Universidade de Aveiro Departamento de Biologia
2019

Daniela Bastos de Freitas

Ontogeny and growth of marine shelled molluscs under climate change scenarios

Ontogenia e crescimento de moluscos marinhos sob cenários de alteração climática

DECLARAÇÃO

Declaro que este relatório é integralmente da minha autoria, estando devidamente referenciadas as fontes e obras consultadas, bem como identificadas de modo claro as citações dessas obras. Não contém, por isso, qualquer tipo de plágio quer de textos publicados, qualquer que seja o meio dessa publicação, incluindo meios eletrónicos, quer de trabalhos académicos.

Universidade de Aveiro Departamento de Biologia
2019

Daniela Bastos de Freitas

Ontogeny and growth of marine shelled molluscs under climate change scenarious

Ontogenia e crescimento de moluscos marinhos sob cenários de alteração climática

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Marinha, realizada sob a orientação científica da Doutora Susana Galante-Oliveira, Investigadora Doutorada do Departamento de Biologia e do Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro, e coorientação da Doutora Isabel Benta Oliveira, Investigadora Doutorada do Departamento de Biologia e do CESAM da Universidade de Aveiro, e do Senhor Professor Doutor Carlos Miguez Barroso, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro.

Apoio financeiro do projeto *CliMaStat* (PTDC/AAG-REC/1600/2014) e *EMSO-PT* (PINFRA/22157/2016) financiados pelo FEDER no âmbito do PT2020 através do COMPETE2020, e por fundos nacionais (OE) através da FCT/MCTES.

Dedico este trabalho aos meus pais.

o júri

presidente	Doutor Ricardo Jorge Guerra Calado Investigador Principal, Universidade de Aveiro
arguente	Doutor Anthony Peter Moreira Investigador em Pós-Doutoramento, Universidade de Aveiro
orientador	Professor Doutor Carlos Miguel Miguez Barroso Professor Auxiliar, Universidade de Aveiro

agradecimentos

Durante esta jornada foram muitos os desafios, as alegrias, as superações e as pessoas que se cruzaram ao longo dela, a quem tenho MUITO a agradecer:

Ao Professor Carlos Miguez por me ter aberto a porta para o grupo de investigação com quem tive o prazer de partilhar muitos momentos, mas sobretudo de aprender e superar-me.

À Su por ser uma orientadora e pessoa incansável. Obrigada pela confiança, pelos desafios e por toda a aprendizagem ao longo destes últimos três anos. Graças a ti, o ClimaStat foi muito mais do que um projeto de investigação.

À Beluxa e ao Pituxa pelas gargalhadas, abraços, cantaroladas, conselhos e palavras de força nos momentos certos.

A todos os que passaram pelo LEME por me mostrarem que se pode ser muito feliz num laboratório.

À Joana, por ter sido a minha companheira nesta e em tantas outras jornadas. Este caminho foi ainda mais bonito por estarmos lado a lado.

Ao Ricardo por estar sempre presente, por ser o meu refúgio e pelo apoio constante durante todos estes anos. Obrigada pelo amor!

Agradeço especialmente à minha mãe por ser o meu pilar e força, ao meu pai por acreditar sempre em mim, à minha tia Ni por ser o meu exemplo a seguir desde sempre, ao meu irmão, à minha avó Júlia e restante Família, que mesmo longe foram um apoio constante.

Por último agradeço aos meus amigos que tornaram Aveiro uma verdadeira casa, na qual construímos todos uma família para a vida.

Que o que aí vem seja tão bonito e feliz como até aqui,

Um grande obrigada a todos!

palavras-chave

Aquecimento Global, Acidificação do Oceano, Temperatura, pH, Gastrópodes, Bivalves, *Tritia reticulata*, *Venerupis corrugata*, Carbonatos biogénicos, Concha, Estatólito, Desenvolvimento, Microscopia Eletrónica de Varrimento (SEM), Espectroscopia de micro-Raman (MRS)

resumo

Os organismos marinhos que produzem carbonatos biogénicos, como os moluscos com concha na Classe Gastropoda e Bivalvia, são especialmente propensos a serem negativamente afetados pela Acidificação (OA) e o Aquecimento (W) do Oceano. Com conchas compostas por polimorfos de carbonato de cálcio mais solúveis, os estágios iniciais da vida desses animais são potencialmente mais vulneráveis à OA-W, um problema sério já que a sobrevivência larvar é crucial para a continuidade das espécies. Nesse sentido, a previsão dos efeitos da OA-W no início da vida dos moluscos marinhos com concha é de extrema importância para a avaliação dos impactos destes fenómenos nos ecossistemas marinhos.

Esta tese descreve os efeitos combinados da OA-W na sobrevivência, desenvolvimento ontogenético, crescimento e integridade dos carbonatos biogénicos dos estágios iniciais da vida de duas espécies modelo de elevada importância ecológica e comercial, respetivamente, o gastrópode *Tritia reticulata* e o bivalve *Venerupis corrugata*. Os primeiros estágios larvares de ambas as espécies foram expostos, durante dois meses, a cenários experimentais de OA-W, com base nas últimas projeções do Painel Intergovernamental sobre Mudanças Climáticas (IPCC) para o final do século.

Os resultados revelaram uma extrema vulnerabilidade dos estágios larvares planctónicos de *T. reticul*ata à ocorrência simultânea da OA-W, com mortalidades severas, taxas de crescimento reduzidas, menor cristalinidade das conchas, maior dissolução, e até perda de concha após apenas 8 dias de exposição ao cenário mais extremo (22°C pHtarget 7.5). Estes resultados sugerem efeitos graves do aquecimento e acidificação do oceano nos estados iniciais de vida de *T. reticulata*, diminuindo consideravelmente as chances de as larvas sobreviverem na natureza, caso as projeções se tornem efetivas.

Por sua vez, o bivalve *V. corrugata* revelou uma aparente resiliência à OA-W nas condições testadas, com maior sobrevivência larvar sob aquecimento e acidificação (22°C pHtarget 7.6). Nesta espécie o desenvolvimento ontogénico foi claramente favorecido pelo aumento da temperatura mesmo sob um efeito antagónico significativo da redução do pH no crescimento. No entanto, mesmo sabendo que os cenários experimentais aplicados não foram exatamente os mesmos e que, portanto, não é possível comparar diretamente os resultados obtidos em ambas as espécies, a amêijoa-macha *V. corrugata* mostrou ser resiliente aos cenários testados. Isto faz da *V. corrugata* um recurso promissor para a indústria da aquacultura, pois parece ser uma espécie capaz de lidar com as condições de OA-W previstas até ao final deste século.

keywords

Global Warming, Ocean Acidification, Temperature, pH, Gastropods, Bivalves, *Tritia reticulata*, *Venerupis corrugata*, Biogenic Carbonates, Shell, Statolith, Development, Scanning Electron Microscopy (SEM), micro-Raman Spectroscopy (MRS)

abstract

Marine organisms that produce biogenic carbonates, such as the shelled molluscs in Class Gastropoda and Bivalvia, are especially prone to be negatively affected by Ocean acidification (OA) and Warming (W). With shells composed of more soluble calcium carbonate polymorphs, these animals' early life stages are potentially more vulnerable to OA-W, a serious problem as larval survival is crucial for the species' persistence. In this sense, the prediction of the effects of OA-W in the early life of the marine shelled molluscs is extremely important for the assessment of the impacts of these phenomena on marine ecosystems.

This thesis describes the combined effects of OA-W on the survival, ontogenic development, growth and integrity of the biogenic carbonates of the early life stages of two model species of high ecological and commercial importance, respectively, the gastropod *Tritia reticulata* and the bivalve *Venerupis corrugata*. The early larval stages of both species were exposed, for two months, to OA-W experimental scenarios, based on the latest projections of the Intergovernmental Panel on Climate Change (IPCC) for the end of the century.

The results revealed an extreme vulnerability of *T. reticulata* planktonic larval stages to the simultaneous occurrence of OA-W, with severe mortalities, reduced growth rates, lower shells' crystallinity, higher dissolution, and even shell loss after only 8 of days exposure to the most extreme scenario (22°C pHtarget 7.5). These results suggest dramatic effects of warming and ocean acidification on *T. reticulata* in early life, lessening considerably the chances of larvae to survive in the wild if projections become effective.

In turn, the bivalve *V. corrugata* revealed an apparent resilience to OA-W under the tested conditions, with increased larval survival under warming and acidification (22°C pHtarget 7.6). In this species, the ontogenic development was clearly favoured by the temperature rise even under a significant antagonistic effect of the pH reduction on growth. However, even knowing that the applied experimental scenarios were not exactly the same and thus, it is not possible to directly compare the results obtained in both species, the pullet carpet shell *V. corrugata* was shown to be resilient to the tested scenarios. This makes *V. corrugata* a very promising resource for the aquaculture industry as it appears to be a species that may be able to cope with future environmental conditions of OA-W predicted until the end of this century.

Index

List of Figures

List of Tables

Chapter 1

General Introduction

1.1. The Molluscs

It is estimated that over a million of animal species have been described to inhabit our planet and, perhaps, as many as 20-50 million more remain to be discovered. Within those described, 95% are invertebrates. This important group, whose common feature is the absence of a vertebral column (or backbone) is, by far, the largest group within the animal kingdom (Brusca and Brusca, 1990), including all animals apart from those in the Subphylum Vertebrata.

Mollusca is the second most numerous and diverse phylum of invertebrate animals (following the arthropods). Regarding size, anatomical structure, behaviour and habitat, Mollusca includes some of the best well-known invertebrates (Morton, 1967). This phylum comprises animals classified in several different classes and able to colonize environments as distinct as land, oceans and freshwater bodies. The taxon includes seven classes (Brusca and Brusca, 1990) three very familiar comprising clams and mussels (Bivalvia), snails and slugs (Gastropoda), squids and octopuses (Cephalopoda), and the other four less well-known: chitons (Polyplacophora), tusk shells (Scaphopoda), *Neopilina* and its relatives (Monoplacophora), solenogasters and caudofoveats (Aplacophora).

Molluscs have in common a series of general characteristics such as bilateral symmetry, coelomate protostomes, an open circulatory system (hemocoel), thick epidermal-cuticular sheet of skin (mantel), cavity to host ctenidia, osphradia, nephridiopores, gonopores and anus (mantel or pallial cavity), and a mantle with shell glands that secretes calcareous epidermal spicules, shell plates or shells. They have also a heart composed of distinct ventricle and atria (inside a pericardial chamber), a large and well-defined muscular foot, buccal region with a radula, complete gut, large and complex metanephridia. Their embryogenesis is typically protostomous, with trochophore larvae usually followed by a veliger larva (Morton, 1967).

Within this widely studied phylum, the most diverse is believed to be Class Gastropoda (with several different marine gastropods in which one of the subject of this study is included), while the most abundant is Class Bivalvia (especially regarding marine bivalves to which the other species included in this work belongs to).

1.1.1 The gastropod *Tritia reticulata* (Linnaeus, 1758)

Gastropods are commonly known as snails and slugs, and belong to the largest and most diverse class within Phylum Mollusca. The netted whelk, *Tritia reticulata* (Linnaeus, 1758), formerly known as *Nassarius reticulatus*, is the most common nassarid in European waters with a wide distribution in the NE Atlantic and throughout the Mediterranean, Baltic and the Black Seas (Fretter and Graham, 1994). This marine snail occurs along the open coast into the outer parts of estuaries, being ubiquitous at both rocky and sandy bottoms (Lambeck, 1984; Barroso and Moreira, 1998). Although this species is not commercially exploited in Portugal, in many other countries it is vulnerable to overfishing and there is interest to develop aquaculture practices for its production (Nasution and Roberts 2004; Carpenter and De Angelis 2016). As many other whelks, *T. reticulata* is a necrophage and has a complex olfactory system which allows a rapid detection of prey, besides being tolerant to environmental changes, such as temperature and salinity (Zupo and Patti, 2009).

This dioecious species has internal fertilization and indirect development; indirect development because its life cycle includes a planktonic phase (Figure 1.1), at the end of which larvae undergoes metamorphosis to become benthic (Quayle, 1952).

Breeding occurs between late spring/early summer and females lay flattened egg capsules containing around 100-120 lecithotrophic embryos (Lebour, 1931; Zupo and Patti, 2009). Capsules are typically laid on hard substrates and seaweeds, in one direction and, normally, in a row (Barnett *et al.*, 1980; Zupo and Patti, 2009). Inside the capsule, organisms pass through a trochophore larval stage before hatching as a veliger, roughly after one month of development (Fretter and Graham, 1994). Veliger

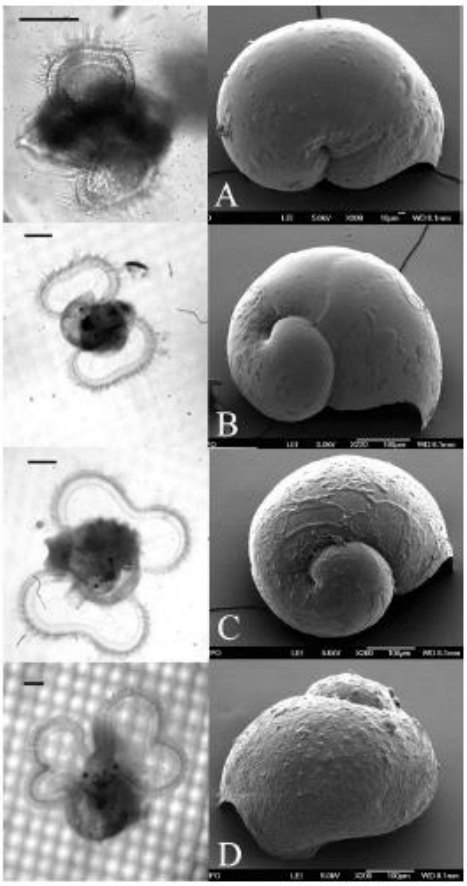

Figure 1.1 - Veliger stages of *Tritia reticulata* under light optical microscopy (on the left) and their shells under Scanning Electron Microscopy (on the right). A - Veliger 1, 2 days after hatching. B - Veliger 2, 8 days after hatching. C - Veliger 3 16 days after hatching. D - Veliger 4, 30 days after hatching, ready for settlement. Scale bars on the left: 60 µm. Extracted from Zupo and Patti (2009).

larvae hatch with a well-formed shell and bilobed velum, on average 12±3 days after capsule laying (Lebour, 1931; Zupo and Patti, 2009) and then passes for four stages, from veliger 1 to veliger 4 (Figure 1.1-A to -D, respectively).

The first hatching veliger is named Veliger 1 and displays an operculum and a circular velum, with thin and long cilia. Veliger 2 is generally observed after 6±2 days under control/laboratory conditions and is characterized by presenting the torsion of the shell and a thicker, larger velum, with shorter cilia and an elongated shape. After 14±3 days, the Veliger 3 already displays a butterfly-shaped, thickened and enlarged velum. The last development stage, which precedes crawling and settlement, is called Veliger 4. It is distinguished by a very thick and tetra-lobated velum, larger pigmented eyespots, shorter cilia and a behavioural modification: this larvae spends more time close to the bottom, retracting the velum into the shell, at a moment it is ready to settle to become a juvenile and, later, an adult (Zupo and Patti, 2009).

1.1.2 The bivalve *Venerupis corrugata* (Gmelin,1791)

Commonly known as "pullet carpet shell", *Venerupis corrugata* (Gmelin, 1791) is a marine bivalve that belongs to the Family Veneridae. This clam species is traditionally harvested for human consumption at the Southwestern and Mediterranean Europe, especially in Portugal, Spain, France and Italy (FAO, 2006). This fact increases the importance of this species that, beyond its ecological relevance as an ecosystems services provider as filter-feeder, it is also a strategic economical resource in a wide geographical area with a high market value (over 18€ per kg) when compared to most shellfish species (Tridge, 2019). *V. corrugata* is a dioecious species, without sexual dimorphism but with external fertilization and indirect development.

The species spawning season is quite long (Cerviño-Otero, 2011) for instance, in the Ria de Aveiro (NW Portugal) it extends from late Winter to the early Summer (Joaquim *et al.*, 2011). During spawning, the sperm and oocytes are emitted to the water column by the exhalant siphon. When the sperm penetrates the oocyte, a fertilization membrane are formed to block the entry of more sperm. During gametogenesis, the newly formed egg has reserves stored to ensure the embryonic development of the larvae. The first larval stage is named

Trochophore, which is characterized by a cilia crown and an apical plume (Cerviño-Otero, 2011).

After 1-2 days post fertilization, a planktonic D-larva with total developed velum appears. The major characteristic of D-larvae is the straight hinge, which gives the D-shape to the shell (Figure 1.2-A). It is at this stage that the larvae starts to feed independently. About 9 days after, the hinge starts to curve forming the umbo. This characteristic is what defines the Umbonate stage (Figure 1.2-B). After 16-18 days, the Pediveliger stage arises when the foot begins to develop while the velum still persists (Figure 1.2-C). At this stage, the larvae shorten the swimming time in the water column and approaches the bottom. During metamorphosis the velum retracts completely, and the gills are formed to ensure the food intake that previously are performed by the velum. About 22 days later, the last stage is reached (Figure 1.2-D): the Postlarva adopts the adult shape and the benthic life starts (Cerviño-Otero, 2011).

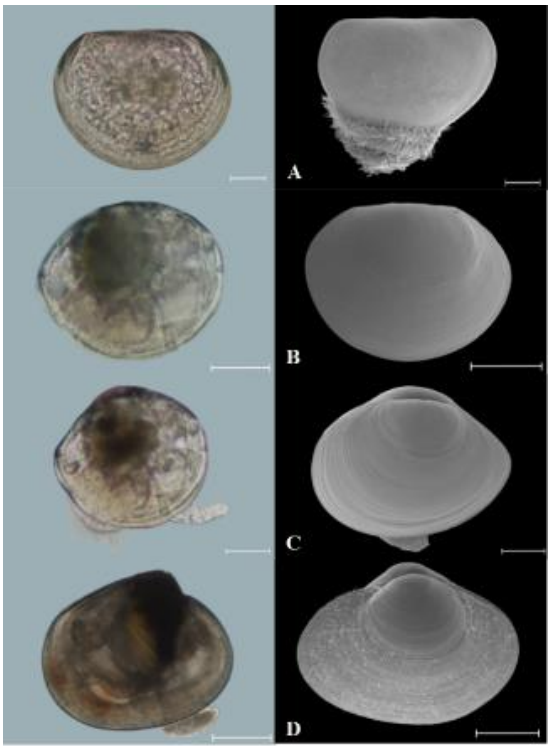

Figure 1.2 - Larval stages of *Venerupis corrugata* under light optical microscopy (on the left) and their shells under Scanning Electron Microscopy (on the right). A – D-larva, 48 hours after fertilization (scale bar: 20 µm). B – Umbonate, 8-9 days after fertilization (scale bar: 50 µm). C – Pediveliger with 16-18 days (scale bar: 50 µm). D – Postlarva, 30 days after fertilization (scale bar: 100 µm). Adapted from Cerviño-Otero (2011).

1.2 The Mollusc Shell

Not all current molluscs bear a shell but most of them, included in Subphylum Conchifera (MolluscaBase, 2019), secrete biogenic carbonate to build that supportive and protective structure. Animals in Class Gastropoda and Bivalvia are among them, and thus the above-mentioned species that share that common characteristic.

The molluscan shell is the primary defence against adverse environmental conditions playing an essential role in feeding, buoyancy control, pH regulation and defence against predation (Comeau *et al.*, 2010; Bednaršek and Ohman, 2015). The most immediate and

obvious difference between both shells is that gastropods, such as *T. reticulata*, have their visceral mass covered by a single piece, usually coiled, shell while bivalves, such as *V. corrugata*, have a shell constituted by two distinct valves. Nevertheless, the composition of gastropod and bivalve shells is identical as it is mostly composed of calcium carbonate ($CaCO_3$), but also of a (comparatively) small amount of organic matrix and other chemical, trace elements (Lowenstam and Weiner, 1989). $CaCO_3$ can be amorphous or occur in three crystalline polymorphs: calcite, aragonite and vaterite. Calcite is the most stable under ambient conditions, aragonite is metastable and vaterite in the most unstable, and rarely found. In fact, mollusc shells consist of aragonite and (or) calcite; the presence of vaterite has been reported on damaged shell areas that underwent regeneration (Wilbur and Watabe, 1963).

1.2.1 Shell's formation

Shell's formation starts on the early trochophore stage, at which the shell gland is formed (Nielsen, 2004). This gland produces an extracellular lamella that will give rise to the periostracum, whose function is to provide the organic support where the minerals will be deposited to build the first shell (Nielsen, 2004). The molluscan shell is highly ordered in a structure that typically presents three layers: (1) the outer layer, called periostracum constituted by organic matter (*P* in Figure 1.3); (2) a prismatic or columnar crystalline layer, added along the edge of the mantle (*PL* in Figure 1.3); and (3) an internal layer, sometimes nacreous, formed from tabular crystals arranged in laminae or columns (*NL* in Figure 1.3), produced by the inner surface of the mantle (Morton, 1967; Wilbur and Saleuddin, 1983; Carter and Clark, 1985).

The entire shell-formation system (Figure 1.3) includes four areas: (1) the external medium; (2) the haemolymph and mantle; (3) the extrapallial fluid, located between the mantel and the inward shell surface; and (4) the shell itself (Wilbur and Saleuddin, 1983; Marin *et al.*, 2012; Suzuki and Nagasawa, 2013). The mantle is the organ that covers the inner shell surface and is directly linked to the formation of the above-mentioned layers, participating in various processes from crystal deposition to the secretion of the shell's organic matrix. This organ is covered by a layer of epithelial cells, the outer and the inner mantle epithelium; the inner is in contact with the external medium (i.e. seawater), which

supplies the haemolymph, pallial muscles, connective tissue and nerve fibres. The outer mantle epithelium, or outer calcifying epithelium, is the one that faces the shell and that secrets all the components needed to its synthesis.

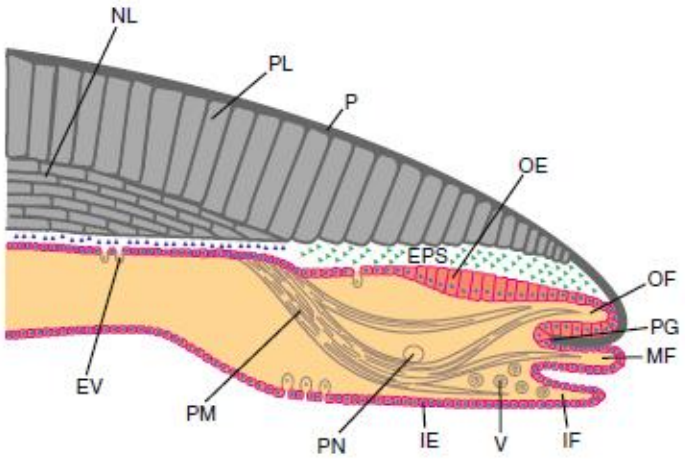

Figure 1.3 - Representation of a transversal section of the shell and mantle of a bivalve extracted from Kocot *et al.* (2016). Green and blue triangles represent molecules secreted by the organism mantle. EPS: extrapallial space, EV: exosome, IE: inner epithelium, IF: inner fold, MF: middle fold, NL: nacreous layer, OE: outer epithelium, OF: outer fold, P: periostracum, PG: periostracal groove, PL: prismatic layer, PM: pallial muscle, PN: pallial nerve, V: vesicles.

To be able to calcify, molluscs need to be supplied by calcium and carbonate ions. The calcium ions (Ca^{2+}) are obtained from the seawater and (or) food, which are absorbed in the inner mantle epithelium, gills and digestive system. Carbonate ions (CO_3^{2-}) are supplied from bicarbonate (HCO^{3-}) available in the mantle or general body tissues and from metabolism (Simkiss and Wilbur, 1989). Figure 1.4 corresponds to a schematic representation of the transference of several ions through different body compartments.

It is on the extrapallial fluid, between the mantle and the inner shell, that shell formation takes place. In fact, it is there that organic and inorganic substances supplied by the mantle are deposited to build the shell. Ion fluxes generally occur inward, towards shell calcification, but the opposite can also happen in case of $CaCO_3$ solubilization (Wilbur and Saleuddin, 1983). Although the calcification process is similar on the vast majority of the

molluscs, the most relevant differences between gastropods and bivalves, are observed on shell microstructure and on the development stages, as explained in the following sections.

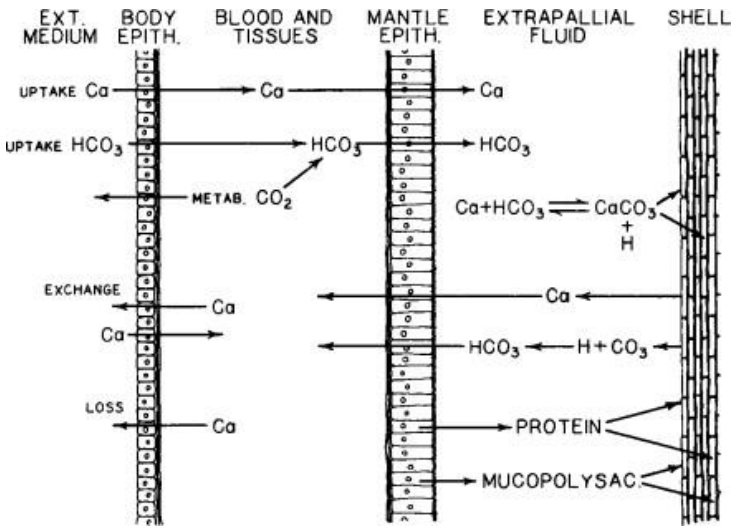

Figure 1.4 – Schematic representation of the mineralizing system of a mollusc. Extracted from Wilbur and Saleuddin (1983).

1.2.2 Shell's microstructure

Together with its composition, shell microstructure creates the observed layer arrangement of the molluscan shell. Thus, molluscan shells contain, at least, two different microstructural types, typically arranged in (1) a prismatic layer and (2) a laminar and more internal layer also called as nacreous layer (while the periostracum or outer layer is mainly constituted by organic matter). According to Carter and Clark (1985), the crystals can be orientated and organized in: prismatic, spherulitic, laminar, crossed, helical, homogenous, isolated spicules and isolated crystal morphotypes. Inside these categories, the shell microstructure may have other types and sub-types, which demonstrates the high complexity of its classification. Nevertheless, it is on the laminar, nacreous layer (or mother-of-pearl layer) that most of studies are focused on (Marin *et al.*, 2012). As an example, the nacreous layer deposition, in bivalves, is made in two dimensions, while in gastropods the crystals "grow" three-dimensionally.

In bivalves, the nacreous layer can be deposited in a so called 'sheet nacre', were the crystals are stacking like stair steps in all vertical sections (Figure 1.5-A). On the other hand,

it may also appear in a ‘row-stack nacre’ form, in which the tablets and stair steps are oriented in the same direction (perpendicular to the length axes), (Figure 1.5-B), (Wilbur and Saleuddin, 1983; Carter and Clark, 1985; Marin *et al.*, 2012). In gastropods, this layer may appear as a ‘columnar nacre’ form, in which flattened tablets deposit above the subjacent tablets and the crystals’ diameter decrease along the pile’s formation conferring a conical form as showed in Figure 1.5-C. It is also shown that the tablets are not completely aligned, derived to a small lateral shift (Wilbur and Saleuddin, 1983; Marin *et al.*, 2012).

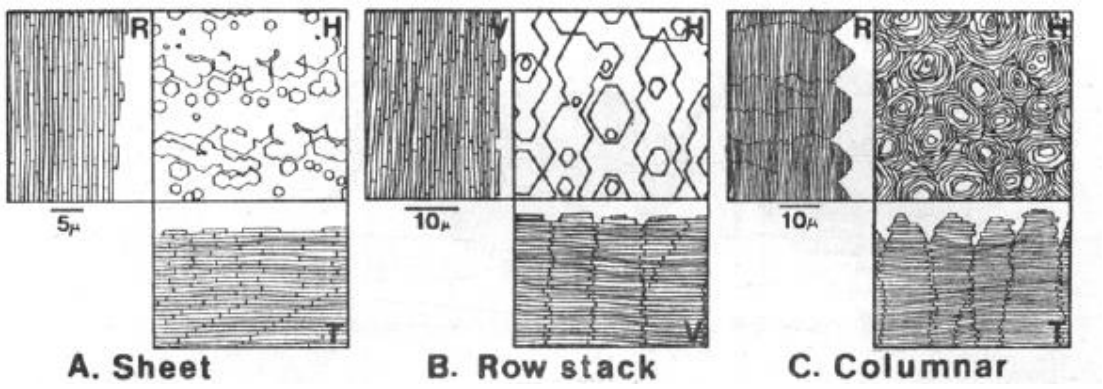

Figure 1.5 – Schematic representation of three types of nacreous layers, extracted from Carter and Clark (1985). A - Sheet nacre, B - Row stack, C - Columnar nacre.

1.2.3 Shell’s ontogeny

As referred above, the first shell is formed in early life, in both gastropods and bivalves (Nielsen, 2004). In gastropods, the early ontogenetic shell is called protoconch I and is built in the late trochophore stage. The protoconch II is deposited during the veliger stage, followed by the teleoconch, which corresponds to the post-metamorphosis shell. This latter shell is characterized by an ornamental change at the terminal edge of the larval shell (Nielsen, 2004; Nützel, 2014). Among bivalves, similar shell stages are found, with a slightly different terminology: the prodissoconch I correspond to the first shell developed during trochophore stage, followed by the prodissoconch II. The latter is formed during the veliger stage and is characterized by concentric growth lines representing a change in the calcifying regime (Marin *et al.*, 2012). After metamorphosis, early juveniles produce the dissoconch, marking that transition with a sharp ridge on the shell surface (Marin *et al.*, 2012).

This subchapter intended to enlighten the complexity and the importance of the molluscan shell. Beyond critical functions, such as support and protection, shells have other extraordinary particularities, among which the possibility to understand the life story of the individual is, because during its formation, shells incorporate information on the physical and chemical characteristics of the environment in which they are formed (see review by Richardson, 2001). Hence, the chemical and microstructural analysis of shells allow us to infer on changes occurring in the environment in which these structures are built. Such particularities also prove the dependency that shells' calcification and mineralization have on the external environment, which is constantly changing and may result in relevant impacts on calcifying organisms such as the gastropods and bivalves.

1.3 Global Climate Change

Throughout the Earth's history, and considering the geological record, the climate has been changing significantly (IPCC, 2007b). These long-term changes, which include variations in temperature and weather patterns, typically at a given place and in a given period of time, are called Climate Change (IPCC, 2013b). When occurring under natural influences (e.g. solar and/or volcanic activity), climate change is a slow process that takes place over hundreds or even thousands of years. However, anthropogenic influence has been accelerating the rate at which such changes are occurring (IPCC, 2007c). This situation has been mostly attributed to the excessive release of Greenhouse Gases (GHGs) into the atmosphere by emissions arising from human activities, altering atmosphere composition (US - EPA, 2013).

The GHGs are constituents of the Earth's atmosphere and can be of natural and/or anthropogenic origin. They comprise natural gases such as Water vapor (H_2O), Carbon dioxide (CO_2), Methane (CH_4), Nitrous oxide (N_2O), Ozone (O_3), but also some synthetic chemicals (e.g., Chlorofluorocarbons – CFCs), and they all enter the atmosphere from many sources: transport and burning of both fossil fuels and other biological materials and wastes, industrial chemical reactions (e.g., metals and chemicals production), agricultural and other land-use practices (US - EPA, 2013). These gases absorb and re-radiate the heat emitted by the Earth's surface, trapping it and originating the Greenhouse Effect (IPCC, 2013b). Even though this effect is determinant for the Life in this planet, keeping the Earth's temperature

about 33ºC above that it would be if no GHGs were present in the atmosphere (AG-DEE, 2019), the heat-trapping gases are currently looked at as deleterious since some of them have been proved to be responsible for the unprecedent rate at which climate change is occurring (Arndt *et al.*, 2019).

A significant increase of the GHGs emissions to the atmosphere has been registered since the Industrial Revolution in the 18th and 19th centuries (White, 2009), namely of CO_2 that is known to be the primary GHG emitted from anthropogenic activities (US - EPA, 2013). Since that period, as CO_2 increased in the atmosphere, the planet's heat content and temperature have also become higher (Harvey, 2014), evidencing the direct relationship between CO_2 concentration in the atmosphere and the Global Warming (IPCC 2007a).

Most of the Earth's heat has, however, been absorbed by the ocean also causing its temperature to rise (Hansen *et al.*, 2011), particularly at the sea surface (Carter *et al.*, 2017). Ocean Warming has critical impacts, not only in the marine ecosystems but at the planetary scale. The phenomenon has been associated with the ice sheets shrinkage, glacial retreat and sea level rise (Fath and Fath, 2014). Moreover, there are evidences that climate change and ocean warming promote more frequent extreme events such as heat/cold waves, storms, hurricanes, droughts, intense rainfalls, but also changes in the oceans' circulation, in the vertical distribution of nutrients (IPCC, 2007b) and Ocean Acidification (Royal Society, 2005).

1.4 Ocean Acidification (OA) and Warming (W)

The rise of atmospheric CO_2 (ρCO_2) from anthropogenic emissions is known to be a critical problem linked with both Ocean Acidification (OA) and Warming (W), endangering marine life (Rhein *et al.*, 2013).

As mentioned above, ocean warming is induced by the absorption of the excess of heat at the interface atmosphere-hydrosphere, resulting in the increase of energy transferred into the water column, increasing its temperature and making the oceans' warmer (Garciá Molinos *et al.*, 2016). Moreover, the oceans make up more than 70% of the surface of planet Earth and play a key role on controlling GHGs concentrations due to the capacity of sequestering most of the atmospheric excess of CO_2 (Carter *et al.*, 2017). In fact, about half of the CO_2 emissions produced since pre-industrial times have been absorbed by the oceans

(Royal Society, 2005). This capacity is related with the carbon flow between and within marine trophic levels, that allow CO_2 transformation especially derived by phytoplankton but also the transference of carbon from the surface layer to the deep ocean (Royal Society, 2005). Therefore, the ocean is a sink not only of the excess of heat but also of the excess of CO_2, helping to reduce its atmospheric concentration and, somewhat, ameliorating the climate impacts (Freedman, 2014). Still, the excess of CO_2 in seawater has created a critical indirect effect of climate change on the oceans that is OA, a phenomenon characterized by the reduction of the pH caused by the dissolution of CO_2 in seawater.

In brief, CO_2 reacts in seawater, hydrating slowly to yield carbonic acid (H_2CO_3) that ionises to form bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions, releasing protons (H^+) and decreasing pH (since $pH=-\log[H^+]$), (Dickson, 2011). Moreover, H^+ is quite unstable and has the tendency to bind CO_3^{2-}, decreasing its availability to react with calcium (Ca^+) and form calcium carbonate ($CaCO_3$), essential to marine calcifiers such as the shelled molluscs (Haugan and Drange, 1996) as described above in this General introduction.

The assessment of the evolution of OA-W throughout time is very important in order to foresee its harmful impacts and future risks, and help the development of measures to protect the Earth and its Life. The Intergovernmental Panel on Climate Change (IPCC) is the body of the United Nations responsible for the assessment of the science related to climate change (IPCC, 2019). It is also responsible for the projections on OA-W occurrence, a role that is as needed as it is difficult, given the huge variability of forcing agents involved in climate change and the uncertainty linked to the path those forcing agents will follow. In this context, the IPCC had developed Representative Concentration Pathways (RCPs) that correspond to scenarios under which climate modeling has been performed to project future climate. Those scenarios are derived from the information available on the multiple driving forces, and correspond to paths that the concentrations of GHGs will potentially follow (and not just simply to their emissions), describing different climatic projections in the near and the long-term (Collins *et al.*, 2013; Kirtman *et al.*, 2013). The most used RCPs are four – RPC2.6, RCP4.5, RCP6.0 and RCP8.5– and their labels correspond to the respective total radiative forcing in year 2100 relative to 1750 (i.e., 2.6, 4.5, 6.0 and 8.5 Wm^{-2}, respectively; Moss *et al.* 2010; IPCC 2013c). Hence, the worst case scenario considered is RCP8.5, in which no additional efforts to constrain GHGs emissions are considered, being the closest scenario to the so called business-as-usual (Riahi *et al.*, 2011; Cubasch *et al.*, 2013).

Based on model simulations forced by the RCPs, the IPCC had projected future conditions of OA-W, considering the near- and the long-term (i.e., the middle and the end of the 21st century; Kirtman *et al.*, 2013 and Collins *et al.* 2013, respectively). Briefly, considering the RCP 8.5, the ocean surface temperature is expected to rise at a rate >0.1 ºC per decade, yielding increases from about 1 to above 3 ºC (Collins *et al.*, 2013; IPCC, 2013a; Kirtman *et al.*, 2013), and the sea surface pH, that had decreased 0.0024 units yr^{-1} since the Industrial Revolution, might drop -0.3 to -0.5 pH units more in the northern hemisphere (Ciais *et al.*, 2013).

The available projections are what currently guarantee the forecast of the conditions of OA-W we will have to face in the future. However, models have always associated a certain degree of uncertainty and it is also known that conditions above temperature and pH global averages have already been registered in some places (Ciais *et al.*, 2013; IPCC, 2014; Allen *et al.*, 2018). Moreover, the current trends are likely to continue, or even accelerate, in the upcoming years, if no effective measures to (1) constrain GHG emissions and (2) mitigate human-induced climate change are applied (Allen *et al.*, 2018). In that case, the harmful effects for the marine life are as unpredictable as potentially negative.

1.4.1 Impacts of OA-W on shelled molluscs

The scientific community has been showing some significant impacts of OA-W on marine shelled molluscs (Byrne, 2011; Kroeker *et al.*, 2013) and their habitats (Queirós *et al.*, 2015). These phenomena occur simultaneously and involve two environmental stressors –pH and temperature– that are known to limit marine invertebrates' distribution, morphology and physiological performance (Ross *et al.*, 2011; Doney *et al.*, 2012; Leung *et al.*, 2017). Most of the studies address these stressors separately (e.g., Zippay and Helmuth, 2012; Wessel *et al.*, 2018). Although, OA-W are concomitant phenomena and, thus, it is critical to investigate their interactive effects as they could induce additive, antagonistic or synergistic effects, better reflecting the reality of their simultaneous occurrence in the oceans. Even though some studies have been addressing the interaction between these stressors (Byrne, 2011; Kroeker *et al.*, 2013; Navarro *et al.*, 2016; Leung *et al.*, 2017) most of the available literature concerns the effects of each stressor separately.

The isolated effect of the pH decrease on marine calcifying organisms has been widely studied (Manríquez *et al.*, 2014; Foo *et al.*, 2018; Wessel *et al.*, 2018; León *et al.*, 2019). In fact, OA is a determinant factor on the secretion of biogenic $CaCO_3$, as it decreases the availability of CO_3^{2-}, and it also induces dissolution by promoting carbonate undersaturation. Although, there is a certain controversy on how carbonate saturation states may influence calcification (Cyronak *et al.*, 2016; Waldbusser *et al.*, 2016), it is generally agreed that a higher energy supply is needed in order to manipulate the physical chemistry at the calcification site. Thus, it will most certainly generate different responses among different organisms. Some authors have revealed negative impacts on growth, calcification, reproduction, fertilization, cleavage and settlement on early developmental stages of several marine calcifiers (e.g., Kurihara, 2008; Milano *et al.*, 2016; Foo *et al.*, 2018). Generally, bivalves have been pointed as being negatively affected by OA, since the pH decrease under elevated concentration of CO_2 has been often proved to decease calcification rates (e.g., Gazeau *et al.*, 2007). However, results are sometimes contradictory and, for instance, while some authors proclaim reduced calcification under acidity, others report resistance to low pH, and this for the same species (e.g., in the bivalve *Crassostrea gigas*; see Gazeau *et al.*, 2007 vs Ko *et al.*, 2013). In gastropods, reports seem to be more consensual, which might be due to their lower economic importance (when compared to the bivalves) or simply because responses are, indeed, in that same direction. For instance, *Nucella lapillus* (Linnaeus, 1758) and *Tritia nitida* (Jeffreys, 1867) calcified structures are affected by OA, revealing lower shell density, corrosion (loss of the characteristic whorls), cracks and some perforations (Keklikoglou *et al.*, 2015). Shell damage by dissolution is widely recognized and there is even a scale for its classification: from Type I - partial dissolution of the prismatic layer to Type III - sever dissolution of the deepest carbonate layer (Bednaršek *et al.*, 2012). Developed using the pteropod *Limacina helicina* (Phipps, 1774) as model, the study that describes that scale also concludes that the most severe shell damage was found on specimens held under carbonate undersaturation (Bednaršek *et al.*, 2012), the same that was also reported in the same species in its natural environment (in the top 100m of the water column with higher percentage of undersaturated water; Kurihara, 2008). More recently, Wessel *et al*. (2018) have exposed *Haliotis tuberculata* (Linnaeus, 1758) larvae to future OA scenarios and not only the survival of abalone early life stages was significantly lower, but

delayed growth and development, and a higher proportion of malformed or unshelled larvae, were registered under acidy.

Some studies on the effects of warming solely are also available in the literature (e.g., Sokolova, 2013; Peng *et al.*, 2016). Temperature is thought to play an important role on the aerobic metabolism of poikilothermic organisms as these are known to be temperature-dependent. Typically, metabolism increases with the rising temperature to cope with the demand for high energy to maintain cellular functions, until it can no longer do so (Sokolova, 2013). When this state is reached, the imbalance on the energy budget may eventually lead to decreased development, growth, and ultimately survival (Sokolova *et al.*, 2012; Sokolova, 2013). Temperature increase is also associated with increased ocean stratification (O_2 is less soluble in warm water), which restricts nutrient supply to photosynthetic organisms in surface water, that in the marine ecosystem are the basis of the trophic chair (Tyson and Pearson, 1991; Keeling *et al.*, 2010).

Due to the marked effects of both environmental stressors involved in OA-W processes, and because marine organisms are being subject to these phenomena simultaneously rather than independently, it is of utmost importance to study their combined effects. The study of OA-W in marine calcifiers are emerging on the scientific literature (e.g., Byrne, 2011; Lischka *et al.*, 2011; Byrne and Przeslawski, 2013; Davis *et al.*, 2013; Zhang *et al.*, 2014; Manríquez *et al.*, 2016). In the green sea urchin, *Lytechinus variegatus* (Lamarck, 1816), the exposure to OA-W scenarios decreases larval fertilization rates but accelerates development, with smaller and more asymmetric individuals being reported. In this case, OA did not affect fertilization, but induced delayed larval development, diminished growth and increased asymmetry; while W decreased fertilization success and accelerated larval development, but had no effect on growth. These results show that the combination of OA-W had addictive effects (Lenz *et al.*, 2019). Furthermore, Lischka *et al.* (2011) studied the impact of OA-W on early juveniles of the pteropod *L. helicina*. Results revealed that temperature had an overriding effect on mortality (and the pH had also some influence on that effect). However, lower shell diameter, lower increment and higher degradation were only promoted by the decreased pH. Another example is the exposure of *Argopecten purpuratus* (Lamarck, 1819) juveniles to OA-W conditions by Lagos *et al.* (2016): at ambient temperature and low pH, scallops showed higher shell dissolution and low growth rates, the same happened on warmer and acidic conditions, although in a lower rate,

revealing that elevated temperature reduces the negative effect caused by acidification. These and many other studies constitute an effort of the scientific community to predict the effects of the expectedly warmer and more acidic future oceans. Typically, the effects disclosed are addictive or synergistic (Pörtner, 2008; Queirós *et al.*, 2015; Leung *et al.*, 2017; Rühl *et al.*, 2017). However, as shown above, different responses are also apparent. Hence, there is a pressing need to investigate the combined effects of these phenomena on more species, particularly those that are admittedly more vulnerable, such as those of shelled molluscs in Class Gastropoda and Bivalvia, and specifically in early life.

1.5 Thesis Rationale and Objective

In the light of the international agenda, climate change and oceans' sustainability have been top priorities. Ocean Acidification (OA) and Warming (W) are ongoing processes, occurring at unprecedented rates and transforming marine ecosystems. Thus, there is a pressing need to understand their combined effects on marine Life, particularly in the most susceptible forms, at realistic/expected levels. The early life stages of marine shelled molluscs are known to be especially prone to OA, reason why are here studied.

These reasons motivated this work, aimed at determining the impacts of OA-W projected scenarios on the performance of the early life stages of gastropods and bivalves using, respectively, *Tritia reticulata* (Linnaeus, 1758) and *Venerupis corrugata* (Gmelin, 1791) as model species.

The dissertation is organized in four chapters, starting with a General introduction (Chapter 1) followed by two original scientific works assessing the concomitant effects of OA-W on the ontogenic development, growth and survival of the early life stages of *T. reticulata* (Chapter 2) and *V. corrugata* (Chapter 3) and, finally, presenting the Main conclusions (Chapter 4).

Chapter 1 introduced the subject of this study, namely by performing (1) a brief description of the model species with special focus on the early larval development and their shell formation and characterization, (2) showing the state of the art regarding OA-W and (3) its known impacts on marine shelled molluscs.

Chapter 2 is focused on the concomitant effects of OA-W on the development, growth, shell integrity and composition, and on *T. reticulata* survival at 12 experimental scenarios for the first 2 months of life. Part of this chapter has been recently submitted for publication on the international journal *Global Change Biology* and yield three presentations in both national and international scientific meetings.

Chapter 3 is aimed at assessing the effects of OA-W on the development, growth, shell malformation and *V. corrugata* survival at 9 experimental scenarios for the first 2 months of life. Part of this chapter also yield two presentations in international scientific meetings.

Chapter 4 summarizes the main conclusions obtained in Chapter 2 and Chapter 3, stating the general effects of OA-W on the performance on early life stages of the marine shelled molluscs' studied.

References

AG-DEE (2019) *Greenhouse effect, Australian Government - Department of the Environment and Energy*. Available at: https://www.environment.gov.au/climate-change/climate-science-data/climate-science/greenhouse-effect (Accessed: 5 December 2019).

Allen, M. R., Dube, O. P., Solecki, W., Aragón-Durand, F., Cramer, W., Humphreys, S., Kainuma, M., Kala, J., Mahowald, N., Mulugetta, Y., Perez, R., Wairiu, M. and Zickfeld, K. (2018) 'Framing and Context', in *Global Warming of 1.5°C. An IPCC Special Report on the impacts of global warming of 1.5°C above pre-industrial levels and related global greenhouse gas emission pathways, in the context of strengthening the global response to the threat of climate change, sustainable development, and efforts to eradicate poverty* [Masson-Delmotte, V., P. Zhai, H.-O. Pörtner, D. Roberts, J. Skea, P.R. Shukla, A. Pirani, W. Moufouma-Okia, C. Péan, R. Pidcock, S. Connors, J.B.R. Matthews, Y. Chen, X. Zhou, M.I. Gomis, E. Lonnoy, T. Maycock, M. Tignor, and T. Waterfield (eds.)]. In Press, pp. 49-91.

Arndt, D. S., Blunden, J. and Dunn, R. J. H. (2019) 'Introduction', in *State of the climate in 2018*. Bulletin of the American Meteorological Society, 100(9), pp. 1–2.

Barnett, P. R. O., Hardy, B. L. S. and Watson, J. (1980) 'Substratum selection and egg-capsule deposition in *Nassarius reticulatus* (L.)', *Journal of Experimental Marine Biology and Ecology*, 45(1), pp. 95–103. doi: 10.1016/0022-0981(80)90072-6.

Barroso, C. M. and Moreira, M. H. (1998) 'Reproductive Cycle of *Nassarius reticulatus* in the Ria De Aveiro, Portugal: Implications for Imposex Studies', *Journal of the Marine Biological Association of the United Kingdom*, 78(4), pp. 1233–1246. doi: 10.1017/s0025315400044453.

Bednaršek, N., Tarling, G. A., Bakker, D. C. E., Fielding, S., Cohen, A., Kuzirian, A., Mccorkle, D., Lézé, B. and Montagna, R. (2012) 'Description and quantification of pteropod shell dissolution: A sensitive bioindicator of ocean acidification', *Global Change Biology*, 18(7), pp. 2378–2388. doi: 10.1111/j.1365-2486.2012.02668.x.

Bednaršek, N. and Ohman, M. D. (2015) 'Changes in pteropod distributions and shell dissolution across a frontal system in the California Current System', *Marine Ecology Progress Series*. Inter-Research, 523, pp. 93–103. doi: 10.3354/meps11199.

Brusca, R. C. and Brusca, G. J. (1990) *Invertebrates*. Sunderland, Massachusetts: Sinauer Associates, pp. 922

Byrne, M. (2011) 'Impact of ocean warming and ocean acidification on marine invertebrate life history stages: vulnerabilities and potential for persistence in a changing ocean', *Oceanography and Marine Biology: An Annual Review*, 49, pp. 1–42.

Byrne, M. and Przeslawski, R. (2013) 'Multistressor impacts of warming and acidification of the ocean on marine invertebrates' life histories', *Integrative and Comparative Biology*, 53(4), pp. 582–596. doi:

10.1093/icb/ict049.

Carpenter, K.E. and De Angelis, N. (2016) 'The living marine resources of the Eastern Central Atlantic. Volume 2: Bivalves, gastropods, hagfishes, sharks, batoid fishes, and chimaeras', in *FAO Species Identification Guide for Fishery Purposes*. Rome, Italy: FAO, pp. 665–1509.

Carter, B. R., Feely, R. A., Mecking, S., Cross, J. N., Macdonald, A. M., Siedlecki, S. A., Talley, L. D., Sabine, C. L., Millero, F. J., Swift, J. H., Dickson, A. G. and Rodgers, K. B. (2017) 'Two decades of Pacific anthropogenic carbon storage and ocean acidification along Global Ocean Ship-based Hydrographic Investigations Program sections P16 and P02', *Global Biogeochemical Cycles*, 31(2), pp. 306–327. doi: 10.1002/2016GB005485.

Carter, J. G. and Clark, G. R. (1985) 'Classification and Phylogenetic Significance of Molluscan Shell Microstructure', *Studies in Geology, Notes for a Short Course*, 13, pp. 50–71. doi: 10.1017/s0271164800001093.

Cerviño-Otero, A. (2011) *Ciclo reproductivo, cultivo en criadero y en el medio natural de la almeja babosa Venerupis pullastra (Montagu, 1803)*. PhD thesis. Santiago de Compostela, Galiza, Spain. Instituto de Acuicultura, Universidade de Santiago de Compostela.

Ciais, P., Sabine, C., Bala, G., Bopp, L., Brovkin, V., Canadell, J., Chhabra, A., DeFries, R., Galloway, J., Heimann, M., Jones, C., Quéré, C. Le, Myneni, R. B., Piao, S. and Thornton, P. (2013) 'Carbon and Other Biogeochemical Cycles', in *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)]. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press, pp. 465–570.

Collins, M., Knutti, R., Arblaster, J., Dufresne, J., Fichefet, T., Friedlingstein, P., Gao, X., Gutowski, W., Johns, T., Krinner, G., Shongwe, M., Tebaldi, C., Weaver, A. and Wehner, M. (2013) 'Long-term Climate Change: Projections, Commitments and Irreversibility', *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)]. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press, pp. 1029-1136.

Comeau, S., Gorsky, G., Alliouane, S. and Gattuso, J. P. (2010) 'Larvae of the pteropod Cavolinia inflexa exposed to aragonite undersaturation are viable but shell-less', *Marine Biology*, 157(10), pp. 2341–2345. doi: 10.1007/s00227-010-1493-6.

Cubasch, U., Wuebbles, D., Chen, D., Facchini, M. C., Frame, D., Mahowald, N. and Winther, J.-G. (2013) 'Introdution', in *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* [Stocker, T.F.,

D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)]. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press, pp. 119-158.

Cyronak, T., Schulz, K. G. and Jokiel, P. L. (2016) 'The Omega myth: what really drives lower calcification rates in an acidifying ocean', *ICES Journal of Marine Science: Journal du Conseil*, 73(3), pp. 558–562. doi: 10.1093/icesjms/fsv075.

Davis, A. R., Coleman, D., Broad, A., Byrne, M., Dworjanyn, S. A. and Przeslawski, R. (2013) 'Complex Responses of Intertidal Molluscan Embryos to a Warming and Acidifying Ocean in the Presence of UV Radiation', *PLoS ONE*, 8(2), p. e55939. doi: 10.1371/journal.pone.0055939.

Dickson, A. G. (2011) 'The carbon dioxide system in seawater: equilibrium chemistry and measurements', in *Guide to best practices for ocean acidification research and data reporting* [U. Riebesell, V. J. Fabry, L. Hansson and J.-P. Gattuso (eds.)]. Luxembourg: Publications Office of the European Union, pp. 17-40.

Doney, S. C., Ruckelshaus, M., Emmett Duffy, J., Barry, J. P., Chan, F., English, C. A., Galindo, H. M., Grebmeier, J. M., Hollowed, A. B., Knowlton, N., Polovina, J., Rabalais, N. N., Sydeman, W. J. and Talley, L. D. (2012) 'Climate Change Impacts on Marine Ecosystems', *Annual Review of Marine Science*, 4(1), pp. 11–37. doi: 10.1146/annurev-marine-041911-111611.

FAO (2006) *Cultured Aquatic Species Information Programme - Venerupis pullastra.* Texted by Figueras, A., in: *FAO Fisheries and Aquaculture Department* [online]. Rome. Updated 26 February 2006. Available at: http://www.fao.org/fishery/culturedspecies/Venerupis_pullastra/en (Accessed: 27 October 2019).

Fath, N. and Fath, B. D. (2014) 'Global climate change, introduction', in *Global Environmental Change* [Freedman, B. (eds.)]. New York, London, UK: Springer Dordrecht Heidelberg, pp. 3–7.

Foo, S. A., Deaker, D. and Byrne, M. (2018) 'Cherchez la femme - impact of ocean acidification on the egg jelly coat and attractants for sperm.', *The Journal of experimental biology*, 221(Pt 13). doi: 10.1242/jeb.177188.

Freedman, B. (2014) *Global Environmental Change*. New York, London, UK: Springer Dordrecht Heidelberg, pp. 940

Fretter, V. and Graham, A. (1994) *British prosobranch molluscs : their functional anatomy and ecology*. London, UK: British Ray Society, pp. 820

Garciá Molinos, J., Halpern, B. S., Schoeman, D. S., Brown, C. J., Kiessling, W., Moore, P. J., Pandolfi, J. M., Poloczanska, E. S., Richardson, A. J. and Burrows, M. T. (2016) 'Climate velocity and the future global redistribution of marine biodiversity', *Nature Climate Change*, 6(1), pp. 83–88. doi: 10.1038/nclimate2769.

Gazeau, F., Quiblier, C., Jansen, J. M., Gattuso, J.-P., Middelburg, J. J. and Heip, C. H. R. (2007) 'Impact of elevated CO_2 on shellfish calcification', *Geophysical Research Letters*, 34(7), p. L07603. doi: 10.1029/2006GL028554.

Hansen, J., Sato, M., Kharecha, P. and von Schuckmann, K. (2011) 'Earth's Energy Imbalance and Implications', *Atmospheric Chemistry and Physics Discussions*, 11(9), pp. 27031–27105. doi: 10.5194/acpd-11-27031-2011.

Harvey, L. D. D. (2014) 'Climatic change and variability - past, present, and future', in *Global Warming: The Hard Science*. New York, London, UK: Routledge, pp. 3–5.

Haugan, P. M. and Drange, H. (1996) 'Effects of CO_2 on the ocean environment', *Energy Conversion and Management*, 37(6–8), pp. 1019–1022. doi: 10.1016/0196-8904(95)00292-8.

IPCC (2007a) *Climate Change 2007: Synthesis Report. Contribution of Working Groups I, II and III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change* [Core Writing Team, Pachauri, R.K and Reisinger, A. (eds.)]. Geneva, Switzerland: IPCC, pp. 104.

IPCC (2007b) *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change* [Solomon, S., D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M. Tignor and H.L. Miller (eds.)]. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press, pp. 996.

IPCC (2007c) 'Summary for Policymakers', in *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change* [Solomon, S., D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M. Tignor and H.L. Miller (eds.)]. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press, pp. 1-18.

IPCC (2013a) 'Annex I: Atlas of Global and Regional Climate Projections [van Oldenborgh, G.J., M. Collins, J. Arblaster, J.H. Christensen, J. Marotzke, S.B. Power, M. Rummukainen and T. Zhou (eds.)]', in *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)]. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press, pp. 1311-1393.

IPCC (2013b) 'Annex III: Glossary [Planton, S. (eds.)]', in *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)]. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press, pp. 1447-1465.

IPCC (2013c) 'Summary for Policymakers', in *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on*

Climate Change [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)]. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press, pp. 3-29.

IPCC (2014) *Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)]. Geneva, Switzerland: IPCC, pp. 151.

IPCC (2019) *The Intergovernmental Panel on Climate Change*. Available at: https://www.ipcc.ch/ (Accessed: 6 December 2019).

Joaquim, S., Matias, D., Matias, A. M., Moura, P., Arnold, W. S., Chícharo, L. and Gaspar, M. B. (2011) 'Reproductive activity and biochemical composition of the pullet carpet shell *Venerupis enegalensis* (Gmelin, 1791) (Mollusca: Bivalvia) from Ria de Aveiro (Northwestern coast of Portugal)', *Scientia Marina*, 75(2), pp. 217–226. doi: 10.3989/scimar.2011.75n2217.

Keeling, R. F., Körtzinger, A. and Gruber, N. (2010) 'Ocean Deoxygenation in a Warming World', *Annual Review of Marine Science*, 2(1), pp. 199–229. doi: 10.1146/annurev.marine.010908.163855.

Keklikoglou, K., Chatzinikolaou, E., Faulwetter, S., Grigoriou, P., Papageorgiou, N., Queirós, A., Rühl, S. and Schmidbaur, H. (2015) 'Revealing effects of ocean acidification on the calcified structures of marine invertebrates through micro-computed tomography (micro-CT)', in *Bruker MicroCT 2015 User Meeting*.

Kirtman, B., Power, S. B., Adedoyin, J. A., Boer, G. J., Bojariu, R., Camilloni, I., Doblas-Reyes, F. J., Fiore, A. M., Kimoto, M., Meehl, G. A., Prather, M., Sarr, A., Schär, C., Sutton, R., G.J. van Oldenborgh, Vecchi, G. and Wang, H. J. (2013) 'Near-term Climate Change: Projections and Predictability', in *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)] Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press, pp. 953-1028

Ko, G. W. K., Vera, C. B. S., R, D., Dennis, C. K. S., Adela, L. J., Yu, Z. and Thiyagarajan, V. (2013) 'Larval and Post-Larval Stages of Pacific Oyster (*Crassostrea gigas*) Are Resistant to Elevated CO_2', *PLoS ONE*, 8(5), p. e64147. doi: 10.1371/journal.pone.0064147.

Kocot, K. M., Aguilera, F., McDougall, C., Jackson, D. J. and Degnan, B. M. (2016) 'Sea shell diversity and rapidly evolving secretomes: insights into the evolution of biomineralization.', *Frontiers in zoology*, 13, p. 23. doi: 10.1186/s12983-016-0155-z.

Kroeker, K. J., Kordas, R. L., Crim, R., Hendriks, I. E., Ramajo, L., Singh, G. S., Duarte, C. M. and Gattuso, J.-P. (2013) 'Impacts of ocean acidification on marine organisms: quantifying sensitivities and interaction with warming', *Global Change Biology*, 19(6), pp. 1884–1896. doi: 10.1111/gcb.12179.

Kurihara, H. (2008) ‘Effects of CO_2-driven ocean acidification on the early developmental stages of invertebrates’, *Marine Ecology Progress Series*, 373, pp. 275–284. doi: 10.3354/meps07802.

Lagos, N. A., Benítez, S., Duarte, C., Lardies, M. A., Broitman, B. R., Tapia, C., Tapia, P., Widdicombe, S. and Vargas, C. A. (2016) ‘Effects of temperature and ocean acidification on shell characteristics of *Argopecten purpuratus*: Implications for scallop aquaculture in an upwelling-influenced area’, *Aquaculture Environment Interactions*, 8, pp. 357–370. doi: 10.3354/AEI00183.

Lambeck, R. H. D. (1984) ‘Dynamics, migration and growth of *Nassarius reticulatus* (Mollusca: Prosobranchia) colonizing saline Lake Grevelingen (SW Netherlands)’, *Netherlands Journal of Sea Research*, pp. 395–417. doi: 10.1016/0077-7579(84)90012-7.

Lebour, M. V. (1931) ‘The Larval Stages of *Nassarius reticulatus* and *Nassarius incrassatus*’, *Journal of the Marine Biological Association of the United Kingdom*, 17(3), pp. 797–817. doi: 10.1017/S0025315400051985.

Lenz, B., Fogarty, N. D. and Figueiredo, J. (2019) ‘Effects of ocean warming and acidification on fertilization success and early larval development in the green sea urchin *Lytechinus variegatus*’, *Marine Pollution Bulletin*, pp. 70–78. doi: 10.1016/j.marpolbul.2019.02.018.

León, P., Bednaršek, N., Walsham, P., Cook, K., Hartman, S. E., Wall-Palmer, D., Hindson, J., Mackenzie, K., Webster, L. and Bresnan, E. (2019) ‘Relationship between shell integrity of pelagic gastropods and carbonate chemistry parameters at a Scottish Coastal Observatory monitoring site’, *ICES Journal of Marine Science*. doi: 10.1093/icesjms/fsz178.

Leung, J. Y. S., Connell, S. D., Nagelkerken, I. and Russell, B. D. (2017) ‘Impacts of Near-Future Ocean Acidification and Warming on the Shell Mechanical and Geochemical Properties of Gastropods from Intertidal to Subtidal Zones.’, *Environmental science & technology*, 51(21), pp. 12097–12103. doi: 10.1021/acs.est.7b02359.

Lischka, S., Büdenbender, J., Boxhammer, T. and Riebesell, U. (2011) ‘Impact of ocean acidification and elevated temperatures on early juveniles of the polar shelled pteropod *Limacina helicina*: mortality, shell degradation, and shell growth’, *Biogeosciences*, 8(4), pp. 919–932. doi: 10.5194/bg-8-919-2011.

Lowenstam, H. A. and Weiner, S. (1989) *On biomineralization*. New York, Oxford, UK: Oxford University Press, pp. 336.

Manríquez, P. H., Jara, M. E., Torres, R., Mardones, M. L., Lagos, N. A., Lardies, M. A., Vargas, C. A., Duarte, C. and Navarro, J. M. (2014) ‘Effects of ocean acidification on larval development and early post-hatching traits in *Concholepas concholepas* (loco)’, *Marine Ecology Progress Series*, 514, pp. 87–103. doi: 10.3354/meps10951.

Manríquez, P. H., Jara, M. E., Seguel, M. E., Torres, R., Alarcon, E. and Lee, M. R. (2016) ‘Ocean Acidification and Increased Temperature Have Both Positive and Negative Effects on Early

Ontogenetic Traits of a Rocky Shore Keystone Predator Species', *PLoS ONE*, 11(3), p. e0151920. doi: 10.1371/journal.pone.0151920.

Marin, F., Le Roy, N. and Marie, B. (2012) 'The formation and mineralization of mollusk shell', *Frontiers in Bioscience - Scholar*, 4 S(3), pp. 1099–1125. doi: 10.2741/s321.

Milano, S., Schöne, B. R., Wang, S. and Müller, W. E. (2016) 'Impact of high pCO_2 on shell structure of the bivalve *Cerastoderma edule*', *Marine Environmental Research*, 119, pp. 144–155. doi: 10.1016/J.MARENVRES.2016.06.002.

MolluscaBase (2019) *MolluscaBase*. *Venerupis corrugata* (Gmelin, 1791), *World Register of Marine Species* [online]. Available at: http://www.marinespecies.org/aphia.php?p=taxdetails&id=18826 (Accessed: 5 December 2019).

Morton, J. E. (1967) *Molluscs*. 4th edn. London, UK: Hutchinson, pp. 244.

Moss, R. H. *et al.* (2010) 'The next generation of scenarios for climate change research and assessment', *Nature*, 463(7282), pp. 747–756. doi: 10.1038/nature08823.

Nasution, S. and Roberts, D. (2004) 'Laboratory trials on the effects of different diets on growth and survival of the common whelk, *Buccinum undatum* L. 1758, as a candidate species for aquaculture', *Aquaculture International*, 12(6), pp. 509–521. doi: 10.1007/s10499-004-5745-7.

Navarro, J. M., Duarte, C., Manríquez, P. H., Lardies, M. A., Torres, R., Acuña, K., Vargas, C. A., Lagos, N. A. and Ocean, N. A. (2016) 'Ocean warming and elevated carbon dioxide: multiple stressor impacts on juvenile mussels from southern Chile'. doi: 10.1093/icesjms/fsv249.

Nielsen, C. (2004) 'Trochophora larvae: cell-lineages, ciliary bands, and body regions. 1. Annelida and Mollusca.', *Journal of experimental zoology. Part B, Molecular and developmental evolution*, 302(1), pp. 35–68. doi: 10.1002/jez.b.20001.

Nützel, A. (2014) 'Larval ecology and morphology in fossil gastropods', *Palaeontology*, 57(3), pp. 479–503. doi: 10.1111/pala.12104.

Peng, T. C., Vengatesen, T. and Hwai, A. T. S. (2016) 'Assessment of temperature effects on early larval development survival of hatchery-reared tropical oyster, *Crassostrea iredalei*', *Tropical Life Sciences Research*, 27, pp. 111–116. doi: 10.21315/tlsr2016.27.3.15.

Pörtner, H. O. (2008) 'Ecosystem effects of ocean acidification in times of ocean warming: A physiologist's view', *Marine Ecology Progress Series*, pp. 203–217. doi: 10.3354/meps07768.

Quayle, D. B. (1952) 'Structure and Biology of the Larva and Spat of *Venerupis pullastra* (Montagu)', *Transactions of the Royal Society of Edinburgh*, 62, pp. 255–297. doi: 10.1017/S0080456800009297.

Queirós, A. M., Fernandes, J. A., Faulwetter, S., Nunes, J., Rastrick, S. P. S., Mieszkowska, N., Artioli, Y., Yool, A., Calosi, P., Arvanitidis, C., Findlay, H. S., Barange, M., Cheung, W. W. L. and Widdicombe,

S. (2015) ‘Scaling up experimental ocean acidification and warming research: From individuals to the ecosystem’, *Global Change Biology*, 21(1), pp. 130–143. doi: 10.1111/gcb.12675.

Rhein, M., Rintoul, S. R., Aoki, S., Campos, E., Chambers, D., Feely, R. A., Gulev, S., Johnson, G. C., Josey, S. A., Kostianoy, A., Mauritzen, C., Roemmich, D., Talley, L. D. and Wang, F. (2013) ‘Observations: Ocean’, in *Climate Change 2013 the Physical Science Basis: Working Group I Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)]. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press, pp. 255-315.

Riahi, K., Rao, S., Krey, V., Cho, C., Chirkov, V., Fischer, G., Kindermann, G., Nakicenovic, N. and Rafaj, P. (2011) ‘RCP 8.5-A scenario of comparatively high greenhouse gas emissions’, *Climatic Change*, 109(1), pp. 33–57. doi: 10.1007/s10584-011-0149-y.

Richardson, C. A. (2001) ‘Molluscs as archives of environmental change’, in *Oceanography and Marine Biology, An Annual Review*. London: Aberdeen University Press/Allen & Unwin, pp. 103–164.

Ross, P. M., Parker, L., O’Connor, W. A. and Bailey, E. A. (2011) ‘The Impact of Ocean Acidification on Reproduction, Early Development and Settlement of Marine Organisms’, *Water*, 3(4), pp. 1005–1030. doi: 10.3390/w3041005.

Royal Society (2005) *Ocean acidification due to increasing atmospheric carbon dioxide Policy document 12/05*. London: The Royal Society, pp. 68.

Rühl, S., Calosi, P., Faulwetter, S., Keklikoglou, K., Widdicombe, S. and Queirós, A. M. (2017) ‘Long-term exposure to elevated pCO_2 more than warming modifies early-life shell growth in a temperate gastropod’, *ICES Journal of Marine Science*, 74(4), pp. 1113–1124. doi: 10.1093/icesjms/fsw242.

Simkiss, K. and Wilbur, K. M. (1989) *Biomineralization*. Elsevier, pp. 337.

Sokolova, I. M., Frederich, M., Bagwe, R., Lannig, G. and Sukhotin, A. A. (2012) ‘Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates’, *Marine Environmental Research*, 79, pp. 1–15. doi: 10.1016/j.marenvres.2012.04.003.

Sokolova, I. M. (2013) ‘Energy-limited tolerance to stress as a conceptual framework to integrate the effects of multiple stressors’, *Integrative and Comparative Biology*, 53(4), pp. 597–608. doi: 10.1093/icb/ict028.

Suzuki, M. and Nagasawa, H. (2013) ‘Mollusk shell structures and their formation mechanism’, *Canadian Journal of Zoology*, 91(6), pp. 349–366. doi: 10.1139/cjz-2012-0333.

Tridge (2019) *Pullet Carpet Shell suppliers, wholesale prices, and global market information.*. Available at: https://www.tridge.com/intelligences/pullet-carpet-shell (Accessed: 23 November 2019).

Tyson, R. V. and Pearson, T. H. (1991) 'Modern and ancient continental shelf anoxia: an overview', *Geological Society, London, Special Publications*, 58(1), pp. 1–24. doi: 10.1144/GSL.SP.1991.058.01.01.

US - EPA (2013) *Overview of greenhouse gases, United States Environmental Protection Agency*. Available at: https://www.epa.gov/ghgemissions/overview-greenhouse-gases (Accessed: 25 November 2019).

Waldbusser, G. G., Hales, B. and Haley, B. A. (2016) 'Calcium carbonate saturation state: on myths and this or that stories', *ICES Journal of Marine Science: Journal du Conseil*, 73(3), pp. 563–568. doi: 10.1093/icesjms/fsv174.

Wessel, N., Martin, S., Badou, A., Dubois, P., Huchette, S., Julia, V., Nunes, F., Harney, E., Paillard, C. and Auzoux-Bordenave, S. (2018) 'Effect of CO2–induced ocean acidification on the early development and shell mineralization of the European abalone (*Haliotis tuberculata*)', *Journal of Experimental Marine Biology and Ecology*, 508, pp. 52–63. doi: 10.1016/j.jembe.2018.08.005.

White, M. (2009) 'The Industrial Revolution', *Historical Journal*. The British Library, p. 543. doi: 10.1017/S0018246X00020380.

Wilbur, K. M. and Saleuddin, A. S. M. (1983) 'Shell Formation', in *The Mollusca*. Elsevier, pp. 235–287.

Wilbur, K. M. and Watabe, N. (1963) 'Experimental studies on calcification in molluscs and the alga *Coccolithus Huxleyi*', *Annals of the New York Academy of Sciences*, 109(1), pp. 82–112. doi: 10.1111/j.1749-6632.1963.tb13463.x.

Zhang, H., Cheung, S. G. and Shin, P. K. S. (2014) 'The larvae of congeneric gastropods showed differential responses to the combined effects of ocean acidification, temperature and salinity', *Marine Pollution Bulletin*, 79(1–2), pp. 39–46. doi: 10.1016/j.marpolbul.2014.01.008.

Zippay, M. L. and Helmuth, B. (2012) 'Effects of temperature change on mussel, *Mytilus*', *Integrative Zoology*, pp. 312–327. doi: 10.1111/j.1749-4877.2012.00310.x.

Zupo, V. and Patti, F. P. (2009) 'Laboratory spawning, larval development and metamorphosis of the marine snail *Nassarius reticulatus* (L.) (Caenogastropoda, Nassariidae)', *Invertebrate Reproduction & Development*, 53(1), pp. 23–31. doi: 10.1080/07924259.2009.9652286.

Chapter 2

Vulnerability of the netted whelk, *Tritia reticulata* (L.) under Ocean Acidification and Warming Projected Scenarios

The data in this chapter yielded the following peer-reviewed presentations

in national and international scientific meetings

(presenting author underlined)

Oliveira IB, Laranjeiro F, Fonseca JG, **Freitas DB**, Rocha RJM, Weston J, Hinzmann M, Machado J, Barroso CM, Galante-Oliveira S (2018) Ocean acidification and warming induce mortality and shell loss in *Nassarius reticulatus* (L.) veligers jeopardizing the species survival. PICES 4th Climate Change International Symposium, 4-8.Jun, Washington DC, USA.

Freitas DB, Fonseca JG, Laranjeiro F, Oliveira IB, Rocha RJM, Galante-Oliveira S, Barroso CM (2018) Efeito do aquecimento oceânico na ontogenia e crescimento de fases iniciais de vida do gastrópode *Nassarius reticulatus*. CNAC'2018, 19-20.Feb, Vila Real, Portugal. *BEST POSTER PRESENTATION*

Most of the data in this chapter are being prepared for submission

as the following original scientific article

(corresponding author underlined)

Galante-Oliveira S, Oliveira IB, **Freitas DB**, Fonseca JG, Laranjeiro F, Rocha RJM, Hinzmann M, Machado J, Barroso CM (in preparation) Vulnerability of *Tritia reticulata* (L.) early life stages to ocean acidification and warming scenarios (submission to *Global Change Biology* scheduled for December 2019).

Abstract

Aquatic organisms that produce biogenic carbonates, such as gastropod molluscs, are especially prone to be negatively impacted by ocean acidification and warming (OA-W). To assess the concomitant effect of the pH decrease and temperature rise on early life stages of *Tritia reticulata* –a common gastropod species in the NE Atlantic coast with a high ecological importance on estuarine and coastal systems– a factorial experimental design of three pH conditions (targeting 8.1, 7.8 and 7.5) at four temperatures (16, 18, 20 and 22ºC) was applied. Newly hatched veligers were exposed to 12 OA-W experimental scenarios and the development, growth and survival followed for two months. Our results show effects of the stressors involved, pH and temperature (TºC), individually or interactively, on development, survival, growth and shell integrity at different exposure times. All endpoints were initially affected by pH, with impaired development and high mortalities being recorded in the first week of exposure, constrained by the most acidic scenarios tested (pH_{target} 7.5). Development was also significantly driven by TºC, resulting in earlier settlement under warming. After two weeks of exposure, larval performance and survival were highly affected by the interaction between pH and TºC: growth was only evident under warming (TºC≥20ºC) and under carbonate saturation (pH≥7.8). In fact, aragonite undersaturation was registered in the most acidic treatments (pH_{target} 7.5), under which critical larval mortality (100%) at 22ºC was recorded and the occurrence of extremely vulnerable, unshelled species was observed at all tested TºC. Shell loss by specimens exposed to the most acidic condition was, perhaps, the most unexpected and striking result of this work. Furthermore, acidity was also proved to alter shell crystallinity, making larval shell mineral matrix more amorphous and, thus, fragile and prone to dissolution. Only 4 OA-W projected scenarios plus the control resisted until settlement. Afterwards, growth and survival stabilized under control pH, but mortality continued to rise under acidity, with less dense shells being recorded in juveniles reared under pH 7.8 (the only pH level under test by that time). These results reveal the extreme sensitivity of this species' early life stages to future OA-W. As recruitment cohorts are the foundation for future populations, our results point towards the extreme vulnerability of this species to OA-W scenarios projected by the Intergovernmental Panel on Climate Change (IPCC) to the end of this century for the northern hemisphere, where *T. reticulata* is ubiquitous. Increased veliger mortality associated with reduced growth rates, shell dissolution and loss under future OA-W will compromise larval performance, jeopardizing *T. reticulata* subsistence.

Keywords: *Tritia reticulata*, gastropod mollusc, climate change, development, growth, shell dissolution, carbonate, mortality, shell loss

2.1 Introduction

Tritia reticulata (Linnaeus, 1758) is a common gastropod with a wide distribution in the Northeast Atlantic –along the European coastline, from the Canaries to Norway– and throughout the Mediterranean, Baltic and the Black Seas (Fretter and Graham, 1994). In the Portuguese coast, the presence of this ubiquitous gastropod extends along the open coast into the outer parts of estuaries, colonizing different habitats from sandy beaches to muddy flats (Lambeck, 1984; Barroso and Moreira, 1998). This species spawns from mid-winter to summer, laying egg capsules onto hard substrates (algae, rocks and/or other molluscs' shells). Inside the capsule, organisms pass through a trochophore larval stage before hatching as veliger, roughly after one month (Fretter and Graham, 1994; Barroso and Moreira, 1998). The veliger stage corresponds to the free-swimming, planktonic larval period typically characterized by the presence of shell, foot and velum. Depending mainly on the temperature (but also, at a lesser extent, on other abiotic factors), this stage may last between 1 to 3 months as larvae reaches competency before settling to the substrate, and metamorphosing to became benthic (Tallmark, 1980; Fretter and Graham, 1994). This transformation comprises the disappearance of the velum, the development of the foot and the organization of the reproductive organs and digestive gland (Marin *et al.*, 2012). Due to its nature as scavenger, *T. reticulata* plays an important role on the environment leading to the transfer of nutrient and energy across different ecosystems as well as on the carbon cycling, distribution and sequestration into the oceans (King *et al.*, 2007).

Oceans' sustainability is a top priority of the international agenda as a number of threats known to cause irreparable damage to both the Earth's climate and to marine ecosystems have been identified. Among them are ocean acidification (OA) and warming (W), recognized as being worsened by human activities, particularly those involving greenhouse gas (GHG) emissions such as carbon dioxide (CO_2). The rise of atmospheric pCO_2 levels from anthropogenic emissions is known to be a critical problem directly linked to an increase of the ocean temperature and acidity (Rhein *et al.*, 2013). Following the Intergovernmental Panel on Climate Change (IPCC) projections, under RCP8.5 (Representative Concentration Pathway 8.5; Riahi *et al.*, 2011; Cubasch *et al.*, 2013), the sea surface temperature (SST) is estimated to rise globally at a rate >0.1ºC per decade, accounting for increases from about 1 to above 3 ºC by the end of the 21st century (Collins

et al., 2013; IPCC, 2013). Regarding sea surface pH, IPCC anticipates a decrease between 0.3 and 0.5 units in the northern hemisphere by the same period (Ciais *et al.*, 2013) although conditions greater than the SST and pH global averages have already been experienced in many regions (Ciais *et al.*, 2013; IPCC, 2014; Allen *et al.*, 2018). These projected trends are likely to continue or accelerate in the upcoming decades if no mitigation actions are taken to constrain GHG emissions and human-induced climate change (Ciais *et al.*, 2013; IPCC, 2014).

Ocean acidification (OA) and warming (W) have a significant impact on marine invertebrates and the ecosystems they inhabit (Ross *et al.*, 2011; Kroeker *et al.*, 2013). These processes alter the seawater carbonate chemistry, affecting carbonate ($CaCO_3$) secretion by marine calcifiers (such as gastropod molluscs), and its dissolution. In this sense, early life stages, whose shells are composed of more soluble $CaCO_3$ polymorphs, have an increased vulnerability to OA-W (Ries, 2011; Ries *et al.*, 2016). Moreover, carbonate statoliths included in these animals' gravireceptors (the statocysts; see Galante-Oliveira *et al.*, 2013 and 2014) are also formed in early life. Such structures are involved in key functions like balance and spatial orientation (Levi *et al.*, 2004) and can also be affected by abnormal calcification under OA. Thus, the panorama for the early life stages is, indeed, of particular concern as their survival is the bottleneck for the species persistence (Byrne, 2011). In fact, OA is thought to be the determinant factor on the formation of biogenic $CaCO_3$ as it decreases the saturation of carbonate ions, thus reducing its availability that is required for calcification (Orr *et al.*, 2005). On the other hand, temperature also plays an important role on the aerobic metabolism that is known to increase with warming to cope with the high energy demand to maintain cellular functions (Sokolova *et al.*, 2012). The assessment of the combined effects of OA-W is therefore essential, as marine organisms, in their natural habitat, will be exposed to these stressors together rather than independently. Moreover, the importance of these studies is even greater due to the discrepancy between results from previous studies. Ko *et al.* (2013) have studied the resistance of larvae and post-larvae of *Crassostrea gigas* (Thunberg, 1793) to elevated CO_2 and no physiological, developmental and calcifying effects were observed. On the other hand, the same team Ko *et al.* (2014) have also reported combined effects of OA-W and reduced salinity, on early-life stages of *C. gigas*, namely impacts on metamorphosis success as well as on larval and juvenile growth. In the case of early juveniles of *Limacina helicina* (Phipps, 1774) elevated temperature and

acidification was proved to affect mortality (with temperature being the overriding effect), shell degradation and growth (mainly affected by OA, and not by temperature), (Lischka *et al.*, 2011). The same was tested in juveniles of *Littorina littorea* (Linnaeus, 1758) and the results suggested that, in a not too distant future (2100), this individuals will have smaller, thinner and rounded shells being, thus, more vulnerable to predators (Melatunan *et al.*, 2013).

The aims of this chapter are to investigate the concomitant effects of OA-W on *T. reticulata* ontogenic development and survival, as well as the growth and integrity of both larvae and juveniles' biogenic carbonates - statoliths and shells - under 12 OA-W experimental scenarios. These scenarios were established as series of progressive environmental conditions based on IPCC near to long-term projections for the oceans, in the northern hemisphere. Conditions to which this species is likely to be exposed in its natural habitat over time, in an attempt to foresee its performance under future OA-W.

2.2 Material and Methods

2.2.1 Sampling

Egg capsules of the netted whelk, *Tritia reticulata* (L.) were collected, by hand at the intertidal during low tide, inside Ria de Aveiro estuarine system (NW Portugal) at 40°38'34.65''N - 8°44'06.80''W. Once in the laboratory, egg capsules were acclimated for 3 days in 1µm-filtered synthetic saltwater (*Ocean Fish*, PRODAC, Italy) at a salinity of 30, 18±1ºC and 12^{L}:12^{D} photoperiod. Following the protocol of Génio *et al.* (2008) mature capsules –containing larvae with well-developed eyes, statocysts and beating velum– were selected under a stereomicroscope (*Leica S8 APO*, Leica Microsystems, Switzerland) and ripped to release nearly hatching veligers. These larvae were kept in 1µm-filtered synthetic saltwater at salinity 30 and maintained as follows.

2.2.2 Maintenance of newly hatched veligers

Veligers were kept at a salinity of 30, 18±1ºC and 12^{L}:12^{D} photoperiod for an additional 24h-acclimation period, at a density of 2 larvae ml^{-1} and fed an appropriate microalgae blend based on the diet applied by Chatzinikolaou and Richardson (2007) but using the following species and cell numbers: *Isochrysis galbana* (20 cells μl^{-1}), *Skeletonema costatum* (10 cells

µl^{-1}), *Rhodomonas salina* (5 cells µl^{-1}) and *Tetraselmis chui* (0.5 cells µl^{-1}). With the exception of *T. chui* (*Phytobloom Prof Tetraselmys*, Necton S.A., Portugal), which was used as a freeze-dried supplement, all other species were produced at our facilities, following the dynamics of a batch culture (Figure 2.1-A to -D): an inoculant per species (provided by C-FOOD clams' farmers, Coimbra, Portugal) was cultured until maximum or near-maximum density was reached, being then harvested until it runs out (Stappen, 1996). This culture protocol was applied to avoid potential contaminations of both the microalgae culture and the experiment itself.

Briefly, Schott-glass bottles of different volumes (500mL, 1L and 2L) were used to culture microalgae cells (Figure 2.1-A). Bottles were supplied with glass pipettes to aerate and, simultaneously, mix the cultures with 0.2µm-filtered ambient air (*Air Venting Filter 50/0.2 PTFE*, Whatman^{TM} ReZist, Germany), allowing light and nutrients equal exposure to all cells, also avoiding sedimentation. Smaller bottles (100mL) or Erlenmeyer flasks (250mL) were used to maintain back-up stock inoculants in the stationary phase (Figure 2.1-D). All the material used was washed with a solution of Alconox detergent (Sigma-Aldrich, Germany) at 10g L^{-1}. Pipettes and smaller material were autoclaved.

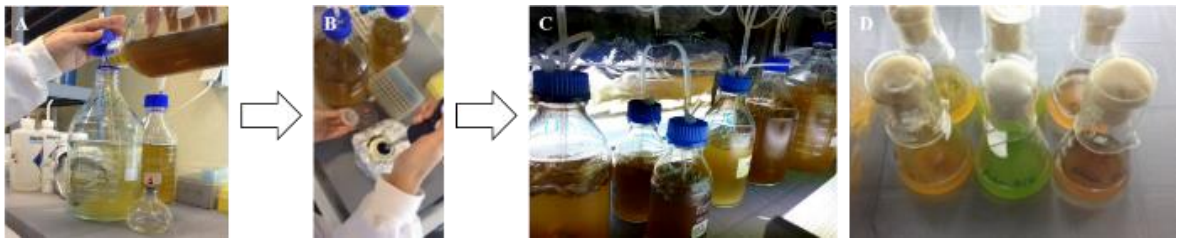

Figure 2.1 - Microalgae batch culture. A – Inoculation of a 2L Schott-glass bottle. B – Addition of nutrient medium to new inoculum. C – Microalgae culture. D – Stock solution on 250mL Erlenmeyer flasks.

One µm-filtered saltwater at a salinity of 30 was used as growing medium after disinfection by 2mL L^{-1} of a solution of 2.5% of sodium hypochlorite (NaClO) for at least 24h. After that period, medium was neutralized under asepsis, by adding 3mL L^{-1} of a autoclaved solution of 2.5% sodium thiosulfate ($Na_2S_2O_3$) and being kept under strong aeration for at least 2 hours. The flasks used to maintain stock inoculants were autoclaved already with the saltwater at salinity 30, in order to maintain the medium as aseptic as possible, avoiding contamination by further manipulation.

After neutralization, the culture medium was inoculated following the protocol showed in Table 2.1. Normally, the culture in 500mL-bottles was used to inoculate 1L-bottles, and this to inoculate 2L-bottles, expanding the culture to the volumes required weekly to feed *T. reticulata* veligers throughout the exposure to OA-W experimental scenarios until settlement. New cultures were supplemented with nutrients (1mL L^{-1} of a pre-prepared nutrient medium; *Nutri Bloom Plus*, Necton S.A., Portugal; Figure 2.1-B) and, in the case of the diatom *S. costatum*, silicates (1mL L^{-1} of a solution of sodium silicate; *Nutri Bloom Silicates Solution*, Necton S.A., Portugal). Finally, the bottles were aerated with 0.2µm-filtered ambient air and kept on shelves, illuminated by fluorescent tubes, at 18±1 ºC and under 12^{L}:12^{D} photoperiod (Figure 2.1-C). These procedures were repeated once every week to avoid culture aging. Stock (back-up) inoculants, which were kept under the same temperature but in the dark (in order to slow growth), were picked to a new medium once a month.

Table 2.1 - Microalgae culture protocol. To a given Schott-glass bottle (Bottle volume), the most appropriate ratio inoculant: medium (Proportion) is indicated, as it is the volume of the Algae inoculum and the Saltwater used.

Bottle volume (mL)	Proportion	Algae inoculum (mL)	Seawater (mL)
500	1:1	200	200
1 000	1:3	200	600
2 000	1:4	400	1 600
100	1:2	30	60
250	1:2	50	100

Culture concentrations per species were assessed by counting the number of cells mL^{-1} on Neubauer counting chambers (Figure 2.2). Larvae were usually fed from culture at 1 and 2L-bottles, after calculation of the volume needed to achieve the concentration mentioned above for each species.

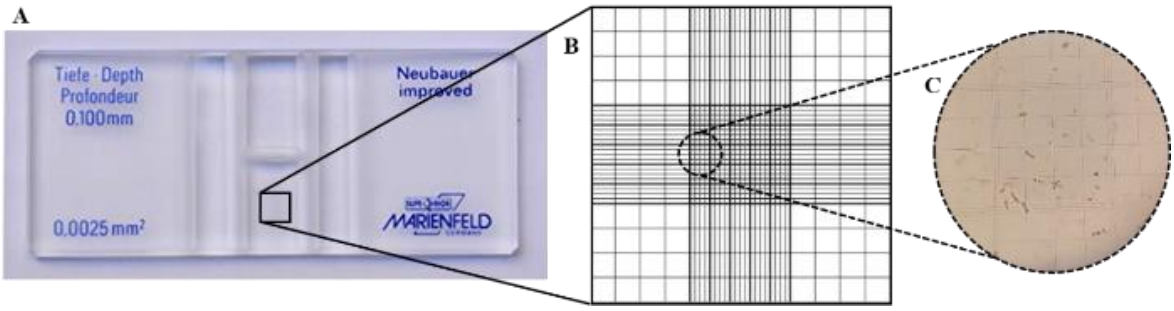

Figure 2.2 – Microalgae cell counting. A – Neubauer-improved counting chamber. B – Scematic representation of the central part of the counting chamber, where counts are made. C – Detail of one square with *Skeletonema costatum* cells observed at 400x magnification.

After the 24-acclimation period, and right before the start of the exposure period (at day 0, T0), veligers were collected in a 100 µm-sieve, back-washed in a 5L beaker to be counted, and aliquoted adequately to be randomly distributed by experimental containers at a density of 1.6 larvae mL^{-1} ($n \approx 4800$ per container) to be exposed to 12 OA-W scenarios as explained above.

2.2.3 Experimental setup, design and treatments

Newly hatched veligers of the netted-whelk *T. reticulata* were exposed for 60 days (from T0 to T60) to 12 OA-W scenarios generated in an Experimental Life Support System (ELSS) developed by Coelho *et al.* (2013). This system was developed to perform microcosm simulation of climate change scenarios, namely of CO_2-induced acidification and warming in coastal and estuarine environments.

Basically, this system consists of four autonomous saltwater reservoirs (each with approximate volume of 230L; see Fig.1 in Coelho *et al.* 2013) in which the pH is independently manipulated through a CO_2 bubbling diffuser. This diffuser is coupled to a water pump allowing an efficient CO_2 gas mixing in seawater. CO_2 injection is controlled by a feedback pH control system that includes a pH electrode connected to a controller (*V^2 control pH controller*, TMC, UK) and a pressure regulator with an integrated solenoid valve (*V^2 pressure regulator pro*, TMC, UK). On the digital display it is possible to adjust the pH value that controls the opening of the solenoid valve. Whenever pH increases above the pre-set value, CO_2 is injected until water pH returns to the value set. To simulate tidal cycles, water is pumped at predetermined time intervals to the microcosms. Temperature in the microcosms is controlled by their partial immersion (at least 2/3) in temperature-controlled

water baths. Diel light cycle simulation is made by 4 programmable luminaires (*ReefSET*®, Aquariofilia Lda., Portugal).

Modifications to its original version were performed in order to: (1) increase the number of pH and temperature (T ºC) levels generated in the system that, in a factorial experimental design, broaden the range of OA-W scenarios simulated from four (two pH * two T ºC levels) to twelve (three pH * four T ºC levels; Figure 2.3); and (2) adapt the microcosms or experimental units to the exposure of planktonic larvae (from 3L-rectangular aquaria with automatic drainage by outflow pumps operated with digital timers, to inverted carboys with aeration and gravity drainage by outlet pipes with 100µm-mesh filter-protected output, at such a height to allow a maximum interior volume of 3L).

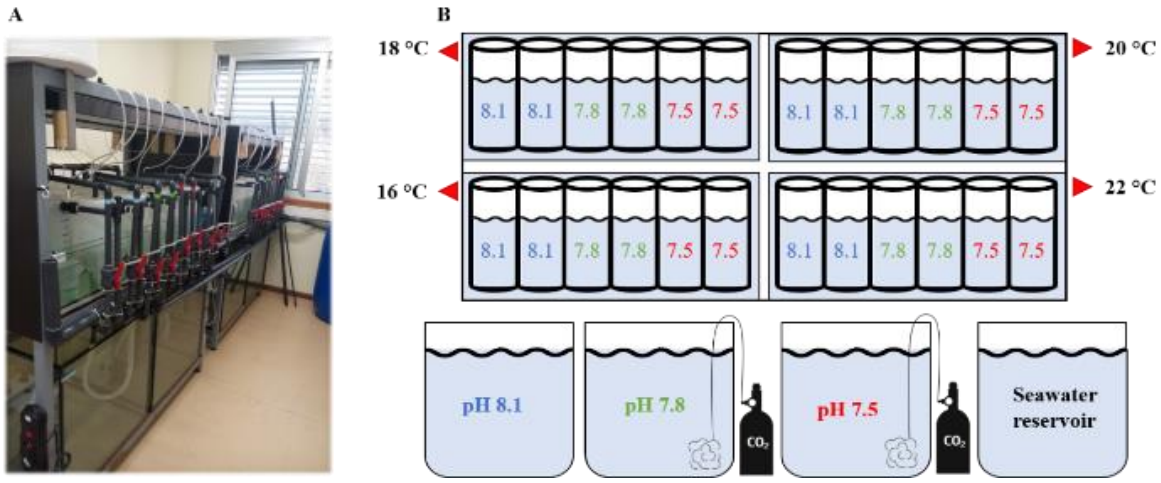

Figure 2.3 - A – Experimental Life Support System (ELSS) modified to generate 12 OA-W experimental scenarios to expose veligers of *Tritia reticulata* in duplicate for 60 days. B – Experimental design for that experiment, including four T ºC (16, 18, 20 and 22ºC) and three pH levels (targeting 8.1, 7.8 and 7.5).

Synthetic saltwater was prepared by mixing freshwater purified by a reverse osmosis (V^2 *Pure 75*, TMC, UK) with a commercial sea salt (*Ocean Fish*, PRODAC, Italy) at 30g L^{-1} (salinity 30). The saltwater was prepared at least 24 h prior to use, in order to allow a complete salt dissolution.

Saltwater pH was manipulated in three of the four reservoirs to generate three distinct pH levels, targeting 8.1, 7.8 and 7.5: a control (~8.1) corresponding to the actual pH measured at the veligers sampling site, plus two acidified conditions of about -0.3 and -0.5 pH units, corresponding to the interval of sea surface pH projected by the IPCC for the end of the 21st century in the northern hemisphere under RCP8.5 scenario (Ciais *et al.*, 2013),

and following the available guidelines applied to OA research (Riebesell *et al.*, 2010). The fourth reservoir was used to keep unmanipulated saltwater to refill the remaining three as needed. Saltwater in all the reservoirs were recirculated for 15 min every two hours to promote water mobilization and to force pH adjustment.

Regarding TºC, the two water baths initially installed (see Fig.1 in Coelho *et al.* 2013) were replaced by four of half of their size. Each new bath was equipped with a temperature control system, similar to the original, but scaled to its dimension. Four TºC levels were then generated (Figure 2.3-B): two possible control conditions –16 and 18°C– corresponding, respectively, to the mean seawater surface temperature (SST) at the Ria de Aveiro mouth and the mean temperature registered during *T. reticulata* spawning season at the site where veligers were obtained (Barroso and Moreira, 1998); plus two warming conditions –20 and 22°C– that, according to the IPCC projection under RCP8.5 of a global increase in the SST of up to 4ºC (Collins *et al.*, 2013; Kirtman *et al.*, 2013), correspond to the end of the century scenarios for controls 16ºC and 18ºC, respectively.

Each of the 12 OA-W scenarios generated were tested in duplicate (and not in triplicate for logistic constrains). The Ria de Aveiro semi-diurnal tidal regime and the water renewal percentage at its central area (Coelho *et al.*, 2013) were simulated by 50% exposure medium exchange twice a day (manually at 9 am and 6 pm), by addition at the bottom of each experimental unit through an inlet pipe, forcing the excess of medium to drain by gravity from the outlet pipe installed at the top. A complete exchange of the medium was performed weekly until settlement and every two weeks thereafter, in order to clean the experimental containers reducing the risk of bacterial contamination by excessive accumulations of organic detritus.

A $12h^{L}$:$12h^{D}$ photoperiod was applied, at half of the light intensities considered by Coelho *et al.* (2013), as well as gentle aeration at 1 bubble seg^{-1}, at intervals of 15 min during the day and 15 min every 2 h at night (reduction to compensate the logistical constraint of effecting media exchanges at 9 am and 6 pm, which implied a further 6 h stay of the media during the night period until the morning exchange). Also, until settlement, food was provided at the end of the day in order to avoid pH imbalance due to microalgae photosynthesis under light, and since veligers of marine bottom invertebrates seems to feed

more actively during the dark (Mileikovsky, 1973). After settlement was first recorded, the diet was supplemented with small pieces of fish *ad libitum* (generally, twice a week).

A pre-assay to monitor the diel cycles of pH and TºC generated in the system under such conditions was carried out and the respective control systems' settings adjusted to avoid treatment overlapping and to allow the predetermined scenarios to be obtained: pH controllers were set to 7.95, 7.35 and 6.95, and cooler/heater thermostats to 16/16, 17/18, 19/20 and 21/22 °C.

The physico-chemical parameters of the exposure media (pH, TºC, salinity, total dissolved oxygen, carbonate chemistry) and biological endpoints (mortality, developmental stage, feeding/nourishment status, statolith and shell growth and integrity) were periodically assessed as follows.

2.2.4 Exposure media physico-chemical analysis

Probe measurements (*Multi 3430 IDS*, WTW, Germany) of pH (in the NBS scale) and TºC were recorded before and after both daily medium exchanges for three consecutive days from T0, to characterize the daily variation to which specimens would be exposed. Then, those parameters were periodically monitored, at least before one of the two daily medium exchanges until day 14 (T14) and at least once a week afterwards. Probe measurements of salinity (SAL) and total dissolved oxygen (DO) were recorded weekly, before the morning medium exchange. Total alkalinity (TA) was determined by manual volumetric titration following Frommlet *et al.* (2015) protocol, in 20mL samples of 0.2µm-filtered exposure medium collected at T0, and before and after the morning medium exchange once a week until T14 and every two weeks from then until T60, in at least one replicate per treatment.

The values of TA and of pH, TºC and SAL read at the time of media sampling, were the inputs to calculate the CO_2 partial pressure (pCO_2) and carbonate (CO_3^{2-}) ion concentration, and the saturation states of calcite (Ω_{Ca}) and aragonite (Ω_{Ar}) using the Microsoft Excel macro CO2Sys_v2.1 (Pierrot *et al.*, 2006), with K1 and K2 carbonate dissociation constants from Mehrbach *et al.* (1973) refitted by Dickson and Millero (1987), and KSO_4 from Dickson (1990).

2.2.5 Analysis of biological endpoints

Larval culture

The performance of the larval culture under OA-W experimental scenarios was evaluated through the analysis of biological endpoints at 3 sampling moments: at day 2 (T2), to assess the randomness of the initial veliger distribution among tanks and the mortality across treatments; and at days 8 (T8) and 14 (T14), to assess, beyond mortality, the developmental stage, the feeding/nourishment status, the shell lenght, growth rates and shell integrity. Due to its nature, growing at a very slower rate than the shell, statolith size and growth from T0 were only assessed at T14.

Accordingly, at T2 veligers were collected in a 100 µm-sieve, back-washed into a beaker, suspended in 3L of the respective exposure medium. The living larvae were counted in three aliquots of 5mL per replicate (i.e., 6 per treatment) to determine survival and calculate mortality. Then, at T8 and T14, larvae were again collected in a 100 µm-sieve but suspended in a volume adjusted to the number of live individuals in each replicate. The living larvae were counted in two aliquots of 10mL per replicate (i.e., 4 per treatment) to determine survival and calculate mortality. Of those larvae, 12 per replicate (i.e. 24 per treatment) were randomly selected and placed in a 24-well plate (1 larva per well). Plates were observed under a stereomicroscope (*Leica S8 APO*, Leica Microsystems, Switzerland) to determine: (1) each specimen developmental stage –categorized into four (I to IV) veliger stages following Zupo and Patti (2009) classification; (2) the presence or absence of gut content (i.e., digesting food in each larvae gut); (3) the presence or absence of shell. Then, and in addition to a representative sample of the initial (T0) pool of recently hatched larvae preserved in absolute ethanol, 20 veligers per replicate were preserved in 70º ethanol to measure the shell length (SL) and the statolith diameter (StD), (Figure 2.4-A and -B, respectively). Other 20 larvae per replicate were preserved in absolute ethanol to study shell microstructural integrity under Scanning Electron Microscopy (SEM).

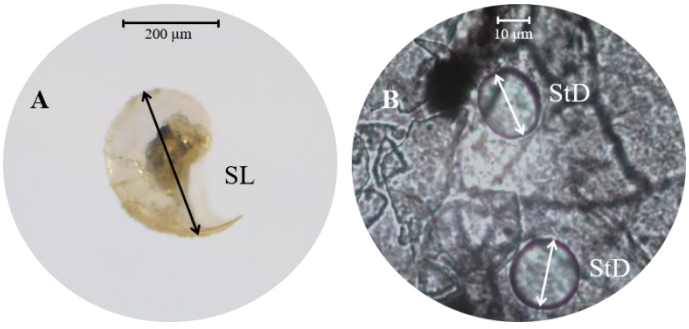

Figure 2.4 - Measurements of *Tritia reticulata* biogenic carbonates after 14 days of exposure. A – Shell length (SL). B – Statolith diameter (StD).

Individuals preserved in 70º ethanol were photographed under the stereomicroscope that is equipped with a digital camera (*MC170 HD*, Leica Microsystems, Switzerland). The SL was measured posteriorly using the equipment software (*Leica Application Suite*, Leica Microsystems, Switzerland; Figure 2.4-A). The mean SL per treatment was used to calculate growth rates, per treatment, in the first (T0-T8) and the second week (T8-T14) of development under OA-W experimental conditions. The same specimens were then rehydrated with distilled water for few minutes, slightly squeezed between a glass slide and a coverslip, and observed under a light optical microscope (*Axiolab E*, Zeiss, Germany) also equipped with a digital camera (*Moticam 2300*, Meyer Instruments, USA) with which were photographed. The StD was, then, measured using the equipment software (*Motic Images Plus v2.0*, Meyer Instruments, USA; Figure 2.4-B).

Finally, the veligers preserved in absolute ethanol were prepared for SEM, following the protocol developed by Bednaršek *et al.* (2012) to characterize juvenile pteropod shell dissolution under acidity but adding an extra step of ethanol 70º overnight prior to the cleaning and drying procedures, in order to rehydrate the samples for easier periostracum removal. Briefly, after hydration by immersion in ethanol 70º overnight, samples were further hydrated by immersion for 3 min. in 50º ethanol and for another 3 min. in distilled water (dH_2O). Then, the periostracum, bacteria and microalgae on the shell's surface were digested by two periods of 2.5 min. in 6% hydrogen peroxide (H_2O_2), after which the solution was removed by keeping samples in dH_2O for 5 min. twice. After cleaning, samples were dried by a series of rinses in several solvents: at first in 50% methanol for two periods of 5 min., then in 80% methanol for 10 min., followed by two distinct periods in 2,2-dimethoxypropane (DMP) for 15 min., an additional period of 10 min. in a solution of DMP and 1,1,1,3,3,3-hexamethyldisilazane (HMDS) 1:1 for 10 min. and, finally, two periods of 20 min. in HMDS. Cleaned and dried shells were left inside a desiccator, in a Petri dish, until being mounted on a conductive double-sided carbon tab on a glass slide for SEM analysis. Larval shells were observed and photographed in a benchtop SEM (*Hitachi TM3030*, Hitachi High-Technologies Corporation, Japan) at 15kV in MIX mode (simultaneous backscattered and secondary electrons imaging) with charge-up reduction.

After SEM analysis, the mineralogy and crystallinity of the shells were assessed by micro-Raman spectroscopy (MRS) in a Horiba Jobin Yvon LabRam 800HR Raman system

(Horiba Ltd., Kyoto, Japan). Two spectra were acquired randomly from cleaned and dried surfaces of two shells per treatment, by the means of a 441.6nm line of a Kimmon He-Cd laser (Kimmon Koha Co. Ltd., Tokyo, Japan) up to ~10mW, a 100× (NA 0.9) objective, a grating with 1,800 grooves mm^{-1} and a pinhole of 250µm. Resolution was of ~2cm^{-1} in the range between 120 and 3,500 cm^{-1}. Repeated acquisitions of each spectrum were accumulated (four scans of 15 s each) to improve the intensity of the signal. Spectra were calibrated using the 520.5 cm^{-1} line of a Si wafer.

A very low number of live individuals were available in some of the replicates after sampling at T14. For that reason, larvae were kept growing under the same conditions until settlement was recorded without further sampling.

Settlement and post-settlement culture

Settlement was first recorded 28 days after T0. At T28, specimens were collected in a 200µm-sieve, back-washed into a beaker and suspended in 500mL of the respective exposure medium. Swimming larvae and settled juveniles –identified by the presence of foot and absence of velum (Zupo and Patti, 2009)– were counted separately in three aliquots of 5mL to determine mortality and settlement percentages. By that time, there were only live specimens at control pH (8.1) at all TºC (16, 18, 20 and 22ºC) and at pH_{target} 7.8 at 18ºC with a very low number of animals per replicate (only one replicate under pH 8.1 at 22ºC. For these reasons, replicates were put together per treatment, and suvivors' development and growth continued to be monitored under the same test conditions, being assessed as follows. At T34, mortality was assessed as at T28 and at T53 - as 100% settlement was recorded, all animals were counted. The experiment ended at T60, moment at which all juveniles were counted to determine mortality and preserved in absolute ethanol to be measured and processed for SEM to assess shell integrity.

Measurements of juvenile SL (Figure 2.5) were taken as described above for larval SL. Likewise, six of the individuals preserved for SEM were prepared under the protocol described above to assess larval shells integrity by SEM, but the times were increased proportionally to the larger size of the juveniles: hydration periods of 15 min. in 50% ethanol and dH_2O were applied; digestion was

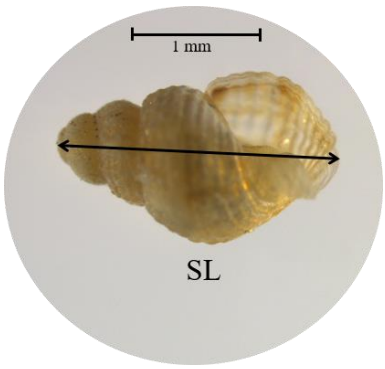

Figure 2.5 - Measurement of *Tritia reticulata*shell length (SL) after 2 months of exposure.

performed using a stronger solution –30% H_2O_2 and sodium hydroxide (NaOH) 0.1M, 1:1– and was accelerated at 50ºC in an oven for 3-4h (controlling surfaces cleaning under a stereomicroscope every hour). Digestion was stopped by rinsing surfaces in dH_2O for 10 min. three times. Drying only required HMDS for two periods of 30 min. Structures were mounted and photographed under the same conditions described above for larvae from T14.

2.2.6 Statistics

Permutational multivariate analysis of variance (PERMANOVA; *Software PRIMER v7*) was used to test for significant effects of pH, TºC and the possible interaction between these factors for each endpoint analysed during the larval culture until T14, since after T28 no statistical treatment was possible (no replication was available at least in one of the treatments). For mortality, one value per replicate (typically the mean of three observations) was used in order to preserve a balanced PERMANOVA design. All other variables were tested per individual organism. Euclidean distance was applied to generate the resemblance matrix. A two-way crossed design (pH * TºC) was applied with a type III partitioning of the sums of squares and the permutation of residuals under a reduced model based on 9999 permutations to obtain the P-value. After a significant PERMANOVA result, a pair-wise t test was used to understand which levels of the factor were responsible for the significant results.

2.3 Results and Discussion

2.3.1 Experimental conditions

Figure 2.6 shows the 12 OA-W scenarios generated in the ELSS (mean temperature vs mean pH probe measurements ± standard deviations). All treatments were significantly different (Pseudo-F=2406.1, p=0.0001) and no difference between replicates from the same treatment was observed (p>0.05). Salinity (30.14±0.18) and dissolved oxygen (7.09±0.309 mg L^{-1}) were registered throughout the experiment, before the morning media exchange. Carbonate chemistry of the exposure media is presented in Table 2.2. “Before” and “After” measurements characterize the variation of the system parameters to which the organisms were exposed during 24h. Temperature (TºC) varied within ±1ºC, mostly due to water baths’ control systems; even so, variation was slightly higher at 22ºC since and initial 15 min.

equilibrium period after media exchange was recorded (Figure 2.6). In its turn, pH tended to increase over time until its re-establishment to target levels upon media exchange and thus, variation was higher in amplitude at lower pH levels (Figure 2.6, Table 2.2). Calcite and aragonite saturation states followed pH variation pattern and understaturation was only observed at the most acidified condition. Moreover, regarding aragonite, undersaturation (Ω<1) was registered both "Before" and "After" medium exchange whilst for calcite, that condition was only observed "After" media exchange.

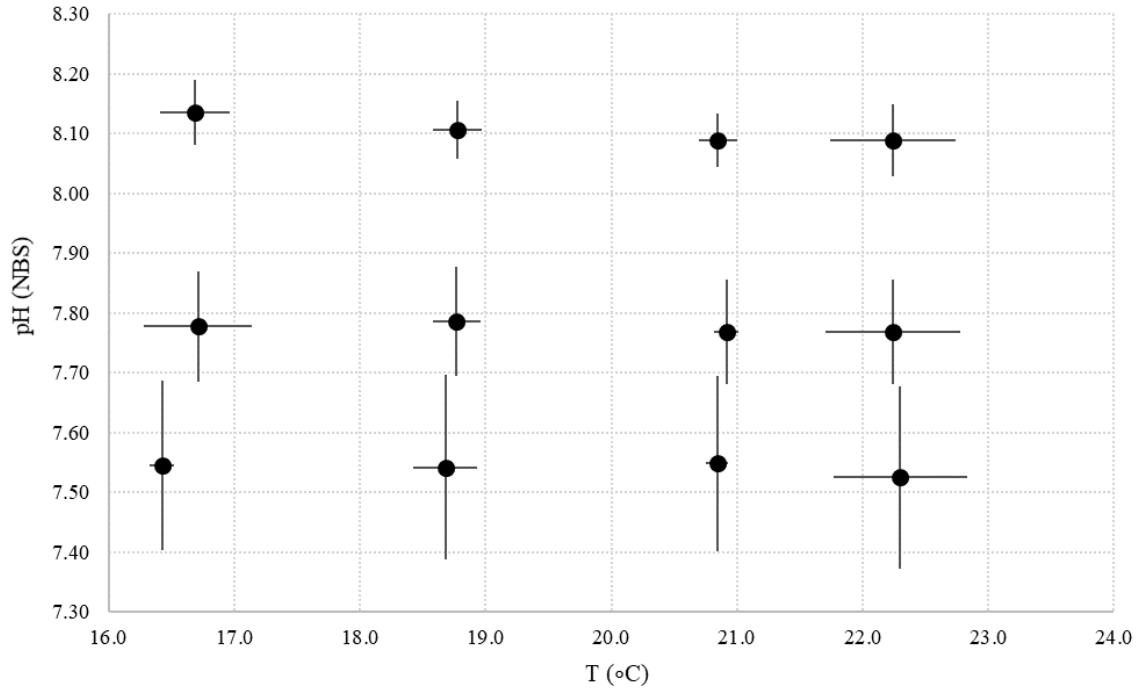

Figure 2.6 - Graphical representation of the 12 OA-W scenarios generated during the experiment corresponding to the temperature (T ºC) and pH daily variation per treatment. Mean values and respective standard deviation of probe measurements of TºC (measured before media exchange twice a day from T0 to T8, once a day until T14 and every two weeks from T15 to T60) and pH (measured before and after media exchange twice a day for 3 consecutive days from T0, once a week from T4 to T14 and every two weeks from T15 until the end of the experiment at T60).

Table 2.2 - Mean carbonate system parameters calculated from samples taken periodically throughout the experiment (see Material and Methods), "Before" and "After" the morning exchange of 50% of the exposure medium in, at least, one replicate per treatment. The treatment column refers to target temperature (T_{target}) and pH (pH_{target}) levels following IPCC (2013) projections as explained in the Material and Methods section. The partial pressure of CO_2 (pCO_2), the carbonate ion concentration (CO_3^{2-}) and the saturation states of calcite (ΩCa) and aragonite (ΩAr) were derived from probe measurements of salinity (SAL), temperature (TºC) and pH, and from total alkalinity (TA) determined by volumetric titration. Values for all carbonate species "After" media exchange were estimated by *CO2Sys* Exel macro from T_{target} levels as output parameter (since T_{target} was achieved after ca. 15 min. from the 50% media exchange). Values in bold evidence calcite/aragonite undersaturation.

Treatment		Probe measurments				Titration		CO2Sys Excel macro							
T_{target} (ºC)	pH_{target} (NBS)	SAL	TºC (ºC)	pH (NBS)		TA (µmol kg-SW^{-1})		ρCO_2 (µatm)		CO_3^{2-} (µmol kg-SW^{-1})		ΩCa		ΩAr	
				Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
16	8.1	30.1	16.6	8.16	8.16	2338	2435	442	457	150	154	3.69	3.79	2.35	2.41
	7.8	30.1	16.7	7.80	7.67	2615	2651	1240	1687	81	61	1.99	1.51	1.27	0.96
	7.5	30.0	16.5	7.57	7.36	2889	2644	2365	3548	56	31	1.38	**0.76**	**0.88**	**0.48**
18	8.1	30.2	18.7	8.09	8.11	2331	2435	533	525	139	149	3.44	3.67	2.20	2.35
	7.8	30.1	18.8	7.83	7.70	2427	2482	1096	1547	85	64	2.10	1.59	1.35	1.01
	7.5	30.0	18.5	7.58	7.35	2625	2661	2202	3745	54	32	1.34	**0.80**	**0.86**	**0.51**
20	8.1	30.3	20.9	8.05	8.09	2387	2464	628	573	142	153	3.51	3.79	2.26	2.43
	7.8	30.1	20.9	7.80	7.64	2615	2635	1285	1863	92	65	2.29	1.61	1.48	1.03
	7.5	30.0	20.7	7.59	7.35	2561	2498	2137	3622	57	32	1.43	**0.79**	**0.92**	**0.50**
22	8.1	30.3	22.2	8.15	8.12	2282	2443	454	533	170	171	4.23	4.23	2.73	2.74
	7.8	30.1	21.5	7.83	7.65	2620	2645	1196	1921	100	69	2.48	1.72	1.60	1.11
	7.5	29.9	21.7	7.67	7.35	2498	2547	1700	3828	68	34	1.68	**0.85**	1.09	**0.55**

2.3.2 Mortality

Figure 2.7 shows the cumulative mortality registered per treatment throughout the two months of exposure. At T2, mortality was proved to be driven by the pH only (Pseudo-F=8.0715, p=0.0073) with higher mortalities observed at the most acidic treatments (pH_{target} 7.5) and significantly differing from the controls (8.1: t-test=3.8953, p=0.0063) and the intermediate pH scenarios (7.8: t-test=3.852, p=0.0062). At T8, not only the pH reduction (Pseudo-F=4.2679, p=0.038) but also the increasing TºC (Pseudo-F=7.1707, p=0.0059) were found to account for higher larval mortality. The highest TºC (22ºC) was significantly different from all others contributing with highest mortalities while the difference between pH levels was only significant between 8.1 and 7.5 treatments (t-test=2.6867, p=0.0296) across temperatures. At both T14 and T28, TºC and pH interactively affected survival (T14: Pseudo-F=11.723, p=0.0002; T28: Pseudo-F=25.817, p=0.0001). At these timepoints, comparisons within the pH_{target} 8.1 showed that at 16ºC a significantly lower mortality was registered. No significant differences were observed within the acidified conditions.

Comparisons in between TºC showed significant differences between control (8.1) and the two other treatments but only at 16 and 20ºC at T14, and at 16ºC at T28.

From T34 onwards, mortality was assessed on the remaining five treatments, without replicates, and thus no statistical comparisons were possible. Four of these treatments correspond to control pH (8.1) at all TºC and only one acidic condition, pH_{target} 7.8 at 18ºC. This situation suggests that 18ºC might be the condition that best mirrors the control TºC, allowing specimens to resist to a slight reduction in pH (7.8) only at this TºC level. Even so, our data must be confirmed by further studies. Yet, mortality at T34 varied from 95.5 at 16ºC to 99.1% at 22ºC and also under pH 7.8 at 18ºC. From then, mortality stabilized under control pH and, by the end of this study (at T53 and T60) about 1% of the total larvae survived under pH 8.1, with 99.8% mortality being registered under acidity.

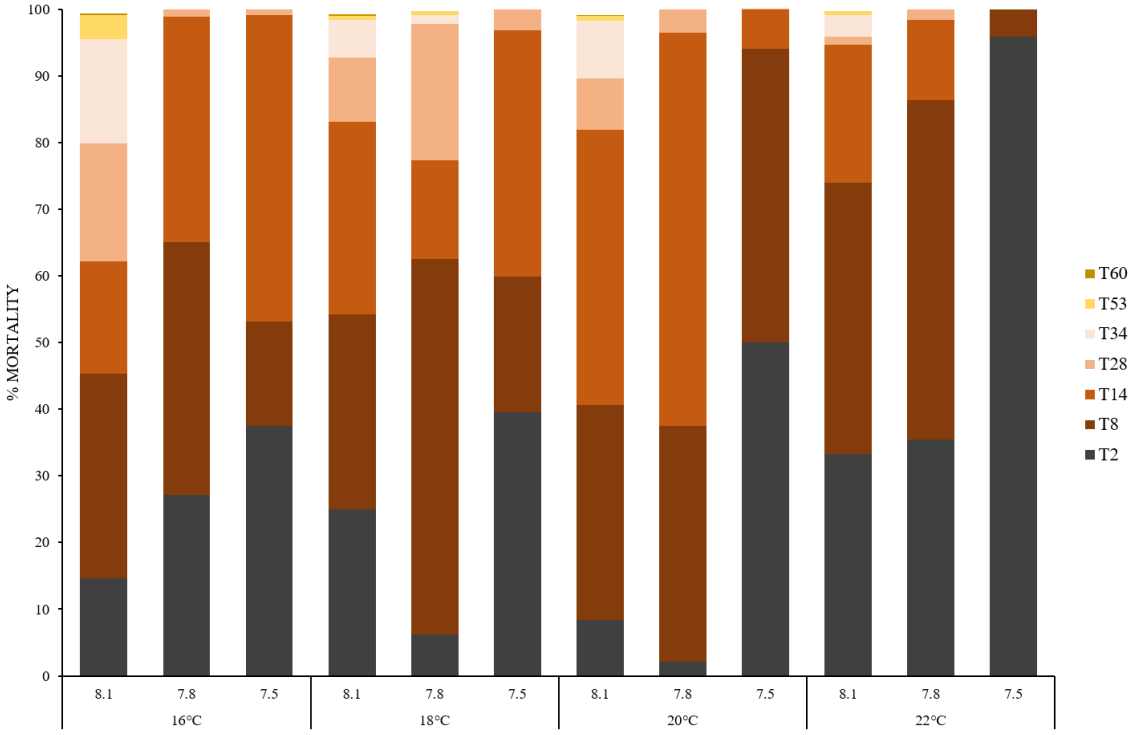

Figure 2.7 - Cumulative mortality calculated for each of the 12 OA-W scenarios throughout the 60 days of exposure at T2, T8, T14, T28, T34, T53 and T60.

Hence, *T. reticulata* survival in early life was proved to be affected by both stressors under study (pH and TºC), individually or in combination. After only two days exposure to acidification and warming, pH reduction was proved to be lethal to early veligers, especially

under the most acidic treatment: ~100% mortality was registered in both replicates at 20ºC and 100% at 22ºC. Species with an open circulatory system, such as gastropods, are known to have low capacity to maintain the acid-base balance of their extracellular fluid under acidified conditions, owing to low metabolic rates and large volumes of extracellular fluid (Pörtner, 2008). As a substantial part of the metabolic energy may be required for acid-base regulation, these animals are vulnerable to developmental delays, hindered growth, hypercapnia and even death, due to energy trade-offs (Pörtner, 2008; Sokolova *et al.*, 2012). This seems to be a plausible explanation for the extreme mortalities registered in *T. reticulata* early veligers.

At T8, T14 and T28, temperature played a significant role on survival, with 22ºC associated to higher mortality when compared with the specimens reared at 16 and 18ºC. It is widely recognized that temperature has a major influence on the development of marine invertebrate larvae, with metabolic depression being suggested at levels before and beyond the species' thermal tolerance range (Pörtner, 2008; Parker *et al.*, 2009; Zhang *et al.*, 2014) Even though, it is not possible to state here that 22ºC correspond to this species tolerance limit; however, this work shows that 22ºC cause a high stress, leading to enhanced mortality.

2.3.3 Larval culture

Development

Results on veligers' development in the larval phase, specifically from 24h post-hatching (Veliger stage I at T0) to T14, are presented in Figure 2.8-A, together with those on the larval nourishment status in Figure 2.8-B.

At T8 (Figure 2.8-A1), the majority of the larvae were still at the first stage of development (veliger I). Even so, a significant effect of both warming (Pseudo-F=4.8099, p=0.0033) and acidification (Pseudo-F=3.9199, p=0.022) at this early stage of development was registered: development was lower at 16°C (16 vs 18ºC: t-test=2.3857, p=0.0164; 16 vs 20ºC: t-test=3.1916, p=0.0017; 16 vs 22ºC: t-test=3.4464, p=0.001) and under pH 7.5 (7.5 vs 7.8: t-test=2.883, p=0.0048; 7.5 vs 8.1: t-test=2.1586, p=0.035). Also, a significant effect of the pH (Pseudo-F=3.916, p=0.0162) and, even greater, of the TºC (Pseudo-F=7.2252, p=0.0004) on veligers' nourishment was evident (Figure 2.8-B1). Approximately 20% of the larvae were not properly fed at 20 and 22ºC since no digesting food was observed inside the

larval gut. The presence of food in the digestive track did not differ significantly between larvae reared at 20 and 22ºC or between 16 and 18ºC. However, significant differences were observed between 16 vs 20ºC (t-test=3.578, p=0.0004), 16 vs 22ºC (t-test=3.2057, p=0.0007), 18 vs 20ºC (t-test=2.9732, p=0.0039) and 18 vs 22ºC (t-test=2.9433, p=0.003). Despite the pH effect, treatments 8.1 and 7.5 were not significantly different (p>0.05). Nonetheless, differences between all other treatments were statistically significant (7.5 vs 7.8: t-test=2.1351, p=0.0322 and 7.8 vs 8.1: t-test=2.7357, p=0.0061).

At T14 (Figure 2.8-A2), TºC was found to be the development single driver (Pseudo-F=12.495, p=0.0001), with the larvae at 22ºC being considerably more developed than the ones reared under all other TºC (16 vs 22ºC: t-test=5.5661, p=0.0001; 18 vs 22ºC: t-test=3.1456 p=0.0028; 20 vs 22ºC: t-test=3.653 p=0.0006). A significant difference on the larvae development reared at 16 and 18ºC was also registered (t-test=1.8402, p=0.0473). Moreover, no significant effects of any of the stressors under study (or their interaction) on the nourishment status were registered at this timepoint (Figure 2.8-B2).

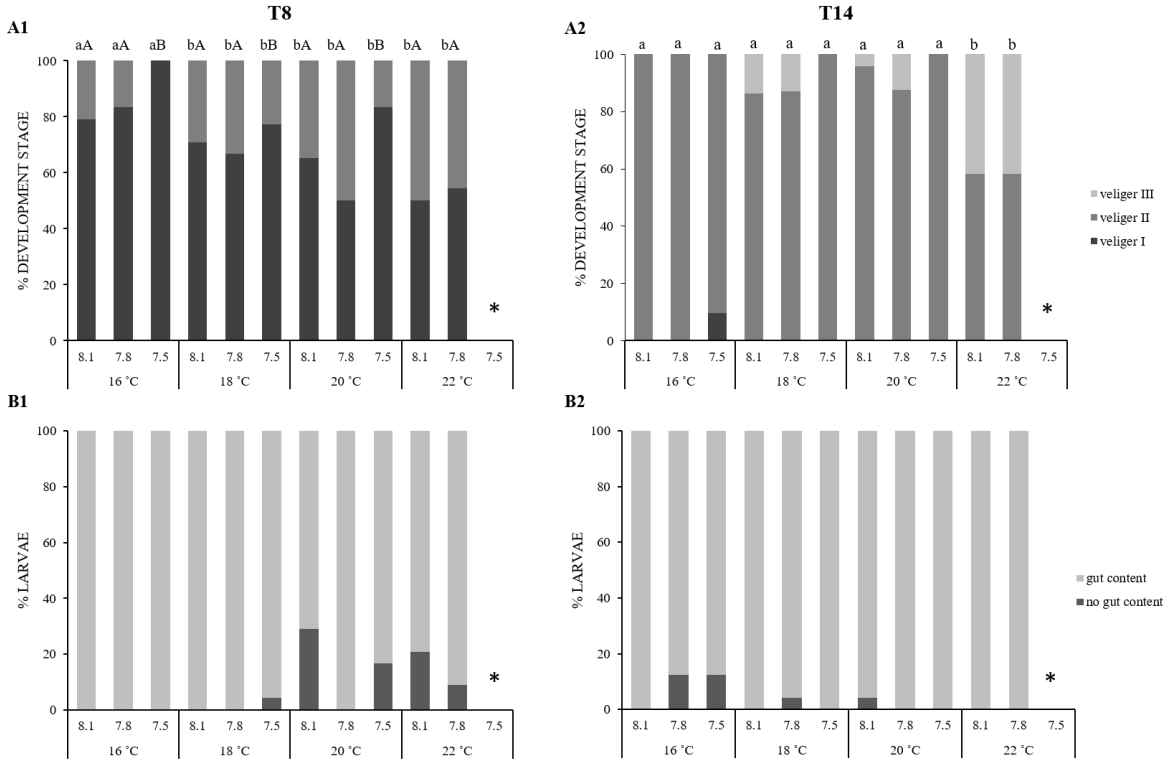

Figure 2.8 - *T. reticulata* larval culture endpoints (shown in different rows: A–Percentage of veligers at development stage I, II or III; B–Percentage of veligers with or without visible gut content) by timepoint (shown in different columns: 1–After 8 days of exposure, at T8; 2–After 14 days of exposure, at T14).*treatment without survivors (100% mortality). Upper case letters represent significant differences between pH levels, at a same temperature. Lower case letters represent significant differences between temperature levels, at a same pH.

The performance of *T. reticulata* larval development is of upmost importance since the planktonic phase is the period during which individuals develop the competence to metamorphose and settle, in order to start the benthic phase and become adults. Larval development was affected by both TºC and pH alone (and not interactively) after 8 days of exposure, although only TºC was proved to have a significant effect at T14. Temperature is known to account for optimized development and growth of marine invertebrates' larvae but, as already mentioned in the *Mortality* results section, within the thermal tolerance window (Pörtner, 2008; Byrne, 2011). For instance, Przeslawski and co-authors (2005) have also shown that temperature might have negative impacts on larval development in the early life stages of three rocky shore gastropods, slowing it down due to physiological stress. Nevertheless, even considering the beneficial effect of TºC on the overall larval performance, it is only possible if enough food is available (Zippay and Helmuth, 2012): in fact, under food limitation, ocean warming might cause death, more than acidification (Mackenzie *et al.*, 2014). However, despite our veligers were proved to be somehow malnourished at T8, specifically at TºC ≥20ºC and pH 8.1 and 7.5, it is relevant to stress that food availability was monitored during the experiment to assure it was kept *ad libitum*; so, the stress induced by higher TºC and low pH must have disturbed larval feeding ability itself. As stated by Parker *et al.* (2013), the simultaneous stress induced by ocean acidification and the low food availability (that here might be attributed to a reduced food intake by larvae rather than to microalgae unavailability) may cause "*greater disturbance in acid-base balance and mediate unfavourable trade-offs in the energy budget*" delaying development and compromising survival.

Looking at Figure 2.8-A2, the same tendency was also apparent at T14, although the results were not statistically conclusive possibly due to the extreme mortalities registered in the most acidic treatments (pH_{target} 7.5). Even so, and as expected for that stressor, it is undisputable that TºC drives larval development after 14 days under warming scenarios.

Biogenic carbonates' growth and shells' integrity

Veligers shell length (SL) was assessed at T0, T8 and T14 in order to understand how pH and temperature affect this species' growth (Figure 2.9). After 8 days of exposure a significant effect of the pH on SL was clearly observed (Pseudo-F=17.763, p=0.0001). Significantly smaller shells were recorded in the most acidic condition (7.5 vs 8.1: t-test=4.5655, p=0.0002; 7.5 vs 7.8: t-test=4.8417, p=0.0001). This was reflected on the lower growth rates of the most acidic treatments (Figure 2.9). Despite low, growth rates were positive, without obvious signs of shell dissolution at this timepoint. However, unshelled larvae were observed under the most acidic condition tested (Figure 2.10-A). These unshelled veligers were specimens actively swimming without a shell. They were first recorded at T8, only at 18 and 20ºC and in a low percentage (4%); although, a significant effect of pH was proved (Pseudo-F=2.4839, p=0.0343). Their occurrence increased at T14, being exclusively observed at pH 7.5 treatments, increasing in percentage, progressively, with increasing TºC (33.3% at 16ºC, 52.6% at 18ºC and 66.7% at 20ºC).

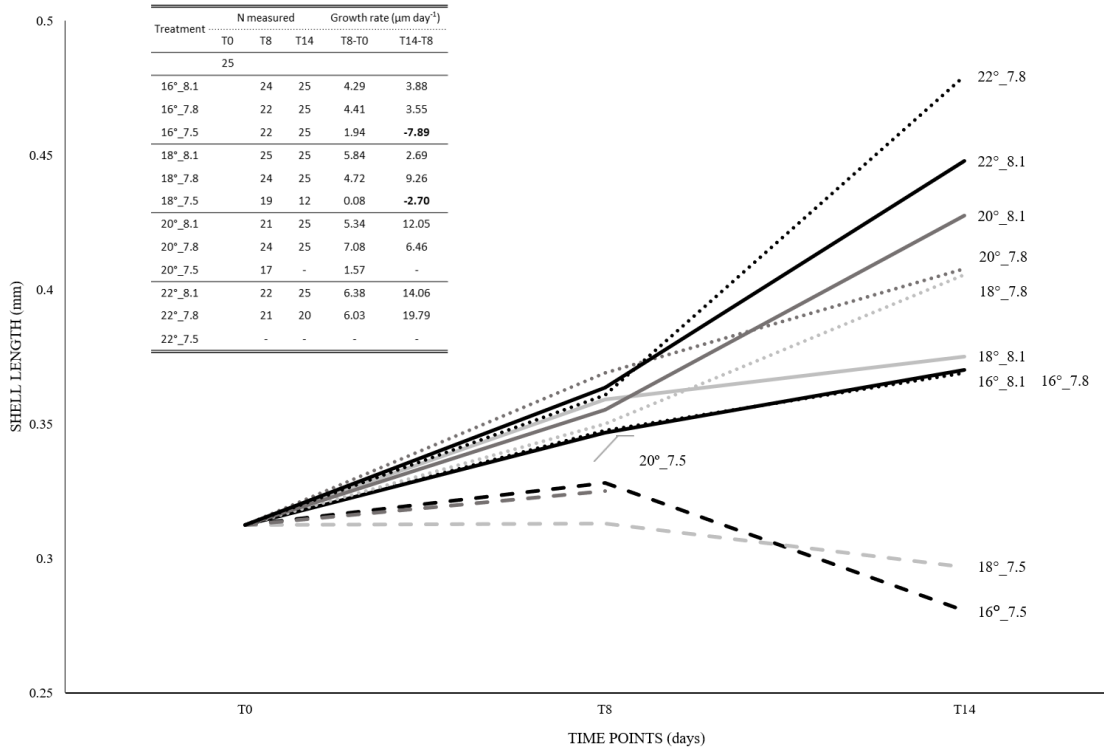

Figure 2.9 - *T. reticulata* mean shell length (SL) evolution across timepoints, namely at the beginning of the experiment (T0), after 8 (T8) and 14 (T14) days of exposure. Solid, dotted and dashed lines correspond to the pH targets 8.1 (control), 7.8 and 7.5, respectively. Temperatures (and pHs) are labelled on the right side of the graph, next to the correspondent line. The insert (table) on the upper left corner compiles the number of animals measured (N measured) per treatment and timepoint, and the growth rate (expressed in µm day^{-1}) on the first (T8-T0) and second (T14-T8) week of exposure. Bold values evidence negative growth rate (reflecting shell dissolution).

Accordingly, a strong significant effect of pH was proved at this timepoint (Pseudo-F=48.079, p=0.0001), with the most acidic treatment being significantly different from the other two (8.1 and 7.8). Even if apparent in Figure 2.10-B, the increasing trend with warming was not significant.

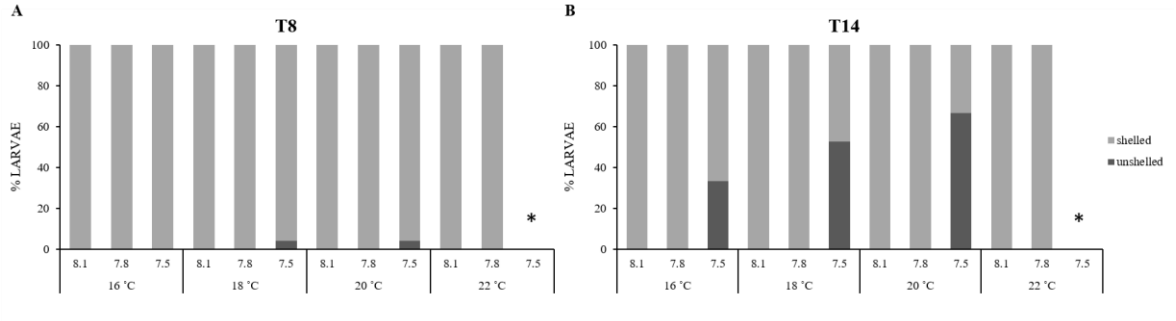

Figure 2.10 - *T. reticulata* shell loss. Percentage of veligers with or without shell (shelled or unshelled, respectively). A – after 8 days of exposure (T8, left plot). B – after 14 days of exposure (T14, right plot).

It is also important to note that shell loss was not due to an abnormal secretion, or even non-secretion, of the shell as reported previously for abalone species (Byrne *et al.*, 2011; Guo *et al.*, 2015; Wessel *et al.*, 2018), nor to their complete dissolution as in pteropods (Comeau *et al.*, 2010). Instead, we observed the literal shell loss, with veligers swimming while their shells were empty on the bottom of the tanks. To confirm this unusual observation, a subsequent experiment in which veligers were individualized in 24-well plates and exposed to pH~7.5 was performed, and shell loss occurred again (Oliveira *et al.*, 2018). This was possibly the most striking result of the current work, which is still under study. Yet, although shell loss under pH 7.5 was not due to dissolution, this effect was also verified.

In fact, negative growth rates were observed at the existing 7.5 treatments (at 16 and 18ºC) between T8 and T14 (Figure 2.9), suggesting significant shell dissolution after only 14 days of exposure. Moreover, a significant interaction of TºC and pH on SL was observed at T14 (Pseudo-F=2.9759, p=0.0201) with animals increasing in size with warming, and diminishing under acidification. Comparisons within the same pH showed that under control pH (8.1), only the two most extreme treatments were not significant in-between them, namely 16 vs 18ºC and 20 vs 22ºC. All other were statistically significant (16 vs 20ºC: t-test=5.1114, p=0.0001; 16 vs 22ºC: t-test=6.744, p=0.0001; 18 vs 20ºC: t-test=4.6456, p=0.0002; 18 vs 22ºC: t-test=6.2894, p=0.0001). Within pH 7.8, SL means differed

significantly between treatments with the single exception of 18 vs 20ºC, and no significant differences were observed within the two existing treatments from pH 7.5.

The growth of another biogenic carbonate was also analysed in the current work: the statolith. The mean diameter of the statoliths at T0 was 18.1±1.7µm (dashed line in Figure 2.11) and the statolith diameter (StD) measured in larvae preserved at T14 is showed per treatment in Figure 2.11. Similarly to that described for shells, an effect of the interaction between TºC and pH on StD was statistically proved (Pseudo-F=5.5336, p=0.0003). However, results are difficult to explain as no clear pattern was registered. Even so, TºC seems to boost statolith growth: comparisons within the same pH showed that under control pH (8.1), only the two most extreme treatments were not significant in-between them, namely 16 vs 18ºC and 20 vs 22ºC (all others were statistically significant – 16 vs 20°C: t-test= 4.1065, p=0.0004; 16 vs 22°C: t-test= 5.2822, p=0.0001; 18 vs 20°C: t-test=5.978, p=0.0001; 18 vs 22°C: t-test=7.5179, p=0.0001). At pH 7.8, StD means differed significantly between treatments, with the single exception of 18 vs 20ºC. Unexpectedly, this intermediate acidification level seems to favour statolith growth, with significant differences recorded between 7.8 and the other two pH levels at 18ºC (8.1 vs 7.8: t-test= 4.1172, p=0.0005 ; 7.8 vs 7.5 : t-test= 3.1152, p=0.0024) and with pH 8.1 at 22º C (t-test= 2.1612, p=0.0334).

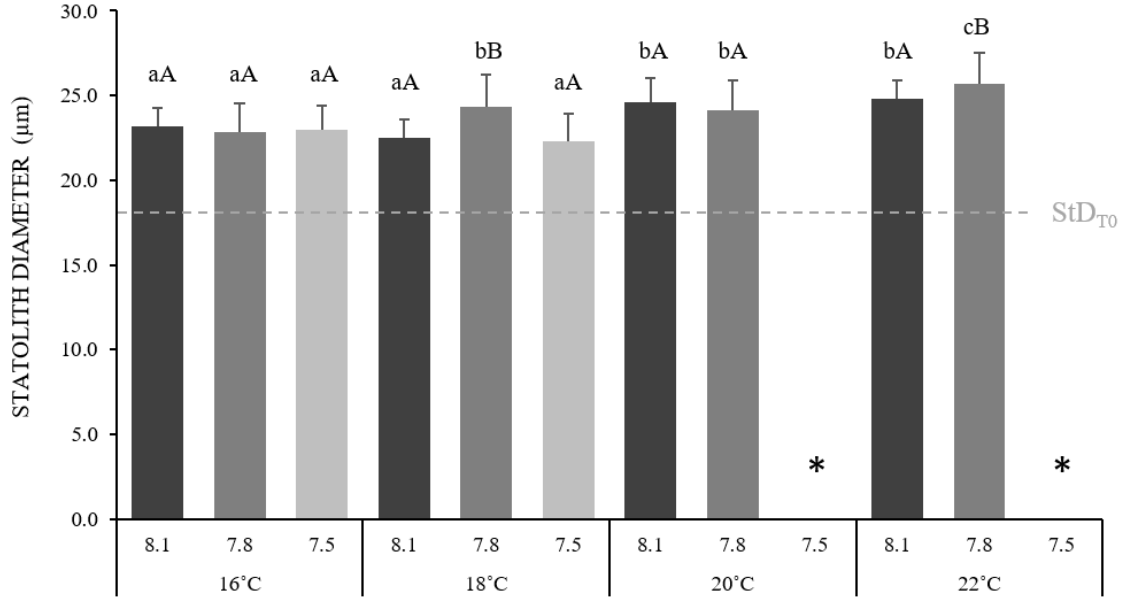

Figure 2.11 - *T. reticulata* statolith diameter (StD) at 14 days of exposure, T14. Upper case letters represent significant differences between pH levels, at a same temperature. Lower case letters represent significant differences between temperatures, at a same pH level. The dashed line crosses the vertical axis at the mean statolith diameter measured in samples preserved at T0 (StDT0). *no available sample due to high mortality at T14.

Furthermore, no significant differences were registered between the two existing treatments at pH_{target} 7.5.

Even though shell dissolution was recorded under the most acidic treatments, this was not the case for the statoliths, whose diameter under pH 7.5 was maintained when compared to pH 8.1. This result was expected since statoliths are internal carbonates, isolated from the exterior and subject to physiological control (Winans and Purcell, 2010), being protected from the direct exposure to undersaturated exposure medium. However, reduced statolith size under OA was not registered in the current work, as it was by Manríquez *et al.* (2014) for the early life stages of the gastropod *Concholepas concholepas* (Bruguière, 1789). By now we can only speculate that it was not confirmed in *T. reticulata* since no samples were available for measurements under pH 7.5 at 20 and 22ºC.

Shell dissolution was then confirmed under SEM (Figure 2.12). Increased porosity corresponding to the type I dissolution, described by Bednaršek *et al.* (2012), was observed

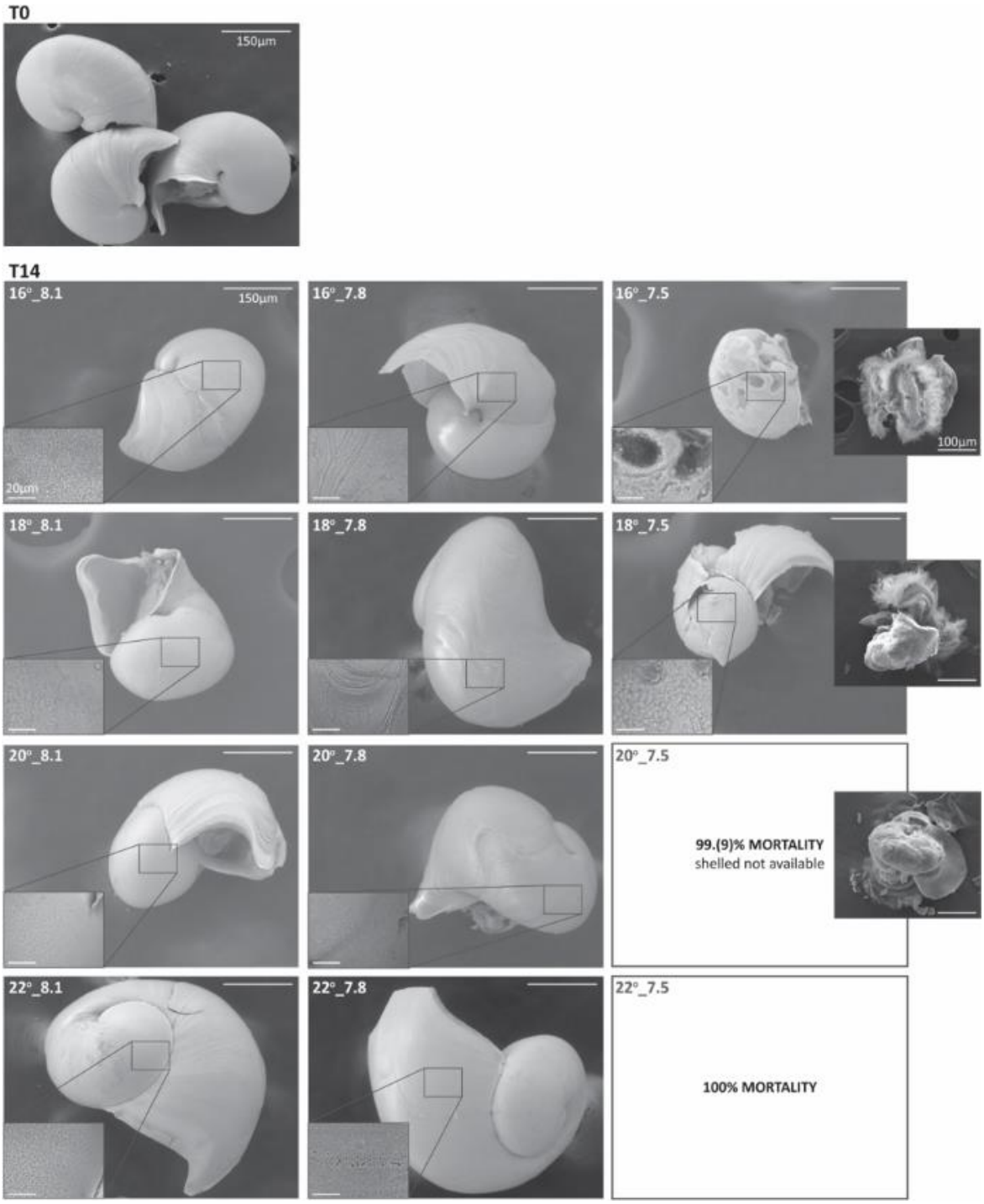

Figure 2.12 - *T. reticulata* larvae under Scanning Electron Microscopy (SEM). Aspect of the veligers' shell collected at T0 (top image) and at T14 after exposure to 12 OA-W experimental scenarios (indicated at the top left of each image; scale bar = 150µm). Inserts in the lower left corner of each image are shells' surface at 1000x magnification (scale bar = 20µm). Pictures of unshelled veligers after critical point drying, detected only at pH~7.5 scenarios, were kindly provided by the colleague Mariana Hinzmann, and are shown overlaid on the right side of the image of the respective treatment (scale bar = 100µm).

under pH 7.8; severe dissolution, of types II and III, were evident in shells built at pH 7.5 were evident. Under undersaturation (pH 7.5), extensive damage was observed: surfaces were covered by numerous dissolved patches, compromising shell integrity and making it extremely fragile.

Shells' structure is directly related with its mineralogy and crystallinity (Marin *et al.*, 2012). It is generally accepted that amorphous calcium carbonate (ACC) is the first mineral polymorph deposited by mollusc veligers (Weiss *et al.*, 2002), followed by aragonite and calcite. It is also known that along these $CaCO_3$ polymorphs, the matrix stability and robustness increase (Galante-Oliveira *et al.*, 2014). Shells' mineralogy and crystallinity was assessed by comparing the standard Raman spectra of biogenic ACC, aragonite and calcite (Figure 2.13) with those acquired from samples preserved at T0 (Figure 2.14) and at T14 (Figure 2.15).

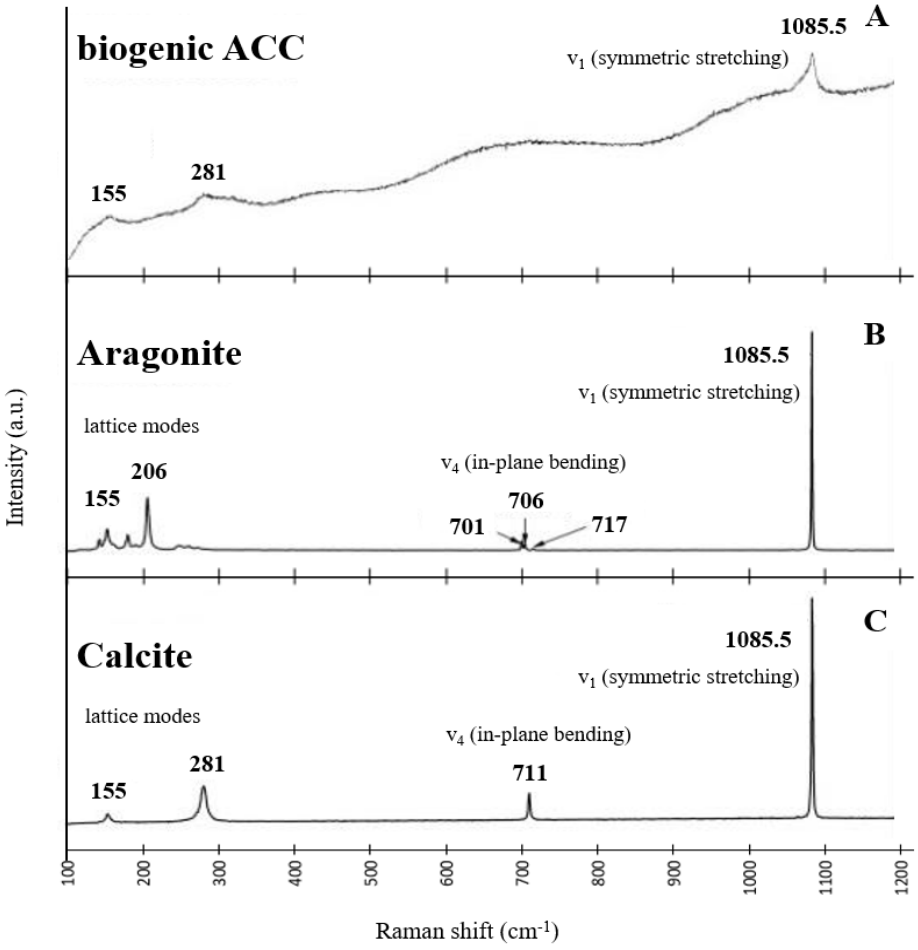

Figure 2.13 - A – Raman spectra of biogenic amorphous calcium carbonate (ACC). B – standard Raman spectra of aragonite. C – standard Raman spectra of calcite. Adapted from Wehrmeister *et al.* (2011)

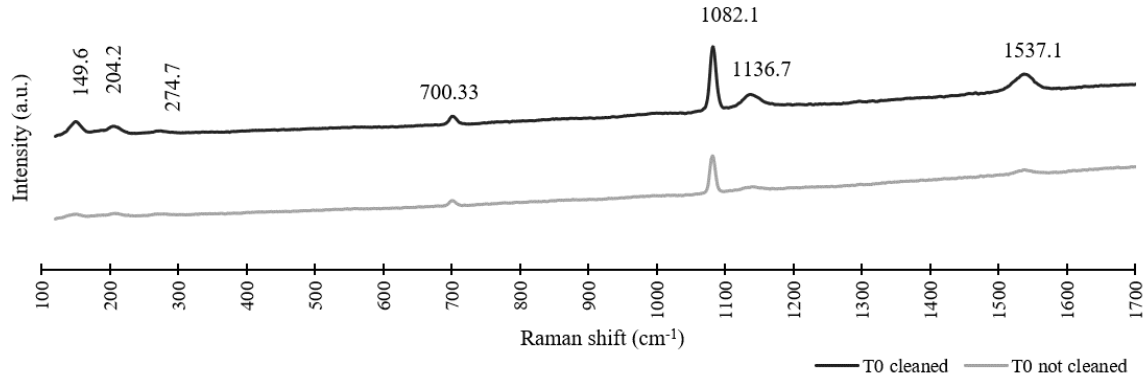

Figure 2.14 - Typical micro-Raman spectra acquired from *Tritia reticulata* larval shells collected at T0. The upper spectrum was acquired from a surface that was digested/cleaned and dried (T0 cleaned). The lower spectrum was acquired from a surface not subject to the cleaning and drying process (T0 not cleaned). The values above the spectrum are the Raman shift value at each visible peak.

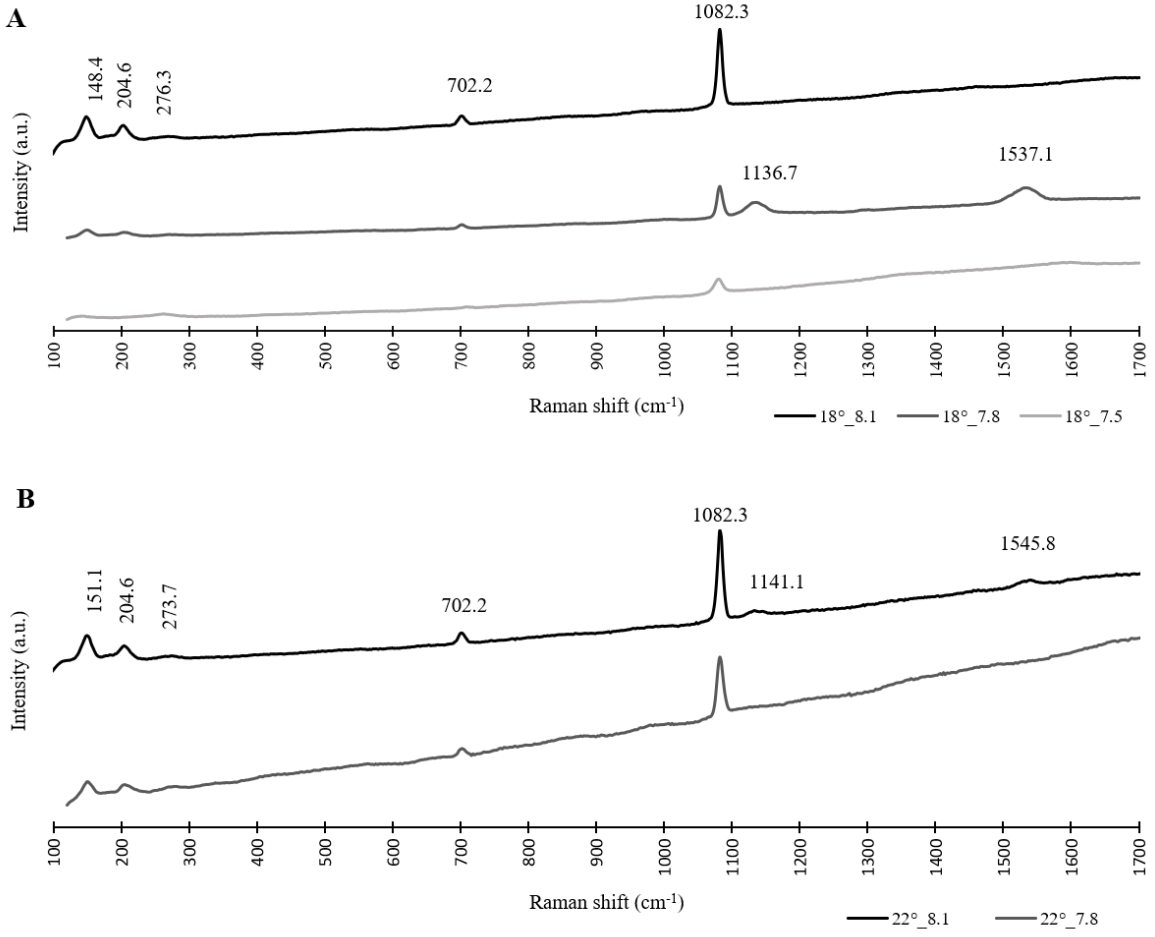

Figure 2.15 - Typical micro-Raman spectra acquired from *Tritia reticulata* larval shell collected at T14. A –Spectra acquired from shells from specimens reared at 18ºC under different acidified treatments (8.1, 7.8 and 7.5). B –Spectra acquired from shells from specimens reared at 22ºC under different acidified treatments (8.1and 7.8). The legend above each plot indicate the correspondence between the line colour and the different pH conditions. The values above the spectrum are the Raman shift value at each visible peak.

Generally, the most intense and characteristic band in the $CaCO_3$ Raman spectrum is the symmetric CO_3 stretching vibration (mode ν_1) at around 1085.5 cm^{-1} (Wehrmeister *et al.*, 2011). Moreover, if present, the above referred $CaCO_3$ polymorphs are characterized by the following additional high intensity peaks: (1) the biogenic ACC at 155cm^{-1} and 281cm^{-1}; (2) the aragonite at 155cm^{-1}, 206cm^{-1} and three distinct signals between 700 and 720cm^{-1}; and (3) the calcite at 155 cm^{-1}, 281cm^{-1} and 711cm^{-1} (Figure 2.13).

At first, the typical spectra acquired from T0 samples (Figure 2.14) proved the carbonate nature of *T. reticulata* veliger shell (possibly protoconch II since T0 was 24h post-hatching) since the sharpest and highest peak in both spectra is the mode ν_1 (1082.1 cm^{-1}). Moreover, in the same figure are shown spectra acquired from samples that were and were not subject to the H_2O_2 digestion, proving that (1) spectra have the same structure, and so cleaning does not seems to alter the sample itself, and (2) the cleaning procedure allows the exposure of the crystalline matrix since more defined and intense peaks are revealed.

The spectra acquired from samples collected at T14 (Figure 2.15) generally confirm the carbonate nature of the samples (sharpest and highest peak at around 1082 cm^{-1}). Moreover, all spectra revealed the same crystalline composition, with three distinct peaks between 100 and 300cm^{-1} and a single one at ~702cm^{-1}. These signals are indicative of the presence of both aragonite and calcite. The formation of the mollusc shell is described in Chapter 1 (*General introduction*) and based on Weiss *et al.* (2002), ACC acts as a precursor for the crystalline polymorph aragonite. The presence of calcite on larval shells are not so common (Eyster, 1986; Jardillier *et al.*, 2008; Auzoux-Bordenave *et al.*, 2010); even so, Carter and Clark (1985) suggested that both crystalline polymorphs –aragonite and calcite– could coexist in the prismatic layer. As a single peak at ~702cm^{-1} was found in all our MRS spectra, here we report admixed fractions of aragonite and calcite in *T. reticulata* veliger shell.

Additionally, other two signals were detected in some of the spectra: ~1136.7cm^{-1} and ~1537.1cm^{-1} (Figure 2.14 and 2.15). According to Komura *et al*. (2018), these peaks are related with nonmethylated polyenes associated to yellow pigments in gastropod shells. We suggest that this might be also the case in *T. reticulata* larval shells that are, in fact, yellowish. These peaks were not present in some of the spectra, possibly due to a non-homogenous shell pigmentation.

A very important result is related to the decreased crystallinity perceived by comparing the gradual reduction in the intensity of the Raman vibrational bands between spectra of decreasing pH (8.1>7.8>7.5) for a given TºC. Analysing the typical spectra in Figure 2.15-A, the intensity of the sharp, well-resolved peaks on the pH 8.1-spectrum decrease progressively in intensity until only broad peaks, typical of biogenic AAC, are the only ones present in the pH 7.5-spectrum. This corroborates the results on the increased fragility and the dissolution of the shells built under pH 7.5 for 14 days (i.e., at T14). Due to the higher solubility of ACC (when compared with the crystalline polymorphs), the organisms that use ACC as a precursor to aragonite and/or calcite are thought to have the final product of their shells' mineralization compromised by acidification (Ries, 2011). Hence, dissolution rate was possibly faster than shells' repair under pH 7.5 since our veligers were not able to move towards a more stable $CaCO_3$ polymorph.

2.3.4 Settlement and post-settlement period

Settlement

Settled juveniles were firstly observed at T28. The percentage of settled individuals from then until 100% was reached in all treatments at T53 as shown in Figure 2.16.

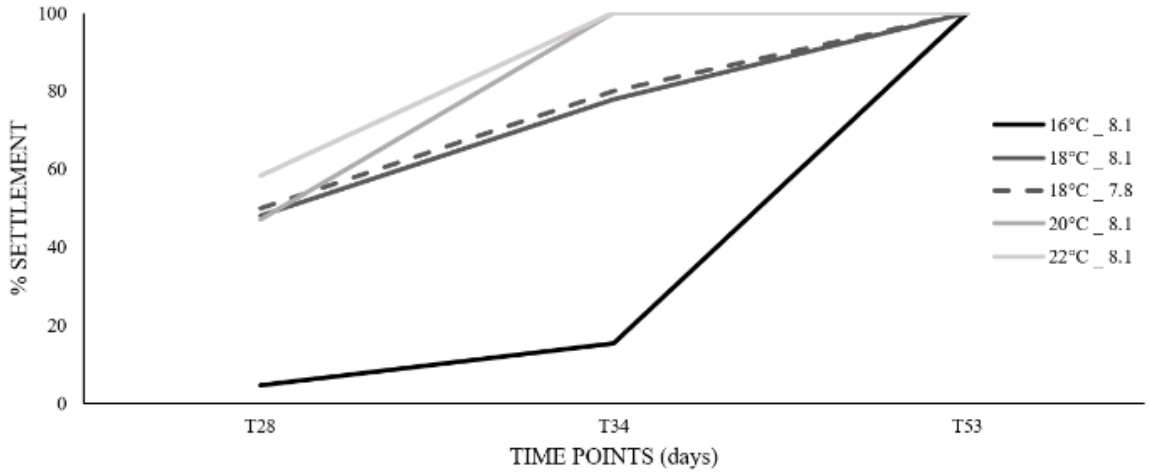

Figure 2.16 - Percentage of settled *T. reticulata* from settlement first record at T28 until 100% was reached at T53. Solid and dashed lines correspond to the pH targets 8.1 (control) and 7.8, respectively. Temperatures (and pHs) are labelled on the right side of the graph

Even though statistical testing was not performed at T28, due to the lack of replication in some of the treatments (8.1_22ºC), it seems graphically obvious a delayed settlement at 16ºC. Only 5 treatments with no replication subsisted from T28 onwards (pH 8.1 at the four different TºC, and a single acidified treatment, pH 7.8 at the most adequate control TºC, 18ºC). Settlement percentage seems to be higher at higher temperatures, achieving 100% at T34 for 20 and 22ºC, and only at T53 for 18 and 16ºC. These results agree with the already referred faster development at higher TºC, and also with several other studies that report favoured development by warming; accordingly, as organisms under warming develop faster, they will settle earlier than at lower temperatures (Byrne, 2011; Byrne *et al.*, 2011).

Shell growth and integrity

After two months of exposure, the SL of the juveniles reared under pH 8.1 at 16, 18, 20 and 22ºC was 1.70±0.39mm, 1.73±0.40mm, 1.94±0.63mm and 1.83±0.31mm, respectively, and under pH 7.8 at 18ºC was 1.43±0.33mm. No significant differences in juvenile mean SL between the different treatments were found.

Juvenile shell integrity was studied by SEM in specimens exposed to control and acidic pH (8.1 and 7.8, respectively) at 18°C. Pictures are compiled in Figure 2.17.

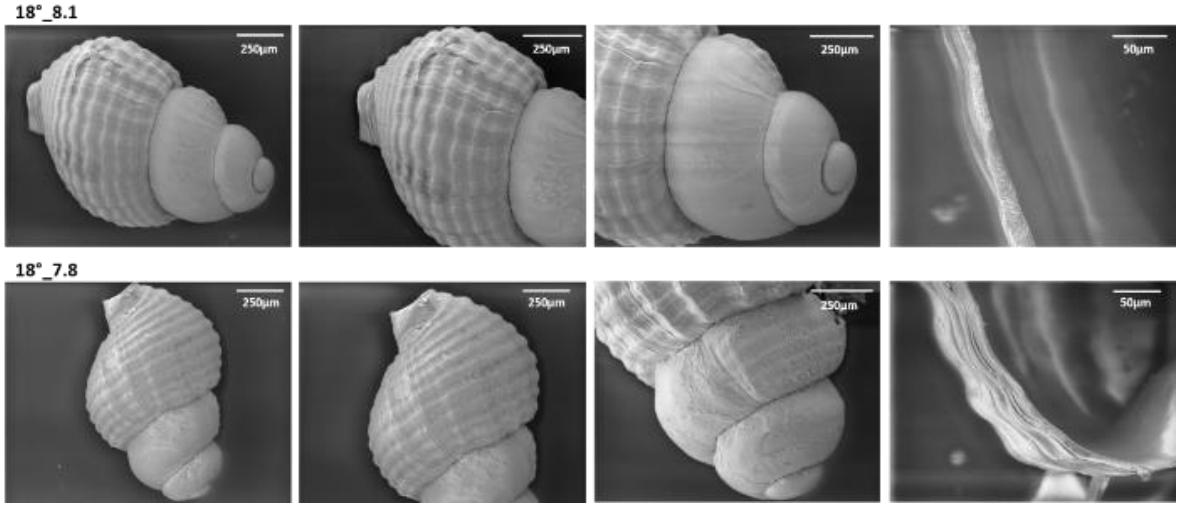

Figure 2.17 - *T. reticulata* juveniles under Scanning Electron Microscopy (SEM). Aspect of the shell of the juveniles collected after two months of exposure to the OA-W experimental scenarios indicated above each line of pictures. From left to right, pictures are of the whole animal, the juvenile shell, the larval shell and the shell growing edge.

T. reticulata larval and juvenile shells are discerned by the difference in the ornamental pattern: juvenile shell is reticulate (Nützel, 2014). Juvenile shells were apparently intact, with no signs of dissolution even under acidity (pH 7.8). However, the larval shell exhibit the type I dissolution of Bednaršek *et al.* (2012) as already mentioned above in the *Larval culture* results section. Similar results were also observed in *Nucella lapillus* (Linnaeus, 1758) juveniles (Rühl *et al.*, 2017). Different degrees of shell damage between life stages may be due to differences in the shell composition: it was already described that $CaCO_3$ shells vary on composition during development, becoming more crystalline (Jardillier *et al.*, 2008). Going back to the Raman spectrum acquired from shells built at 18ºC under pH 8.1 and 7.8 (Figure 2.14-A), larval shells were proved to be of both calcite and aragonite, but less crystalline under pH 7.8 than under 8.1.

When the teleoconch is formed, the calcifying regime changes (Marin *et al.*, 2012) and the juvenile shell becomes more robust. However, even though the external surface of the juvenile shell does not appear to lack any integrity change, the growing edge of the shells built under pH 7.8 reveal lower density than the compact edge at pH 8.1 (Figure 2.17). This less dense edge should confer less resistance to the juvenile shell compromising its integrity even at this intermediate acidified scenario.

2.4 Conclusion

This study proves the extreme vulnerability of *T. reticulata* early life stages to projected OA-W. All the studied endpoints were significantly affected by either each stressor individually or interactively: (1) survival was affected by both pH and TºC in a synergistic effect, since warming and acidification were proved to increase mortality; (2) larvae development was accelerated by warming, causing premature settlement relatively to control TºC, being delayed by acidification in an antagonistic effect of both stressors involved; (3) pH and TºC also seem to have an antagonistic effect on larval growth, with larger shells at warmer treatments but smaller, dissolved and chemically soluble shells under acidity; (4) statolith growth followed shell growth but no dissolution was verified; (5) besides shell dissolution, shell loss under aragonite undersaturation was recorded; and (6) the only specimens surviving to until settlement were those reared under pH control and under intermediate acidity under control temperature (18ºC); (7) after settlement, growth and survival stabilized

under pH control but continued to rise under acidity; and (8) less dense shells were recorded in juveniles reared under the only available acidic scenario.

Critical impacts of the OA-W experimental scenarios tested were evident, particularly on the planktonic stages, lessening considerably the chances of larvae to survive in nature. The negative effects documented here, especially those occurring at the most extreme scenarios –lowest pH (7.5) and highest temperature (22ºC)– that correspond to IPCC projections to the end of the current century, were proved to prevent this species survival only after a week of exposure. Even considering that adaptive processes to OA-W cannot be disregarded, this study shows the extreme vulnerability of *T. reticulata* larvae to the concomitant effects of these phenomena. Our results highlight the risk of climate change-induced OA-W to marine gastropods and calls for the urgency of the mitigation actions towards biodiversity protection.

References

Allen, M. R., Dube, O. P., Solecki, W., Aragón-Durand, F., Cramer, W., Humphreys, S., Kainuma, M., Kala, J., Mahowald, N., Mulugetta, Y., Perez, R., Wairiu, M. and Zickfeld, K. (2018) 'Framing and Context', in *Global Warming of 1.5°C. An IPCC Special Report on the impacts of global warming of 1.5°C above pre-industrial levels and related global greenhouse gas emission pathways, in the context of strengthening the global response to the threat of climate change, sustainable development, and efforts to eradicate poverty* [Masson-Delmotte, V., P. Zhai, H.-O. Pörtner, D. Roberts, J. Skea, P.R. Shukla, A. Pirani, W. Moufouma-Okia, C. Péan, R. Pidcock, S. Connors, J.B.R. Matthews, Y. Chen, X. Zhou, M.I. Gomis, E. Lonnoy, T. Maycock, M. Tignor, and T. Waterfield (eds.)]. In Press, pp. 49-91.

Auzoux-Bordenave, S., Badou, A., Gaume, B., Berland, S., Helléouet, M. N., Milet, C. and Huchette, S. (2010) 'Ultrastructure, chemistry and mineralogy of the growing shell of the European abalone *Haliotis tuberculata*', *Journal of Structural Biology*, 171(3), pp. 277–290. doi: 10.1016/j.jsb.2010.05.012.

Barroso, C. M. and Moreira, M. H. (1998) 'Reproductive Cycle of *Nassarius reticulatus* in the Ria De Aveiro, Portugal: Implications for Imposex Studies', *Journal of the Marine Biological Association of the United Kingdom*, 78(4), pp. 1233–1246. doi: 10.1017/s0025315400044453.

Bednaršek, N., Tarling, G. A., Bakker, D. C. E., Fielding, S., Cohen, A., Kuzirian, A., Mccorkle, D., Lézé, B. and Montagna, R. (2012) 'Description and quantification of pteropod shell dissolution: A sensitive bioindicator of ocean acidification', *Global Change Biology*, 18(7), pp. 2378–2388. doi: 10.1111/j.1365-2486.2012.02668.x.

Byrne, M. (2011) 'Impact of ocean warming and ocean acidification on marine invertebrate life history stages: vulnerabilities and potential for persistence in a changing ocean', *Oceanography and Marine Biology: An Annual Review*, 49, pp. 1–42.

Byrne, M., Ho, M., Wong, E., Soars, N. A., Selvakumaraswamy, P., Shepard-Brennand, H., Dworjanyn, S. A. and Davis, A. R. (2011) 'Unshelled abalone and corrupted urchins: Development of marine calcifiers in a Changing Ocean', *Proceedings of the Royal Society B: Biological Sciences*, 278(1716), pp. 2376–2383. doi: 10.1098/rspb.2010.2404.

Carter, J. G. and Clark, G. R. (1985) 'Classification and Phylogenetic Significance of Molluscan Shell Microstructure', *Studies in Geology, Notes for a Short Course*, 13, pp. 50–71. doi: 10.1017/s0271164800001093.

Chatzinikolaou, E. and Richardson, C. A. (2007) 'Evaluating growth and age of netted whelk *Nassarius reticulatus* (Gastropoda: Nassariidae) using statolith growth rings', *Marine Ecology Progress Series*, 342, pp. 163–176. doi: 10.3354/meps342163.

Ciais, P., Sabine, C., Bala, G., Bopp, L., Brovkin, V., Canadell, J., Chhabra, A., DeFries, R., Galloway, J.,

Heimann, M., Jones, C., Quéré, C. Le, Myneni, R. B., Piao, S. and Thornton, P. (2013) 'Carbon and Other Biogeochemical Cycles', in *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)].Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press, pp. 465-570.

Coelho, F. J. R. C., Rocha, R. J. M., Pires, A. C. C., Ladeiro, B., Castanheira, J. M., Costa, R., Almeida, A., Cunha, Â., Lillebø, A. I., Ribeiro, R., Pereira, R., Lopes, I., Marques, C., Moreira-Santos, M., Calado, R., Cleary, D. F. R. and Gomes, N. C. M. (2013) 'Development and validation of an experimental life support system for assessing the effects of global climate change and environmental contamination on estuarine and coastal marine benthic communities', *Global Change Biology*, 19(8), pp. 2584–2595. doi: 10.1111/gcb.12227.

Collins, M., Knutti, R., Arblaster, J., Dufresne, J., Fichefet, T., Friedlingstein, P., Gao, X., Gutowski, W., Johns, T., Krinner, G., Shongwe, M., Tebaldi, C., Weaver, A. and Wehner, M. (2013) 'Long-term Climate Change: Projections, Commitments and Irreversibility', in *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)]. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press, pp. 1029-1136.

Comeau, S., Gorsky, G., Alliouane, S. and Gattuso, J. P. (2010) 'Larvae of the pteropod *Cavolinia inflexa* exposed to aragonite undersaturation are viable but shell-less', *Marine Biology*, 157(10), pp. 2341–2345. doi: 10.1007/s00227-010-1493-6.

Cubasch, U., Wuebbles, D., Chen, D., Facchini, M. C., Frame, D., Mahowald, N. and Winther, J.-G. (2013) 'Introdution', in *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)]. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press, pp. 119-158.

Dickson, A. G. (1990) 'Standard potential of the reaction: $AgCl(s) + 1\ 2H_2(g) = Ag(s) + HCl(aq)$, and and the standard acidity constant of the ion HSO_4^- in synthetic sea water from 273.15 to 318.15 K', *The Journal of Chemical Thermodynamics*, 22(2), pp. 113–127. doi: 10.1016/0021-9614(90)90074-Z.

Dickson, A. G. and Millero, F. J. (1987) 'A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media', *Deep Sea Research Part A, Oceanographic Research Papers*, 34(10), pp. 1733–1743. doi: 10.1016/0198-0149(87)90021-5.

Eyster, L. S. (1986) 'Shell inorganic composition and onset of shell mineralization during bivalve and gastropodn embryogenesis', *The Biological Bulletin*, 170(2), pp. 211–231. doi: 10.2307/1541804.

Fretter, V. and Graham, A. (1994) *British prosobranch molluscs : their functional anatomy and ecology*. London, UK: British Ray Society, pp. 820.

Frommlet, J. C., Sousa, M. L., Alves, A., Vieira, S. I., Suggett, D. J. and Serôdio, J. (2015) 'Coral symbiotic algae calcify ex hospite in partnership with bacteria', *Proceedings of the National Academy of Sciences*, 112(19), pp. 6158–6163. doi: 10.1073/pnas.1420991112.

Galante-Oliveira, S., Marçal, R., Ribas, F., Machado, J. and Barroso, C. (2013) 'Studies on the morphology and growth of statoliths in Caenogastropoda', *Journal of Molluscan Studies*, 79(4), pp. 340–345. doi: 10.1093/mollus/eyt028.

Galante-Oliveira, S., Marçal, R., Guimarães, F., Soares, J., Lopes, J. C., Machado, J. and Barroso, C. (2014) 'Crystallinity and microchemistry of *Nassarius reticulatus* (Caenogastropoda) statoliths: Towards their structure stability and homogeneity', *Journal of Structural Biology*, 186(2), pp. 292–301. doi: 10.1016/j.jsb.2014.03.023.

Génio, L., Sousa, A., Vaz, N., Dias, J. M. and Barroso, C. (2008) 'Effect of low salinity on the survival of recently hatched veliger of *Nassarius reticulatus* (L.) in estuarine habitats: A case study of Ria de Aveiro', *Journal of Sea Research*, 59(3), pp. 133–143. doi: 10.1016/j.seares.2007.09.001.

Guo, X., Huang, M., Pu, F., You, W. and Ke, C. (2015) 'Effects of ocean acidification caused by rising CO_2 on the early development of three mollusks', *Aquatic Biology*, 23(2), pp. 147–157. doi: 10.3354/ab00615.

IPCC (2013) *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)]. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press, pp. 1535.

IPCC (2014) *Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)]. Geneva, Switzerland: IPCC, pp. 151.

Jardillier, E., Rousseau, M., Gendron-Badou, A., Fröhlich, F., Smith, D. C., Martin, M., Helléouet, M. N., Huchette, S., Doumenc, D. and Auzoux-Bordenave, S. (2008) 'A morphological and structural study of the larval shell from the abalone *Haliotis tuberculata*', *Marine Biology*, 154(4), pp. 735–744. doi: 10.1007/s00227-008-0966-3.

King, N., Bailey, D. and Priede, I. (2007) 'Role of scavengers in marine ecosystems', *Marine Ecology Progress Series*, 350, pp. 175–178. doi: 10.3354/meps07186.

Kirtman, B., Power, S. B., Adedoyin, J. A., Boer, G. J., Bojariu, R., Camilloni, I., Doblas-Reyes, F. J., Fiore, A. M., Kimoto, M., Meehl, G. A., Prather, M., Sarr, A., Schär, C., Sutton, R., G.J. van Oldenborgh, Vecchi, G. and Wang, H. J. (2013) 'Near-term Climate Change: Projections and Predictability', in

Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)]. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press, pp. 953-1028.

Ko, G. W. K., Vera, C. B. S., R, D., Dennis, C. K. S., Adela, L. J., Yu, Z. and Thiyagarajan, V. (2013) 'Larval and Post-Larval Stages of Pacific Oyster (*Crassostrea gigas*) Are Resistant to Elevated CO_2', *PLoS ONE*, 8(5), p. e64147. doi: 10.1371/journal.pone.0064147.

Komura, T., Kagi, H., Ishikawa, M., Yasui, M. and Sasaki, T. (2018) 'Spectroscopic Investigation of Shell Pigments from the Family Neritidae (Mollusca: Gastropoda)', in *Biomineralization* [Endo, K., Kogure, T., Nagasawa, H. (eds.)]. Singapore, China: Springer Nature Singapore. pp. 73–82.

Kroeker, K. J., Kordas, R. L., Crim, R., Hendriks, I. E., Ramajo, L., Singh, G. S., Duarte, C. M. and Gattuso, J.-P. (2013) 'Impacts of ocean acidification on marine organisms: quantifying sensitivities and interaction with warming', *Global Change Biology*, 19(6), pp. 1884–1896. doi: 10.1111/gcb.12179.

Lambeck, R. H. D. (1984) 'Dynamics, migration and growth of *Nassarius reticulatus* (Mollusca: Prosobranchia) colonizing saline Lake Grevelingen (SW Netherlands)', *Netherlands Journal of Sea Research*, pp. 395–417. doi: 10.1016/0077-7579(84)90012-7.

Levi, R., Varona, P., Arshavsky, Y. I., Rabinovich, M. I. and Selverston, A. I. (2004) 'Dual Sensory-Motor Function for A Molluskan Statocyst Network', *Journal of Neurophysiology*, 91(1), pp. 336–345. doi: 10.1152/jn.00753.2003.

Lischka, S., Büdenbender, J., Boxhammer, T. and Riebesell, U. (2011) 'Impact of ocean acidification and elevated temperatures on early juveniles of the polar shelled pteropod *Limacina helicina*: mortality, shell degradation, and shell growth', *Biogeosciences*, 8(4), pp. 919–932. doi: 10.5194/bg-8-919-2011.

Mackenzie, C. L., Ormondroyd, G. A., Curling, S. F., Ball, R. J., Whiteley, N. M. and Malham, S. K. (2014) 'Ocean warming, more than acidification, reduces shell strength in a commercial shellfish species during food limitation', *PLoS ONE*, 9(1). doi: 10.1371/journal.pone.0086764.

Manríquez, P. H., Jara, M. E., Torres, R., Mardones, M. L., Lagos, N. A., Lardies, M. A., Vargas, C. A., Duarte, C. and Navarro, J. M. (2014) 'Effects of ocean acidification on larval development and early post-hatching traits in *Concholepas concholepas* (loco)', *Marine Ecology Progress Series*. Inter-Research, 514, pp. 87–103. doi: 10.3354/meps10951.

Marin, F., Le Roy, N. and Marie, B. (2012) 'The formation and mineralization of mollusk shell', *Frontiers in Bioscience - Scholar*, 4(3), pp. 1099–1125. doi: 10.2741/s321.

Mehrbach, C., Culberson, C., Hawley, J. and Pytkowicx, R. (1973) 'Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure', *Limnology and Oceanography*, 18(6), pp. 897–907. doi: 10.4319/lo.1973.18.6.0897.

Melatunan, S., Calosi, P., Rundle, S., Widdicombe, S. and Moody, A. (2013) 'Effects of ocean acidification and elevated temperature on shell plasticity and its energetic basis in an intertidal gastropod', *Marine Ecology Progress Series*, 472, pp. 155–168. doi: 10.3354/meps10046.

Mileikovsky, S. A. (1973) 'Speed of active movement of pelagic larvae of marine bottom invertebrates and their ability to regulate their vertical position', *Marine Biology*, 23(1), pp. 11–17. doi: 10.1007/BF00394107.

Nützel, A. (2014) 'Larval ecology and morphology in fossil gastropods', *Palaeontology*, 57(3), pp. 479–503. doi: 10.1111/pala.12104.

Oliveira, I., Laranjeiro, F., Fonseca, J., Freitas, D., Barroso, C. and Galante-Oliveira, S. (2018) 'Exposure of *Nassarius reticulatus* veligers in 24-well plates to assess effects of ocean acidification and warming projections on larval survival and behavior', *Frontiers in Marine Science*, 5. doi: 10.3389/conf.fmars.2018.06.00158.

Orr, J. C., Fabry, V. J., Aumont, O., Bopp, L., Doney, Scott C., Feely, R. A., Gnanadesikan, A,. Gruber, N., Ishida, A., Joos, F., Key, R. M., Lindsay, K., Maier-R., E., Matear, R., Monfray, P., Mouchet, A., Najjar, R. G., Plattner, G. K., Rodgers, K. B., Sabine, C. L., Sarmiento, J. L., Schlitzer, R., Slater, R. D., Totterdell, I. J., Weirig, M. F. Yamanaka, Y., Yool, A. (2005) 'Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms', *Nature*, 437(7059), pp. 681–686. doi: 10.1038/nature04095.

Parker, L. M., Ross, P. M., O'Connor, W. A., Pörtner, H. O., Scanes, E. and Wright, J. M. (2013) 'Predicting the response of molluscs to the impact of ocean acidification', *Biology*, pp. 651–692. doi: 10.3390/biology2020651.

Parker, L. M., Ross, P. M. and O'Connor, W. A. (2009) 'The effect of ocean acidification and temperature on the fertilization and embryonic development of the Sydney rock oyster *Saccostrea glomerata* (Gould 1850)', *Global Change Biology*, 15(9), pp. 2123–2136. doi: 10.1111/j.1365-2486.2009.01895.x.

Pierrot, D., Lewis, E. and Wallace, D. W. R. (2006) 'MS Excel program developed for CO2 system calculations', *Carbon Dioxide Information Analysis Center*. Oak Ridge National Laboratory, U.S. Department of Energy, Oak Ridge, Tennessee ORNL/CDIAC-105a. doi: 10.3334/CDIAC/otg.CO2SYS_XLS_CDIAC105a.

Pörtner, H. O. (2008) 'Ecosystem effects of ocean acidification in times of ocean warming: A physiologist's view', *Marine Ecology Progress Series*, pp. 203–217. doi: 10.3354/meps07768.

Przeslawski, R., Davis, A. R. and Benkendorff, K. (2005) 'Synergistic effects associated with climate change and the development of rocky shore molluscs', *Global Change Biology*, 11(3), pp. 515–522. doi: 10.1111/j.1365-2486.2005.00918.x.

Rhein, M., Rintoul, S. R., Aoki, S., Campos, E., Chambers, D., Feely, R. A., Gulev, S., Johnson, G. C., Josey,

S. A., Kostianoy, A., Mauritzen, C., Roemmich, D., Talley, L. D. and Wang, F. (2013) 'Observations: Ocean', in *Climate Change 2013 the Physical Science Basis: Working Group I Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)]. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press, pp. 255-315.

Riahi, K., Rao, S., Krey, V., Cho, C., Chirkov, V., Fischer, G., Kindermann, G., Nakicenovic, N. and Rafaj, P. (2011) 'RCP 8.5-A scenario of comparatively high greenhouse gas emissions', *Climatic Change*, 109(1), pp. 33–57. doi: 10.1007/s10584-011-0149-y.

Riebesell, U., Fabry, V. J., Hansson, L. and Gattuso, J.-P. (2010) *Guide to best practices for ocean acidification research and data reporting*, *Environment*. Luxembourg: Official Publications of the European Communities, pp. 260.

Ries, J. B. (2011) 'Skeletal mineralogy in a high-CO_2 world', *Journal of Experimental Marine Biology and Ecology*, 403(1–2), pp. 54–64. doi: 10.1016/j.jembe.2011.04.006.

Ries, J. B., Ghazaleh, M. N., Connolly, B., Westfield, I. and Castillo, K. D. (2016) 'Impacts of seawater saturation state (ΩA = 0.4–4.6) and temperature (10, 25 °C) on the dissolution kinetics of whole-shell biogenic carbonates', *Geochimica et Cosmochimica Acta*, 192, pp. 318–337. doi: 10.1016/j.gca.2016.07.001.

Ross, P. M., Parker, L., O'Connor, W. A. and Bailey, E. A. (2011) 'The Impact of Ocean Acidification on Reproduction, Early Development and Settlement of Marine Organisms', *Water*, 3(4), pp. 1005–1030. doi: 10.3390/w3041005.

Rühl, S., Calosi, P., Faulwetter, S., Keklikoglou, K., Widdicombe, S. and Queirós, A. M. (2017) 'Long-term exposure to elevated pCO_2 more than warming modifies early-life shell growth in a temperate gastropod', *ICES Journal of Marine Science*, 74(4), pp. 1113–1124. doi: 10.1093/icesjms/fsw242.

Sokolova, I. M., Frederich, M., Bagwe, R., Lannig, G. and Sukhotin, A. A. (2012) 'Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates', *Marine Environmental Research*, 79, pp. 1–15. doi: 10.1016/j.marenvres.2012.04.003.

Stappen, G. Van (1996) 'Introduction, biology and ecology of Artemia', in *Manual on the production and use of live food for aquaculture. FAO Technical Paper* [Lavens, P. and Sorgeloos, P. (eds.)]. FAO, pp.79-106.

Tallmark, B. (1980) 'Population Dynamics of *Nassarius reticulatus* (Gastropoda, Prosobranchia) in Gullmar Fjord, Sweden', *Marine Ecology Progress Series*, 3, pp. 51–62. doi: 10.3354/meps003051.

Wehrmeister, U., Jacob, D. E., Soldati, A. L., Loges, N., Häger, T. and Hofmeister, W. (2011) 'Amorphous, nanocrystalline and crystalline calcium carbonates in biological materials', *Journal of Raman*

Spectroscopy, 42(5), pp. 926–935. doi: 10.1002/jrs.2835.

Weiss, I. M., Tuross, N., Addadi, L. and Weiner, S. (2002) 'Mollusc larval shell formation: amorphous calcium carbonate is a precursor phase for aragonite.', *The Journal of experimental zoology*, 293(5), pp. 478–91. doi: 10.1002/jez.90004.

Wessel, N., Martin, S., Badou, A., Dubois, P., Huchette, S., Julia, V., Nunes, F., Harney, E., Paillard, C. and Auzoux-Bordenave, S. (2018) 'Effect of CO_2–induced ocean acidification on the early development and shell mineralization of the European abalone (*Haliotis tuberculata*)', *Journal of Experimental Marine Biology and Ecology*, 508, pp. 52–63. doi: 10.1016/j.jembe.2018.08.005.

Winans, A. K. and Purcell, J. E. (2010) 'Effects of pH on asexual reproduction and statolith formation of the scyphozoan, *Aurelia labiata*', *Hydrobiologia*, 645(1), pp. 39–52. doi: 10.1007/s10750-010-0224-9.

Zhang, H., Cheung, S. G. and Shin, P. K. S. (2014) 'The larvae of congeneric gastropods showed differential responses to the combined effects of ocean acidification, temperature and salinity', *Marine Pollution Bulletin*, 79(1–2), pp. 39–46. doi: 10.1016/j.marpolbul.2014.01.008.

Zippay, M. L. and Helmuth, B. (2012) 'Effects of temperature change on mussel, *Mytilus*', *Integrative Zoology*, pp. 312–327. doi: 10.1111/j.1749-4877.2012.00310.x.

Zupo, V. and Patti, F. P. (2009) 'Laboratory spawning, larval development and metamorphosis of the marine snail *Nassarius reticulatus* (L.) (Caenogastropoda, Nassariidae)', *Invertebrate Reproduction & Development*, 53(1), pp. 23–31. doi: 10.1080/07924259.2009.9652286

Chapter 3

Resilience of the pullet carpet shell, *Venerupis corrugata* (G.) to Ocean Acidification and Warming Projected Scenarios

The data in this chapter yielded the following peer-reviewed presentations

in international scientific meetings

(presenting author underlined)

Galante-Oliveira S, Fonseca JG, **Freitas DB**, Oliveira IB, Rocha RJM, Barroso CM (2020) Productive potential of the clam *Venerupis corrugata* under ocean acidification and warming scenarios. Aquaculture America 2020, 9-12.Feb, Honolulu, Hawaii, USA. (Accepted)

Rocha RJM, **Freitas DB**, Fonseca JG, Oliveira IB, Barroso CM, Galante-Oliveira (2019) High performance of *Venerupis corrugata* larval culture under projected acidification and warming scenarios unveils the species' resilience to future climate change. Poster presentation, Aquaculture Europe 2019, 7-10.Oct, Berlin, Germany

Abstract

The simultaneous increase in the global temperature and carbon dioxide in seawater, alters the physical and chemical properties of the oceans. In this chapter, early life stages of the pullet carpet shell, *Venerupis corrugata* (Gmelin, 1791) –a common bivalve traditionally harvested and produced at the Southwestern and Mediterranean Europe– were reared for two months under experimental conditions correspondent to Ocean Acidification (OA) and Warming (W) projected scenarios, generated by applying a factorial experimental design of three pH conditions (targeting 8.1, 7.8 and 7.6) at three temperatures (18, 20 and 22 ºC). The combined effects of OA-W experimental scenarios were investigated by assessing the ontogenic development, growth, shell malformation and survival during the exposure period (i.e., throughout all larval stages, metamorphosis, settlement and until one month post-settlement). The results confirm a significant effect of pH and temperature (TºC) individually on mortality and development at different exposure times. An additive effect of OA-W on mortality was registered specifically at the moment when complete settlement was recorded (100% settlement in all treatments at 28 days of exposure), favouring survival. Moreover, development and settlement were significantly faster at the warmest condition tested (22 ºC). Both larvae and juveniles were proved to grow more and better at 22 ºC, although the growth rates under acidity were generally lower than at control pH (8.1), an effect attenuated by the concomitant warming. These results prove that the warmer scenarios tested are within the thermal tolerance range of this species, and suggest that *V. corrugata* larval culture might be favoured, in both growth and survival, under such conditions. Production at higher TºC than the currently applied in hatcheries is also suggested as a way of improving its profitability. However, results also show that acidification has a significant antagonistic effect, delaying growth, suggesting that the acid-base unbalance might force an additional energetic demand to maintain basal functions towards survival, not allowing the organism to allocate enough energy to all the other biological requisites such as calcification and growth. Still, the OA-W scenarios projected to the end of this century by the Intergovernmental Panel on Climate Change for this species' geographical range, tested within the experimental conditions of this study, point towards the resilience of *V. corrugata* early life stages to warming, even if the worst acidification scenario become effective. In addition, the pullet carpet shell is suggested as a very promising species to aquaculture in a future environment, since warming will potentially compensate growth rates' decrease caused by acidification.

Keywords: *Venerupis corrugata*, bivalve mollusc, aquaculture, climate change, development, growth, shell, malformation, mortality

3.1 Introduction

The pullet carpet shell, *Venerupis corrugata* (Gmelin, 1791) is a clam species traditionally harvested at the Southwestern and Mediterranean Europe, mainly in Portugal, Spain, France and Italy (FAO, 2006). This lamellibranch can be found from the mean tide level to the lower shore up to 35 meters in depth, buried up to 5 cm deep in mixed sandy substrates (Rayment, 2007). It is also adapted to live in habitats with high amount of organic detritus in suspension due to wave action (Johannessen, 1973). *V. corrugata* spawns massively; spawning is seasonal and dependent on many biotic and abiotic factors but it occurs, generally, from late Winter to the early Summer in the Ria de Aveiro (NW Portugal) (Joaquim *et al.*, 2011). Even so, mature specimens can be found almost all year round (Cerviño-Otero, 2011). Fertilization is external, so females and males emit their gametes to the water column. After fertilization a high number of newly planktotrophic larvae are formed. Larval development is external and includes several ontogenic stages –trochophore, D-Larvae, Umbonate, Pediveliger– until it undergoes metamorphosis and settlement, becoming Postlarva (Cerviño-Otero, 2011). This transition from the planktonic to the benthic life comprises (1) the complete retraction of the velum and the formation of the gills, which from this moment assumes the feeding function; (2) the development of the byssal gland, whose function ensures that spat will adhere and fix to the substrate (this is especially important on specimens at coastal environments under greater wave activity) and (3) formation of the dissoconch that, being more robust that larval shell, provides additional protection (Quayle, 1952). This change, from the free-living larvae to a sedentary juvenile form, is recognized as the most critical stage along this bivalve life-history.

Poor management and pathogen-induced mortality determined natural stocks' reduction, forcing *V. corrugata* aquaculture production in order to respond to a high market demand. With an actual high market value (wholesale price over 18€ per kg; Tridge, 2019) when compared to most shellfish species, intensification of *V. corrugata* aquaculture production is very likely, in order to support stocks' recovery under high consumption and increased mortality due to disease outbreaks and extreme climatic events, predictably more frequent under future environmental reality in a changing climate. This includes several undergoing global phenomena such as ocean acidification (OA) and warming (W), recognized as being worsened by human activities, particularly those involving greenhouse

gas (GHG) emissions such as carbon dioxide (CO_2). The rise of the atmospheric CO_2 (i.e., the pCO_2 levels) from anthropogenic emissions is known to be a critical problem directly linked to an increase in the ocean temperature and acidity (Rhein *et al.*, 2013). The latest long-term projections of OA-W, to occur by the end of the 21st century, were presented by the Intergovernmental Panel on Climate Change (IPCC) considering several Representative Concentration Pathway (RCP) scenarios (Cubasch *et al.*, 2013): under RCP8.5 –scenario without additional efforts to constrain current emissions (Riahi *et al.*, 2011; Cubasch *et al.*, 2013)– the sea surface temperature (SST) might increase from about 1 to above 3 ºC (Collins *et al.*, 2013; IPCC, 2013), while sea surface pH might decrease 0.3 to 0.5 units in the northern hemisphere by the same period (Ciais *et al.*, 2013). Nevertheless, conditions greater than the SST and pH global averages have already been felt in many regions (Ciais *et al.*, 2013; IPCC, 2014; Allen *et al.*, 2018) and it is certain that the projected trends are likely to continue or accelerate in the upcoming decades if no mitigation actions are taken to constrain GHG emissions and human-induced climate change and OA-W (Ciais *et al.*, 2013; IPCC, 2014).

The oceans have been change gradually for decades (IPCC, 2013); although, nowadays, the change is happening much faster than in the geological past (Byrne, 2011). Therefore, it is important to determine the potential impacts of that changes and, if possible, to assess if that adaptation might occur at that same rate, in order to avoid local populations and species extinctions (Byrne, 2011). In that sense, many studies have been performed and many descriptions of harmful effects of OA-W on marine invertebrates (and their habitats) are available in the literature (e.g., Przeslawski *et al.*, 2005; Kroeker *et al.*, 2013; Queirós *et al.*, 2015). The study of the impacts of OA-W in early life is even more relevant, not only because their survival is the bottleneck for the species persistence (Byrne, 2011) but also because many authors have concluded that marine invertebrates' larvae and early juveniles are more sensitive, and suffer more damaging effects when exposed to temperature and/or pH changes (Pankhurst and Munday, 2011; Talmage and Gobler, 2011; León *et al.*, 2019). Effects caused by OA are mainly due to the fact that the phenomenon alters seawater carbonate chemistry, affecting carbonate ($CaCO_3$) biogenic deposition by marine calcifies, such as the bivalve molluscs (Orr *et al.*, 2005). Moreover, the constant exposure to acidified conditions can also induce shell dissolution, particularly concerning early life stages, whose shells are composed of amorphous calcium carbonate (ACC), the most soluble of the $CaCO_3$ polymorphs (Jardillier *et al.*, 2008; Ries, 2011). On the other hand, temperature plays a very

important role on the aerobic metabolism that is known to increase with warming. This happens since organisms need to keep the ATP supply enough to maintain several functions beyond the basal metabolism, like development, growth, among others (Sokolova *et al.*, 2011 and 2012). When the stress induced is considered moderate, all the ATP metabolized is directed to basal maintenance (i.e., survival), so the other functions are jeopardized (Pörtner, 2012). For this reason, if warming is the one inducing an extreme stress situation, the temperature rise leads to decrease development, growth and ultimately survival (Sokolova, 2013).

Many studies on the isolated effects of temperature (TºC) and pH mention the need and urgency of assessments of the combined effects of OA-W (Zippay and Helmuth, 2012; Gazeau *et al.*, 2013; Sokolova, 2013; Milano *et al.*, 2016; Lenz *et al.*, 2019). According to Folt *et al.* (1999), the combination of environmental stressors can have simple additive effects (both have or not a significant interaction) or have complex interactive effects, presenting synergistic (increasing stress) or antagonistic (decreasing stress) effects on biological processes. All types of effects have already been described when OA-W are studied interactively: Talmage and Gobler (2011) demonstrated additive effects on bivalve larvae, just like Lenz *et al.* (2019) on green sea urchin larvae; however, synergistic (Przeslawski *et al.*, 2005; Lagos *et al.*, 2016) and antagonistic (Mackenzie *et al.*, 2014; Clements and Darrow, 2018) effects have also been reported. Nevertheless, according to Byrne and Przeslawski (2013), additive negative effects are most commonly reported, followed by antagonistic effects, in which TºC reduce the negative effect of acidification, and less commonly, synergistic.

In this context, this study aims to investigate if there are any concomitant effects of OA-W on *V. corrugata* ontogenic development, growth and survival and, if so, which are its nature and extent. With that purpose, experimental scenarios of OA-W were generated after being established as a series of progressive environmental conditions based on IPCC near to long-term projections for the oceans in the northern hemisphere (where this species is currently distributed). Thus, the experimental treatments are intended to correspond to conditions to which this species is likely to be exposed in its natural habitat over time, in an attempt to foresee its performance and resilience to future OA-W.

3.2 Material and Methods

3.2.1 Spawning induction and fertilization

Local *V. corrugata* broodstock was obtained from a shellfish farmer from Ria de Aveiro (NW Portugal). Approximately sixty adults were transported to the laboratory and kept dry during 48h at 18±1ºC. After that period, animals were washed with running tap water and, then, with 1µm-filtered and UV-sterilized (*V2 Vecton 600*, TMC, UK) artificial seawater (*Red Sea Salt*, Red Sea Europe, France) at salinity 35 to remove all remaining sediment and epibionts from the shells' surface.

Cleaned clams were placed in a temperature-controlled water bath at 22±1ºC (Figure 3.1A) for spawning stimulation. Two consecutive thermal shocks were applied, alternating immersion in the hot bath at 22±1ºC for 2h and in a cold bath at 11±1ºC for other 2h. First gamete emission was noticed after almost 8 hours. About 50% of the breeders had spawned. Spawning males and females were individualized in 1L-glass flasks kept at the same TºC as the main hot bath (Figure 3.1B) as soon as spawning was noticed.

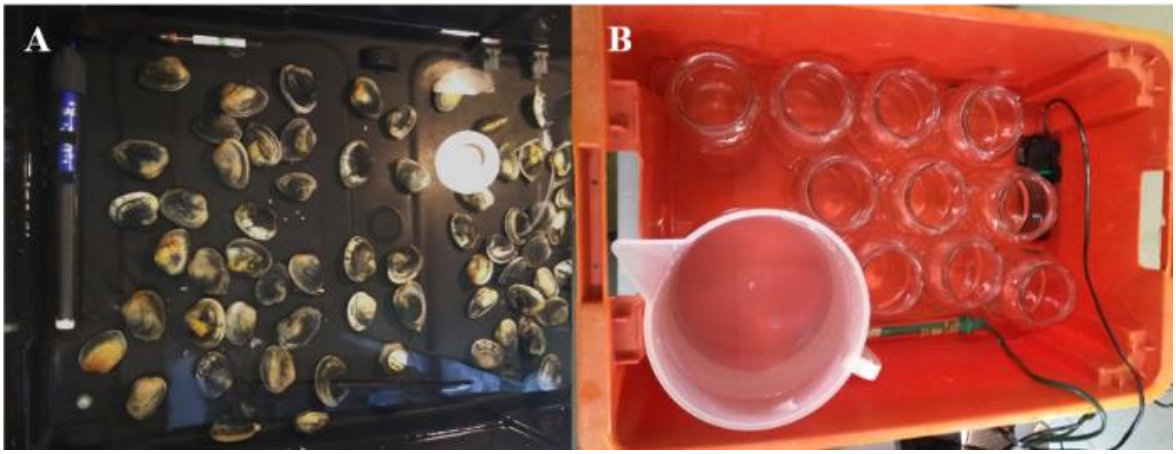

Figure 3.1 - Thermal stimulation of *Venerupis corrugata* broodstock. A – Individuals in the hot bath at 22ºC. B – Individual containers kept at 22ºC to individualize specimens after spawning is detected.

Oocytes from ten females were mixed, passed through a 100µm-mesh and suspended in 10L of 1µm-filtered and UV-sterilized seawater at 21°C. The eggs were fertilized with about 200mL of a mixture of sperm from six males and kept at room temperature, at about 20°C. Thirty minutes later, the fertilization rate was assessed in two aliquots of 1mL using a Sedgewick rafter counting chamber under a light optical microscope (*Axiolab E*, Zeiss,

Germany), and the quantity of spermatozoids per oocyte was checked in order to keep it at 10:1 avoiding low fertilization rates (if under that value) or polyspermy (if above). The container was placed again in the water bath at 22°C and the fertilization continued for 30 min. more. After that period, the number of fertilized eggs (identified by having polar body or already in cell division; Cerviño-Otero, 2011) were counted in four aliquots of 1mL and their total number was determined. Fertilized eggs were, then, cultured to obtain D-larvae as follows.

3.2.2 Culture of fertilized eggs to obtain D-larvae

Fertilized eggs were kept in 30L-plastic tanks at a density of 50 fertilized eggs mL^{-1} in 1µm-filtered and UV-sterilized seawater, at salinity 35, 18±1°C and 12^{L}:12^{D} photoperiod. Twenty-four hours after fertilization, 75 cells μL^{-1} of *Isochrysis aff. galbana* (*T-iso*) was added to the incubation medium since D-larvae might develop earlier and need to be fed (Cerviño-Otero, 2011). Microalgae used in this experiment – *T-iso* and also *Chaetoceros calcitrans* (*Cha*), used to feed individuals during the exposure to OA-W scenarios as described below – were produced at our laboratory, following the batch culture protocol detailed in Chapter 2. However, here, the inoculants were obtained from Aqualgae, SL – Portugal, as was the culture medium (*Gold Medium*, Aqualgae, Spain) and the 40% sodium silicate solution (*Sodio Silicato QP*, Quimipur, Spain).

Seventy-two hours (i.e., 3 days) post-fertilization, D-larvae were collected using a 60µm-sieve (Figure 3.2A). Those retained were resuspended in 4L of 1µm-filtered and UV-sterilized seawater at salinity 35 and counted in four aliquots of 1mL (Figure 3.2B). The D-larvae were then concentrated in 1L and kept ready to be aliquoted adequately and randomly distributed by the experimental containers for their exposure to OA-W scenarios.

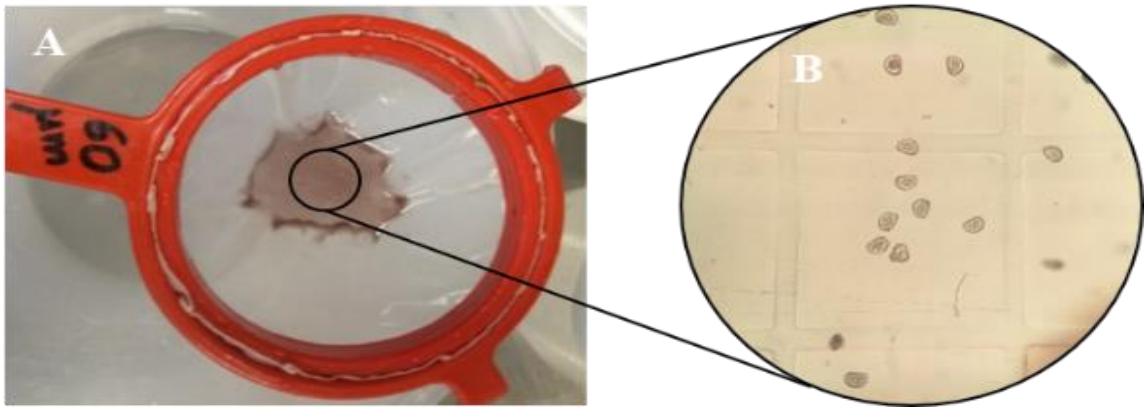

Figure 3.2 - *Venerupis corrugata* D-larvae. A – Larvae retained in the 60µm-sieve, 3 days after fertilization. B – D-larvae observed under the light optical microscope (at 100x magnification).

3.2.3 Experimental setup, design and treatments

The Experimental Life Support System (ELSS) available at the Centre for Environmental and Marine Studies (CESAM) of the University of Aveiro (Portugal) was used to expose the D-larvae of *V. corrugata* to 9 OA-W scenarios, in triplicate, for two months (Figure 3.3).

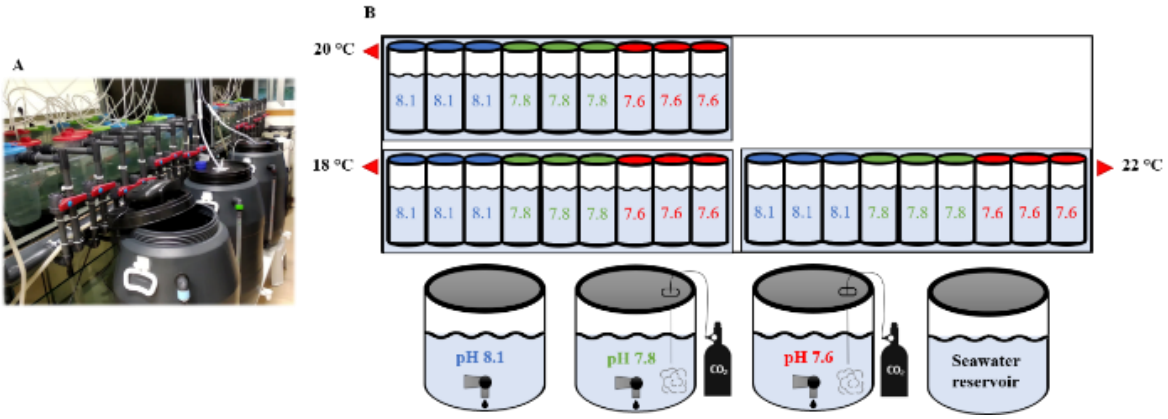

Figure 3.3 - Experimental setup and design used on the exposure of *V. corrugata* early life stages to 9 projected scenarios of OA-W. A – Experimental Life Support System (ELSS) after the modifications needed for this experiment. B – Factorial design of 3 temperatures (18, 20 and 22ºC) and 3 pH target levels (8.1, 7.8 and 7.6) resulting in the 9 OA-W treatments to which D-larvae will be exposed in triplicate. The ELSS alteration included the possibility of experimental containers' randomization that is not shown in the scheme but was performed weekly.

The ELSS (Figure 3.3-A), originally developed by Coelho *et al.* (2013), was adapted for the exposure of veligers of the gastropod *Tritia reticulata* to 12 OA-W scenarios in Chapter 2 (*Material and Methods* section). The expanded version, after the modifications described in Chapter 2, was also able to accommodate the factorial design of 3 TºC and 3

pH levels (3x3) of the current experiment (Figure 3.3-B). However, as D-larvae rearing is optimized for the production of bivalves' seed in hatcheries, and the protocol includes slow and continuous aeration and the exchange of 100% of the culture medium with the sanitation of the tanks in which larvae are grown two to three times a week (Helm and Bourne, 2004; Cerviño-Otero, 2011), additional modifications were needed. Accordingly, the experimental containers of the ELSS were changed by new ones, hermetically sealed, avoiding the exchange of CO_2 at the interface ambient air-exposure medium, keeping the pH stable, at target levels, for three days. These new containers were smaller (1.65L) than the used in Chapter 2 since the density recommended for the culture of *V. corrugata* D-larvae is about ten times higher than the required to culture *T. reticulata* veligers. Moreover, as these larvae need to be continuously aerated, the reservoirs used to manipulate seawater pH were also changed by hermetically sealed ones, allowing to generate a CO_2-enriched atmosphere inside the reservoir while pH equilibration by CO_2 bubbling is performed. This equilibrated atmosphere was then captured and pumped (with common aquaria pumps) to circulate the medium inside experimental containers of the respective treatment, with the desired pCO_2, keeping pH at target levels for three days.

Synthetic saltwater was produced on a fourth reservoir of 250L, by mixing reverse osmosis purified freshwater (*V^2 Pure 75*, TMC, UK) with Red Sea Salt (Red Sea Europe, France) to obtain salinity 35. The water was prepared and UV-sterilized for at least 24 h prior to use. Saltwater was then distributed by the three new, hermetically sealed reservoirs (50L/each) and manipulated to generate three distinct pH levels: control (pH ~8.1) plus two acidified conditions of -0.3 and -0.5 pH units, corresponding to the interval of sea surface pH projected by the IPCC for the end of the century in the northern hemisphere under the RCP8.5 (Ciais *et al.*, 2013), and following the available guidelines applied to OA research (Riebesell *et al.*, 2010). The fourth reservoir (of 250L) was used to keep unmanipulated saltwater and refill the remaining three as needed. Saltwater in all the reservoirs was kept under constant recirculation to promote water mobilization and force pH adjustment (by the same equipment used in Chapter 2; *V^2 control pH controller/monitor and V^2 pressure regulator pro*, TMC, UK). Regarding TºC, three levels were then generated: one control condition –18°C– corresponding to the mean SST registered during *V. corrugata* spawning season in Ria de Aveiro (Joaquim *et al.*, 2011) and described as the optimum TºC for the species production in laboratory/hatchery (Cerviño-Otero, 2011); plus two warming

conditions –20 and 22°C– corresponding, respectively, to near and long-term scenarios according to the IPCC projection of a global increase in the SST of up to 4ºC under RCP8.5 by the end of the century (Collins *et al.*, 2013; IPCC, 2013). The 1.65L experimental containers were partially (2/3) immerged in temperature-controlled water baths at this TºC in order to keep it constant (as in Chapter 2). Each of the 9 OA-W scenarios generated were tested in triplicate, under continuous aeration of 3 bubbles s^{-1} (by CO_2-enriched air obtained as explained above) and under $12h^L$:$12h^D$ photoperiod. Exposure medium was renewed completely every three to four days and the tanks were washed with a brush soaked in a 1:1 mixture of soap (*Alconox® detergent*, Sigma-Aldrich, Germany) and commercial bleach at 5%, rinsed off thoroughly with tap water.

A pre-assay to monitor the pH and TºC generated in the system under such conditions, during three days, was carried out. The pH and TºC control systems' settings were adjusted to avoid treatments' overlapping and allow the predetermined OA-W scenarios to be obtained: pH controllers were set to 7.68 and 7.30, and cooler/heater thermostats to 17/18, 19/20 and 21/22°C.

At the beginning of the exposure (at T0), the experimental containers were filled with the respective exposure medium, and equilibrated during the previous 24h. The D-larvae retained in the 60µm-sieve (Figure 3.2), which were counted and concentrated in a volume of 1L, were aliquoted adequately and distributed randomly for a density of 16 larvae mL^{-1} (n≈26 400 per container, following the densities suggested by Joaquim *et al.* 2014). Tanks' location in each water bath was randomly assigned by using *Microsoft Exel*, a procedure that was repeated weekly.

Larvae were fed *ad libitum* with a blend of equal proportions of *T-Iso* and *Cha*, following the diets applied by Joaquim *et al.* (2014) and Fernández-Pardo *et al.* (2016). The availability of microalgae in the exposure media was guaranteed by adjusting the diet in terms of feeding time (morning/late afternoon) and quantity (ration), according to the specimens' development in different treatments and the stability of the pH of the exposure medium. Those adjustments were generally applied to all tanks under a same TºC, at least until complete settlement (i.e., 100% of settled individuals at all treatments, registered after 28 days of exposure) as no differences between different pH treatments for a given TºC were registered by then. The rations applied until day 28 (T28) are indicated in Table 3.1. During

this period, microalgae were provided in the late afternoon, in order to avoid pH imbalance due to photosynthesis during the day and as larvae of marine benthic invertebrates are considerably active in the water column during dark hours, favouring feeding (Mileikovsky, 1973). However, from T21 onwards, the larvae at 22°C were fed in the morning and a supplement (25 *T-Iso* cells μL^{-1} + 25 *Cha* cells μL^{-1}) was needed and provided in the late afternoon.

Table 3.1 - *Venerupis corrugata* diet applied during the exposure of D-larvae to OA-W experimental scenarios, before settlement.

Time period (days)	Diet
T0 – T2	75 *T-Iso* cells μL^{-1}
T2 – T4	75 *T-Iso* cells μL^{-1}
T5 – T11	50 *T-Iso* cells μL^{-1} + 30 *Cha* cells μL^{-1}
T12 – T17	50 *T-Iso* cells μL^{-1} + 40 *Cha* cells μL^{-1}
T18 – T20	50 *T-Iso* cells μL^{-1} + 50 *Cha* cells μL^{-1}
Until T28	22 ºC bath was supplemented with more ½ ration

After complete settlement (Table 3.2) the differences in the specimens' development between treatments were more marked and varied. The diet was ajusted as a function of the

Table 3.2 - *Venerupis corrugata* diet applied during the exposure of D-larvae to OA-W experimental scenarios, after complete settlement.

Time period (days)	Temperature	Diet
T29 – T32	18 and 20 ºC	50 *T-Iso* cells μL^{-1} + 50 *Cha* cells μL^{-1}
	22 ºC	75 *T-Iso* cells μL^{-1} + 75 *Cha* cells μL^{-1}
T33 – T38	18 ºC	50 *T-Iso* cells μL^{-1} + 50 *Cha* cells μL^{-1}
	20 ºC	75 *T-Iso* cells μL^{-1} + 75 *Cha* cells μL^{-1}
	22 ºC	100 *T-Iso* cells μL^{-1} + 100 *Cha* cells μL^{-1}
T39 and T40	18 ºC	50 *T-Iso* cells μL^{-1} + 50 *Cha* cells μL^{-1}
	20 ºC	100 *T-Iso* cells μL^{-1} + 100 *Cha* cells μL^{-1}
	22 ºC	125 *T-Iso* cells μL^{-1} + 125 *Cha* cells μL^{-1}
T41 – T44	18 ºC	50 *T-Iso* cells μL^{-1} + 50 *Cha* cells μL^{-1}
	20 ºC	100 *T-Iso* cells μL^{-1} + 100 *Cha* cells μL^{-1}
	22 ºC	150 *T-Iso* cells μL^{-1} + 150 *Cha* cells μL^{-1}
T45 – T47	18 ºC	75 *T-Iso* cells μL^{-1} + 75 *Cha* cells μL^{-1}
	20 ºC	125 *T-Iso* cells μL^{-1} + 125 *Cha* cells μL^{-1}
	22 ºC	150 *T-Iso* cells μL^{-1} + 150 *Cha* cells μL^{-1}
T48 – T56	18 ºC	100 *T-Iso* cells μL^{-1} + 100 *Cha* cells μL^{-1}
	20 ºC	150 *T-Iso* cells μL^{-1} + 150 *Cha* cells μL^{-1}
	22 ºC	150 *T-Iso* cells μL^{-1} + 150 *Cha* cells μL^{-1}

TºC and individuals were feed in the morning, with the exception of the ones at 18ºC, visibly less developed, to which half of the ration was added in the morning and the other half in late afternoon (otherwise pH imbalance would occur due to the consumption of CO_2 through photosynthesis during the day by an excess of microalgae in the medium).

These adjustments were done along the experiment with the constant monitoring of the exposure medium physico-chemical parameters performed as follows.

3.2.4 Exposure media physico-chemical analysis

Probe measurements (*Multi 3430 IDS*, WTW, Germany) of pH (NBS scale) and TºC were taken three times per day, for six consecutive days from T0, to characterize the daily variation to which larvae would be exposed for the period of three days between maintenance. Then, those parameters were periodically monitored, at least once a day until the end of the experiment (after 56 days, at T56). Measurements were taken in early morning and/or late afternoon, always before feeding. In addition, probe measurements of salinity (SAL) and total dissolved oxygen (DO) were recorded at least once a week in the experimental containers (although salinity was checked every three days, at the time saltwater was produced). Total dissolved inorganic carbon (DIC) was quantified in 15mL of 0.2µm-filtered exposure medium collected after the morning measurement at day 1 (T1), once a week until day 14 (T14) and every two weeks from then until T56, in at least one replicate per treatment. Analyses were performed immediately after samples collection, in CESAM's Carbon analyser (*Multi N/C 3100*, Analytik Jena, Germany), following the manufacturer instructions and standard protocols applied on OA research (Goyet and Snover, 1993; Dickson, 2011). The values of DIC and of pH, TºC and SAL measured at the time of media sampling, were the inputs to calculate the CO_2 partial pressure (pCO_2), bicarbonate (HCO^{3-}) and carbonate (CO_3^{2-}) ion concentrations, and the saturation states of calcite (Ω_{Ca}) and aragonite (Ω_{Ar}) with the *Microsoft Excel* macro CO2Sys_v2.1 (Pierrot *et al.*, 2006), using K1 and K2 carbonate dissociation constants from Mehrbach *et al.* (1973) refitted by Dickson and Millero (1987), and KSO_4 from Dickson (1990).

3.2.5 Analysis of biological endpoints

The survival, the ontogenic development and the growth of *V. corrugata*, reared under the experimental OA-W scenarios from D-larvae to the juvenile stage, was assessed throughout exposure at different timepoints. Monitoring and/or specimens' sampling were performed every three to four days until complete settlement was recorded, and once a week thereafter [i.e., at all times of tanks' maintenance at: day 3 (T3), day 7 (T7), day 10 (T10), day 14 (T14), day 18 (T18), day 22 (T22), day 25 (T25), T28, day 35 (T35), day 43 (T43), day 49 (T49) and at the end of the experiment, at T56].

Due to the species indirect development, including irreversible morphological alterations during metamorphosis, undergone at the end of the planktonic (larval) phase to become benthic (after settlement), some biological endpoints are only meaningful in certain stages of the animal life and so were not assessed at all timepoints (e.g, settlement). Accordingly, the mortality (calculated from survival), the development stage and shells' malformation were assessed throughout exposure at all timepoints. Settlement was determined until 100% was registered in all treatments (calculated from the number of postlarvae recorded at all timepoints until T28). Growth rates (calculated from shell length measurements in preserved specimens) was determined for both the larval culture (between T0 and T14), and the benthic, post-settlement period (between T28 and T56). The protocol for the assessment of all the endpoints is detailed below, in chronological order.

Larval culture

A sample of the initial pool of D-larvae was collected at T0 and preserved in 70% ethanol for posterior measurement of the shell length (SL, Figure 3.4-A). After three days of exposure, at T3, the randomness of the initial distribution of D-larvae and the survival across

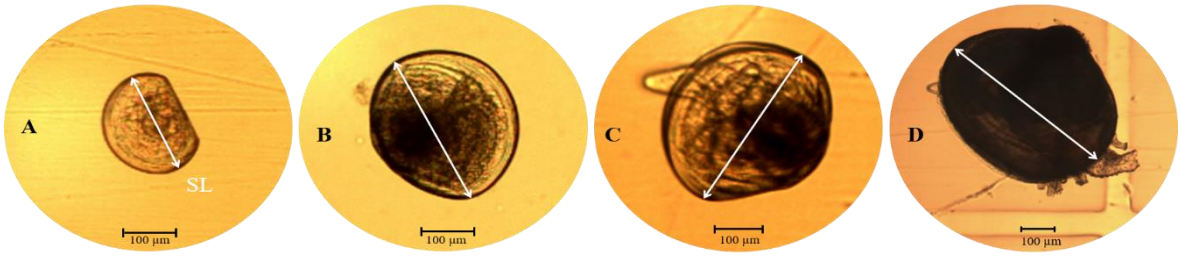

Figure 3.4 - *Venerupis corrugata* pictures taken during the exposure to 9 OA-W scenarios for the measurement of the shell length (SL) and the classification of the development stage at different time points. A – D-larva. B – Umbonate. C – Pediveliger. D – Postlarva.

treatments were monitored. Larvae were collected in a 60µm-sieve, back-washed into a beaker, resuspended in 400mL of the respective exposure medium and counted in two aliquots of 1mL per replicate (i.e., 6 per treatment). This procedure was repeated at all moments of larval culture maintenance, adapting the volume in which the larvae were resuspended to the number of specimens available, and also using and adequate mesh of the sieve to the size of the specimens (60µm was used until T18, 80µm from T22 to T25). In addition, while counting the specimens, their development stage was categorized into four different stages (D-larva, Umbonate, Pediveliger or Postlarva) following Cerviño-Otero (2011) classification (Figure 3.4) and the number of specimens exhibiting malformed shells was also registered and those specimens were photographed. Furthermore, at T14, beyond counting the live larvae in two aliquots of 1mL, classifying their development stage and registering any shells' malformation, the specimens observed were also preserved in 70% ethanol to assess growth. For that, animals were observed under the light optical microscope equipped with a digital camera (*Moticam 2300*, Meyer Instruments, USA) and photographed for posterior measurement of SL (figure 3.4), using *Software Motic Images Plus v2.0*. The growth rate between T0 and T14 was then calculated.

Post-settlement culture

Settlement was achieved in all treatments at T28. Different densities between treatments, and even between replicates, were registered at this timepoint, forcing density adjustment due to the consequent imbalance of the pH conditions. Density was normalized throughout replicates by collecting the postlarvae in a 100 µm-sieve, counting to determine mortality, and returning only 600 individuals to the respective tank (new density of 0.36 larvae mL^{-1}). The remaining specimens were preserved in 70% ethanol to assess growth as described above. The replicates in which the number of live animals were not enough, ceased at T28.

From T28 to the end of exposure (at T56), it was possible to discern live from dead individuals under a stereomicroscope (*Leica® S8 APO*, Leica Microsystems, Germany). However, some of the specimens were too small at T35, reason why mortality was not determined at this timepoint, but it was at T43 as already described. From T28 to T43, animals were collected in a 200µm-sieve.

At T49 the specimens reared under pH 7.6 at 18ºC were still collected in the 200 µm-sieve while the 300µm-mesh was used in the remaining treatments. Animals were then counted and the mortality determined. At this timepoint, the densities were again normalized (new density 0.12 individuals mL^{-1}). The remaining juveniles were preserved in 70% ethanol to be analysed if necessary.

The exposure ended at T56 (59 days post fertilization). All the specimens were collected in the 300µm-sieve, placed on filter paper and photographed with a 12MP camera (*M1805D1SG*, Xiaomi, China) to measure SL (Figure 3.5) using the open source *Software ImageJ* (National Institutes of Health, USA). Finally, juveniles were preserved in 100% (absolute) ethanol, to be posteriorly processed for Scanning Electron Microscope (SEM) to study shells' microstructural damage, if necessary.

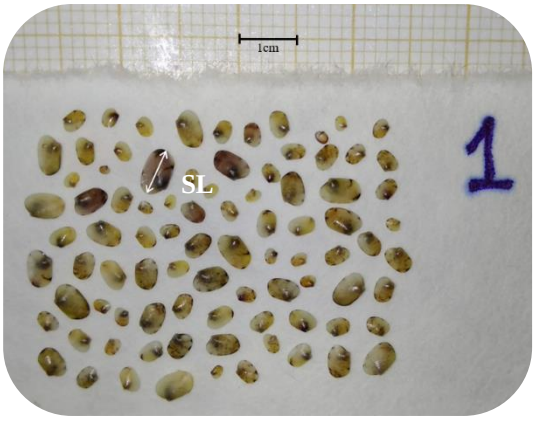

Figure 3.5 - *Venerupis corrugata* juveniles, photographed at T56 for the posterior measurement of the shell length (SL), distance indicated by the blanck arrow.

3.2.6 Statistics

Permutational multivariate analysis of variance (PERMANOVA; *Software PRIMER v7*) was used to test for significant effects of pH, TºC and the possible interaction between these factors at each endpoint analysed. For mortality, development stage, shell length and shells' malformation, three mean values per treatment were used (one mean per replicate). From T28 onwards, and since some replicates have ceased, the mean of all observations per treatment was considered. Euclidean distance was applied to generate resemblance matrices. A two-way crossed design (pH * TºC) was applied with a type III partitioning of the sums of squares and the permutation of residuals under a reduced model based on 9999 permutations to obtain the p-value. After a significant PERMANOVA result, a pair-wise t-test (or Monte Carlo test –MC– if the number of unique permutations were less than 20) was used to understand which levels of the factor were responsible for the significant results.

3.3 Results and Discussion

This study was designed to investigate the effects of the concomitant Ocean Acidification (OA) and Warming (W) on the performance of *Venerupis corrugata* early life stages, in an attempt to foresee the resilience of this ecologically and economically relevant species to future environmental conditions they are expected to face. In that sense, we determined the mortality, followed the ontogenic development registering shells' malformation, and determined the growth rates in both the planktonic and the early benthic phase, while rearing the animals under OA-W projected scenarios. The results obtained and its discussion is presented below.

3.3.1 Experimental conditions

Figure 3.6 corresponds to the graphical representation of the 9 OA-W experimental scenarios to which specimens were exposed for two months, while the carbonate chemistry of the exposure media is compiled in Table 3.3. Moreover, the average salinity and total dissolved

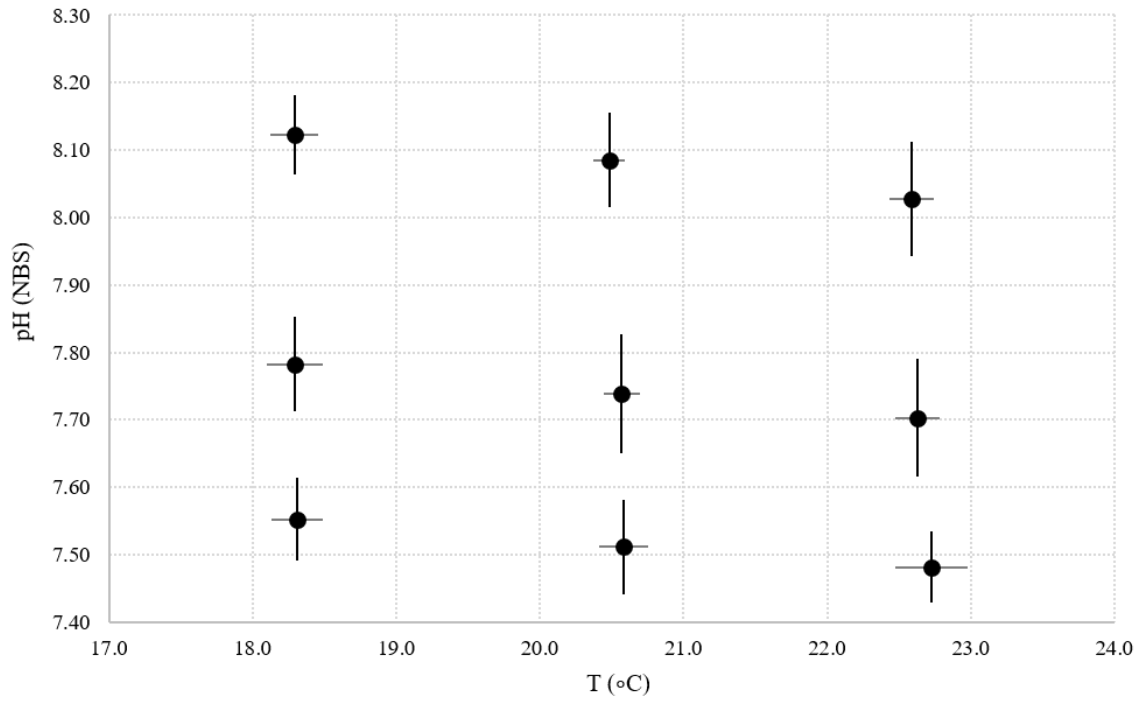

Figure 3.6 - Graphical representation of the 9 OA-W scenarios generated in the ELSS during the experiment. Values correspond to the temperature (TºC) and pH daily variation per treatment and are means and respective standard deviations of probe measurements of TºC and pH measured three times per day from T0 to T6 and twice a day from then until T55 (the day before the end of the experiment).

oxygen registered throughout the experiment were 34.31±0.62 and 9.05±0.69 mg L^{-1}, respectively.

Table 3.4 - Mean carbonate system parameters determined in samples taken at T1, T4, and every two weeks from T8 until T55 (in two replicates per treatment). The treatment column refers to target temperature (T_{target}) and pH (pH_{target}) levels following IPCC (2013) projections as explained in the *Material and Methods* section. The partial pressure of CO_2 (pCO_2), the bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ion concentrations, and the saturation states of calcite (ΩCa) and aragonite (ΩAr) were derived from probe measurements of salinity (SAL), temperature (TºC) and pH, and from total dissolved inorganic carbon (DIC) quantified by acidification, gas stripping and infrared detection in a Carbon analyser. Values for all carbonate species were estimated by the Microsoft Exel macro *CO2Sys* from T_{target} levels as output parameter.

Treatment		Probe measurements		Quantified	CO2Sys Excel macro					
T_{target} (ºC)	pH_{target} (NBS)	SAL	TºC (ºC)	pH (NBS)	DIC (µmol kg-SW^{-1})	ρCO_2 (µatm)	HCO_3^- (µmol kg-SW^{-1})	CO_3^{2-} (µmol kg-SW^{-1})	ΩCa	ΩAr
18	8.1	34.3	18.4	8.15	3112	634	2836	255	6.11	3.95
	7.8	34.1	18.5	7.76	3220	1696	3053	110	2.63	1.70
	7.6	34.1	18.5	7.53	3449	3079	3275	69	1.66	1.07
20	8.1	34.7	20.4	8.12	3020	693	2749	249	5.97	3.88
	7.8	34.2	20.4	7.74	3166	1776	2997	112	2.69	1.75
	7.6	34.2	20.6	7.51	3312	3173	3140	70	1.68	1.09
22	8.1	34.7	22.3	8.07	2955	766	2693	239	5.75	3.76
	7.8	34.3	22.4	7.72	3147	1892	2974	116	2.79	1.82
	7.6	34.3	22.4	7.50	3262	3244	3091	73	1.77	1.16

Although variability between replicates within the same treatment has been registered, all treatments were proved to be significantly different (Pseudo-F=2823.8, p=0.0001). Temperature varied less than ±1ºC (see Figure 3.6), while the pH, which was supposed to vary ±0.05 due to the controller specifications, showed higher variation, decreasing over time until re-establishment to target levels upon CO_2 injection on the reservoirs, a more marked tendency at higher TºC (Figure 3.6). As expected, calcite and aragonite saturation states followed the pH variation pattern (Table 3.3). However, despite the acidification of the exposure media, undersaturation (Ω<1) was never registered regarding both calcite and aragonite. Although total alkalinity was not quantified, the commercial salt mixture used (*Red Sea Salt*) is known to provide elevated alkalinity (see the manufacturer description of the product; Red Sea, 2019). Also, this mixture is optimized to maintain calcifying organisms in closed systems, reason why high DIC and both bicarbonate

and carbonate ions concentrations in the exposure medium were evident (Table 3.3), avoiding carbonate undersaturation even in the most acidic conditions (at pH~7.6).

Even so, saturation was reduced in our acidified scenarios, and very close to 1 regarding aragonite in the most acidic ones (of pH_{target} 7.6). In this sense, our experimental treatments can be used as proxies of the environmental conditions expected under some of the OA-W scenarios projected for the near and long-term future.

3.3.2 Mortality

Figure 3.7 shows the cumulative mortality of *V. corrugata* early life stages per experimental treatment, throughout the 2 months' exposure. Significant differences between treatments were only found at T28. At T28, TºC (Pseudo-F=5.1061, p=0.0182) and pH (Pseudo-F=5.6073, p=0.0131) were proved to significantly affect mortality, although no significant interaction between the stressors was found. Control TºC (18ºC) revealed significantly higher mortality than both warmer treatments: 20ºC (t-test=2.8649, p=0.0152) and 22ºC (t-test=2.5943, p=0.0253) while higher acidity (pH_{target} 7.6) revealed lower mortality than the specimens reared under the other two conditions: pH_{target} 8.1 (t-test=2.6652, p=0.0222) and 7.8 (t-test=2.6692, p=0.0227).

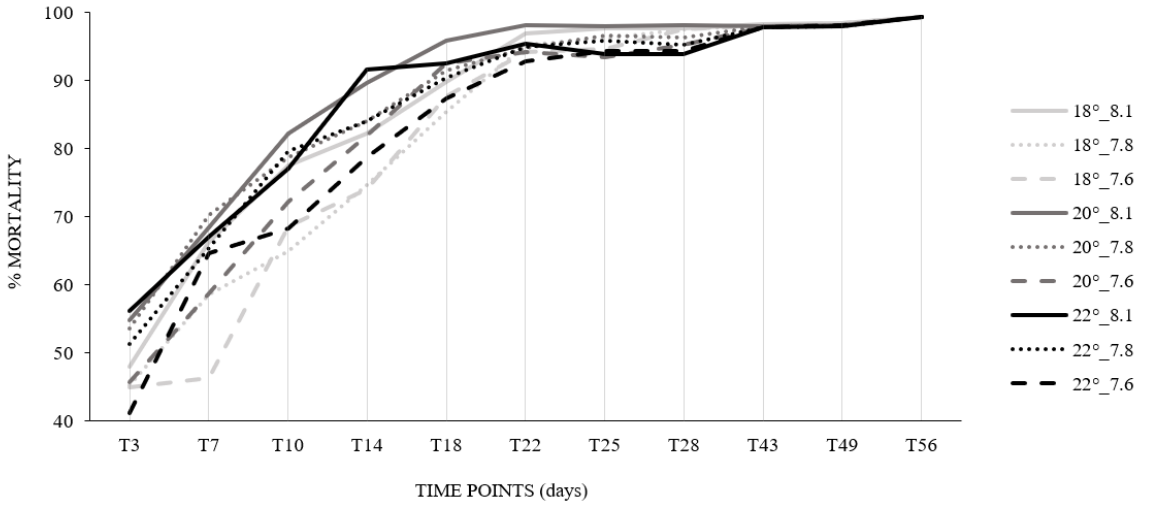

Figure 3.7 - *V. corrugata* cumulative mortality calculated from survival determined throughout the exposure to 9 OA-W scenarios at T3, 7, 10, 14, 18, 22, 28, 43, 49 and 56. Solid, dotted and dashed lines correspond to pH_{target} levels (8.1, 7.8 and 7.6, respectively) and different colours, from light to intermediate to dark grey indicate the increasing TºC tested (18, 20 and 22ºC, respectively). Treatments are labelled on the right side of the graph.

Based on these results, *V. corrugata* early life stages seem to be resilient to the OA-W scenarios tested. The high mortality registered until settlement (>95%) was expected as reported by other authors (Cerviño-Otero, 2011; Joaquim *et al.*, 2016), and also due to the species reproductive strategy of emitting large number of gametes originating high numbers of larvae that suffer high mortality in the water column (Thorson, 1950). Moreover, the frequent larvae handling to change the exposure medium and clean the small experimental containers (1.65L) might have caused higher losses than in hatchery (Cerviño-Otero, 2011).

Regarding the absence of significant effects of the stressors under study during the larval phase (before T28), it is not completely unreasonable to assume some error associated with sampling implying high variability within replicates: at first because estimates were needed, despite their associated error, but also since the resuspension of postlarvae (after settlement was first recorded at T10; see below) yielded higher variance within groups that might have masked some of the effects of the experimental treatments. Then, as complete settlement was recorded at T28 and the density was normalised among treatments, the variance was reduced, as no estimates were necessary (all animals were counted and the determined mortality was, so to say, "real").

Nevertheless, significant effects of TºC and pH, individually, were proved at T28. This species revealed to be resilient to warming, at least under the tested temperatures. This was somehow expected since other authors have reported remarkable resistance of this species' juveniles to higher TºC. Although not completely comparable, as older/larger individuals were used rather than newly settled postlarvae, Santos (2018) has exposed *V. corrugata* juveniles to different TºC (from 16 to 28ºC) and mortality was only significantly increased under the warmest; the author has also registered about 90% survival among the other TºC tested, showing that in a 4ºC-range (16-20ºC) the individuals resist to temperature fluctuations. The most surprising was that, in this work, mortality was significantly higher at 18ºC than under the warmer conditions tested (20 and 22ºC), suggesting that warming, in fact, favours survival. Thus, it seems that all the TºC values tested here are within the species thermal range but that 20 and 22ºC should be the optimal conditions for *V. corrugata* in early life. It is also important to stress that both estuarine and sedentary organisms, such as our model, have naturally higher adaptability to environmental fluctuations due to the constant exposure to variable conditions, namely regarding salinity, temperature, pH and tide (Kroeker *et al.*, 2013). Thus, a certain adaptation to variable thermal conditions can expand

the optimal thermal range, enhancing thermal tolerance (Sokolova *et al.*, 2012). Also, as mentioned above in *Introduction* , when organisms are exposed to moderate stress, energy is allocated to survival instead of being used to perform other functions (Pörtner, 2012). These two last explanations are also valid to justify a significantly higher percentage of survivors under our most acidified treatment (pH 7.6) shown at T28. Following Cunha *et al.* (2005) pH values at Ria de Aveiro's water could range between 7.6 and 8.5 and in sediment it could reach even lower values (6.6-6.9). As *V. corrugata* broodstock were obtained at Ria de Aveiro it is likely that they were adapted to pH variations. Thomsen *et al.* (2017) compared two populations of *Mytilus edulis* (Linnaeus, 1758) – one that habit in a CO_2-enriched habitat and the other in an nonenriched habitat - and they have documented that larvae from CO_2-enriched habitat as successfully settled and presented higher fitness under acidified conditions, than the larvae originate from the nonenriched population. Another study performed by Baria *et al.* (2015) hypothesises that parents that have been exposed or acclimatized to OW could transfer to offspring this adaptation (e.g. cellular protective mechanism and high amount of heat shock proteins (HSP)). Moreover, taking into account the results reported by other authors (O'Brien *et al.*, 2016; Kandler *et al.*, 2018) we can also speculate that changes in the microbiome might have been the source of the increased survival registered under lower pH. Even possible, this hypothesis requires confirmation by further studies.

3.3.3 Malformations

The occurrence of shells' malformations was periodically assessed throughout the experiment. A sample of the deformities detected are shown in Figure 3.8. The percentage of malformation was highly variable among exposure times and no significant differences between treatments at each timepoint and among timepoints were revealed ($p>0.05$). The maximum value of 25% was detected only once (T7) and in a single treatment (18ºC_pH 7.8). Thus, malformations were apparently random, not caused by the experimental conditions but rather by natural causes or due to the specimens handling. Even so, some of the deformities showed in Figure 3.8 have already been reported as being caused by exposure to OA scenarios (Kurihara, 2008; Byrne and Przeslawski, 2013; Guo *et al.*, 2015). Thus, although no significant differences between treatments were obtained, a slightly lower average percentage of malformation (~3%) was registered under control pH (among all

temperatures and during the entire experiment) when compared to that registered under the highest acidic treatment (~6% at pH_{target} 7.6).

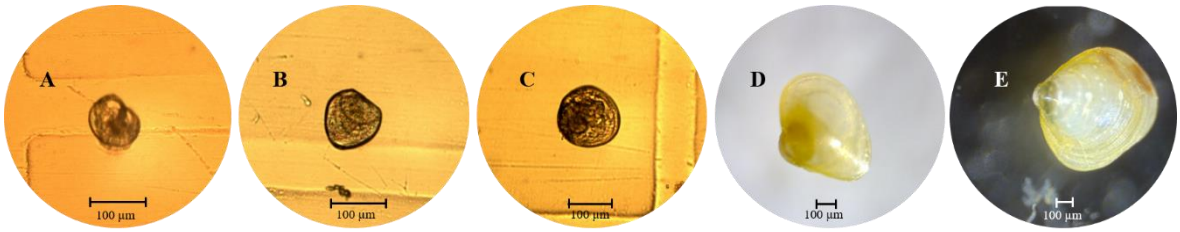

Figure 3.8 - *V. corrugata* shell malformations observed along the exposure to 9 OA-W experimental scenarios. A -convex hinge (at T3), B -disproportional height due to triangular form(at T7), C –rounded shell with no marked umbo (at T18), D –disproportional length due to lateral elongation (at T43) and E –ventral deformation (at T49).

3.3.4 Larval culture performance

The assessment of the larval culture performance was carried out by following the veligers' development and growth until complete settlement was recorded at T28 (i.e., from T0 to T28 at T3, T7, T10, T14, T18 and T22). Until T28, the development was exclusively driven by TºC being confirmed a significant acceleration after 14 and 18 days exposure (T14: Pseudo-F=13.755, p=0.0003 and T18, Pseudo-F=8.1121, p=0.0016). As pH did not reveal a significant effect on development, Figure 3.9 presents the results on our classification per TºC and timepoint.

Significant differences were found between the development registered at 22ºC and the two lower levels tested at both T14 (18 vs 22ºC: t-test=6.7313, p=0.0001; 20 vs 22ºC: t-test=2.7551, p=0.0175) and T18 (18 vs 22ºC: t-test: 6.536, p=0.0001; 20 vs 22ºC: t-test=2.7304, p=0.0104). It is widely recognized that TºC has a major influence on development: warmer temperatures seem to accelerate the transition between stages resulting in earlier settlement (Byrne, 2011), exactly what happened in the present study. At T14, ~30% of postlarvae were recorded at 22ºC while at 18 and 20ºC only ~2% and ~13% were registered, respectively. As postlarvae corresponds to the development stage immediately after larval metamorphosis, it indicates that the development was indeed significantly accelerated at 22ºC. The same scenario was apparent at T18, with 80% of postlarvae at 22ºC, whereas at 18 and 20ºC approximately 60% of larvae had not yet undergone metamorphosis (i.e., were umbonates and pediveligers). Hence, the warmest temperature tested (22ºC) seems to favour development, being within the thermal tolerance

range of the species *V. corrugata*, otherwise the effects would have caused metabolic depression (Pörtner, 2008) compromising the development.

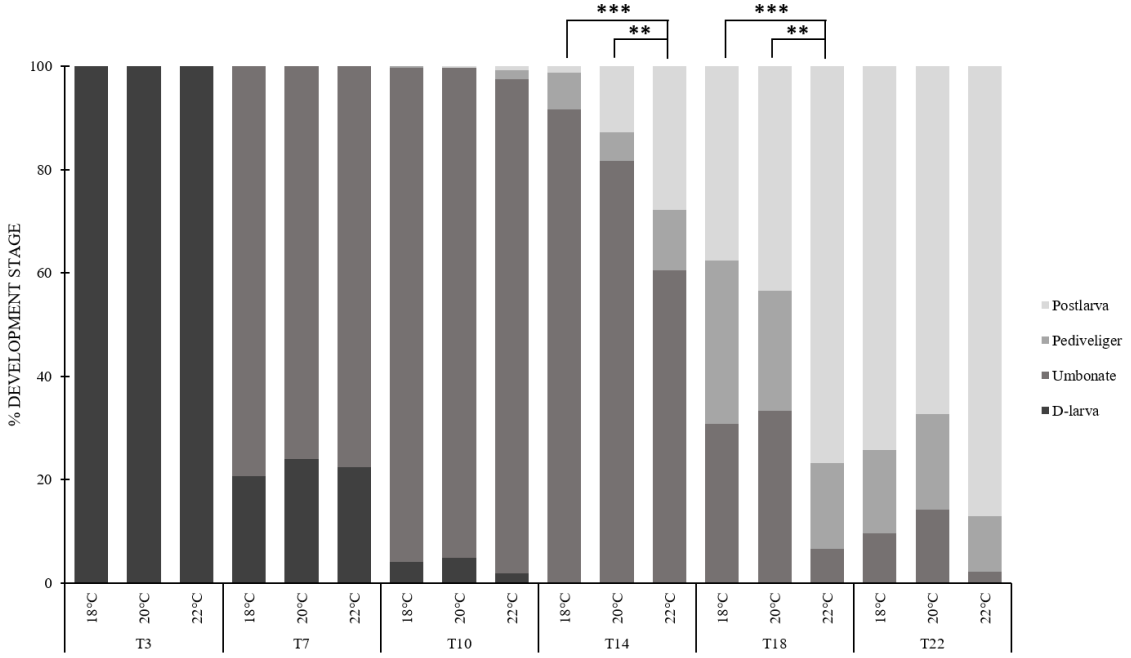

Figure 3.9 - *V. corrugata* development until complete settlement was recorded at T28 by timepoint and per temperature, categorized into the four stage classification by Cervino-Otero (2011): D-larva, Umbonate, Pediveliger and Postlarva. ** p<0.01, ***p<0.001.

In face of these results, the shell length (SL) was measured in specimens collected at T0 and T14 in order to (1) calculate growth rates per treatment during the early planktonic life, and (2) define if and how the environmental stressors under study –pH and TºC– affect this species' growth. Results on the SL and growth rates are compiled in (Figure 3.10).

A significant effect of the interaction between pH and TºC on the SL at T14 was proved (Pseudo-F=5.6481, p=0.0031). At pH control (8.1), specimens reared at 22ºC grew significantly more (18 vs 22ºC: t-test=0.1036, p(MC)=0.0118; 20 vs 22ºC: t-test=0.1007, p(MC)=0.004) while at pH 7.8 significant differences between 20 and 22º C were detected (t-test=0.1024, p(MC)=0.0487). Also, significant differences in the SL of specimens reared in different pH treatments at 22º were found, evidencing higher SL at pH control (8.1 vs 7.8: t-test=3.4475, p(MC)=0.028; 8.1 vs 7.6: t-test=3.1773, p(MC)=0.038) as shown in Figure 3.10). This is in accordance with the growth rates calculated that are highest at pH control (8.1) at the warmest TºC tested (22ºC): 16.6 μm day^{-1} between T0 and T14. The growth input

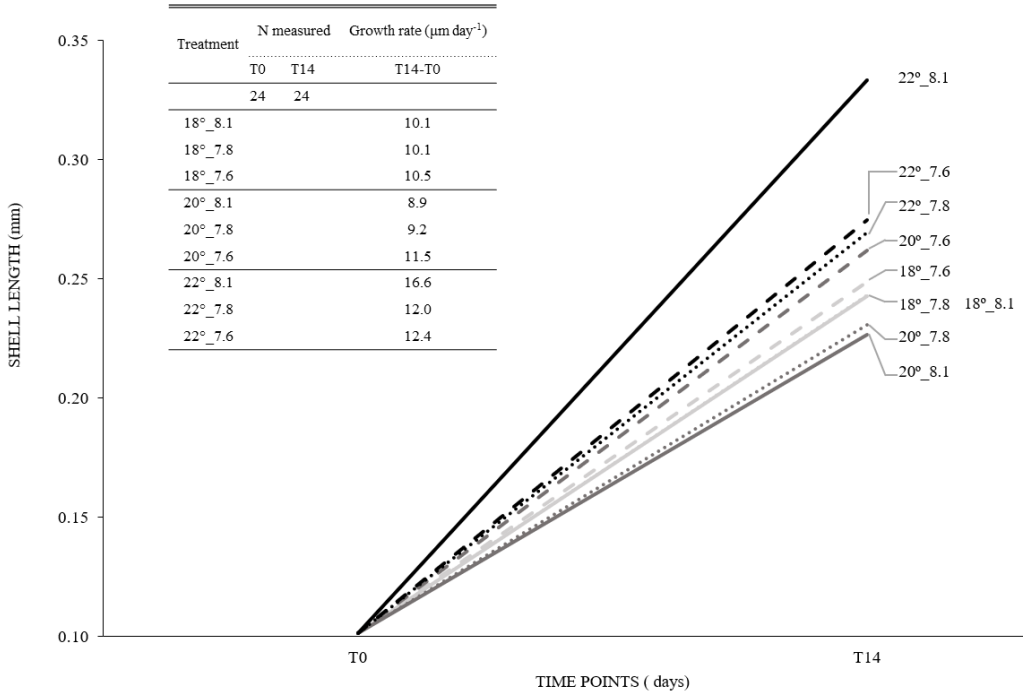

Figure 3.10 - *V. corrugata* mean shell length (SL) evolution from T0 to T14. Solid, dotted and dashed lines correspond to pH target levels (8.1, 7.8 and 7.6, respectively) and different colours, from light to intermediate to dark grey indicate the increasing TºC tested (18, 20 and 22ºC, respectively). Treatments are labelled on the right side of the graph, next to the correspondent line. The insert (table) on the upper left corner compiles the number of animals measured (N measured) per treatment and timepoint, and the growth rate (expressed in µm day^{-1}) during the first two weeks of exposure.

caused by temperature is possibly due to an increase in metabolism that consequently, increase filtration rate and absorption (Sokolova, 2013). However, this would only be possible if the temperature increase was within the thermal tolerance range of the species (Pörtner, 2008) and if food was available in in the required amount (Zippay and Helmuth, 2012). Based on studies developed by Peng *et al.* (2016) and Lazo and Pita (2012) both *Crassostrea iredalei* (Faustino, 1932) and *Mytilus galloprovincialis* (Lamarck, 1819) larvae, respectively, showed higher growth rates when exposed to warmer conditions (within the favour to the species). The negative effect of OA on bivalves' growth could be caused by a metabolic depression that affects acid-base balance calling for a higher energy demand to restore the normal condition (Pörtner, 2008). In line with the results presented here, *Crassostrea gigas* (Thunderg, 1793) larvae also reveal lower growth rates under acidified conditions (Barros *et al.*, 2013). However, it is also evident in this work that the proved significant interaction between TºC and pH on SL correspond to antagonistic effects of these stressors on *V. corrugata* growth (since, for instance, the growth rate of specimens reared under pH 7.6 at 22º was higher than the one at under the same pH at 18º). In this sense, it

seems obvious that here, similarly to what was described by other authors (Byrne and Przeslawski, 2013), warming counteracts the negative effects of acidification, reducing it.

3.3.5 Settlement and post-settlement culture

The first postlarvae were recorded at T10 for TºC ≥20ºC, in a very low percentage (less than ~1%). The pH was proved to have no significant effect ($p>0.05$) on the number of postlarvae registered per treatment at any timepoint until complete settlement was recorded at T28. As so, Figure 3.11 presents the percentage of settled animals from T10 to T28 only by TºC. By a simple observation of the plot, it seems clear that warmer treatments induced an earlier/faster settlement, as expected since the development throughout the larval culture (see above) was also increased at higher TºC.

Statistically, a significant effect of TºC on settlement was proved at T14 and T18 (T14: Pseudo-F=13.858, p=0.0006; T18, Pseudo-F=9.9636, p=0.0021). Although, while the percentage of settled animals was significantly different among all TºC at T14 (18 vs 20ºC: t-test=2.4663, p=0.03; 18 vs 22ºC: t-test=6.5313, p=0.0001; 20 vs 22ºC: t-test=2.4241, p=0.0342), the differences at T18 were only registered between the warmest and the other two levels (18 vs 22ºC: t-test=7.058, p=0.0003; 20 vs 22ºC: t-test=2.95, p=0.0142), indicating that settlement was only significantly increased under 22ºC at this timepoint. Once more, these results corroborate not only our data on the larval development, but also the data in the literature, since marine benthic calcifiers (such as bivalves) under warming, as long as within their thermal window, are known to settle sooner than those at lower temperatures (Pörtner, 2008; Byrne, 2011). Examples of this were reported in *C. gigas* larvae by Kheder *et al.* (2010) but also on larval stages of *Limnoperna fortune* (Dunker, 1857) by

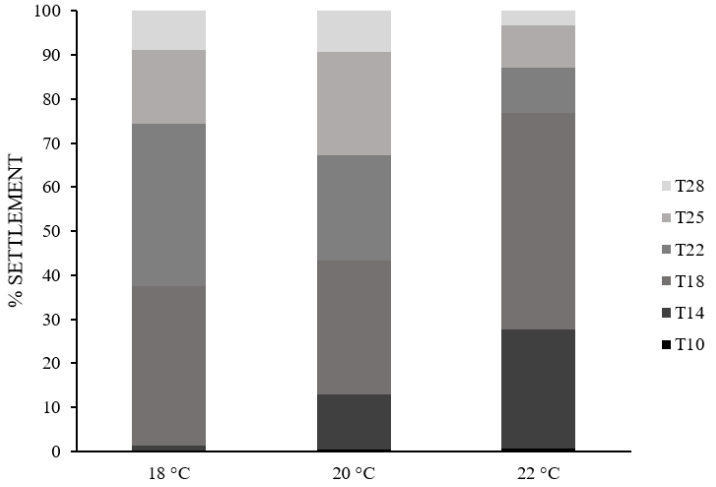

Figure 3.11 - *V. corrugata* settlement registered throughout the exposure to 9 OA-W scenarios. As no significant effects of pH were registered, the percentage of settled individuals is presented by temperature (18, 20 and 22ºC) from the first time it was registered, at T10, to the complete settlement recorded at T28.

Cataldo *et al.* (2005) – in which the metamorphosis phase that precedes settlement, have occurred earlier in larvae exposed to higher temperatures.

The SL of *V. corrugata* juveniles was measured at T28 –timepoint at which 100% settlement was recorded in all treatments and so only postlarvae were recorded– and at the end of the experiment, at T56. Its evolution between both timepoints and the growth rates calculated per treatment in that period are compiled in Figure 3.12.

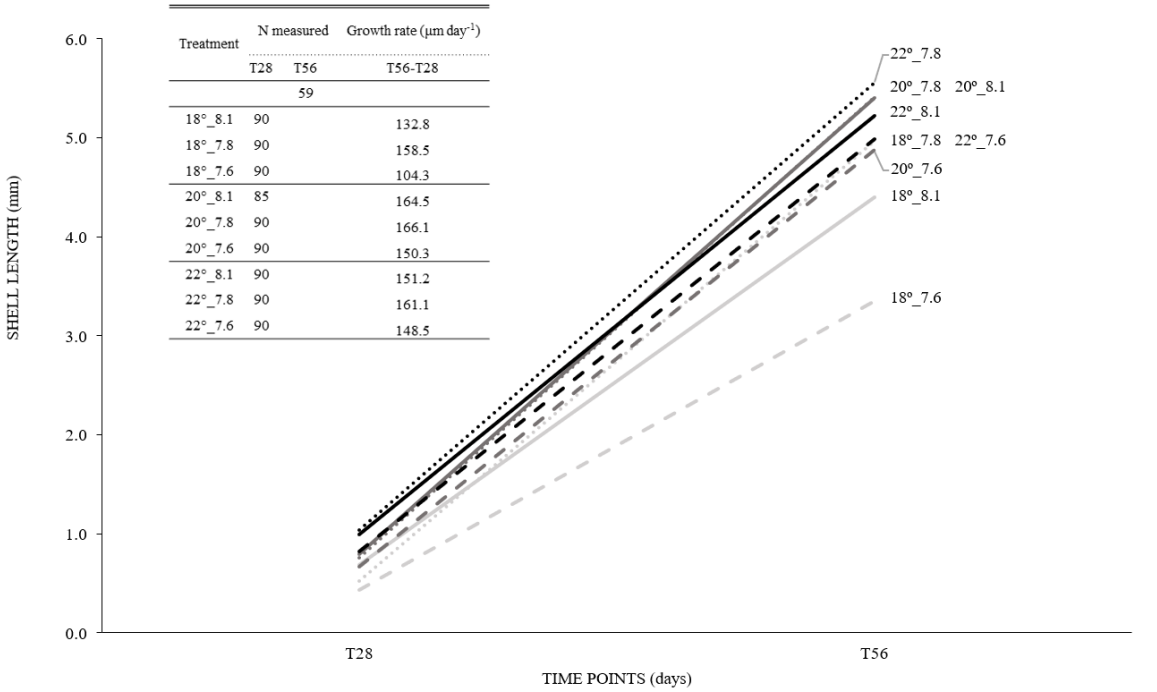

Figure 3.12 - *V. corrugata* mean shell length (SL) evolution between complete settlement (at T28) and after 56 (T56) days of exposure. Solid, dotted and dashed lines correspond to pH target levels (8.1, 7.8 and 7.6, respectively) and different colours, from light to intermediate to dark grey indicate the increasing TºC tested (18, 20 and 22ºC, respectively). Treatments are labelled on the right side of the graph, next to the correspondent line. The insert (table) on the upper left corner compiles the number of animals measured (N measured) per treatment and timepoint, and the growth rate (expressed in µm day^{-1}) during the last four weeks of exposure.

After 28 days of exposure, both the TºC (Pseudo-F=54.324, p=0.0001) and the pH (Pseudo-F=11.497, p=0.0012) were proved to have significant effects on the SL, but no significant interaction between both stressors was found. The SL of the specimens reared at all TºC levels was significantly different from each other (18 vs 20ºC: t-test=4.6396, p=0.0003; 18 vs 22ºC: t-test=13.212, p=0.0002; 20 vs 22ºC: t-test=4.911, p=0.0008) and the most acidic treatment (pH_{target} 7.6) was significantly different from the other two (8.1 vs 7.6: t-test=4.4715, p=0.0013, 7.8 vs 7.6: t-test=4.6789, p=0.0008).

These results follow the pattern exhibited by the larval growth described above regarding the fact that warming was proved to favour clams' size while acidification had the opposite, antagonistic effect. However, in this early benthic life, these stressors act separately and not interactively, exactly as reported by Navarro *et al.* (2016) for juvenile mussels exposed to comparable levels of warming (within the range of thermal tolerance) and acidification. As shown by those authors for *Mytilus chilensis* (Hupé, 1854), the positive effect of TºC on *V. corrugata* early juvenile growth could also be related to a potential energy gain (for instance, from the increased rates of processes like feeding, visibly increased under warming; see Table 3.2), energy that was possibly allocated for growth. In turn, the lower pH (at levels inducing moderate stress) might have reduced the energy allocated for growth by redirecting it to vital functions, as survival, instead of secondary issues such as growth (Pörtner, 2012). Thus, under most acidic treatments (pH_{target} 7.6), *V. corrugata* juveniles might have allocated a considerable part of the energy budget to basal functions, compromising growth. Even though, the antagonistic effect of the stressors under study, in this species, after settlement, at T28, is translated into a negative pressure of acidification masked by a positive pressure of TºC at 22ºC.

The positive effect of warming was also verified at the end of the exposure at T56: TºC was proved to affect significantly juveniles' size (Pseudo-F=9.717, p=0.0366) and the growth rates calculated per treatment for the benthic period (see the insert in Figure 3.12) were similar under warming (20 and 22ºC) and lower at control 18ºC. However, differently to the registered at T28 and even being close to the significance (p=0.0524), pH did not have a significant effect on SL after 2 months of exposure. Accordingly, the growth rates calculated for that period reveal variable results between different pH levels tested. This can be a sign that, as referred before to explain this species survival under lower pH, *V. corrugata* is possibly adapted to environmental fluctuations of pH in its benthic life, due to the constant exposure to variable conditions (of pH but also salinity, temperature, tide, etc) in its estuarine habitat (Ria de Aveiro -NW Portugal; see Cunha *et al.* 2005) in which the range of pH that the specimens should be prepared to face are within the range tested in our experiment.

3.4 Conclusion

Ocean acidification (OA) and Warming (W) are unequivocally changing our oceans' dynamic and, thus, marine life. However, within some limits and under certain conditions, some species might be resilient to OA-W projected scenarios as shown in the current work.

V. corrugata survival and growth were proved to be significantly affected by either warming and acidification, separately or interactively, at different phases of the specimens' early development. Even so, both the planktonic and benthic early life stages seem to be significantly favored by the concomitant OA-W, with additive positive effects of both stressors involved (TºC and pH) on larval survival, and antagonistic effects on larvae and juvenile growth, with the higher temperature mitigating the negative effects of lower pH. The ontogenic development was proved to be accelerated by warming and a significant reduction of the most fragile, planktonic life period, by premature settlement at higher temperature was evident. Furthermore, shell malformation was not significantly different between and within treatments in the first two months of life under OA-W projected scenarios.

Despite being promising results, pointing towards the species resilience under the tested scenarios, increased survival and growth under such conditions are dependent on its occurrence within the range of the species tolerance and with higher food intake under warming, requisites that might limit such a response in some regions, both in the wild and in productive environments (hatcheries and nurseries). The same being applied to acidification, since only carbonate saturation was tested here.

Nevertheless, this chapter documents the response of *V. corrugata* early life stages under several possible future scenarios of OA-W, showing their better performance under the levels projected for the end of the century at the species geographical range. These results are not only important as indicative of the resilience of the species to the environmental TºC and pH levels it is expected to face in its natural habitat; it is also very relevant to the aquaculture industry once, contrarily to other shellfish species proved to be severely affected by future OA-W, *V. corrugata* high productive potential under the tested conditions suggests that the species is a potential profitable resource to invest.

References

Allen, M. R., Dube, O. P., Solecki, W., Aragón-Durand, F., Cramer, W., Humphreys, S., Kainuma, M., Kala, J., Mahowald, N., Mulugetta, Y., Perez, R., Wairiu, M. and Zickfeld, K. (2018) 'Framing and Context', in *Global Warming of 1.5°C. An IPCC Special Report on the impacts of global warming of 1.5°C above pre-industrial levels and related global greenhouse gas emission pathways, in the context of strengthening the global response to the threat of climate change, sustainable development, and efforts to eradicate poverty* [Masson-Delmotte, V., P. Zhai, H.-O. Pörtner, D. Roberts, J. Skea, P.R. Shukla, A. Pirani, W. Moufouma-Okia, C. Péan, R. Pidcock, S. Connors, J.B.R. Matthews, Y. Chen, X. Zhou, M.I. Gomis, E. Lonnoy, T. Maycock, M. Tignor, and T. Waterfield (eds.)]. In Press, pp. 49-91.

Baria, M. V. B., Kurihara, H. and Harii, S. (2015) 'Tolerance to Elevated Temperature and Ocean Acidification of the Larvae of the Solitary Corals *Fungia fungites* (Linnaues, 1758) and *Lithophyllon repanda* (Dana, 1846)', *Zoological Science*, 32(5), p. 447. doi: 10.2108/zs150036.

Barros, P., Sobral, P., Range, P., Chícharo, L. and Matias, D. (2013) 'Effects of sea-water acidification on fertilization and larval development of the oyster *Crassostrea gigas*', *Journal of Experimental Marine Biology and Ecology*, 440, pp. 200–206. doi: 10.1016/j.jembe.2012.12.014.

Byrne, M. (2011) 'Impact of ocean warming and ocean acidification on marine invertebrate life history stages: vulnerabilities and potential for persistence in a changing ocean', *Oceanography and Marine Biology: An Annual Review*, 49, pp. 1–42.

Byrne, M. and Przeslawski, R. (2013) 'Multistressor impacts of warming and acidification of the ocean on marine invertebrates' life histories', *Integrative and Comparative Biology*, 53(4), pp. 582–596. doi: 10.1093/icb/ict049.

Cataldo, D., Boltovskoy, D., Hermosa, J. L. and Canzi, C. (2005) 'Temperature-dependent rates of larval development in *Limnoperna fortunei* (Bivalvia: Mytilidae)', *Journal of Molluscan Studies*, 71(1), pp. 41–46. doi: 10.1093/mollus/eyi005.

Cerviño-Otero, A. (2011) *Ciclo reproductivo, cultivo en criadero y en el medio natural de la almeja babosa Venerupis pullastra (Montagu, 1803)*. PhD thesis. Santiago de Compostela, Galiza, Spain. Instituto de Acuicultura, Universidade de Santiago de Compostela

Ciais, P., Sabine, C., Bala, G., Bopp, L., Brovkin, V., Canadell, J., Chhabra, A., DeFries, R., Galloway, J., Heimann, M., Jones, C., Quéré, C. Le, Myneni, R. B., Piao, S. and Thornton, P. (2013) 'Carbon and Other Biogeochemical Cycles', in *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)]. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press, pp. 465-570.

Clements, J. C. and Darrow, E. S. (2018) 'Eating in an acidifying ocean: a quantitative review of elevated CO_2 effects on the feeding rates of calcifying marine invertebrates', *Hydrobiologia*. doi: 10.1007/s10750-018-3665-1.

Coelho, F. J. R. C., Rocha, R. J. M., Pires, A. C. C., Ladeiro, B., Castanheira, J. M., Costa, R., Almeida, A., Cunha, Â., Lillebø, A. I., Ribeiro, R., Pereira, R., Lopes, I., Marques, C., Moreira-Santos, M., Calado, R., Cleary, D. F. R. and Gomes, N. C. M. (2013) 'Development and validation of an experimental life support system for assessing the effects of global climate change and environmental contamination on estuarine and coastal marine benthic communities', *Global Change Biology*, 19(8), pp. 2584–2595. doi: 10.1111/gcb.12227.

Collins, M., Knutti, R., Arblaster, J., Dufresne, J., Fichefet, T., Friedlingstein, P., Gao, X., Gutowski, W., Johns, T., Krinner, G., Shongwe, M., Tebaldi, C., Weaver, A. and Wehner, M. (2013) 'Long-term Climate Change: Projections, Commitments and Irreversibility', in *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)]. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press, pp. 1029-1136.

Cubasch, U., Wuebbles, D., Chen, D., Facchini, M. C., Frame, D., Mahowald, N. and Winther, J.-G. (2013) 'Introdution', in *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)]. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press, pp. 119-158.

Cunha, M. A., Pedro, R., Almeida, M. A. and Silva, M. H. (2005) 'Activity and growth efficiency of heterotrophic bacteria in a salt marsh (Ria de Aveiro, Portugal)', *Microbiological Research*, 160(3), pp. 279–290. doi: 10.1016/j.micres.2005.02.001.

Dickson, A. G. (1990) 'Standard potential of the reaction: AgCl(s) + 1 $2H_2$(g) = Ag(s) + HCl(aq), and and the standard acidity constant of the ion HSO_4^- in synthetic sea water from 273.15 to 318.15 K', *The Journal of Chemical Thermodynamics*, 22(2), pp. 113–127. doi: 10.1016/0021-9614(90)90074-Z.

Dickson, A. G. (2011) 'The carbon dioxide system in seawater: equilibrium chemistry and measurements', in *Guide to best practices for ocean acidification research and data reporting* [U. Riebesell, V. J. Fabry, L. Hansson and J.-P. Gattuso (eds.)]. Luxembourg: Publications Office of the European Union, pp. 17–40.

Dickson, A. G. and Millero, F. J. (1987) 'A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media', *Deep Sea Research Part A, Oceanographic Research Papers*, 34(10), pp. 1733–1743. doi: 10.1016/0198-0149(87)90021-5.

FAO © (2006) *Cultured Aquatic Species Information Programme - Venerupis pullastra.* Texted by Figueras, A., in: *FAO Fisheries and Aquaculture Department* [online]. Rome. Updated 26 February 2006. Available at: http://www.fao.org/fishery/culturedspecies/Venerupis_pullastra/en (Accessed: 27 October 2019)..

Fernández-Pardo, A., da Costa, F., Rial, D., Nóvoa, S., Martínez-Patiño, D. and Vázquez, J. A. (2016) 'Use of response surface methodology to determine optimum diets for *Venerupis corrugata* larvae: effects of ration and microalgal assemblages', *Aquaculture*. Elsevier, 452, pp. 283–290. doi: 10.1016/J.AQUACULTURE.2015.11.005.

Folt, C. L., Chen, C. Y., Moore, M. V. and Burnaford, J. (1999) 'Synergism and antagonism among multiple stressors', *Limnology and Oceanography*, 44(3 II), pp. 864–877. doi: 10.4319/lo.1999.44.3_part_2.0864.

Gazeau, F., Parker, L. M., Comeau, S., Gattuso, J. P., O'Connor, W. A., Martin, S., Pörtner, H. O. and Ross, P. M. (2013) 'Impacts of ocean acidification on marine shelled molluscs', *Marine Biology*, 160(8), pp. 2207–2245. doi: 10.1007/s00227-013-2219-3.

Goyet, C. and Snover, A. K. (1993) 'High-accuracy measurements of total dissolved inorganic carbon in the ocean: comparison of alternate detection methods', *Marine Chemistry*, 44(2–4), pp. 235–242. doi: 10.1016/0304-4203(93)90205-3.

Guo, X., Huang, M., Pu, F., You, W. and Ke, C. (2015) 'Effects of ocean acidification caused by rising CO_2 on the early development of three mollusks', *Aquatic Biology*, 23(2), pp. 147–157. doi: 10.3354/ab00615.

Helm, M. M. and Bourne, N. (Neil) (2004) *Hatchery culture of bivalves : a practical manual* [A. Lovatelli (eds.)]. Rome, Italy: Food and Agriculture Organization of the United Nations.

IPCC (2013) *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)]. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press, pp. 1535

IPCC (2014) *Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)]. Geneva, Switzerland: IPCC, pp. 151

Jardillier, E., Rousseau, M., Gendron-Badou, A., Fröhlich, F., Smith, D. C., Martin, M., Helléouet, M. N., Huchette, S., Doumenc, D. and Auzoux-Bordenave, S. (2008) 'A morphological and structural study of the larval shell from the abalone *Haliotis tuberculata*', *Marine Biology*, 154(4), pp. 735–744. doi: 10.1007/s00227-008-0966-3.

Joaquim, S., Matias, D., Matias, A. M., Moura, P., Arnold, W. S., Chícharo, L. and Gaspar, M. B. (2011)

‘Reproductive activity and biochemical composition of the pullet carpet shell *Venerupis enegalensis* (Gmelin, 1791) (Mollusca: Bivalvia) from Ria de Aveiro (Northwestern coast of Portugal)’, *Scientia Marina*, 75(2), pp. 217–226. doi: 10.3989/scimar.2011.75n2217.

Joaquim, S., Matias, D., Matias, A. M., Leitão, A., Soares, F., Cabral, M., Chícharo, L. and Gaspar, M. B. (2014) ‘The effect of density in larval rearing of the pullet carpet shell *Venerupis corrugata* (Gmelin, 1791) in a recirculating aquaculture system’, *Aquaculture Research*, 47(4), pp. 1055–1066. doi: 10.1111/are.12561.

Joaquim, S., Matias, D., Matias, A. M., Leitão, A., Soares, F., Cabral, M., Chícharo, L. and B., G. M. (2016) ‘The effect of density in larval rearing of the pullet carpet shell *Venerupis corrugata* (Gmelin, 1791) in a recirculating aquaculture system’, *Aquaculture Research*, 47(4), pp. 1055–1066. doi: 10.1111/are.12561.

Johannessen, O. H. (1973) ‘Population structure and individual growth of *Venerupis pullastra* (Montagu) (Lamellibranchia)’, *Sarsia*, 52(1), pp. 97–116. doi: 10.1080/00364827.1973.10411234.

Kandler, N. M., Abdul Wahab, M. A., Noonan, S. H. C., Bell, J. J., Davy, S. K., Webster, N. S. and Luter, H. M. (2018) ‘In situ responses of the sponge microbiome to ocean acidification’, *FEMS microbiology ecology*, 94(12). doi: 10.1093/femsec/fiy205.

Kheder, R. Ben, Moal, J. and Robert, R. (2010) ‘Impact of temperature on larval development and evolution of physiological indices in *Crassostrea gigas*’, *Aquaculture*, 309(1–4), pp. 286–289. doi: 10.1016/j.aquaculture.2010.09.005.

Kroeker, K. J., Kordas, R. L., Crim, R., Hendriks, I. E., Ramajo, L., Singh, G. S., Duarte, C. M. and Gattuso, J.-P. (2013) ‘Impacts of ocean acidification on marine organisms: quantifying sensitivities and interaction with warming’, *Global Change Biology*, 19(6), pp. 1884–1896. doi: 10.1111/gcb.12179.

Kurihara, H. (2008) ‘Effects of CO_2-driven ocean acidification on the early developmental stages of invertebrates’, *Marine Ecology Progress Series*, 373, pp. 275–284. doi: 10.3354/meps07802.

Lagos, N. A., Benítez, S., Duarte, C., Lardies, M. A., Broitman, B. R., Tapia, C., Tapia, P., Widdicombe, S. and Vargas, C. A. (2016) ‘Effects of temperature and ocean acidification on shell characteristics of *Argopecten purpuratus*: Implications for scallop aquaculture in an upwelling-influenced area’, *Aquaculture Environment Interactions*, 8, pp. 357–370. doi: 10.3354/AEI00183.

Lazo, C. S. and Pita, I. M. (2012) ‘Effect of temperature on survival, growth and development of *Mytilus galloprovincialis* larvae’, *Aquaculture Research*, 43(8), pp. 1127–1133. doi: 10.1111/j.1365-2109.2011.02916.x.

Lenz, B., Fogarty, N. D. and Figueiredo, J. (2019) ‘Effects of ocean warming and acidification on fertilization success and early larval development in the green sea urchin *Lytechinus variegatus*’, *Marine Pollution Bulletin*, pp. 70–78. doi: 10.1016/j.marpolbul.2019.02.018.

León, P., Bednaršek, N., Walsham, P., Cook, K., Hartman, S. E., Wall-Palmer, D., Hindson, J., Mackenzie, K., Webster, L. and Bresnan, E. (2019) 'Relationship between shell integrity of pelagic gastropods and carbonate chemistry parameters at a Scottish Coastal Observatory monitoring site', *ICES Journal of Marine Science*. doi: 10.1093/icesjms/fsz178.

Mackenzie, C. L., Ormondroyd, G. A., Curling, S. F., Ball, R. J., Whiteley, N. M. and Malham, S. K. (2014) 'Ocean warming, more than acidification, reduces shell strength in a commercial shellfish species during food limitation', *PLoS ONE*, 9(1). doi: 10.1371/journal.pone.0086764.

Mehrbach, C., Culberson, C., Hawley, J. and Pytkowicx, R. (1973) 'Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure', *Limnology and Oceanography*, 18(6), pp. 897–907. doi: 10.4319/lo.1973.18.6.0897.

Milano, S., Schöne, B. R., Wang, S. and Müller, W. E. (2016) 'Impact of high pCO_2 on shell structure of the bivalve *Cerastoderma edule*', *Marine Environmental Research*, 119, pp. 144–155. doi: 10.1016/J.MARENVRES.2016.06.002.

Mileikovsky, S. (1973) 'Speed of active movement of pelagic larvae of marine bottom invertebrates and their ability to regulate their vertical position', *Marine Biology*, 23(1), pp. 11–17. doi: 10.1007/BF00394107.

Navarro, J. M., Duarte, C., Manríquez, P. H., Lardies, M. A., Torres, R., Acuña, K., Vargas, C. A., Lagos, N. A. and Ocean, N. A. (2016) 'Ocean warming and elevated carbon dioxide: multiple stressor impacts on juvenile mussels from southern Chile'. doi: 10.1093/icesjms/fsv249.

O'Brien, P. A., Morrow, K. M., Willis, B. L. and Bourne, D. G. (2016) 'Implications of ocean acidification for marine microorganisms from the free-living to the host-associated', *Frontiers in Marine Science*. doi: 10.3389/fmars.2016.00047.

Orr, J. C., Fabry, V. J., Aumont, O., Bopp, L., Doney, Scott C., Feely, R. A., Gnanadesikan, A,. Gruber, N., Ishida, A., Joos, F., Key, R. M., Lindsay, K., Maier-R., E., Matear, R., Monfray, P., Mouchet, A., Najjar, R. G., Plattner, G. K., Rodgers, K. B., Sabine, C. L., Sarmiento, J. L., Schlitzer, R., Slater, R. D., Totterdell, I. J., Weirig, M. F. Yamanaka, Y., Yool, A. (2005) 'Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms', *Nature*, 437(7059), pp. 681–686. doi: 10.1038/nature04095.

Pankhurst, N. W. and Munday, P. L. (2011) 'Effects of climate change on fish reproduction and early life history stages', *Marine and Freshwater Research*, 62(9), pp. 1015–1026. doi: 10.1071/MF10269.

Peng, T. C., Vengatesen, T. and Hwai, A. T. S. (2016) 'Assessment of temperature effects on early larval development survival of hatchery-reared tropical oyster, *Crassostrea iredalei*', *Tropical Life Sciences Research*, 27, pp. 111–116. doi: 10.21315/tlsr2016.27.3.15.

Pierrot, D., Lewis, E. and Wallace, D. W. R. (2006) 'MS Excel program developed for CO2 system

calculations', *Carbon Dioxide Information Analysis Center*, (3), p. ORNL/CDIAC-105a. doi: 10.3334/CDIAC/otg.CO2SYS_XLS_CDIAC105a.

Pörtner, H. O. (2008) 'Ecosystem effects of ocean acidification in times of ocean warming: A physiologist's view', *Marine Ecology Progress Series*, pp. 203–217. doi: 10.3354/meps07768.

Pörtner, H. O. (2012) 'Integrating climate-related stressor effects on marine organisms: Unifying principles linking molecule to ecosystem-level changes', *Marine Ecology Progress Series*, 470, pp. 273–290. doi: 10.3354/meps10123.

Przeslawski, R., Davis, A. R. and Benkendorff, K. (2005) 'Synergistic effects associated with climate change and the development of rocky shore molluscs', *Global Change Biology*, 11(3), pp. 515–522. doi: 10.1111/j.1365-2486.2005.00918.x.

Quayle, D. B. (1952) 'Structure and Biology of the Larva and Spat of *Venerupis pullastra* (Montagu)', *Transactions of the Royal Society of Edinburgh*, 62, pp. 255–297. doi: 10.1017/S0080456800009297.

Queirós, A. M., Fernandes, J. A., Faulwetter, S., Nunes, J., Rastrick, S. P. S., Mieszkowska, N., Artioli, Y., Yool, A., Calosi, P., Arvanitidis, C., Findlay, H. S., Barange, M., Cheung, W. W. L. and Widdicombe, S. (2015) 'Scaling up experimental ocean acidification and warming research: From individuals to the ecosystem', *Global Change Biology*, 21(1), pp. 130–143. doi: 10.1111/gcb.12675.

Rayment, W. J. (2007) *Venerupis corrugata Pullet carpet shell*, *Marine Life Information Network: Biology and Sensitivity Key Information Reviews* [online] [Tyler-Walters H, Hiscock K (eds.)]. Plymouth: Marine Biological Association of the United Kingdom, Available at: https://www.marlin.ac.uk/species/detail/1558 (Accessed: 2 December 2019).

Red Sea (2019) *Red Sea Salt -Ideal for mature reef and low nutrient systems*. Available at: https://www.redseafish.com/red-sea-salts/red-sea-salt/ (Accessed: 2 December 2019).

Rhein, M., Rintoul, S. R., Aoki, S., Campos, E., Chambers, D., Feely, R. A., Gulev, S., Johnson, G. C., Josey, S. A., Kostianoy, A., Mauritzen, C., Roemmich, D., Talley, L. D. and Wang, F. (2013) 'Observations: Ocean', in *Climate Change 2013 the Physical Science Basis: Working Group I Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)]. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press, pp. 225-315.

Riahi, K., Rao, S., Krey, V., Cho, C., Chirkov, V., Fischer, G., Kindermann, G., Nakicenovic, N. and Rafaj, P. (2011) 'RCP 8.5-A scenario of comparatively high greenhouse gas emissions', *Climatic Change*, 109(1), pp. 33–57. doi: 10.1007/s10584-011-0149-y.

Riebesell, U., Fabry, V. J., Hansson, L. and Gattuso, J. P. J.-P. (2010) *Guide to best practices for ocean acidification research and data reporting*, *Environment*. Luxembourg: Official Publications of the

European Communities, pp. 260.

Ries, J. B. (2011) 'Skeletal mineralogy in a high-CO_2 world', *Journal of Experimental Marine Biology and Ecology*, 403(1–2), pp. 54–64. doi: 10.1016/j.jembe.2011.04.006.

Santos, A. (2018) *Mortalidade de juvenis de Venerupis corrugata (Gmelin, 1791) por stresse salino e térmico*. MSc thesis. Aveiro, Portugal. Departamento de Biologia, Universidade de Aveiro.

Sokolova, I. M., Frederich, M., Bagwe, R., Lannig, G. and Sukhotin, A. A. (2012) 'Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates', *Marine Environmental Research*, 79, pp. 1–15. doi: 10.1016/j.marenvres.2012.04.003.

Sokolova, I. M. (2013) 'Energy-limited tolerance to stress as a conceptual framework to integrate the effects of multiple stressors', *Integrative and Comparative Biology*, 53(4), pp. 597–608. doi: 10.1093/icb/ict028.

Sokolova, I. M., Sukhotin, A. A. and Lannig, G. (2011) 'Stress Effects on Metabolism and Energy Budgets in Mollusks', in *Oxidative Stress in Aquatic Ecosystems* [Abele, D., Vázquez-Medina, J. P., and Zenteno-Savín., T. (eds.)]. Blackwell Publishing Ltd., pp. 261–280.

Talmage, S. C. and Gobler, C. J. (2011) 'Effects of elevated temperature and carbon dioxide on the growth and survival of larvae and juveniles of three species of northwest Atlantic bivalves', *PLoS ONE*, 6(10), p. e26941. doi: 10.1371/journal.pone.0026941.

Thomsen, J., Stapp, L. S., Haynert, K., Schade, H., Danelli, M., Lannig, G., Wegner, K. M. and Melzner, F. (2017) 'Naturally acidified habitat selects for ocean acidification–tolerant mussels', *Science Advances*, 3(4). doi: 10.1126/sciadv.1602411.

Thorson, G. (1950) 'Reproductive and Larval Ecology of Marine Bottom Invertebrates', *Biological Reviews*, 25(1), pp. 1–45. doi: 10.1111/j.1469-185X.1950.tb00585.x.

Tridge (2019) *Pullet Carpet Shell suppliers, wholesale prices, and global market information.*. Available at: https://www.tridge.com/intelligences/pullet-carpet-shell (Accessed: 23 November 2019).

Zippay, M. L. and Helmuth, B. (2012) 'Effects of temperature change on mussel, *Mytilus*', *Integrative Zoology*, pp. 312–327. doi: 10.1111/j.1749-4877.2012.00310.x.

Chapter 4

Main conclusions and Final remarks

On a global scale, human-induced climate change is altering the composition, diversity and several services provided by marine ecosystems. Ocean acidification (OA) and warming (W) are two of the most important climate change-related consequences, altering the physical and the chemical properties of seawater, with particular effects on marine shelled organisms such as the gastropods and the bivalves. In fact, OA-W are now recognized as a joint massive disturbance on marine ecosystems, impacting marine biodiversity with difficult, or even with no, return.

The urgency in understanding the nature and the extent of such impacts was the motivation for this dissertation. Here were tested the effects of the combined occurrence of OA-W in two species of marine shelled molluscs: the gastropod, *Tritia reticulata*, a well-known bioindicator of environmental quality and, as a scavenger, a species of high ecological importance in coastal ecosystems; and the bivalve, *Venerupis corrugata*, a food resource with high market value, and a filter-feeder, thus providing economically and ecologically valuable ecosystem services.

In *T. reticulata* (Chapter 2) the environmental stressors involved –temperature and pH– were proved to have dramatic effects, particularly in the early planktonic life, lessening considerably the chances of larvae to survive in nature. Severe mortalities, reduced growth, shells' dissolution and loss are amongst the deleterious effects of even short-term exposure of the species' early life stages to some of the OA-W scenarios tested, showing its vulnerability under expected environmental conditions. Contrarily, *V. corrugata* (Chapter 3) was proved to be resilient to the same stressors, under the conditions tested. Even acknowledging that experimental scenarios applied were not exactly the same, and so no direct comparisons between the results obtained in the different species are correct, higher survival of *V. corrugata* larvae under warm and acidic conditions was real, and the development was clearly favored by the temperature increase even under a significant antagonistic effect of the pH reduction on growth.

The results here presented report, to my best knowledge and for the first time to science, the impacts of future OA-W on these species development, growth and survival. Moreover, the data in this thesis are original and contribute to better understand the effects

of human-induced climate change on coastal and marine ecosystems, of which many shelled molluscs are part.

Future work should consider that, in nature, organisms are exposed to many more environmental stressors than pH and temperature. In this sense, it is of upmost importance to consider other stressors (e.g., salinity, dissolved oxygen, nutrients) in factorial experimental designs, bringing research closer to the complexity of the environmental reality in which these animals live. Acclimatization is also a relevant topic to take into account, since organisms can adapt to the expected environmental conditions faster than it becomes effective, never forgetting that adaptation is dependent on a series of species-specific factors such as the genetic background. Therefore, it is necessary to study carefully selected species so that the conclusions drawn can clarify what will happen in the future for a larger number of species and ecosystems.