *Carcinus maenas*, Hardness, Predatory behaviour, Metabolic rate, IDH, LDH, AChE

Resumo

As alterações climáticas estão gradualmente a aumentar o aquecimento e acidificação da água do mar. A literatura disponível indica respostas variadas de diferentes espécies à exposição aos níveis indicados pelas previsões climáticas. Assim, perceber as respostas de espécies-chave às mudanças climáticas é fundamental para determinar como o ambiente marinho poderá ser afetado no futuro e planear possíveis medidas de proteção que aumentem a sua resiliência a estas alterações. No presente estudo foram investigados os efeitos combinados de níveis aquecimento e acidificação do oceano nas propriedades mecânicas da carapaça de *Carcinus maenas* e também no comportamento predatório dos caranguejos, na sua taxa metabólica (consumo de O_2) e na atividade de enzimas relacionada com a neurotransmissão colinérgica (acetilcolinesterase, AChE) e a produção de energia (lactato desidrogenase, LDH, e isocitrato desidrogenase, IDH). Os caranguejos foram expostos por 30 dias a seis tratamentos resultantes da combinação de dois níveis de pH (ambiente: 8.02; baixo: 7.88) e três temperaturas (18 °C, 22 °C e 26 °C). Os níveis de pH e temperatura foram selecionados a partir das previsões recentes efetuadas pelo Painel Internacional sobre Mudanças Climáticas (IPCC). Durante a exposição, foram analisadas a mortalidade, o comportamento predatório e a taxa metabólica. A avaliação da taxa metabólica focou-se em duas condições de consumo de O_2: quando os animais eram alimentados e quando eles eram privados de comer por 48h antes das medições de consumo. A avaliação do comportamento predatório consistiu em investigar o número de tentativas de alimentação de *C. maenas* sobre *Mytillus* sp*,* a partir de vídeos noturnos obtidos com animais de cada tratamento, após 12, 18 e 24 dias de exposição nos mesocosmos. Após a exposição, também foram recolhidas amostras biológicas: a carapaça foi usada para avaliar os efeitos na estrutura calcificante e os órgãos e tecidos foram removidos para determinar a atividade das enzimas AChE (em gânglio e músculo), LDH (músculo) e IDH (músculo). Os resultados obtidos revelaram efeitos significativos de diminuição de pH na dureza e no crescimento da carapaça, e diminuição na atividade da IDH e na sobrevivência do caranguejo. Este estudo revelou efeitos negativos sinergéticos em algumas das respostas devido a estes fatores de mudança climáticas. No entanto, em geral, a resiliência desta espécie será provavelmente alta o suficiente para lidar com o stress resultante de tendências de aquecimento e acidificação num futuro próximo.

Palavras-chave: Alterações climáticas, pH, Temperatura, CO_2, *Carcinus maenas*, Dureza, Comportamento de predação, Taxa metabólica, IDH, LDH, AChE

Contents

Abbreviation List

AChE - Acetylcholinesterase

ATP - Adenosine triphosphate

Ca – calcium

cm – Centimeter

CO_2 – Carbon dioxide

FW – Fresh Weight

GHG - Greenhouse emission gases

H_2O_2 – Hydrogen Peroxide

IDH - $NADP^+$- dependent Isocitrate dehydrogenase

IPCC - Intergovernmental Panel on Climate Change

L – Liter

LDH - Lactate dehydrogenase

ln – Natural logarithmic function

LPO – Lipid peroxidation

Mg - Magnesium

mM – Millimolar

nm – Nanometers

nmol - Nanomol

NOAA - National Oceanic and Atmospheric Administration

O_2 – Oxygen

PAH - Polycyclic aromatic hydrocarbon

ppm – Parts per million

ppt – Parts per trillion

ROS – Reactive oxygen species

ZJ – Zettajoule

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Introduction

Ocean and Climate Change

Since ancient times, the ocean and its seemingly endless resources have been fundamental to mankind by providing essential supporting services. Humans have traditionally explored the ocean for food, transport and trade, and even for recreational and leisure purposes (Inniss et al., 2016). However, the ocean also plays an important role in Earth's equilibrium, being determinant as climate regulator, distributing heat and driving weather systems. The ocean stores heat trapped in the atmosphere originated from increasing emissions of greenhouse gases, it regulates atmosphere temperature and slows surface warming, it stores excess carbon dioxide and is a fundamental component of global biogeochemical cycles (Bindoff et al., 2019). Furthermore, it is a source of life reaching up to 80% of the planet's biodiversity and providing habitats for a million species of animals and plants, from single-cell organisms to massive creatures, containing unique life forms and genetic resources (Sala et al., 2021).

In the last decades, oceans and particularly coastal areas are being disproportionately impacted by increasing carbon dioxide (CO_2) and other greenhouse gas (GHG) emissions resulting from human activities. Since the industrial revolution the concentration of carbon dioxide in atmospheric levels has doubled, primarily as result of the combustion of fossil fuels, as well as industrial and agricultural activities (Feely et al., 2008). In the last decades the emissions accelerated dramatically and between 1970 and 2004 the annual emissions of GHG grew by about 80% (Pachauri and Reisinger, 2007). In the following years a similar trend in the CO_2 accumulation in the atmosphere was observed (Figure 1). According to NASA, even with the global shutdowns related to the current COVID-19 pandemic, which reduced the amount of carbon dioxide released into the atmosphere last year, the trends in CO_2 concentrations did not register any reduction (NASA, 2021).

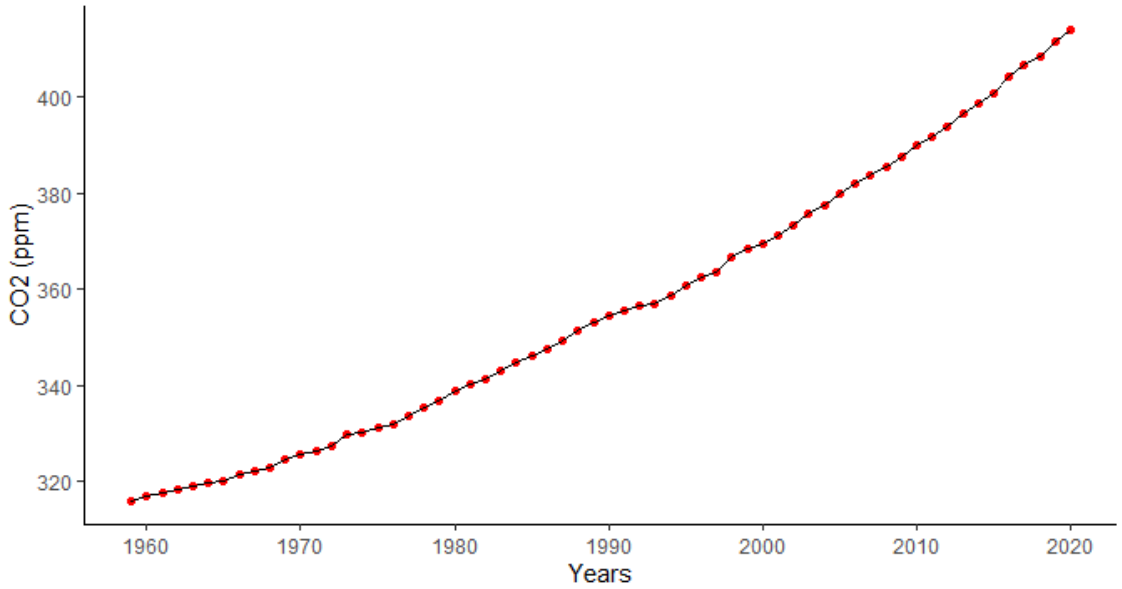

Figure 1: Global Annual mean CO_2 concentrations (ppm) in the atmosphere since 1958 until 2020. Based on data collected from NOAA (National Oceanic and Atmospheric Administration) database of the Mauna Loa Observatory. Carbon dioxide emissions are expressed as a mole fraction in dry air, µmol/mol, abbreviated as ppm. The estimated uncertainty in the annual mean is the standard deviation of the differences of annual mean values determined independently by NOAA/ESRL and the Scripps Institution of Oceanography. Permission to reproduce results from Pieter Tans - NOAA Federal.

The ocean has removed up about 30 % of the anthropogenic CO_2 released into the atmosphere since the industrial revolution (Ciais et al., 2013). This process of CO_2 absorption reduces GHG concentrations in the atmosphere and minimizes the effects of global warming (Feely et al., 2008). However, it impacts the ocean chemistry equilibrium, leading to ocean acidification. Ocean pH decreased by 0.1 units since the beginning of the industrial era, corresponding to an increase in acidity of 26% (Abram et al., 2019). Ocean acidification refers to this decline in the ocean pH (hydrogen potential of a solution). When carbon dioxide dissolves in the ocean it combines with the seawater forming carbonic acid (H_2CO_3), a weak acid that dissociates into hydrogen ions (H^+) and bicarbonate ions ($HCO3^-$), thus lowering the water pH (-log10 [H^+]) (Gattuso and Hansson, 2011, Ryan, 2019). Bicarbonate can in turn dissociate into H^+ and carbonate ions (CO_3^{2-}), which are used by many marine organisms to form the calcium carbonate ($CaCO_3$) incorporated in their shells or skeletal elements. The reactions occurring are as follows:

$$CO_2 + H_2O \rightarrow H_2CO_3$$

$$H_2CO_3 \rightarrow HCO_3^- + H^+$$

$$HCO_3^- \rightarrow H^+ + CO_3^{2-}$$

Due to water's specific properties (high density and specific heat) the uptake of heat by the ocean is 4000 times more effective than by the air, volume to volume, and so it can be transported and stored in large quantities (Laffoley and Baxter, 2016). In fact, oceans have adsorbed more than 90% of the extra energy from the enhanced greenhouse effect between 1971 and 2010, a very significant increment of energy in the climate system (Stocker et al., 2013). Obviously, the atmosphere is also warming up with additional effect on coastal communities. Atmospheric warming is fostering the melting of inland glaciers and ice, causing rising sea levels with dramatic impacts on coastlines. Projections from the IPCC point to a mean increase of the sea level from 0.40 m to 0.63 m by 2100, depending on the emission scenarios (Abram et al., 2019). Extreme weather events are also predicted to increase in frequency due to rising GHG emissions (Abram et al., 2019).

Based on the prediction models and all plausible scenarios presented in the Special Report on the ocean and cryosphere in a changing climate of the Intergovernmental Panel on Climate Change (IPCC), it is certain that oceans will continue uptaking the heat in the coming decades (Bindoff et al., 2019) (Figure 2), at a rate reliant on the reduction scenarios for global emissions.

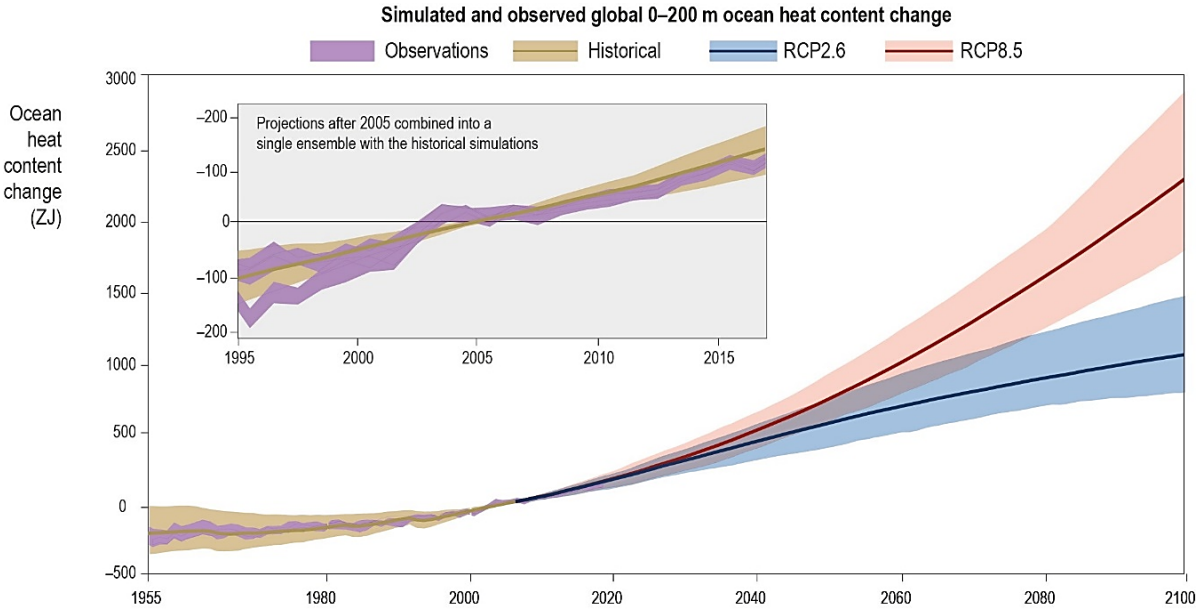

Figure 2: Historical, Present and Predicted Ocean heat content change (ZJ) until 2100. Figure 5.1: Time series of globally integrated upper 2000 m ocean heat content changes in ZJ, relative to the 2000–2010 period average, as inferred from observations (magenta) and as simulated for historical (tan), Representative Concentration Pathway (RCP)2.6 (blue) and RCP8.5 (red) forcing by a 25-member ensemble of Coupled Model Intercomparison Project Phase 5 (CMIP5) Earth System Models (ESMs) (Cheng et al. 2019). The shaded magenta in the outer panel is the very likely range determined by combining data from 4 long-term estimates (Palmer et al. 2007; Levitus et al. 2012; Lyman and Johnson, 2014; Cheng and Chen, 2017; Cheng et al. 2017; Ishii et al.2017) processed as in Johnson et al. (2018). The tan, blue and red lines are the ESM ensemble means, while shading shows each ensemble's 5th to 95th percentile range. In the inset subpanel, the four different shaded magenta areas are the reported very likely range of heat content changes as inferred from observations by four independent groups (Magenta shading; Palmer et al. 2007; Lyman and Johnson, 2014; Cheng and Chen, 2017; Cheng et al. 2017; Ishii et al. 2017) processed as in Johnson et al. (2018).In the inset subpanel the RCP2.6 and RCP8.5 projections after 2005 are combined into a single ensemble with the historical simulations, from: Bindoff, N.L., W.W.L. Cheung, J.G. Kairo, J. Arístegui, V.A. Guinder, R. Hallberg, N. Hilmi, N. Jiao, M.S. Karim, L. Levin, S. O'Donoghue, S.R. Purca Cuicapusa, B. Rinkevich, T. Suga, A. Tagliabue, and P. Williamson, 2019: Changing Ocean, Marine Ecosystems, and Dependent Communities. In: IPCC Special Report on the Ocean and Cryosphere in a Changing Climate [H.-O. Pörtner, D.C. Roberts, V. Masson-Delmotte, P. Zhai, M. Tignor, E. Poloczanska, K. Mintenbeck, A. Alegría, M. Nicolai, A. Okem, J. Petzold, B. Rama, N.M. Weyer (eds.)]. In press. Permission to reproduce from IPCC.

Temperature is one of the most important environmental drivers, it influences physical and chemical properties of water, such as CO_2 concentration, oxygen solubility or salinity (Yao and Somero, 2014). Current rises in ocean temperature reduce water density and consequently enhances the stratification and circulation of water masses, which in turn can alter the distribution of organisms and the transport of nutrients (Cavicchioli et al., 2019). In addition, ocean warming can affect plankton, creating phenological shifts in the dynamics of food webs (Southward et al., 1995), promoting a "cascade" of alterations in many other organisms.

These chemical changes in ocean chemistry can have large impacts on the biotic component of the ocean. Considering marine animals, acidification can diminish the reproductive, behavioural and growing performance especially in organisms occupying

lower trophic levels, once they have a minor capacity of ionic and acid-base status compensation and are metabolically sensitive (Pörtner, 2008). Elevated partial pressure of CO_2, known as hypercapnia, can also have impacts on marine fauna by changes in calcification rates and disturbances in acid-base physiology (Fabry et al., 2008). However, in the most studied species, the more common negative effects include a reduction in larval development and size, and alterations in shell integrity (Ross et al., 2011). Increased energetic costs with the maintenance of $CaCO_3$ structures can also be a consequence of ocean acidification, which can compel the organisms to deviate resources from other biological processes to accomplish internal equilibrium (Keppel et al., 2012). Temperature is also fundamental for ectothermic organisms because their body temperatures fluctuate with environmental temperatures and the rates of most biochemical reactions and biological processes increase approximately exponentially with temperature (Zuo et al., 2012, Yao and Somero, 2014).

Invertebrates on a changing environment

A great portion of all animals inhabiting our planet are invertebrates. Previous works have shown that invertebrates can be affected by temperature and ocean acidification. In crustaceans like our target species, *Carcinus maenas*, previous laboratory work has shown that temperature can influence the acid-base status of crustacean haemolymph and the extracellular pH, which vary inversely (Henry and Wheatly, 1992). Significant higher rates of oxygen consumption and enhanced overall metabolism were other responses recorded through experimental procedures (Henry and Wheatly, 1992, Salomon et al., 2000). Although temperature can influence several biological responses, acidification seems to play a more important role in the life of crustaceans even when they are considered to be tolerant to changes in this parameter. Primary responses to ocean acidification were described at an individual level and rely on physiological adjustments when facing changes in seawater chemistry (Whiteley, 2011). As water-breathers, this is quite important because it can compromise the excretion of CO_2 through the gills and, consequently, lower the haemolymph pH which is internally a highly controlled intracellular parameter and important to maintain oxygen purveyance (Whiteley, 2011). Tissue O_2 content can also decrease once oxygen cannot successfully bind to hemocyanin at lower pH values (Taylor and Whiteley, 1989). This will, eventually, affect respiration rates (Conlan, 1994) with the less tolerant species (poorer osmoregulators) developing a compensating acidosis, from accumulating lactic acid, and facing metabolic disruption when exposed to decreasing pH values (Whiteley, 1999).

Acidification can also alter morphological features and the duration of crucial events. According to Keppel et al., 2012, *Homarus americanus* larvae showed a significant decrease in the carapace length at all developmental stages when exposed to an experimentally acidified environment (pH=7.7). Also, the number of days to achieve the next molt was twice as longer than that of the control group. The lower growth rates observed were suggested to result from reallocation of energy to other processes. Similar responses seem to happen in the Red Crab (*Paraliyhodes camtschaticus*) in which a reasonable amount of energy is spent in osmoregulation and calcification when exposed to acidified conditions (Long et al., 2013). This seems to be a crab species particularly vulnerable to acidification.

Most intertidal organisms exhibit a negative response to reduced pH in one or several of the physiological and morphological traits commonly measured (Devries et al., 2016, Melzner et al., 2009). Generally, acute and drastic decreases in pH are associated with reduced growth, increased metabolic activity, cellular damage and mortality in crustaceans (Whiteley, 2011, Hernroth et al., 2012). Since crustaceans rely on calcium carbonate to build their exoskeleton, an alteration in the patterns of mineralization due to acidification phenomena could deeply affect the properties and functioning of the structure (Devries et al., 2016) and significantly disturb the fitness in decapod species (Coffey et al., 2017). Differences in the carapace hardness may be a result of changes in the availability of specific carbonate species due to altered seawater chemistry. These may affect carapace formation and functionality either by abnormal incorporation of carbonate species into the skeletal matrix or by weakening the ion gradient crucial to its formation (Glandon et al., 2018). During the calcification process, protons are released as a result of calcium carbonate creation. By transporting these protons in a more acidified environment, additional costs in carapace formation (Glandon et al., 2018) could influence other physiological parameters, negatively affecting disproportionately the calcifying structures (Coffey et al., 2017). Ocean acidification was also found to affect marine chemical communication by changing the structure and function of peptide signalling molecules (Roggatz et al., 2016). Furthermore, the investigation carried out in *C. maenas* suggest an impaired functionality of the altered signalling peptides at low pH.

Like in many other marine ectotherms, temperature effects on crustaceans have been extensively studied, especially on intertidal species exposed to highly variable thermal conditions. The responses of crustaceans to temperature are consistent with Shelford's law of tolerance (Shelford, 1931), illustrated by a bell-shaped curve depicting the relationship between environmental factors´ intensity and its favourability for species.

Beyond the species optimum, temperature changes have a profound impact on animals' metabolism, leading to a switch from aerobic to anaerobic metabolism because of the limited capacity for ventilation and circulation at extreme temperatures causing insufficient oxygen supply. At the cellular level, one of the most significant effects of temperature is membrane leakiness, which results in higher ATP demand, leading to a mismatch between the oxygen demand and supply system (Pörtner and Knust, 2007). Other studies suggest a shift in the oxygen carrying capacity of blood and concomitant decreased thermal tolerance in these benthic decapod crustaceans (Schiffer et al., 2014). However, it has been claimed that responses of marine organisms, namely crustaceans, to ocean warming may be somewhat less coherent and predictable than initial analysis (Faulkner et al., 2014). More recent studies have shown that temperature modulates compensatory responses to food limitation at metamorphosis in *C. maenas* (Torres and Giménez, 2020). Indeed, higher temperatures (>20 °C) were found to increase the effect of food limitation on larvae mortality and the developmental time, while reducing body mass, carbon and nitrogen content of metamorphosing larvae. Additionally, increased temperature was found to enhance larvae capacity to osmoregulate in *C. maenas*, while low temperature may constraint the capacity of osmoregulatory mechanisms to work properly and sustain haemolymph osmolality at appropriate levels (Torres et al., 2021).

Aim of the study

Despite the increase in knowledge in this area, namely in the last decade, there are knowledge gaps that still need to be filled to better understand the resilience of marine ecosystems to climate changes and to provide environmental managers with information useful for designing efficient adaptation measures. In particular, information is still lacking about the combined impacts of ocean warming and acidification on the fundamental traits and fitness related processes like survival, mechanical properties of crustaceans shell, predatory behaviour and metabolic rates. In this study we hypothesized that warmer temperatures and lower pH would negatively impact *Carcinus maenas*. Furthermore, these impacts would be synergistic upon species fitness. To investigate this, crab specimens were exposed to different temperature and pH conditions for 30 days. The predatory behaviour, shell hardness, metabolic rate (O_2 consumption) and the activity of energy production and neurotransmission enzymes (aerobic and anaerobic pathways) were measured. The methods employed, results obtained and their discussion and main conclusions are presented in the following chapters.

Methods

Model Species

Commonly known as green crab (Figure 3), *Carcinus maenas* is a mid-intertidal crab and one of the most studied decapods (Reid et al., 1997). As native and one of the most common crustaceans in Europe, this species is widely distributed and can be found both on hard and soft intertidal and shallow subtidal substrates, especially in wave protected areas such as harbors and estuarine areas (Moksnes et al., 1998, Hampton and Griffiths, 2007). They also usually live in association with estuarine and marine sediments where many pollutants and chemicals can accumulate rising toxicity levels (Rodrigues and Pardal, 2014). Due to their physiological plasticity, green crabs are highly adaptable, which makes this one of the worst invasive species in the world (Young and Elliott, 2020). In consequence, the green crab has established populations on every continent except Antarctica but is primarily native from the north-western Europe and North of Africa (Moksnes et al., 1998, Young and Elliott, 2020).

Belonging to the Portunidae family, *C. maenas* has a body divided in two segments (cephalothorax and abdomen), with two pairs of antennae, one pair of mandibles, two pairs of maxillae and five pairs of walking legs, the first of them are the chelae used for offense/defence purposes (Crothers, 1967). Their solid skeleton offers protection and support to the body including muscle attachment sites. This integument, generally composed by four layers, presents a massive endocuticle with outer coatings of impregnated calcium salts, a particular aspect of this phylum (Crothers, 1967). The gas exchange is carried out by 9 pairs of gills and their corporal fluid – haemolymph – assures O_2/CO_2 transportation, as well as that of nutrients and excretion products (Crothers, 1967). Although an invertebrate, their nervous system in consolidated and based on an all-or-nothing response meaning that any stimulus can produce a simple, but complex reflex, if the threshold is reached (Crothers, 1967).

Figure 3: *Carcinus maenas* specimen

As an eurythermal species, *C. maenas* can tolerate a wide range of temperatures of 0-35 °C (Klassen and Locke, 2007, Tepolt and Somero, 2014) and survive in environments of low (4‰) to high salinities (52‰) (Klassen and Locke, 2007). However, they prefer environmental temperatures between 3 °C to 26 °C, with minimum and maximum critical temperatures below 3 °C (low rates of survival) and above 35 °C, respectively (Rodrigues et al., 2015). Critical thermal maximum in Portugal for this species was established at 35.1 ºC (Madeira et al., 2012). This species is mainly active during the night and high tide (Baeta et al., 2006).

Both native and indigenous populations are considered ecosystem engineers holding the ability to modify entire ecosystems (Young and Elliott, 2020). Based on its omnivorous (plants, algae, bivalves, gastropods, other decapods and fishes) and detritivorous diet, it is considered a species with a key ecological role in benthic communities (Moksnes et al., 1998, Klassen and Locke, 2007) of estuarine and coastal ecosystems. Due to its ubiquity, easy capture and maintenance, this species is suitable for laboratory work. Considering the knowledge on this species biology, it is a regular model organism used in several studies, especially in ecology and ecotoxicology (Mesquita et al., 2011, Rodrigues and Pardal, 2014, Rodrigues et al., 2014).

Collection and acclimatization

Crabs were collected in Ria de Aveiro, Centre of Portugal, and kindly offered for this experiment by MARE EST NOSTER firm (Matosinhos, Portugal). The 579 crabs were placed at the animal facilities at CIIMAR, where the experiment was conducted, and distributed by 250 and 300 litre tanks for a three-week acclimatization. The tanks were filled with filtered seawater, with a pH range of 7.8-8.0 and salinity 34-35‰. Room

temperature was maintained at 16-17 °C. To prevent water quality degradation, mechanic water filters and aeration were installed, water parameters were monitored every day and the seawater was renewed every other day. Crabs were fed every other day with squid and PVC tubes were placed in the acclimation tanks to provide hideouts and increase animal comfort.

Experimental design and setup

The experiment was conducted in a controlled temperature room using a semi-continuous flow-through seawater system composed by 24 mesocosms, each filled with 60 litres of seawater (Figure 4). The bottom of each mesocosm was filled with a layer of beach sand to mimic sea-shore substrate and provide hiding places to the crabs. In addition, each tank was equipped with a submersible heater (SICCE SCUBA 50W) and aeration.

Four head-tanks were used as a reservoir tanks. Each head tank fed six experimental mesocosms with a microcomputer system that provide approx. 0.5 L of new seawater at each mesocosm every five minutes. Two of these head-tanks were CO_2 mixing tanks, where the pH was lowered, the other two head reservoirs were control conditions for the pH treatment.

Figure 4: Experimental setup display

Three different temperatures and two pH conditions were chosen to simulate future seawater parameters, based on the Intergovernmental Panel on Climate Change's RCP2.6 and RCP8.5 projections (Bindof et al., 2019) and on the database collected from NOAA at the Mauna Loa Observatory since 1958 until 2020. In total, six different experimental conditions, with four mesocosms replicates each, were tested as can be seen on Figure 5. The control setup was defined as 18 °C and pH 8.0 – 8.1. The water pH was lowered through automatic CO_2 injection regulated to maintain the values between 7.8 and 7.9. pH in the two acid head tanks. This regulation was done using two independent Aqua Medic pH Controllers. Temperatures were controlled individually at each experimental mesocosms using thermostats STC 1000 connected to 50 W seawater heaters. Temperature levels were 18 °C, 22 °C and 26 °C.

The temperature, pH, salinity and dissolved oxygen from each experimental tank were measured daily using a digital temperature controller (INKBIRD ITC-1000F) and environmental probes (Multi meter HACH HQ40d). Alkalinity, as the capacity for an aqueous system to neutralize strong acids, was also measured according to the method of (Sarazin et al., 1999) during the last week of the experiment. Data is presented graphically and shows that alkalinity (molarity) of each water sample, collected from each tank, does not have major deviations from the interval 2-2.5mM (Appendix, I).

Fifteen female crabs (4.47 ± 0.42 cm mean shell width ± SD) were exposed to experimental conditions per tank for 30 days. The crabs were marked on the right side of the dorsal carapace with numbers impressed in water resistant paper (Figure 6). A one-week acclimation in the mesocosms was allowed prior to the start of the experiments. The photoperiod was set at 10 h light : 14 h dark, representing a short-day. Crabs were fed with squid every other day. Crab mortality and the water parameters were checked daily.

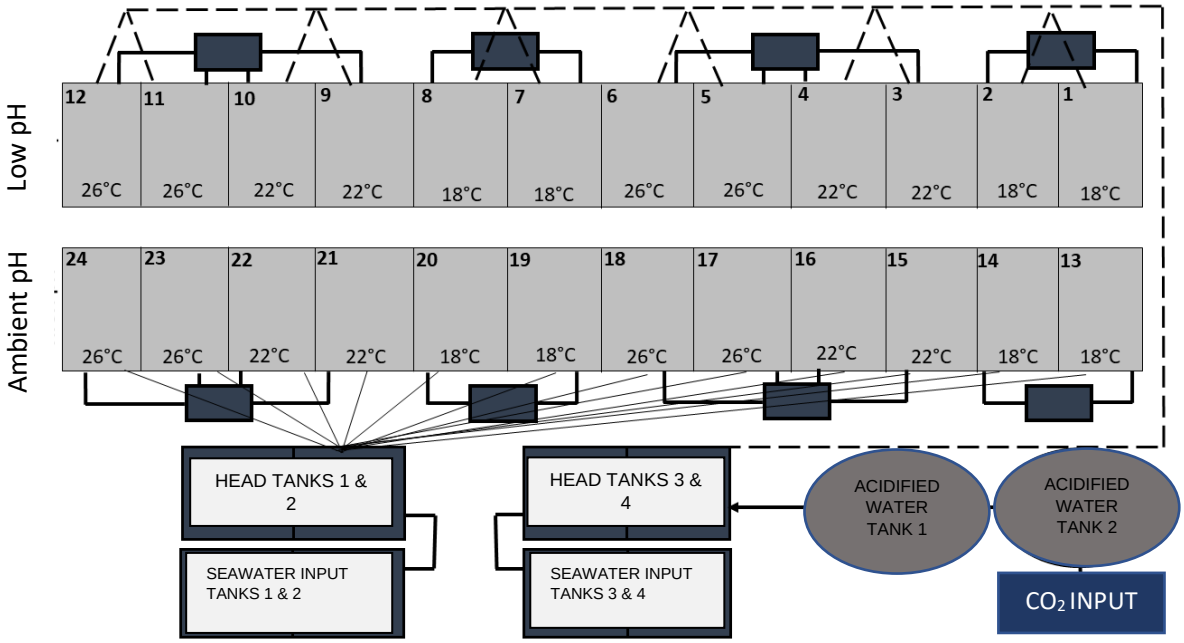

Figure 5: Experimental setup. In the figure,  corresponds to temperature control thermostats. Lines from mother tanks connected to the experimental tanks represent water distribution tubes.

Figure 6: Marked female crab with number 26a

Data collection

Survival and growth rates

Survival rates per treatment were assessed according to the number of living individuals of *Carcinus maenas* present at the end of the experiment. The animals were considered dead when their limbs were immobile for more than 30 seconds and their mandibular pieces were permanently open. Growth was assessed by comparing the final shell length (after 30 days of experiment) and the initial length at the beginning of the experiment.

Data was further transformed using the natural logarithm function to estimate relative growth. Measurements were made using a calliper (0.1 ± 0.05 cm).

Shell Hardness

At the end of the exposure the animals were dissected and their dorsal shells were kept at -80 °C until measurement of their hardness. The hardness test consisted in measuring the hardness of each carapace in four different areas as shown in Figure 7. Each area was measured five times with a SHORE A durometer (Figure 8). The durometer was placed perpendicularly to the carapace surface and pressed instantly and then released. The hardness value is dimensionless, expressed in UA (units of measure) (value range 0-100), and was read in one second. All measurements were done after the shells were gradually unfrozen on ice.

Figure 7: Hardness measurement areas

Figure 8: SHORE A durometer and placement on the crab shell

Predatory behaviour

Four crabs were randomly chosen from each experimental condition and used to assess the predatory behaviour. Assessments took place at 12, 18 and 24 days of exposure. The trials were carried out in individual boxes filled with 10 cm height of seawater matching their exposure condition. These crabs were not fed for 48 h prior to the test to increase their hunger and predatory efforts. *Mytilus spp.*, a common food item in *Carcinus maenas* diet, was selected as prey. Four mussels, ranging in range size from 3.5 to 4 cm were provided to each crab (Figure 9). Since crabs differed in claw size, to prevent preferential choices on prey that could influence results, a trial experiment took place before with different crabs which had different claw sizes, to understand which range of mussel lengths would be the best option. To investigate the predatory behaviour (i.e. approaches to prey, mussel opening and ingestion) the crabs were filmed overnight using two webcams (AUKEY 1080p). The cameras were placed above the boxes, so that the whole area in each box could be filmed. The computer program ISPYCONNECT was used to record continuously the videos during the 12 hours that each trial lasted. The next day, the number of mussels opened and the type of opening (*i.e.* breaking of the shell or pulling the valves apart) were registered.

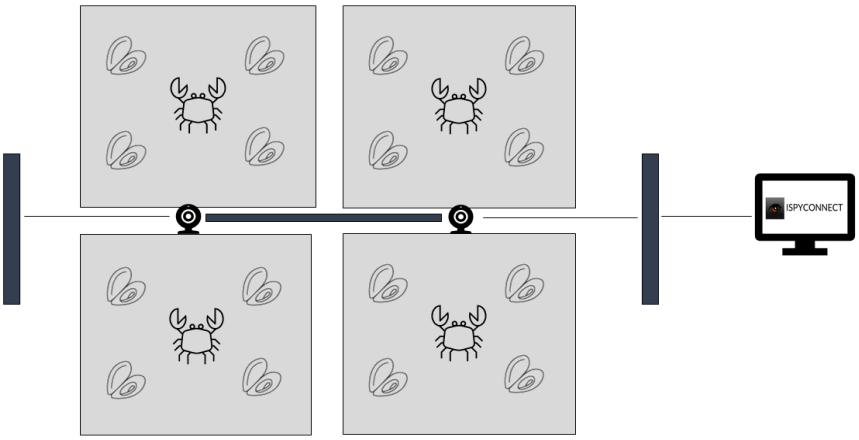

Figure 9: Schematic representation of the filming behaviour setup. Mussel silhouette is from Freepik.com

Metabolic rates – Respirometry

Estimation of the respiration rate on fed crabs and crabs starved for 48h was done through the measurement of oxygen consumption within closed chambers. For the measurements, crabs were placed individually in acrylic chambers containing seawater

from the corresponding experimental condition and previously filtered using a 5 microns filter to reduce the contributions of microorganisms to the oxygen fluxes. The chambers were submerged in water baths to keep the temperature stable. The O_2 consumption was measured in four crabs per experimental condition (one animal from each replicate). Blank incubations with no crabs were simultaneously performed to control for seawater respiration rates due to microorganisms. To measure oxygen fluxes we used one PreSens OXY-4 multi-channel fiber optic oxygen meter with four optical DO sensors supported by the software PreSens Measurement Studio 2. Each metabolic incubation ran for 40 minutes and the sensor provides an oxygen reading each 20 seconds. At the end of each measurement, crabs were returned to their original tanks, and the volume of water remaining in each chamber was estimated for water volume correction during the calculations of the metabolic rate. Respiration rates were scaled by the wet body mass of each crab at the end of the exposure period (determined to 0.01 g) so that the metabolic rate was expressed as μmol O_2 min^{-1} g^{-1}.

Biochemical measurements

At the end of the experiment, three randomly selected crabs were removed from each tank for the collection of tissue samples. The crabs were anaesthetised on ice before dissection. Samples of gills, muscle and the thoracic ganglion (Figure 10) were collected from each crab into microtubes and snap frozen in liquid nitrogen. These samples and the remaining crabs were stored at -80 °C until analysis.

Figure 10: Biological sampling procedure. 1-Gill 2- Digestive Gland 3- Nervous Ganglion 4- Muscle

The samples of muscle were used to determine the activity of the enzymes acetylcholinesterase (AChE), $NADP^+$-dependent isocitrate dehydrogenase (IDH) and lactate dehydrogenase (LDH) using the methods described and adapted by Rodrigues et al., 2013; AChE was also assayed in the thoracic ganglion. The enzyme AChE acts on cholinergic neurotransmission, which in this species is involved in locomotion, a behaviour essential for predation and food catching (Mesquita et al., 2011 and references therein). The enzymes IDH and LDH are involved in the aerobic and anaerobic pathways of energy production, respectively (Lee et al., 2002; Rodrigues et al., 2012)

All the reagents used for enzymatic analyses were purchased at Sigma-Aldrich Chemical (Steinheim, Germany), except for the Bradford reagent that was acquired at Bio-Rad (Munich, Germany). For sample homogenization a Precellys24 homogenizer was used and afterwards the VWR Micro Star 17R centrifuge for the centrifugation of homogenates. All enzyme activities were assayed in a microplate reader (BioTek Power Wave 340 spectrophotometer) and the results were expressed in nmol per minute per mg of protein. The concentration of protein in the samples was determined according to the method of Bradford (1976), using the Bio-Rad reagent. For the protein standard solution bovine γ-globulin (1 mg ml^{-1}) was used (Rodrigues et al., 2013). Absorbance readings were performed at 600 nm and the final protein concentration was expressed in mg mL^{-1}.
For determination of AChE activity, the thoracic ganglion and a portion of muscle were homogenized in 700 µL each of 100 mM phosphate buffer, 150 mM KCl, 1 mM Na_2EDTA (pH 7.4), at 5600 rpm for 2x5 seconds, and centrifuged at 6000 g, at 4 ºC, for 5 minutes. The supernatant was recovered for quantification of AChE activity using the method described by Ellman et al., 1961. According to this method, acetylthiocoline supplied as a substrate is hydrolyzed by the AChE present in the sample into thiocoline and acetate. The thiocoline produced reacts with 5,5-dithiobis-2-nitrobenzene (DTNB) forming a yellow colored compound, which production can be measured as an increase in absorbance at 412 nm wavelenght.

For determination of IDH, a portion of muscle was homogenized in Tris / NaCl buffer (0.1 M, pH 7.8). This homogenate was centrifuged at 15000 g, for 15 min, at 4 ºC, and the supernatant was collected for analysis of IDH activity through the method described by Ellis and Goldberg, 1971. In this method, the activity is evaluated by monitoring the increase in absorbance due to the reduction in $NADP^+$ at 340 nm. This reduction leads to the regeneration of NADPH which also provides the energy needed to maintain the redox balance of the cell. For LDH quantification, a portion of muscle tissue was homogenized in Tris / NaCl (0.1 M, pH 7.2) buffer and centrifuged at 6000 g, for 3 min, at 4 ºC. The

supernatant collected was used for the analysis of LDH activity using the method described by Vassault, 1983. This method is based on the determination of the amount of pyruvate converted to lactate by LDH in the samples, and consequently oxidation of NADH into NAD^+. The NADH consumption in the reaction was measured at 340 nm.

Data Analysis

Experimental results were examined using linear models through analyses of variance (ANOVA). The experimental design included three factors: Temperature (fixed factor with three levels), pH (fixed factor with two levels) and mesocosm (random factor with four levels and nested in the interaction temperature x pH). We used this ANOVA design to detect effects of the treatments in the response variables measured in the experiment: growth, survival, shell hardness, predatory behaviour and enzyme activity. Homogeneity of variances was assessed by Bartlett's Test and normality of the data was evaluated with the Shapiro-Wilk test. All graphics and statistical analysis were performed with the R software (v 4.0.5), using the "GAD" function package (Sandrini-Neto and Camargo, 2010) for ANOVA and ggplot2 (Wickham, 2009) for plotting. GAD contains functions for the analysis of many complex ANOVA models and is based on general principles described by Underwood et al. (1997). Once differences were detected, the post-hoc Least Square Mean test (LS Means), using Multiple Comparisond of Means based on Tukey-test contrats, was performed using the "emmeans" package (Lenth et al., 2019) to find which treatments were significantly different. The significance level was set at $p<0.05$.

Results

Physical variables in the experimental mesocosms

Throughout the 30 days of the experiment, the physical variables were carefully measured twice a day. The results indicated that the experimental conditions were kept stable throughout the exposure, with a variation lower than 1 unit for Temperature, pH and dissolved oxygen (Table 1, Appendix). In the acidified tanks (1-12) the pH was kept at 7.88 ± 0.09 (mean ± S.D) and in the non-acidified tanks (13-24) it was kept at 8.02 ± 0.08 (mean ± S.D), corresponding to the ambient pH from incoming seawater. As for temperature, the mesocosms with the lowest experimental temperature (18 ºC) varied on average between 17.92 ºC and 18.38 ºC. The mesocosms with the intermediate temperature (22 ºC) varied on average between 21.75 ºC and 21.89 ºC, and in the

mesocosms with the highest temperature (26 ºC) ranged on average between 25.65 ºC and 25.80 ºC.

Mortality and changes in carapace width

Mortality was assessed every day to prevent scavenging by the other crabs on the tank and water quality degradation. The mortality rate and the relative width calculated for the different experimental conditions are presented in Figure 11.

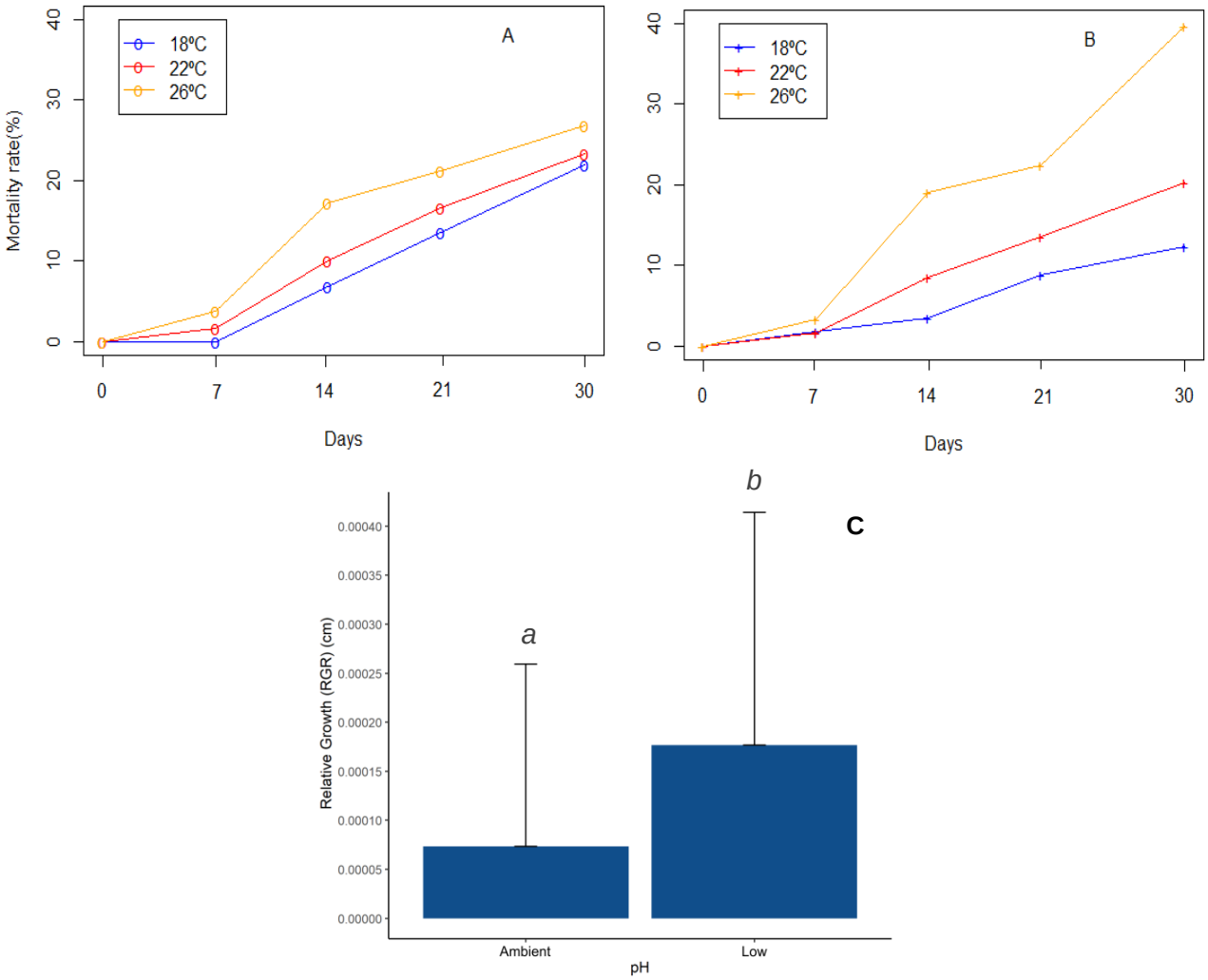

Figure 11: Cumulative mortality and relative growth of crabs exposed to six treatments for 30 days. **A**: mortality at low pH (7.88 ± 0.09, mean ± S.D) at 18°C, 22°C and 26°C; **B**: mortality at ambient pH (8.02 ± 0.08 , mean ± SD) at 18°C, 22°C and 26°C; **C**: Relative growth rate of crabs (mean ±SD), measured as the relative elongation in shell length, for the ambient and low pH treatments.

The results of the statistical analysis suggested a marginally no significant effect of temperature in mortality rates (ANOVA, p=0.0508), with mortality increasing as temperature rises (Table 2, Figure 11). This effect would be significant if the interaction effect is pooled and only the two main sources of variation are kept in the ANOVA. No significant effects of pH, or the interaction between Temperature and pH, on mortality were observed (Table 2).

Table 2: Results of the statistical analysis performed to investigate the effects of temperature and pH on the mortality of crabs exposed for 30 days to 6 pH and temperature treatments: Ambient pH (8.02 ± 0.08 , mean ± S.D) and low pH (7.88 ± 0.09, mean ± S.D) at 18 ºC, 22 ºC and 26 ºC.

Response	Variation Source	dF	SS	MS	F	P
Mortality Rate (%)	pH	1	13.1	13.05	0.0677	0.7977
	Temperature	2	1362.9	681.45	3.5325	0.0508
	Temperature x pH	2	379.0	189.50	0.9823	0.3936
	Residual	18	3472.4	192.91		

In contrast, the crabs relative growth (RGR), measured as the relative increased in shell length, was significant and positively affected by the exposure to low pH, as indicated by the three-Way ANOVA (Table 3). The RGR was higher in the lower pH treatment (Figure 11). No statistically significant effects were found for temperature or the interaction between Temperature and pH.

Table 3: Results of the statistical analysis ANOVA performed to investigate the effects of temperature and pH on the growth of crabs exposed for 30 days to different pH and temperature treatments: ambient pH (8.02 ± 0.08 , mean ± S.D) and low pH (7.88 ± 0.09, mean ± S.D) at 18 ºC, 22 ºC and 26 ºC. pH and Temperature were considered as fixed factors. Tank number was considered a nested factor in the pH x Temperature interaction. Significant effects (p<0.05) in bold.

Response	Variation Source	dF	SS	MS	F	P
Relative growth rate	pH	1	1.9248e-07	1.9248e-07	4.9941	**0.03835**
	Temperature	2	1.5006e-07	7.5029e-08	1.9467	0.17165
	Temperature x pH	2	1.1212e-07	5.6062e-08	1.4546	0.25967
	Tank (pHxTemp)	18	6.9375e-07	3.8542e-08	0.8329	0.65458
	Residual	48	2.2211e-06	4.6273e-08		

Shell Hardness

Area 1 presented lower hardness values in all treatments (<75 UA). All other areas showed similar range of hardness values. In general, the ANOVA analysis revealed significant effects of the interaction temperature x pH on the hardness of Area 1 and of the pH on the hardness of Area 4 and Area 5 (Table 4). For area 1, significantly lower shell hardness was found in crabs exposed to low pH and 18 ºC, compared to the remaining test conditions (Figure 12 *top*). For area 4 and area 5, shell hardness was significantly lower (by 3.2% and 4.2%, respectively) in crabs exposed to low pH than in crabs exposed to ambient pH (Figure 12 *middle* and *bottom*).

Table 4: Results of the statistical analysis ANOVA performed to examine the effects of temperature and pH on the shell hardness of crabs exposed for 30 days to different pH and temperature treatments: ambient pH (8.02 ± 0.08 , mean ± S.D) and low pH (7.88 ± 0.09, mean ± SD) at 18 ºC, 22ºC and 26 ºC. pH and Temperature were considered as fixed factors. Tank number was considered a nested factor in the interaction pH x Temperature. Significant effects (p<0.05) in bold.

Response	Variation Source	dF	SS	MS	F	P
Hardness Area 1	pH	1	25.68	25.681	2.0340	0.1709
	Temperature	2	48.20	24.102	1.9089	0.1770
	Temperature x pH	2	91.10	15.552	3.6079	**0.0481**
	Tank (pHxTemp)	18	35.65	5.942	0.9950	0.4813
	Residual	48	609.08	12.689		
Hardness Area 3	pH	1	33.62	33.620	1.1383	0.2642
	Temperature	2	23.21	11.604	0.4585	0.6394
	Temperature x pH	2	40.55	20.274	0.8010	0.4643
	Tank (pHxTemp)	18	455.60	25.311	1.4463	0.1538
	Residual	48	840.05	17.501		
Hardness Area 4	pH	1	160.50	160.503	11.0005	**0.00383**
	Temperature	2	8.94	4.472	0.3065	0.73980
	Temperature x pH	2	33.35	16.676	1.1429	0.34097
	Tank (pHxTemp)	18	262.63	14.591	1.3457	0.20357
	Residual	48	520.42	10.842		
Hardness Area 5	pH	1	274.56	274.561	13.3162	**0.00183**
	Temperature	2	30.55	15.273	0.7408	0.49073
	Temperature x pH	2	83.00	41.502	2.0128	0.16258
	Tank (pHxTemp)	18	371.13	20.619	1.8465	**0.04650**
	Residual	48	535.97	11.166		

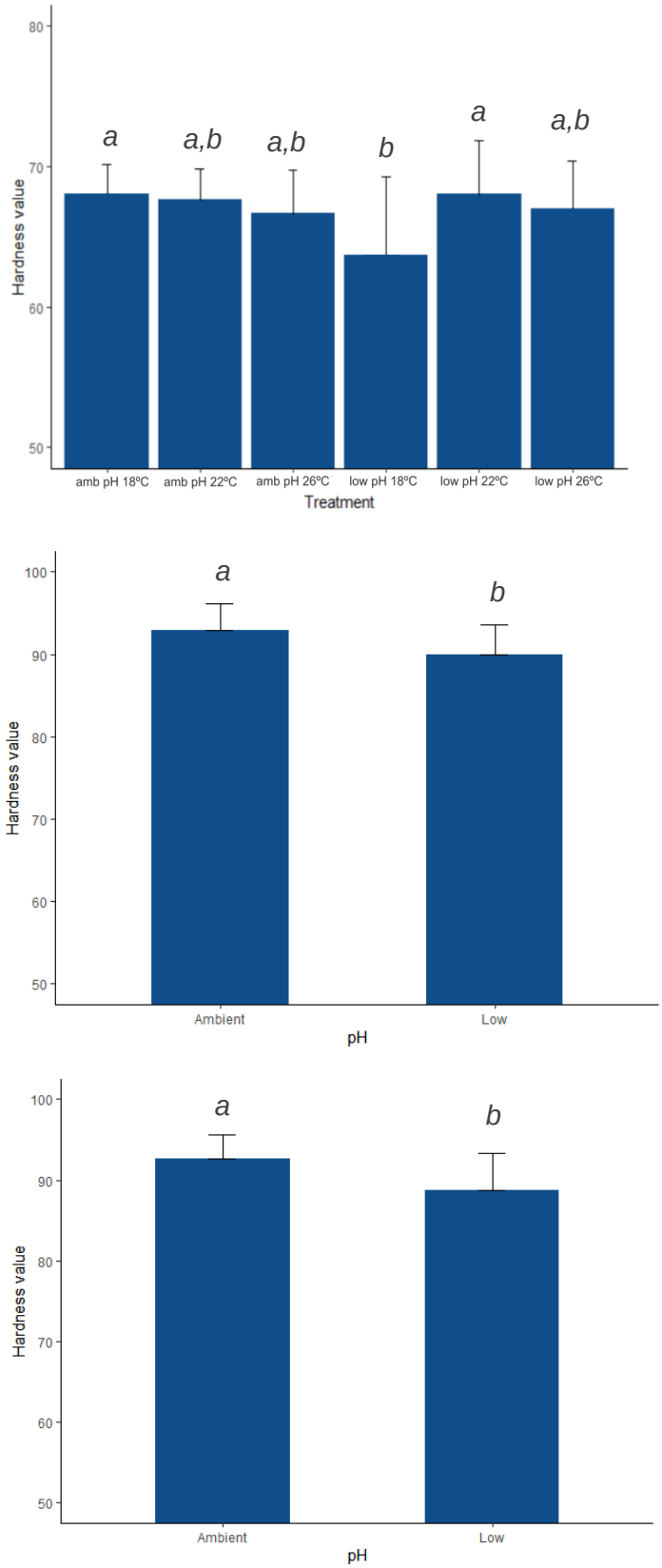

Figure 12: Shell hardness (mean ± SD) in area 1 (top), area 4 (middle) and area 5 (bottom) of crabs exposed for 30 days to 6 pH and temperature treatments: ambient pH (amb) (8.02 ± 0.08 , mean ± S.D) at 18ºC, 22ºC and 26ºC, and low pH (7.88 ± 0.09, mean ± S.D) at 18ºC, 22ºC and 26ºC; n=12 crabs per treatment. Hardness values are expressed in UA (units of measure). Different letters indicate statistically significant differences between treatments (Mixed ANOVA and LS Means, $p<0.05$).

Predatory Behaviour

Predation rate trials were done three times along the experiment. In the first trial after 12 days of exposure to experimental conditions, ANOVA test indicated a significant effect of temperature and the interaction between temperature and pH (Table 5). At 12 days of exposure the number of interactions of the crabs with their prey was lower at 18 ºC. In particular, it was significantly lower in the combined 18 ºC and low pH treatment compared to treatments at 22 ºC, ambient pH and 26 ºC, low pH as indicated by the LS Means test (Figure 13). While some variation was observed in the number of interactions of the predators with their prey, no statistically significant differences among groups were observed after 12 days of exposure, suggesting that after this first response the crabs were able to adapt to the low pH and low temperature exposure conditions.

Table 5: Results of the ANOVA performed to investigate the effects of temperature and pH on the predatory behaviour (total number of interactions with the prey; mean and standard error of the mean) of crabs (n=4 per treatment and time point, except for total interaction analysis) exposed for 12, 18 and 24 days to six combined treatments of pH (low (7.88 ± 0.09, mean ± S.D) and ambient (8.02 ± 0.08 , mean ± S.D)) and temperature (18 ºC, 22 ºC and 26 ºC). pH and temperature were considered as fixed factors. Significant effects (p<0.05) in bold.

Response	Variation Source	dF	SS	MS	F	P
Total Interaction	pH	1	861.1	861.12	3.0288	0.0864
	Temperature	2	1561.6	780.79	2.7462	0.0715
	Temperature x pH	2	1182.2	591.12	2.0791	0.1331
	Residual	66	18764.9	284.32		
Total Interaction After 12 days	pH	1	433.50	433.50	4.0630	0.0590
	Temperature	2	1668.08	834.04	7.8171	**0.0036**
	Temperature x pH	2	937.75	468.87	4.3946	**0.0279**
	Residual	18	1920.50	106.69		
Total Interaction After 18 days	pH	1	315.4	315.37	1.0763	0.3133
	Temperature	2	456.1	228.04	0.7783	0.4741
	Temperature x pH	2	1247.2	623.62	2.1283	0.1480
	Residual	18	5274.2	293.01		
Total Interaction After 24 days	pH	1	150.0	150.0	0.4167	0.5267
	Temperature	2	394.1	197.04	0.5474	0.5878
	Temperature x pH	2	190.7	95.37	0.2650	0.7702
	Residual	18	6479.0	359.94		

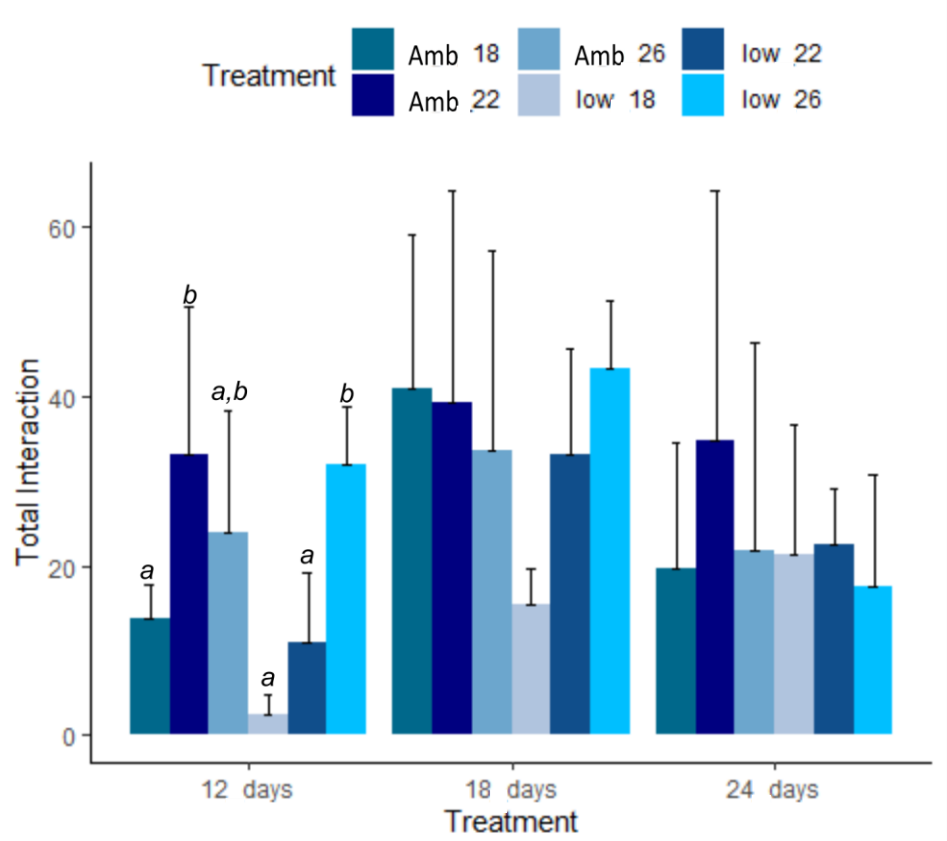

Figure 13: Total number of interactions between *C. maenas* and *Mytillus sp*. (mean ± SD) exposed for 12, 18 and 24 days to 6 pH and temperature treatments: ambient pH (amb) (8.02 ± 0.08 , mean ± SD) and low pH (7.88 ± 0.09, mean ± SD) at 18ºC, 22ºC and 26ºC (Two-way Nested ANOVA). Bars with different letters indicate significant differences in the tests a posteriori ($p<0.05$).

Regarding the real number of mussels consumed by the crabs, we found a significant effect again in the first trial (Table 6). In this trial the number of eaten mussels was significantly higher at ambient pH (0.91±0.51, mean ± SD) versus low pH treatment (0.25±0.45) (Figure 14). No effects were found for the other three factors.

Table 6: Results of the ANOVA performed to investigate the effects of temperature and pH on number of eaten mussels (n=4 per treatment and time point except the case of total eaten mussels) exposed for 12, 18 and 24 days and the six combined treatments of pH (low (7.88 ± 0.09, mean ± S.D) and ambient (8.02 ± 0.08 , mean ± S.D) and temperature (18 ºC, 22 ºC and 26 ºC). pH and temperature were considered as fixed factors. Significant effects ($p<0.05$) in bold.

Response	Variation Source	dF	SS	MS	F	P
Total eaten mussels	pH	1	0.500	0.500	1.3171	0.2830
	Temperature	2	1.695	0.847	2.2317	0.1455
	Temperature x pH	2	1.583	0.791	2.0854	0.1645
	Residual	66	28.166	0.444	0.8542	

Total eaten mussels After 12 days	pH	1	2.667	2.666	10.666	**0.0042**
	Temperature	2	0.333	0.166	0.666	0.5256
	Temperature x pH	2	0.333	0.166	0.666	0.5256
	Residual	18	4.500	0.250		
Total eaten mussels After 18 days	pH	1	1.041	1.0416	3.00	0.1003
	Temperature	2	2.083	1.0417	3.00	0.0750
	Temperature x pH	2	0.583	0.291	0.84	0.4479
	Residual	18	6.250	0.347		
Total eaten mussels After 24 days	pH	1	2.041	2.041	3.973	0.0616
	Temperature	2	1.083	0.541	1.054	0.3690
	Temperature x pH	2	1.583	0.791	1.540	0.2412
	Residual	18	9.250	0.513		

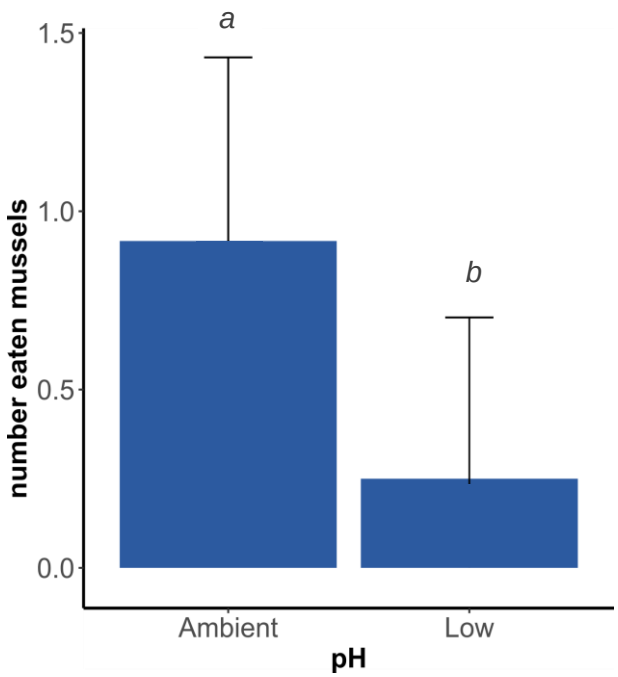

Figure 14: Number eaten mussels by *C. maenas* (mean ± SD) after 12 days exposed to 2 pH treatments: ambient pH (8.02 ± 0.08 , mean ± S.D) and low pH (7.88 ± 0.09, mean ± S.D) (Two-way ANOVA). Bars with different letters indicate significant differences in the tests a posteriori ($p<0.05$).

Metabolic Rate

Metabolic rates were measured through oxygen consumption across all treatments and two food intake conditions: fed and starved (for 48 hours prior measurements). On

average apparently higher values of oxygen consumption were observed on the starved conditions at 22 ºC (5.96 ± 1.55 µmol O_2.min^{-1}.g^{-1} FW, mean ± standard deviation) followed by 18 ºC (5.90 ± 3.73 µmol O_2.min^{-1}.g^{-1} FW) and 26 ºC (4.36 ± 2.05 µmol O_2.min^{-1}.g^{-1} FW). The lowest metabolic rate mean values was obtained for animals exposed to ambient pH at 22 ºC. The results of the statistical analysis revealed a borderline significance for the effect of the feeding condition (FE, p=0.075) and the interaction between temperature and FE (p=0.078) on the metabolic rates of the exposed crabs (Figure 15, Table 7). Indeed, fed animals exposed to ambient pH at 22º C showed a two to four fold decrease in their metabolic rate compared to crabs exposed to the remaining test conditions.

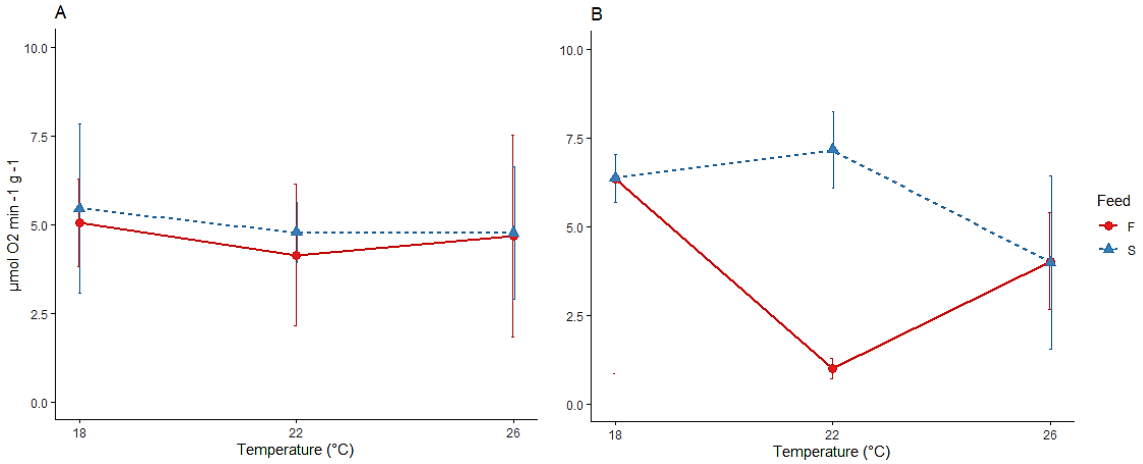

Figure 15*:* Metabolic rates of *C. maenas* (mean ± SD) at the end of a 30 days exposure to pH and temperature: ambient pH (8.02 ± 0.08 , mean ± SD) (**B**) and low pH (7.88 ± 0.09, mean ± SD*)* (**A**) at 18ºC, 22ºC and 26ºC. Feed indicates the feeding condition of the animals*:* red circles indicate measurements with fed (F) crabs and blue triangles indicate measurements crabs starved (S) crabs for 48h prior to the assessments (n=4 per treatment).

Table 7: Results of the ANOVA performed to investigate differences in the metabolic rate of *C. maenas* exposed to pH and temperature treatments: ambient pH (8.02 ± 0.08 , mean ± S.D) and low (7.88 ± 0.09, mean ± SD) pH at 18 ºC, 22 ºC and 26 ºC. The variable feeding condition (FE) had two categories: fed crabs or crabs starved for 48 h prior to the assessments (n=4 per treatment). Significant effects (p<0.05) in bold.

Response	Variation Source	dF	SS	MS	F	P
	pH	1	0.0000	0.000	0.0000	0.9997
	Temperature	2	23.669	11.8343	2.2547	0.1195
	FE	1	17.613	17.6131	3.3557	0.0752
Metabolic	pH x temperature	2	7.520	3.7601	0.7164	0.4953
Rate	pH x FE	1	8.442	8.4420	1.6084	0.2128
	Temperature x FE	2	28.752	14.3761	2.7390	0.0781
(O_2	pH x Temperature x FE	2	22.178	11.0891	2.1128	0.1356
consumption)	Residual	36	188.951	5.2486		

Biochemical measurements

The activity of the enzymes AChE, LDH and IDH determined in the experimental animals is presented in Figure 16. On average, AChE activity in the ganglion varied between a minimum of 314.60 ± 126.18 nmol.min^{-1}.mg^{-1} protein (mean ± SD) and a maximum of 400.84 ± 141.04 nmol.min^{-1}.mg^{-1} protein. The enzyme activity in the muscle ranged from 33.78 ± 10.47 to 42.48 ± 18.43 nmol.min^{-1}.mg^{-1} protein. For LDH, the enzyme activity varied between 176.13 ± 58.72 and 211.27 ± 37.81 nmol.min^{-1}.mg^{-1} protein, while for IDH the activity values ranged from 3.79 ± 1.25 to 5.86 ± 3.33 nmol.min^{-1}.mg^{-1} protein. No statistically significant differences among groups were found for the activities of AChE or LDH. The statistical analysis revealed a significant effect of the exposure temperature on the activity of IDH enzyme (Table 8). The post-hoc analysis indicated a decrease in the enzyme activity with temperature increase (Figure 16). On average, IDH activity was about 44% lower in crabs exposed at 26 ºC than in those exposed at 18 ºC.

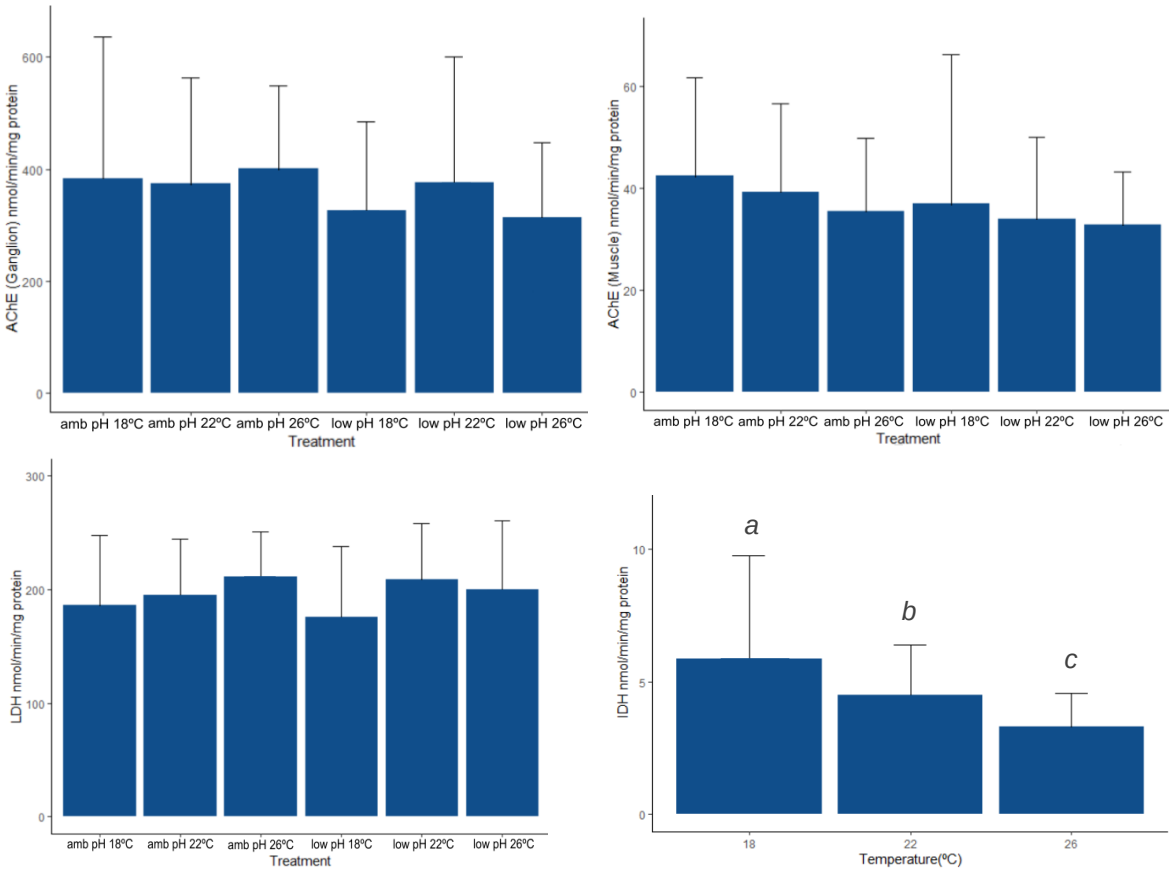

Figure 16: Enzymatic activity of the enzymes acetylcholinesterase (AChE) determined in the thoracic ganglion and muscle tissue, lactate dehydrogenase (LDH) determined in muscle tissue and NADP$^+$-dependent isocitrate dehydrogenase (IDH) determined in muscle tissue of crabs exposed for 30 days to six experimental conditions: ambient (Amb) (8.02 ± 0.08 , mean ± S.D) and low pH (7.88 ± 0.09, mean ± S.D) at 18ºC, 22ºC and 26ºC. Values correspond to group means and respective standard errors. Different letters indicate significant differences between treatments (ANOVA followed by the LS Means test).

Table 8: Results of mixed ANOVA performed to investigate the effects of temperature and pH on the activities of acetylcholinesterase (AChE, assayed in ganglion and muscle tissue), lactate dehydrogenase (LDH) and $NADP^+$-dependent isocitrate dehydrogenase (IDH). The crabs were exposed to six conditions: ambient (8.02 ± 0.08 , mean ± SD) or low pH (7.88 ± 0.09, mean ± SD) at 18ºC, 22ºC and 26ºC. Significant effects ($p<0.05$) in bold.

Response	Variation Source	dF	SS	MS	F	P
AChE Ganglion	pH	1	51015	51015	1.7071	0.2078
	Temperature	2	10388	5194	0.1738	0.8419
	Temperature x pH	2	28649	14324	0.4793	0.6269
	Tank(Temperature x pH)	18	537911	29884	0.8176	0.6713
	Residual	48	1754458	36551		
AChE Muscle	pH	1	368.8	368.81	0.9787	0.3356
	Temperature	2	382.5	191.26	0.5076	0.6103
	Temperature x pH	2	31.6	15.79	0.0419	0.9591
	Tank(Temperature x pH)	18	6782.8	376.82	1.1298	0.3550
	Residual	48	16010.1	333.54		
LDH	pH	1	104	104.2	0.0481	0.8288
	Temperature	2	8362	4181.0	1.9316	0.1738
	Temperature x pH	2	2519	1259.6	0.5819	0.5690
	Tank(Temperature x pH)	18	38960	2164.5	0.6885	0.8050
	Residual	48	150908	3143.9		
IDH	pH	1	5.083	5.083	0.5803	0.4560
	Temperature	2	78.466	39.233	4.4793	**0.0263**
	Temperature x pH	2	16.893	8.446	0.9643	0.4000
	Tank(Temperature x pH)	18	157.658	8.759	1.4794	0.1399
	Residual	48	284.189	5.921		

Discussion

Marine organisms can adapt to local temperature changes demonstrating good physiological responses matching the average environmental temperature (Hoegh-Guldberg and Bruno, 2010). Crustaceans, as ectothermic animals, experience body temperature variation and have mechanisms of physiological adaptation to adjust for varying environmental conditions (Haupt, 2006). However, continuous deviations from optimal temperature conditions will eventually surpass the threshold thermic limits affecting the fitness and survival of these organisms (Dissanayake, 2014). Additionally, ocean acidification is projected to cause a decrease in the pH of surface waters between 0.14 and 0.35 unit by the end of the 21st century (Meehl et al., 2007). High mortality resulting from low pH (7.5) exposure can impact negatively population abundance and productivity in crab juveniles (Long et al., 2013, Long et al., 2021). Nevertheless, the synergistic effects of these two climate drivers (elevated CO_2, and temperature) are still largely unexplored for many marine organisms.

In the present study, the largest mortality rates (up to 35.17% ± 6.1, mean ± SE) were observed in the warmest conditions through the entire experiment, however those differences were marginally no significant. Information about negative impacts of ocean warming on crustaceans is not scarce but responses seem to be more idiosyncratic than expected (Faulkner et al., 2014). In the experimental work of Chevaldonné and Lejeusne, 2003, 90% of *Hemimysis speuncula* crabs died at 31°C. This species is native from caves on the Mediterranean sea, which has been registering thermal anomalies over the past years due mostly to global warming. Other studies suggest that warmer temperatures can severely affect larval dispersion and the settlement of crustacean larvae (Hoegh-Guldberg and Bruno, 2010, Mao et al., 2019, Marochi et al., 2021). Mao et al., 2019 for example, estimated larval settlement of the snow crab (*Chionoecetes opilio*) and reported that in the scenario of warmer temperatures, larval settlement and its distribution would be negatively impacted.

Studies on *Carcinus maenas* thermal tolerance are abundant (reviewed in Young & Elliot 2020). The species is eurythermal and is able to survive short-term exposure to temperatures between 0 and 33–35 ºC. A critical thermal maximum (CTMax) of 35.1 ºC was estimated for crabs collected in Portugal (Madeira et al., 2014) and of 35.8 ºC for North Sea crabs caught in the fall and acclimated to 22 ºC (Cuculescu et al., 1998). These values are much higher than those tested in our populations. CTmax experiments are fast experiments with abrupt temperature increases (1 ºC h^{-1} in Madeira et., al 2014) and their values as indicator of the thermal niche of the species is relative. Our observations

suggest that adults are highly eurythermal and this capacity may help govern the biogeographic range of the species. However, the fact that there are no populations of *C. maenas* in tropical or sub-tropical environments (despite being introduced repeatedly in many of such locations), suggests that the species may be sensitive to high seawater temperatures in certain phases of its life-cycle.

In the present work no effect of pH or the interaction between temperature and pH on mortality was found in crabs exposed for 30 days to the different treatments, suggesting the animals are able to cope with the lower pH level (7.8) in the short-term. Similar results were found for the exposure of blue king crab (*Paralithodes platypus*) juveniles to pH 7.8 for a year (Long et al., 2016). The authors reared the juvenile crabs in seawater at three pH levels: ambient (control, pH ~8.1), pH 7.8, and pH 7.5. According to their findings, exposure at pH 7.8 had no effect on morphology or mortality and had only a minor effect on growth compared with the ambient treatment (Long et al., 2016). In contrast, exposure at pH 7.5 was found to cause initial high mortality (and decreased growth), when compared with the ambient pH, which subsequently dropped to levels comparable to those recorded for higher pH rates, suggesting acclimation could occur in this species. Jeeva Priya et al., 2017 also observed that a 0.5 unit fall in pH over a 15 day period could cause significant mortality of the brachyuran crustacean *Portunus pelagicus*. More recently, Whiteley et al., 2018 conducted a study investigating the interaction between water acidification and a 20% seawater dilution. The authors concluded that *Carcinus maenas* was able to hyperosmoregulate, maintaining the haemostasis of its haemolymph pH for at least a year, while the dible crab *Cancer pagurus* had limited ion-transporting capacities, exhibiting disrupted homeostasis of haemolymph pH following elevated CO_2 exposure for nine months. Furthermore, the authors alerted for the need to understand the full spectrum of species-related vulnerabilities given the differences in plasticity, to avoid erroneous predictions of the impacts of a changing marine climate (Whiteley et al., 2018). Interactive effects of temperature and pH on survival of stone crabs (*Menippe mercenaria*) were investigated by Gravinese et al., 2018. The authors exposed larvae of stone crabs for 30 days to predicted values of ocean warming and acidification. Seawater acidification was found to decrease larvae survival by 37%, while elevated temperature (32 ºC) caused a 71% reduction and the combination of these two factors elicited a reduction of 80%, relative to the control.

The responses of marine organisms to ocean acidification can vary largely in what concerns biological aspects such as dissolution and calcification of calcareous skeletons (Kroeker et al., 2010). Previous studies have shown that alteration of the seawater carbonate chemistry due to ocean acidification may negatively affect the deposition and

dissolution rates of calcium carbonate ($CaCO_3$) structures in tropical corals and planktonic organisms (reviewed by Kroeker et al., 2010). However, the sensitivity of calcification to ocean acidification appears to be related to the mineral form of calcium carbonate used by the organism. A review of available studies has shown that calcification of animals using calcite high in magnesium content suffered a slight positive effect of ocean acidification or was not was affected at all (Kroeker et al., 2010). Such is the case of several crustacean species, which in general utilize high-magnesium calcite. In the present study a slight increase in the carapace width of adult *Carcinus maenas* was found after a 30 days exposure to pH 7.8, suggesting that the low pH treatments favoured carapace calcification. A short-term exposure (21 days) of the caridean shrimp *Lysmata californica* found no differences in exoskeleton increase at reduced pH (7.53), compared to ambient pH (7.99), and a significant increase in the calcium weight percent of the cuticle, resulting in a greater Ca:Mg ratio (Taylor et al., 2015). Species able to control their haemolymph pH, as the green crab (with developed intracellular/extracellular pH regulatory mechanisms), appear to cope better with ocean acidification (Berry et al., 2002, Cohen and McConnaughey, 2003, Qadri et al., 2007). Additionally, some marine invertebrates are able to compensate for changes in carbonate chemistry by increasing calcification rates (Gutowska et al., 2008). Elemental composition of the carapace, coupled with detailed studies of the crabs' haemolymph, will help to confirm the occurrence of such a biogenic calcification process and better predict potential growth impacts derived from the deviation of energy to calcification and/or haemolymph homeostasis.

The exoskeleton of decapods is a multifunctional weapon of defence and attack. It is crucial for physical protection, determination of physical shape (Horst and Freeman, 1993), musculature attachment to facilitate swimming and gill movement (Glandon et al., 2018) and to support the external load (Fabritius et al., 2012). Thus crustaceans depend on their calcified exoskeleton for many critical functions and the mechanical properties of the exoskeleton are fundamental (Taylor et al., 2015). According to Young's modulus (Coffey et al., 2017) hardness can be defined as the maximum stress (strength/unit per area) that a material can hold before suffering permanent deformation. A lower value of hardness means the material will be able to hold reduced stress. In this study, the measurements of shell hardness indicated that pH caused lower hardness in carapace areas 4 and 5 and that low pH and low temperature conditions also reduced hardness in carapace area 1. This may suggest that ocean acidification may render the crabs' carapace more susceptible to damage. Declines in shell thickness can have the potential to affect the ability to protect the crab against predation and crash prey, affecting survival and feeding (Coffey et al., 2017, Glandon et al., 2018). Differential hardness of shell areas

were previously measured in animals exposed to low pH. Coffey et al., 2017 exposed juvenile king crabs (*Paraliyhodes*) to a future ocean acidification scenario; blue crabs (*P. platypus)* were exposed for a year to three pH levels (8.1, ambient; 7.8 and 7.5); red crabs (*P. camtschaticus*) were exposed for ~6 months to pH 8.0 and 7.8, at ambient temperature, ambient +2°C and ambient +4°C. At low pH, the authors observed reduced cuticle microhardness (or Vickers hardness, a measure of resistance to permanent or plastic mechanical deformation) in the chela, but not in the carapace, of both species. Interestingly, they also found the calcium content was significantly elevated in the carapace of blue king crabs and in the chela of red king crabs exposed to low pH at ambient temperature, compared to ambient pH. Increased calcification associated to acidification was also found in other crustaceans (Taylor et al., 2015). This suggested the reductions in micro-hardness would not be driven by lower calcium level in the shell and that the calcium content per se may not be an effective proxy for mechanical properties (Coffey et al., 2017). In fact, increasing mineral content may make exoskeleton more brittle because the increase of rigidity favours crack propagation (Taylor et al., 2015). We also found some evidences of significant interaction between pH and temperature. This type of interactions has been described in other crustaceans, for example it was also detected for the magnesium content in shell of the red king crab. Longmire, 2021 investigated combined effects of low pH (7.0-7.2 vs 8.0-8.3) and salinity (16 ppt vs 30 ppt) on juveniles of *Callinectes sapidus* and the clam *Mercenaria mercenaria* co-exposed twice for about 10 weeks. The author found the hardness (as measured with a durometer) of the crabs' carapace had increased after exposure to low pH or to the low pH and low salinity treatment, relative to the other treatments.

Regarding the predation behaviour, knowledge gaps on the influence of climate change in predator-prey relations are noticeable, and the detected direction and magnitude of the recorded effects are highly variable and idiosyncratic (Landes and Zimmer, 2012, Miller et al., 2014, Marangon et al., 2019). However, it is largely recognized that climate change has the potential to affect both spatial and temporal coexistence of organisms which might lead to disruptions between predator-prey relations (Bretagnolle and Gillis, 2010). South et al., 2017 found that the predation of the lionfish (*Pterois volitans*) on the prey *Paleomocetes varians* (shrimp) was higher at 26 °C rather than at 22 °C, mostly by decreasing the handling time on prey. Lord et al., 2019 examined the predation relation between three gastropod species (including invasive and native) and *C. maenas* under acidified conditions. Results found that gastropods with primarily aragonite shells were negatively impacted by pH due to their corrosion, which obviously favoured *C. maenas* predatory behaviour. The study hinted that this could lead to an altered native and

invasive community modulation. In Appelhans et al., 2012, a study of the effects of seawater acidification on growth, feeding and behaviour of *C. maenas* showed a decreased consumption of prey in more acidified condition (pH=7.7) during an experimental phase. Veiga et al., 2011 found no effect of temperature on the consumption rates of *Carcinus maenas* in mussels. Our results found significant temperature x pH effects in the number of predator-prey interactions for the first experimental trial. When comparing the 18 °C and 22 °C versus 26 ºC treatments both at low pH, a possible explanation for the increase of interactions with temperature could be related to higher metabolic rates as previously reported by Marangon et al., 2019. However, the same pattern was not found at ambient pH for the same trial and those effects vanished in the subsequent trials. In fact, apparently there was a bigger number of attempts to open and consume *Mytilus* sp. mussels by *C. maenas* after 18 days and even 24 days of experiment. Rates seemed to stabilize for the different treatments with time, suggesting some kind of acclimation to the experimental conditions. Interestingly, in Ferrari et al., 2015 a synergistic interaction was found between temperature and pH. In this experiment, two closely related species of damfish and a predatory dottybak were used under predicted climate change conditions to explore their predatory relation. pH alone was responsible to revert prey selection when comparing to normal conditions where the number of preys selected were the same. However, when adding high temperature influence an increase on predation rate from 30% to 70% was observed. Although predator success was actually improved as in the previous example, the community effects can still emerge if the easier preys preferred are affected by stressor negative impacts. Also, Landes and Zimmer, 2012 in a 5 month experiment found that acidification negatively affected the closer muscle length of the crusher chela and correspondingly the claw-strength increment in *C. maenas*. However, no changes in the predator-prey interactions were found. Although it can be difficult to predict the dynamic of predator-prey interactions under climate change due to multiple direct and indirect causes, it could be essential to improve our knowledge on the changes of strength and directions of these multispecies interactions in order to better forecast future population dynamics and trends (Ockendon et al., 2014).

Oxygen consumption rate can be defined as the amount of O_2 that an animal removes from the environment per unit of time, which is linearly equivalent to the rate of energy utilization (Nespolo et al., 2011). It is considered one of the most accurate and forthright measures of anaerobic metabolism in animals. Normally, higher temperatures induce higher metabolic rates as it is driven directly from the kinetic energy of the cell (Clarke and Fraser, 2004) and regularly leads to high metabolic rates in marine animals if the

temperature experienced is within their range of tolerance (Miller et al., 2014). When there is food intake, nutrients from the digestive tract are absorbed by tissue which stimulates the rapid increase of metabolism and consequently O_2 consumption (Carter and Mente, 2014). Particularly, in crustaceans, protein synthesis appeals to a process of high energy demand that is affected by both abiotic and biotic factors. Acidification seems as well to be capable to impair metabolic rates, as high values of pCO_2 are normally associated to high metabolic rates since diffusion gradients need to increase in order to excrete quantities proportional to the ones the organisms have consumed (Melzner et al., 2009). In Kim et al. (2016), hermit crabs (*Pagurus tanneri*) were submitted to two pH treatments (ambient: 7.6, acidified: 7.1). After 3 weeks immersed in the low pH treatment higher metabolic rates were verified, which suggests that for more acidified environments imposes energetic costs possibly related to acid-base regulation. However, in our study, we found no significant effects of temperature or pH on the metabolic rates of the experimental crabs. No effects were found either for the feeding conditions. Despite the abundant literature linking metabolic rates with temperature and pH in ectothermic organisms (Deutsch et al., 2015, Semsar-kazerouni et al., 2020) our lack of effect suggest a high capacity for acclimation to different environmental conditions for this species which match with the large ability to invade numerous seashores across the oceans.

On overall, the enzyme levels determined in this study were within the range previously determined for control animals of this species, either in field or laboratory experiments (Rodrigues et al., 2013). The enzyme AChE is involved in cholinergic transmission and has been broadly applied in laboratory research and monitoring studies to assess neurotransmission and neurotoxic effects of abiotic stressors and chemical contaminants on vertebrate and invertebrate species (Mesquita et al., 2011, Oliva et al., 2012, Rodrigues et al., 2012, Rodrigues et al., 2013, Rodrigues et al., 2014, Camacho et al., 2020). In cholinergic transmission, this enzyme acts on the hydrolysis of acetylcholine, causing its release from the post-synaptic receptors allowing the cell to recover to its resting potential. Decreased levels of the enzyme can result either from a modulation of cholinergic transmission or from inhibition of the enzyme activity. The latter can cause muscle overstimulation, which when can eventually lead to death of the animal. Changes in AChE activity can be elicited by different stressors, from temperature or salinity to exposure to chemical contaminants (Mesquita et al., 2011, Oliva et al., 2012, Rodrigues et al., 2012, Rodrigues et al., 2013, Rodrigues et al., 2014, Camacho et al., 2020, Rodrigues and Pardal, 2014). In the present study, no effects of the treatments on AChE activity were found either in the muscle or the ganglion, suggesting the animals are able to cope with the short-term exposure to these combined climate change factors. Studies on the

impact of ocean acidification and warming on relevant biochemical parameters of crustacean species are still scarce. Nevertheless, a recent study (Jeeva Priya et al., 2017), investigated the effect of a 15-day exposure to water acidification on AChE activity of the decapod *Portunus pelagicus*. Although the enzyme activity was observed to decrease with the increase in pH, no relevant differences were found between crabs exposed to 7.5 and 8.1 pH, in line with the findings of the present work.

Lactate dehydrogenase is a central enzyme in the anaerobic pathway of energy metabolism. Anaerobic metabolism is less efficient in the production of ATP molecules, compared to the aerobic pathway, but it is a faster pathway of production, with LDH providing additional energy to cope with the effects of the exposure to chemical and abiotic stress (Wu and Lam, 1997, Menezes et al., 2006, Rodrigues et al., 2012, Rodrigues et al., 2013). This enzyme It is widely distributed in living organisms and their tissues exhibiting higher levels in the muscles and the liver. It catalyses the reversible conversion of pyruvate and NADH into lactic acid and NAD^+, which can then be regenerated through oxidative phosphorylation by the electron transport chain (Valvona et al., 2016). In the present work no significant effects of warming and/or acidification on LDH activity could be detected. Studies investigating the impact of these factors, either single or combined, on the energy metabolism of crustacean species are scarce. In a previous study set to examine the effects of predicted acidification trends (pH ~7.8) on larval stages of the European lobster (Rato et al., 2017), no significant differences in LDH activity, relative to ambient pH, could be found either. In contrast, Menu-Courey et al. (2019) found that the increase in acidification elicited a slight decrease in the activity of this enzyme related to the anaerobic pathway of glycolytic capacity in juvenile recruits of the American lobster (*Homarus americanus*).

On the other hand, isocitrate dehydrogenase (IDH) is a mitochondrial enzyme that participates in the citric acid cycle. It is a NADP-dependent enzyme, which provides NADPH for the regeneration of the glutathione molecule, thus playing an key role in cellular defence against oxidative stress mediated by reactive oxygen species (Napierska and Podolska, 2008). In this work, IDH was significantly affected by temperature, with the results showing a decrease in activity (of about 15 to 30%) with increasing temperature. Although temperature usually enhances enzymatic reactions to a certain extent, if the temperature is too high, above optimal levels, the enzyme can undergo denaturation and stop working. The observed IDH activity decreases with increasing temperature may indicate that cellular NADPH, which is crucial in antioxidant and detoxification systems, may not be obtained. A recent study about the impact of warming and acidification on the American lobster (*Homarus americanus*) also observed an alteration of the aerobic energy

pathway (Klymasz-Swartz et al., 2019). The authors did a short exposure (14-16 days) of adults to ambient temperature and pH (16 °C and pH 8.10) and to levels predicted for the year 2300 (20 °C and pH 7.10). They found a slight increase in the whole animal oxygen consumption and a decrease in the activity of muscle citrate synthase, a Krebs-cycle enzyme, in the combined warming and acidification treatment. The authors tested a pH level much lower than the one tested in the present work. Nevertheless, citrate synthase is the first enzyme involved in the citric acid cycle, acting upstream of IDH. Further, experiments investigating long-lasting effects of ocean warming and acidification, will help understand if *Carcinus maenas* is able to compensate for such alterations in the anaerobic energy pathway or if these will reflect in increased oxygen consumption.

Conclusion

Climate change is gradually resulting in ocean warming and acidification. Invertebrates can show varying responses to these changes, which difficult predictions of how marine ecosystems will respond to such alterations and, consequently, establishing adequate mitigation measures. In the present study, a battery of biomarkers, including biochemical, physiological and ecological parameters was employed to investigate short-term responses of the decapod *Carcinus maenas* to ocean warming and acidification. Overall, the results suggest the animals may experience some early negative impacts of the exposure, but appear to be fairly resilient to carapace dissolution and able to cope with the exposure over time. The most relevant responses found here were a significantly lower ability of the crabs to interact with their prey at 12 days of exposure to low pH and temperature, which was no longer observed after 24 days of exposure, and a slight decrease in aerobic energy metabolism with the increase in temperature, that appeared to have no relevant impact on oxygen consumption, suggesting the animals were probably capable of restoring their internal homeostasis. This is consistent with the known high ability of this species to adapt to changing environmental conditions, which makes it one of the worst invasive species worldwide. Nevertheless, findings on lower hardness values of the carapace in acidified treatments and lower IDH enzymatic activity with increasing temperature suggest that this species can be more susceptible to damages and be compromised at cellular/molecular detoxification and antioxidant systems, respectively, in a near future considering the predicted climate change effects. Future long-term exposures, specially focused in multiple stressors experiments, will bring further insight about the species resilience to predicted climate change scenarios.

Appendix

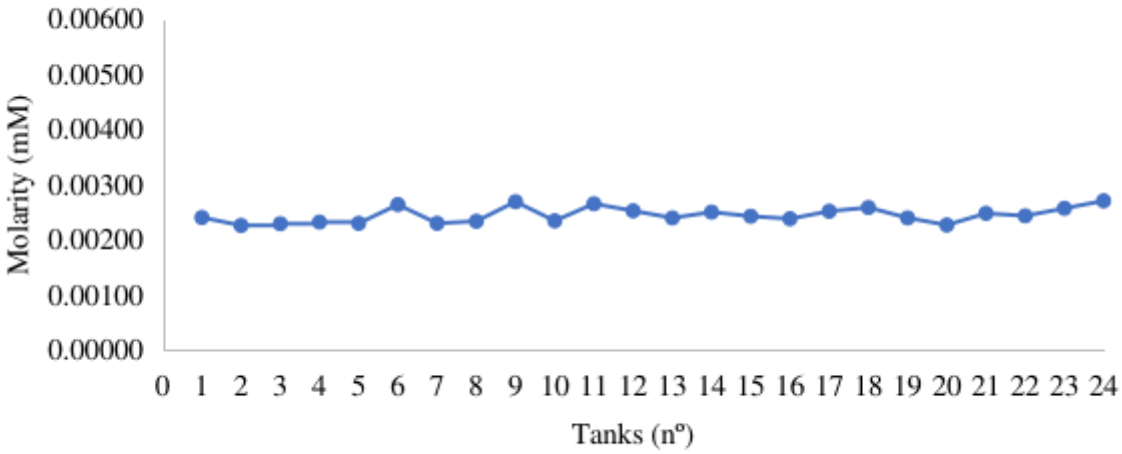

Figure 1: Alkalinity in mM of a water sample retrieved from each tank

Table 1: Water chemistry (mean and standard deviation of the measured parameters considering all days of the experiment).

Physical Variables	pH	Temperature	Salinity	DO (mg/L)	DO (%)
Tank	Mean ± S.D				
1	7.81 ± 0.05	17.92 ± 0.27	33.40 ± 1.35	8.68 ± 0.20	104.29 ± 1.59
2	7.87 ± 0.08	18.24 ± 0.25	33.76 ± 1.35	8.72 ± 0.19	104.12 ± 1.79
3	7.86 ± 0.09	21.81 ± 0.28	34.51 ± 1.43	8.66 ± 0.23	104.88 ± 2.29
4	7.87 ± 0.09	21.87 ± 0.16	34.46 ± 1.47	8.32 ± 0.29	101.91 ± 3.06
5	7.94 ± 0.10	25.77 ± 0.30	35.61 ± 1.56	8.36 ± 0.32	104.43 ± 3.45
6	7.92 ± 0.09	25.65 ± 0.28	35.23 ± 1.49	8.06 ± 0.27	102.04 ± 3.01
7	7.89 ± 0.08	18.32 ± 0.27	33.58 ± 1.11	7.99 ± 0.35	99.30 ± 3.63
8	7.88 ± 0.07	18.11 ± 0.27	33.59 ± 1.15	8.14 ± 0.42	99.29 ± 4.69
9	7.88 ± 0.08	21.90 ± 0.14	34.82 ± 1.10	8.56 ± 0.32	104.90 ± 3.04
10	7.91 ± 0.07	21.89 ± 0.11	34.66 ± 1.19	8.40 ± 0.26	103.83 ± 2.68
11	7.83 ± 0.08	25.80 ± 0.14	35.79 ± 1.07	8.18 ± 0.28	102.59 ± 2.75
12	7.85 ± 0.08	25.73 ± 0.18	35.93 ± 1.16	7.84 ± 0.27	99.89 ± 3.56
13	7.98 ± 0.08	17.98 ± 0.17	33.34 ± 1.09	8.03 ± 0.29	100.31 ± 2.68
14	8.01 ± 0.08	18.15 ± 0.17	33.59 ± 1.12	8.43 ± 0.15	103.40 ± 0.95
15	7.96 ± 0.06	21.88 ± 0.19	34.44 ± 1.22	8.36 ± 0.20	102.89 ± 2.00
16	7.97 ± 0.08	21.83 ± 0.13	34.32 ± 1.14	8.22 ± 0.26	102.24 ± 2.65
17	8.02 ± 0.05	25.72 ± 0.38	35.07 ± 1.03	8.42 ± 0.32	106.76 ± 3.60
18	8.03 ± 0.06	25.69 ± 0.38	34.79 ± 1.18	8.12 ± 0.24	103.92 ± 3.42
19	8.05 ± 0.05	18.38 ± 0.62	32.96 ± 1.48	8.13 ± 0.15	101.69 ± 2.30
20	8.04 ± 0.05	18.10 ± 0.13	33.06 ± 1.51	8.39 ± 0.16	103.08 ± 1.77
21	8.03 ± 0.06	21.88 ± 0.17	33.86 ± 1.77	8.66 ± 0.21	106.51 ± 2.24
22	8.07 ± 0.05	21.75 ± 0.44	33.52 ± 1.60	8.54 ± 0.20	105.88 ± 2.40
23	8.01 ± 0.08	25.78 ± 0.18	34.99 ± 1.57	8.36 ± 0.23	105.79 ± 2.65
24	8.02 ± 0.11	25.71 ± 0.38	34.71 ± 1.39	8.17 ± 0.20	105.12 ± 2.11

References

ABRAM, N., GUTTUSO, J.-P., PRAKASH, A., CHENG, L., CHIDICHIMO, M. P., CRATE, S., ENOMOTO, H., GARSCHAGEN, M., GRUBER, N. & HARPER, S. 2019. Framing and Context of the Report-Intergovernmental Panel on Climate Change Special Report on Oceans and Cryosphere in a Changing Climate.

APPELHANS, Y. S., THOMSEN, J., PANSCH, C., MELZNER, F. & WAHL, M. 2012. Sour times: seawater acidification effects on growth, feeding behaviour and acid–base status of *Asterias rubens* and *Carcinus maenas*. *Marine Ecology Progress Series*, 459, 85-98.

BAETA, A., CABRAL, H. N., MARQUES, J. C. & PARDAL, M. A. 2006. Feeding ecology of the green crab, *Carcinus maenas* (L., 1758) in a temperate estuary, Portugal. *Crustaceana*, 1181-1193.

BERRY, L., TAYLOR, A. R., LUCKEN, U., RYAN, K. P. & BROWNLEE, C. 2002. Calcification and inorganic carbon acquisition in coccolithophores. *Functional Plant Biology*, 29, 289-299.

BINDOFF, N. L., CHEUNG, W. W., KAIRO, J. G., ARÍSTEGUI, J., GUINDER, V. A., HALLBERG, R., HILMI, N. J. M., JIAO, N., KARIM, M. S. & LEVIN, L. 2019. Changing ocean, marine ecosystems, and dependent communities. *IPCC special report on the ocean and cryosphere in a changing climate,* 477-587. [H.-O. Pörtner, D.C. Roberts, V. Masson-Delmotte, P. Zhai, M. Tignor, E. Poloczanska, K. Mintenbeck, A. Alegría, M. Nicolai, A. Okem, J. Petzold, B. Rama, N.M. Weyer (eds.)]. In press.

BRETAGNOLLE, V. & GILLIS, H. 2010. Predator–prey interactions and climate change. *Effects of climate change on birds*, 227-248.

CAMACHO, C., MAULVAULT, A. L., SANTOS, M. T., BARBOSA, V., FOGAÇA, F. H. S., POUSÃO-FERREIRA, P., NUNES, M. L., ROSA, R. & MARQUES, A. 2020. Mercury in Juvenile Solea senegalensis: Linking Bioaccumulation, Seafood Safety, and Neuro-Oxidative Responses under Climate Change-Related Stressors. *Applied Sciences*, 10, 1993.

CARTER, C. & MENTE, E. 2014. Protein synthesis in crustaceans: a review focused on feeding and nutrition. *Open Life Sciences*, 9, 1-10.

CAVICCHIOLI, R., RIPPLE, W. J., TIMMIS, K. N., AZAM, F., BAKKEN, L. R., BAYLIS, M., BEHRENFELD, M. J., BOETIUS, A., BOYD, P. W. & CLASSEN, A. T. J. N. R. M. 2019. Scientists' warning to humanity: microorganisms and climate change. 17, 569-586.

CHEVALDONNÉ, P. & LEJEUSNE, C. 2003. Regional warming-induced species shift in north-west Mediterranean marine caves. *Ecology Letters*, 6, 371-379.

CIAIS, P., SABINE, C., BALA, G., BOPP, L., BROVKIN, V., CANADELL, J., CHHABRA, A., DEFRIES, R., GALLOWAY, J. & HEIMANN, M. 2013. Carbon and other biogeochemical cycles. *Climate change 2013: the physical science basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge University Press.

CLARKE, A. & FRASER, K. 2004. Why does metabolism scale with temperature? *Functional ecology*, 18, 243-251.

COFFEY, W. D., NARDONE, J. A., YARRAM, A., LONG, W. C., SWINEY, K. M., FOY, R. J. & DICKINSON, G. H. 2017. Ocean acidification leads to altered micromechanical properties of the mineralized cuticle in juvenile red and blue king crabs. *Journal of Experimental Marine Biology and Ecology*, 495, 1-12.

COHEN, A. L. & MCCONNAUGHEY, T. A. 2003. Geochemical perspectives on coral mineralization. *Reviews in mineralogy and geochemistry*, 54, 151-187.

CONLAN, K. E. 1994. Amphipod crustaceans and environmental disturbance: a review. *Journal of Natural History*, 28, 519-554.

CROTHERS, J. J. F. S. 1967. The biology of the shore crab *Carcinus maenas* (L.) 1. The background-anatomy, growth and life history. 2, 407-434.

CUCULESCU, M., HYDE, D. & BOWLER, K. 1998. Thermal tolerance of two species of marine crab, *Cancer pagurus* and *Carcinus maenas*. *Journal of Thermal Biology*, 23, 107-110.

DEUTSCH, C., FERREL, A., SEIBEL, B., PÖRTNER, H.-O. & HUEY, R. B. 2015. Climate change tightens a metabolic constraint on marine habitats. *Science*, 348, 1132-1135.

DEVRIES, M. S., WEBB, S. J., TU, J., CORY, E., MORGAN, V., SAH, R. L., DEHEYN, D. D. & TAYLOR, J. R. A. 2016. Stress physiology and weapon integrity of intertidal mantis shrimp under future ocean conditions. *Scientific Reports*, 6, 38637.

DISSANAYAKE, A. 2014. Ocean acidification and warming effects on Crustacea: possible future scenarios. *The Mediterranean Sea*. Springer.

ELLIS, G., BELFIELD, A. & GOLDBERG, D. M. 1971. Colorimetric determination of serum acid phosphatase activity using adenosine 3′-monophosphate as substrate. *Journal of clinical pathology*, 24, 493-500.

ELLMAN, G. L., COURTNEY, K. D., ANDRES JR, V. & FEATHERSTONE, R. M. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical pharmacology*, 7, 88-95.

FABRITIUS, H.-O., KARSTEN, E. S., BALASUNDARAM, K., HILD, S., HUEMER, K. & RAABE, D. 2012. Correlation of structure, composition and local mechanical properties in the dorsal carapace of the edible crab *Cancer pagurus. Zeitschrift für Kristallographie - Crystalline Materials*, 227, 766-776.

FABRY, V. J., SEIBEL, B. A., FEELY, R. A. & ORR, J. C. 2008. Impacts of ocean acidification on marine fauna and ecosystem processes. *ICES Journal of Marine Science*, 65, 414-432.

FAULKNER, K. T., CLUSELLA-TRULLAS, S., PECK, L. S. & CHOWN, S. L. 2014. Lack of coherence in the warming responses of marine crustaceans. *Functional Ecology*, 28, 895-903.

FEELY, R. A., SABINE, C. L., HERNANDEZ-AYON, J. M., IANSON, D. & HALES, B. 2008. Evidence for Upwelling of Corrosive "Acidified" Water onto the Continental Shelf. *Science*, 320, 1490-1492.

FERRARI, M. C. O., MUNDAY, P. L., RUMMER, J. L., MCCORMICK, M. I., CORKILL, K., WATSON, S.-A., ALLAN, B. J. M., MEEKAN, M. G. & CHIVERS, D. P. 2015. Interactive effects of ocean acidification and rising sea temperatures alter

predation rate and predator selectivity in reef fish communities. *Global Change Biology*, 21, 1848-1855.

GATTUSO, J.-P. & HANSSON, L. 2011. *Ocean acidification*, Oxford University Press.

GLANDON, H. L., KILBOURNE, K. H., SCHIJF, J. & MILLER, T. J. 2018. Counteractive effects of increased temperature and pCO2 on the thickness and chemistry of the carapace of juvenile blue crab, *Callinectes sapidus*, from the Patuxent River, Chesapeake Bay. *Journal of Experimental Marine Biology and Ecology*, 498, 39-45.

GRAVINESE, P. M., ENOCHS, I. C., MANZELLO, D. P. & VAN WOESIK, R. 2018. Warming and pCO2 effects on Florida stone crab larvae. *Estuarine, Coastal and Shelf Science,* 204, 193-201.

GUTOWSKA, M. A., PÖRTNER, H. O. & MELZNER, F. 2008. Growth and calcification in the cephalopod *Sepia officinalis* under elevated seawater pCO2. *Marine Ecology Progress Series*, 373, 303-309.

HAMPTON, S. L. & GRIFFITHS, C. L. 2007. Why *Carcinus maenas* cannot get a grip on South Africa's wave-exposed coastline. *African Journal of Marine Science*, 29, 123-126.

HAUPT, L. 2006. Temperature Tolerance In Crustaceans: Critical Temperatures And The Heart. All theses and dissertations, University of New England. Retrieved from http://dune.une.edu/theses/3

HENRY, R. P. & WHEATLY, M. G. 1992. Interaction of respiration, ion regulation, and acid-base balance in the everyday life of aquatic crustaceans. *American Zoologist*, 32, 407-416.

HERNROTH, B., SKÖLD, H. N., WIKLANDER, K., JUTFELT, F. & BADEN, S. 2012. Simulated climate change causes immune suppression and protein damage in the crustacean *Nephrops norvegicus*. *Fish & Shellfish Immunology*, 33, 1095-1101.

HOEGH-GULDBERG, O. & BRUNO, J. F. 2010. The impact of climate change on the world's marine ecosystems. *Science*, 328, 1523-1528.

HORST, M. N. & FREEMAN, J. A. 1993. *The crustacean integument: morphology and biochemistry*, CRC Press.

INNISS, L., SIMCOCK, A., AJAWIN, A. Y., ALCALA, A. C., BERNAL, P., CALUMPONG, H. P., ARAGHI, P. E., GREEN, S. O., HARRIS, P. & KAMARA, O. K. 2016. The first global integrated marine assessment. United Nations. Accessed at on 5th February.

JEEVA PRIYA, R., ANAND, M., MARUTHUPANDY, M. & HAMEEDHA BEEVI, A. 2017. Biomarker response of climate change-induced ocean acidification and hypercapnia studies on brachyurian crab *Portunus pelagicus*. *Global Journal of Environmental Science and Management*. 3, 165-176.

KEPPEL, E. A., SCROSATI, R. A. & COURTENAY, S. C. J. J. O. N. A. F. S. 2012. Ocean acidification decreases growth and development in American lobster (*Homarus americanus)* larvae. *J. Northw. Atl. Fish*. *Sci.,* 44, 61-66.

KIM, T. W., TAYLOR, J., LOVERA, C. & BARRY, J. P. 2016. CO2-driven decrease in pH disrupts olfactory behaviour and increases individual variation in deep-sea hermit crabs. *ICES Journal of Marine Science*, 73, 613-619.

KLASSEN, G. J. & LOCKE, A. 2007. A biological synopsis of the European green crab, *Carcinus maenas*, Fisheries and Oceans Canada Moncton, New Brunswick, Canada.

KLYMASZ-SWARTZ, A. K., ALLEN, G. J., TREBERG, J. R., YOON, G. R., TRIPP, A., QUIJADA-RODRIGUEZ, A. R. & WEIHRAUCH, D. 2019. Impact of climate change on the American lobster (*Homarus americanus*): Physiological responses to combined exposure of elevated temperature and pCO2. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 235, 202-210.

KROEKER, K. J., KORDAS, R. L., CRIM, R. N. & SINGH, G. G. 2010. Meta-analysis reveals negative yet variable effects of ocean acidification on marine organisms. *Ecology Letters*, 13, 1419-1434.

LAFFOLEY, D. D. A. & BAXTER, J. 2016*. Explaining ocean warming: Causes, scale, effects and consequences*, IUCN Gland, Switzerland.

LANDES, A. & ZIMMER, M. 2012. Acidification and warming affect both a calcifying predator and prey, but not their interaction. *Marine Ecology Progress Series*.

LENTH, R., SINGMANN, H., LOVE, J., BUERKNER, P. & HERVE, M. 2019. R Package emmeans. CRAN.

LONG, W. C., SWINEY, K. M. & FOY, R. J. 2021. Effects of ocean acidification on young-of-the-year golden king crab (*Lithodes aequispinus*) survival and growth. *Marine Biology*, 168, 126.

LONG, W. C., SWINEY, K. M., HARRIS, C., PAGE, H. N. & FOY, R. J. 2013. Effects of ocean acidification on juvenile red king crab (*Paralithodes camtschaticus*) and Tanner crab (*Chionoecetes bairdi*) growth, condition, calcification, and survival. *PloS one*, 8, e60959-e60959.

LONG, W. C., VAN SANT, S. B., SWINEY, K. M. & FOY, R. J. 2016. Survival, growth, and morphology of blue king crabs: effect of ocean acidification decreases with exposure time. *ICES Journal of Marine Science*, 74, 1033-1041.

LONGMIRE, K. S. 2021. Effects of Acidification and Salinity on *Callinectes sapidus*, *Mercenaria mercenaria*, and Their Interactions. *Dissertations, Theses, and Masters Projects*. William & Mary. Paper 1616444437 .

LORD, J. P., HARPER, E. M. & BARRY, J. P. 2019. Ocean acidification may alter predator-prey relationships and weaken nonlethal interactions between gastropods and crabs. *Marine Ecology Progress* Series, 616, 83-94.

MADEIRA, D., NARCISO, L., CABRAL, H. N., DINIZ, M. S. & VINAGRE, C. 2014. Role of thermal niche in the cellular response to thermal stress: Lipid peroxidation and HSP70 expression in coastal crabs. *Ecological indicators*, 36, 601-606.

MADEIRA, D., NARCISO, L., CABRAL, H., VINAGRE, C. & DINIZ, M. 2012. HSP70 production patterns in coastal and estuarine organisms facing increasing temperatures. *Journal of sea research*, 73, 137-147.

MAO, X., GUO, X., WANG, Y. & TAKAYAMA, K. 2019. Influences of Global Warming on the Larval Survival and Transport of Snow Crab (*Chionoecetes opilio*) in the Sea of Japan. *Sustainability*, 11, 2198.

MARANGON, E., GOLDENBERG, S. U. & NAGELKERKEN, I. 2019. Ocean warming increases availability of crustacean prey via riskier behavior. *Behavioral Ecology*, 31, 287-291.

MAROCHI, M. Z., COSTA, T. M. & BUCKLEY, L. B. 2021. Ocean warming is projected to speed development and decrease survival of crab larvae. Estuarine, Coastal and *Shelf Science*, 259, 107478.

MEEHL, G. A., COVEY, C., DELWORTH, T., LATIF, M., MCAVANEY, B., MITCHELL, J. F., STOUFFER, R. J. & TAYLOR, K. E. 2007. The WCRP CMIP3 multimodel dataset: A new era in climate change research. *Bulletin of the American meteorological society*, 88, 1383-1394.

MELZNER, F., GUTOWSKA, M., LANGENBUCH, M., DUPONT, S., LUCASSEN, M., THORNDYKE, M., BLEICH, M. & PÖRTNER, H.-O. 2009. Physiological basis for high CO2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? *Biogeosciences*, 6, 2313-2331.

MENEZES, S., SOARES, A. M., GUILHERMINO, L. & PECK, M. R. 2006. Biomarker responses of the estuarine brown shrimp *Crangon crangon* L. to non-toxic stressors: temperature, salinity and handling stress effects. Journal of *Experimental Marine Biology and Ecology*, 335, 114-122.

MESQUITA, S. R., GUILHERMINO, L. & GUIMARÃES, L. 2011. Biochemical and locomotor responses of *Carcinus maenas* exposed to the serotonin reuptake inhibitor fluoxetine. *Chemosphere*, 85, 967-976.

MILLER, L. P., MATASSA, C. M. & TRUSSELL, G. C. 2014. Climate change enhances the negative effects of predation risk on an intermediate consumer. *Global Change Biology*, 20, 3834-3844.

MOKSNES, P. O., PIHL, L. & MONTFRANS, J. V. 1998. Predation on postlarvae and juveniles of the shore crab *Carcinus maenas*: importance of shelter, size and cannibalism. *Marine Ecology Progress Series*, 166, 211-225.

NAPIERSKA, D. & PODOLSKA, M. 2008. Relationship between biomarker responses and contaminant concentration in selected tissues of flounder (Platichthys flesus) from the Polish coastal area of the Baltic Sea. *Oceanologia*, 50, 421-442.

NASA, 2021. 2020 Tied for Warmest Year on Record, NASA Analysis Shows. [Online] Available at < https://climate.nasa.gov/news/3061/2020-tied-for-warmest-year-on-record-nasa-analysis-shows/ > [Accessed on February 2021]

NESPOLO, R. F., BACIGALUPE, L. D., FIGUEROA, C. C., KOTEJA, P. & OPAZO, J. C. 2011. Using new tools to solve an old problem: the evolution of endothermy in vertebrates. *Trends in Ecology & Evolution*, 26, 414-423.

NOAA, 2017. What is eutrophication? [Online] Available at <https://oceanservice.noaa.gov/facts/eutrophication.html> [Accessed on February 2021]

OCKENDON, N., BAKER, D. J., CARR, J. A., WHITE, E. C., ALMOND, R. E. A., AMANO, T., BERTRAM, E., BRADBURY, R. B., BRADLEY, C., BUTCHART, S. H. M., DOSWALD, N., FODEN, W., GILL, D. J. C., GREEN, R. E., SUTHERLAND, W. J., TANNER, E. V. J. & PEARCE-HIGGINS, J. W. 2014. Mechanisms underpinning climatic impacts on natural populations: altered species interactions are more important than direct effects. *Global Change Biology*, 20, 2221-2229.

OLIVA, M., PERALES, J. A., GRAVATO, C., GUILHERMINO, L. & GALINDO-RIAÑO, M. D. 2012. Biomarkers responses in muscle of Senegal sole (*Solea senegalensis*) from a heavy metals and PAHs polluted estuary. *Marine Pollution Bulletin,* 64, 2097-2108.

PACHAURI, R. & REISINGER, A. 2007. Synthesis report: Contribution of working groups I, II and III to the fourth assessment report of the intergovernmental panel on climate change.

PÖRTNER, H. O. & KNUST, R. 2007. Climate change affects marine fishes through the oxygen limitation of thermal tolerance. *Science*, 315, 95-97.

PÖRTNER, H.-O. J. M. E. P. S. 2008. Ecosystem effects of ocean acidification in times of ocean warming: a physiologist's view. *Progress Series,* 373, 203-217.

QADRI, S. A., CAMACHO, J., WANG, H., TAYLOR, J. R., GROSELL, M. & WORDEN, M. K. 2007. Temperature and acid–base balance in the American lobster *Homarus americanus*. *Journal of Experimental Biology*, 210, 1245-1254.

RATO, L. D., NOVAIS, S. C., LEMOS, M. F. L., ALVES, L. M. F. & LEANDRO, S. M. 2017. *Homarus gammarus* (Crustacea: Decapoda) larvae under an ocean acidification scenario: responses across different levels of biological organization. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 203, 29-38.

REID, D. G., ABELLÓ, P., KAISER, M. J. & WARMAN, C. G. 1997. Carapace Colour, Inter-moult Duration and the Behavioural and Physiological Ecology of the Shore Crab*Carcinus maenas*. *Estuarine, Coastal and Shelf Science*, 44, 203-211.

RODRIGUES, A. P., OLIVEIRA, P. C., GUILHERMINO, L. & GUIMARÃES, L. 2012. Effects of salinity stress on neurotransmission, energy metabolism, and anti-oxidant biomarkers of *Carcinus maenas* from two estuaries of the NW Iberian Peninsula. *Marine Biology*, 159, 2061-2074.

RODRIGUES, A. P., SANTOS, L. H. M. L. M., OLIVA-TELES, M. T., DELERUE-MATOS, C. & GUIMARÃES, L. 2014. Joint effects of salinity and the antidepressant sertraline on the estuarine decapod *Carcinus maenas*. *Aquatic Toxicology*, 156, 169-178.

RODRIGUES, A., LEHTONEN, K., GUILHERMINO, L. & GUIMARÃES, L. 2013. Exposure of *Carcinus maenas* to waterborne fluoranthene: accumulation and multibiomarker responses. *Science of the Total Environment*, 443, 454-463.

RODRIGUES, E. T. & PARDAL, M. Â. 2014. The crab *Carcinus maenas* as a suitable experimental model in ecotoxicology. *Environment International*, 70, 158-182.

RODRIGUES, E. T., MORENO, A., MENDES, T., PALMEIRA, C. & PARDAL, M. Â. 2015. Biochemical and physiological responses of *Carcinus maenas* to temperature and the fungicide azoxystrobin. *Chemosphere*, 132, 127-134.

ROGGATZ, C. C., LORCH, M., HARDEGE, J. D. & BENOIT, D. M. 2016. Ocean acidification affects marine chemical communication by changing structure and function of peptide signalling molecules. *Global Change Biology*, 22, 3914-3926.

ROSS, P. M., PARKER, L., O'CONNOR, W. A. & BAILEY, E. A. J. W. 2011. The impact of ocean acidification on reproduction, early development and settlement of marine organisms. *Water*, 3, 1005-1030.

RYAN, P. 2019. *Environmental and Low-Temperature Geochemistry*, John Wiley & Sons.

SALA, E., MAYORGA, J., BRADLEY, D., CABRAL, R. B., ATWOOD, T. B., AUBER, A., CHEUNG, W., COSTELLO, C., FERRETTI, F. & FRIEDLANDER, A. M. 2021. Protecting the global ocean for biodiversity, food and climate. *Nature*, 592, 397-402.

SALOMON, M., BUCHHOLZ, F. J. C. B., BIOCHEMISTRY, P. P. B. & BIOLOGY, M. 2000. Effects of temperature on the respiration rates and the kinetics of citrate synthase in two species of Idotea (Isopoda, Crustacea). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 125, 71-81.

SANDRINI-NETO, L. & CAMARGO, M. 2010. GAD (General ANOVA Design): an R package for ANOVA designs from the general principles. Available on CRAN.

SARAZIN, G., MICHARD, G. & PREVOT, F. 1999. A rapid and accurate spectroscopic method for alkalinity measurements in sea water samples. *Water Research*, 33, 290-294.

SCHIFFER, M., HARMS, L., LUCASSEN, M., MARK, F. C., PÖRTNER, H.-O. & STORCH, D. 2014. Temperature tolerance of different larval stages of the spider crab *Hyas araneus* exposed to elevated seawater PCO2. *Frontiers in Zoology*, 11, 87.

SEMSAR-KAZEROUNI, M., BOERRIGTER, J. G. J. & VERBERK, W. C. E. P. 2020. Changes in heat stress tolerance in a freshwater amphipod following starvation: The role of oxygen availability, metabolic rate, heat shock proteins and energy reserves. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 245, 110697.

SHELFORD, V. E. 1931. Some Concepts of Bioecology. *Ecology*, 12, 455-467.

SOUTH, J., DICK, J. T. A., MCCARD, M., BARRIOS-O'NEILL, D. & ANTON, A. 2017. Predicting predatory impact of juvenile invasive lionfish (*Pterois volitans*) on a crustacean prey using functional response analysis: effects of temperature, habitat complexity and light regimes. *Environmental Biology of Fishes*, 100, 1155-1165.

SOUTHWARD, A., HAWKINS, S. & BURROWS, M. J. J. O. T. B. 1995. Seventy years' observations of changes in distribution and abundance of zooplankton and

intertidal organisms in the western English Channel in relation to rising sea temperature. *Journal of thermal Biology,* 20(1-2), 127-155.

STOCKER, T. F., QIN, D., PLATTNER, G.-K., TIGNOR, M., ALLEN, S. K., BOSCHUNG, J., NAUELS, A., XIA, Y., BEX, V. & MIDGLEY, P. M. 2013. Climate change 2013: the physical science basis. Intergovernmental panel on climate change, working group I contribution to the IPCC fifth assessment report (AR5). *New York*.

TAYLOR, E. & WHITELEY, N. 1989. Oxygen transport and acid-base balance in the haemolymph of the lobster, *Homarus gammarus*, during aerial exposure and resubmersion. *Journal of Experimental Biology*, 144, 417-436.

TAYLOR, J. R., GILLEARD, J. M., ALLEN, M. C. & DEHEYN, D. D. 2015. Effects of CO 2-induced pH reduction on the exoskeleton structure and biophotonic properties of the shrimp *Lysmata californica. Scientific reports*, 5, 1-12.

TEPOLT, C. K. & SOMERO, G. N. 2014. Master of all trades: thermal acclimation and adaptation of cardiac function in a broadly distributed marine invasive species, the European green crab, *Carcinus maenas*. *Journal of Experimental Biology*, 217, 1129-1138.

TORRES, G. & GIMÉNEZ, L. 2020. Temperature modulates compensatory responses to food limitation at metamorphosis in a marine invertebrate. *Functional Ecology*, 34, 1564-1576.

TORRES, G., CHARMANTIER, G., WILCOCKSON, D., HARZSCH, S. & GIMÉNEZ, L. 2021. Physiological basis of interactive responses to temperature and salinity in coastal marine invertebrate: Implications for responses to warming. *Ecology and Evolution*, 11, 7042-7056.

UNDERWOOD, A. J., UNDERWOOD, A. L., UNDERWOOD, A. J. & WNDERWOOD, A. 1997. *Experiments in ecology: their logical design and interpretation using analysis of variance*, Cambridge university press.

VALVONA, C. J., FILLMORE, H. L., NUNN, P. B. & PILKINGTON, G. J. 2016. The Regulation and Function of Lactate Dehydrogenase A: Therapeutic Potential in Brain Tumor. *Brain Pathology*, 26, 3-17.

VASSAULT, A. 1983. Lactate dehydrogenase. Methods of enzymatic analysis, enzymes: oxireductases, transferase. Academic Press, New York.

VEIGA, P., RUBAL, M., ARENAS, F., INCERA, M., OLABARRIA, C. & SOUSA-PINTO, I. 2011. Does *Carcinus maenas* facilitate the invasion of *Xenostrobus securis*? *Journal of Experimental Marine Biology and Ecology*, 406, 14-20.

WHITELEY, N., EGGINTON, S., TAYLOR, E. & RAVEN, J. Acid-base regulation in crustaceans: the role of bicarbonate ions. SEMINAR SERIES-SOCIETY FOR EXPERIMENTAL BIOLOGY, 1999. 233-256.

WHITELEY, N. J. M. E. P. S. 2011. Physiological and ecological responses of crustaceans to ocean acidification. *Marine Ecology Progress Series*, 430, 257-271.

WHITELEY, N. M., SUCKLING, C. C., CIOTTI, B. J., BROWN, J., MCCARTHY, I. D., GIMENEZ, L. & HAUTON, C. 2018. Sensitivity to near-future CO2 conditions in marine crabs depends on their compensatory capacities for salinity change. *Scientific Reports*, 8, 15639.

WICKHAM, H. 2009. Elegant graphics for data analysis. *Media*, 35, 10.1007.

WU, R. S. & LAM, P. K. 1997. Glucose-6-phosphate dehydrogenase and lactate dehydrogenase in the green-lipped mussel (*Perna viridis*): possible biomarkers for hypoxia in the marine environment. *Water Research*, 31, 2797-2801.

YAO, C.-L. & SOMERO, G. N. 2014. The impact of ocean warming on marine organisms. *Chinese Science Bulletin*, 59, 468-479.

YOUNG, A. M. & ELLIOTT, J. A. 2020. Life History and Population Dynamics of Green Crabs (*Carcinus maenas*). *Fishes*, 5(1), 4.

ZUO, W., MOSES, M. E., WEST, G. B., HOU, C. & BROWN, J. H. 2012. A general model for effects of temperature on ectotherm ontogenetic growth and development. *Proceedings of the Royal Society B: Biological Sciences,* 279, 1840-1846.