**Universidade de Aveiro** Departamento de Biologia
**2019**

**Joana Gomes Fonseca**

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# Efeitos do aquecimento e da acidificação oceânica no comportamento de estádios iniciais de vida de moluscos marinhos

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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 do Senhor Prof. Doutor Carlos Miguez Barroso, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro, e da Doutora Mariana Hinzmann, Investigadora em Pós-Doutoramento do Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR) da Universidade do Porto.

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Aos meus pais, mano e avós.
Consegui.

**o júri**

| | |
|---|---|
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**palavras-chave**

Alteração climática, Temperatura, pH, Gastropódes, Bivalves, *Tritia reticulata*, *Venerupis corrugata*, locomoção, mobilidade, natação, alimentação, histologia, microscopia

**resumo**

Ao longo das últimas décadas o clima da Terra tem vindo a alterar-se de um modo sem precedentes. A temperatura da superfície do planeta tem aumentado (Aquecimento Global) devido à crescente emissão de gases com efeito de estufa provenientes de atividades antropogénicas, especialmente o dióxido de carbono ($CO_2$). As alterações globais têm afetado também os oceanos, provocando um aumento da sua temperatura – *Aquecimento do Oceano* (W) – e uma diminuição do pH da água do mar em consequência do aumento da concentração de $CO_2$ – *Acidificação do Oceano* (OA). Os gastrópodes e os bivalves com concha são potencialmente sensíveis a cenários de OA-W pelo facto de serem calcificadores e por terem ciclos de vida complexos. Estes ciclos de vida compreendem, geralmente, uma fase larvar planctónica seguida de uma profunda metamorfose conducente a um modo de vida bentónico. Desta forma, os estados iniciais de desenvolvimento podem ser eventualmente mais suscetíveis ao aquecimento e acidificação da água do mar.

Com o objetivo de avaliar o impacto da OA-W sobre o comportamento e a sobrevivência dos estádios iniciais da vida dos moluscos com concha, as fases larvares do gastrópode *Tritia reticulata* (Linnaeus, 1758) – uma espécie necrófaga essencial nos ecossistemas costeiros europeus – e do bivalve *Venerupis corrugata* (Gmelin, 1791) – filtrador e importante recurso pesqueiro no sudeste europeu – foram expostas a cenários de OA-W projetados pelo Painel Intergovernamental de Mudanças Climáticas (IPCC) para o final deste século.

A natação e a sobrevivência larvar de *T. reticulata* foram negativamente afetadas pela exposição a condições experimentais de OA-W durante 14 dias. Para além do efeito sinérgico do pH e da temperatura na atividade, velocidade e distância percorrida pelas velígeras de *T. reticulata*, foi verificada a existência de dano morfo-histológico do epitélio do pé, com potenciais implicações funcionais na fase larvar e bentónica. Por sua vez, após 14 dias de exposição a cenários de OA-W, a amêijoa-macha (*V. corrugata)* apresentou uma maior sobrevivência e desenvolvimento larvares, e não se observaram alterações comportamentais. Apesar do efeito antagónico da temperatura e do pH, a sua ocorrência em simultâneo poderá diminuir os impactos da OA no desenvolvimento das populações naturais e na produção de semente em maternidade, muito embora a exposição a cenários de OA, durante 60 dias, tenha demonstrado afetar a capacidade da amêijoa juvenil se enterrar no sedimento e de produzir bisso, ficando possivelmente mais vulnerável à predação.

Em suma, os resultados apresentados nesta tese demonstram que a sobrevivência de *T. reticulata* parece estar ameaçada pelas condições de OA-W

projetadas pelo IPCC para o final do século, contrariamente a *V. corrugata*, cujos estágios iniciais de vida aparentam uma certa resiliência às condições ambientais em estudo, sugerindo um efeito benéfico do aquecimento que pode vir a compensar os efeitos negativos da acidificação caso os cenários testados se tornem efetivos.

**keywords**

Climate change, Temperature, pH, Gastropods, Bivalves, *Tritia reticulata*, *Venerupis corrugata*, locomotion, mobility, swimming, feeding, histology, microscopy

**abstract**

Over the last few decades the Earth's climate change has been changing in an unprecedent way. The planet's surface temperature has increased (*Global Warming*) due to the increasing emission of greenhouse gases from anthropogenic activities, especially carbon dioxide ($CO_2$). Global changes have also affected the oceans causing an increase in its temperature – Ocean warming (W) – and a decrease in the seawater pH as a consequence of the increased concentration of $CO_2$. Shelled gastropods and bivalves are potentially sensitive to OA-W since are calcifiers and have complex life cycles. These life cycles generally comprise a planktonic larval phase followed by a profound metamorphosis leading to a benthic way of life. In this way, early development stages may eventually be more susceptible to seawater acidification and warming.

In order to assess the impact of OA-W on the behaviour and survival of the early stages of shelled molluscs, the larval phases of the gastropod *Tritia reticulata* (Linnaeus, 1758) – a necrophage essential in european coastal ecosystems – and of the bivalve *Venerupis corrugata* (Gmelin, 1791) – a filter-feeder and important fishery resource – were exposed to OA-W scenarios predicted by the Intergovernmental Panel on Climate Change (IPCC) for the end of this century.

*T. reticulata* swimming and larval survival were negatively affected by the exposure to OA-W experimental conditions for 14 days. In addition to the synergistic effect of pH and temperature on the activity, velocity and distance traveled by *T. reticulata* veligers, there was evidence of morpho-histological damage of the foot epithelium with potential functional implications on both the larval and benthic phases. In turn, after 14 days of exposure to AO-W scenarios, the pullet carpet shell *Venerupis corrugata* showed greater larval survival and development, and no behavioural changes were observed. Despite the antagonistic effect of temperature and pH, their simultaneous occurrence may reduce the impacts of OA on natural populations' development and seed production in hatchery, although exposure to OA scenarios for 60 days has been shown to affect the ability of juvenile clams to bury in the sediment and to produce byssus, possibly becoming more vulnerable to predation.

In short, the results presented in this thesis demonstrate that *T. reticulata* survival seems to be threatened by late-century OA-W conditions projected by the IPCC, contrarily to *V. corrugata,* whose early life stages appear somewhat resilient to the environmental conditions studied, suggesting a beneficial effect of warming that may offset the negative effects of acidification if the tested scenarios become effective.

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# Chapter 1: General Introduction

## 1.1 Global Climate Change

According to the Intergovernmental Panel on Climate Change (IPCC), *Climate* is the average weather (i.e., the mean and the variability of surface temperature, precipitation, wind, etc.) over a given period of time, typically 30 years as defined by the World Meteorological Organization (IPCC 2013b). Still, in a wider sense, it is considered the statistical description of the state of the *Climate System*, which include the Earth's five "spheres" –atmosphere, hydrosphere, cryosphere, lithosphere and biosphere– and the complex interactions between them (IPCC 2013b).

The climate system dictates our long-term living conditions on Earth. Its internal dynamics progresses over time and is modulated by external forcing of both natural (e.g., volcanic eruptions, solar variations) and anthropogenic origin (composition of the atmosphere and land use). Whereas changes in the climate dynamics would always be expected and taken as "normal" evolution over long periods of time (typically decades or more), *Climate change* is now on the top of the international agenda because the climate system is changing at a very high and unprecedent rate (IPCC 2014). This situation has been mostly attributed to the excessive release of greenhouse gases (GHGs) into the atmosphere by emissions arising from human activities, altering irredeemably the atmosphere composition (IPCC 2007).

Greenhouse gases (GHGs) include primary natural gases such as Water vapor ($H_2O$), Carbon dioxide ($CO_2$), Methane ($CH_4$), Nitrogen monoxide ($N_2O$) and Ozone ($O_3$), which enter the atmosphere from many sources, namely the transport and burning of fossil-fuels and other biological materials and wastes, many industrial chemical reactions (e.g., manufacture of cement), livestock and other agricultural practices (IPCC 2013b, Lin 2014). In addition, fluorinated synthetic gases (e.g., Hydrofluorocarbons – HFCs, Perfluorocarbons – PFCs, Chlorofluorocarbons – CFCs, etc.) are also emitted from a variety of industrial processes and, although in smaller quantities, are potent GHGs (sometimes referred to as "High Global Warming Potential Gases"; US EPA (2017)).

Beyond the unquestionable importance of GHGs on the control of the planet's temperature, once they allow the Earth's surface heating through the absorption and retention of radiant energy within the infrared range (Kautzman 2014), such "heat-trapping" gases

have today a very negative connotation due to their proved responsibility on the high rate at which global climate change is taking place (Boesch 2014). The excessive release of GHGs and the long life some of them have in the atmosphere are inducing the so called *Global warming* (Doney et al. 2012) and, taking into account the inertia of the climate system (Hansen et al. 2011), surface temperature is likely to continue increasing for decades or centuries, even in the case GHGs emissions' reduce or stabilize (Hansen et al. 2013, Allen et al. 2018).

Industrial revolution (1760-1830) represents the beginning of the critical increment of GHGs release. Since then, human activities contribute with huge amounts of GHGs to the Earth's atmosphere (Brierley & Kingsford 2009), namely of $CO_2$ that is the primary GHG emitted from anthropogenic sources (Hansen et al. 2013). From 1760 (right before industrial revolution) to 2008, $CO_2$ concentration in the atmosphere ($CO_{2[atm]}$) increased from 280 to 385 ppm (Brierley & Kingsford 2009). Actual global $CO_2$ average concentration, obtained from the National Oceanic & Atmospheric Administration (NOAA) of the United States Department of Commerce (NOAA 2019), is 411.10 ppm (retrieved on November 8, 2019 and relative to the average of daily measurements during October 2019, taken at four baseline observatories: Barrow, in Alaska; Mauna Loa, in Hawaii; American Samoa Observatory; and South Pole, in Antarctica).

Of the cumulative anthropogenic $CO_2$ released between 1750 and 2011, about 40% remained in the atmosphere, while the other 60% were stored in plants, soil and in the ocean (IPCC 2014). However, even though $CO_2$ storage could be seen as a way to bypass the excess of $CO_2$ released into the atmosphere, the global increment of $CO_2$ has changed the balance between the Earth's "spheres" (atmosphere, hydrosphere, cryosphere, lithosphere and biosphere) and, thus, the climate system equilibrium (Hansen et al. 2013), with many implications at the planetary level (Fath & Fath 2014).

As $CO_2$ increased, the planet's heat content (and, thus, temperature) became higher, an evidence of the relationship between $CO_2$ concentration in the atmosphere and the so called *Global warming* (Brierley & Kingsford 2009). In fact, global warming is a major aspect of global climate change and its occurrence is undeniable: the last three decades presented temperature values successively higher than any preceding decade since 1850 (IPCC 2013c).

Moreover, the period from 1983 to 2012 was *likely* the warmest 30-year period of the last 1400 years in the Northern hemisphere (IPCC 2013c).

Furthermore, global warming is also directly related with the rise of the sea surface temperature (SST) and the warming of the worlds' oceans, phenomena linked with the shrinkage of ice sheets, glacial retreat and sea level rise (Fath & Fath 2014). In addition, there are evidences that climate change, and the consequent ocean warming, indirectly promote the increase of the frequency of extreme events like tornadoes and hurricanes, heat waves and droughts, cold waves and hail storms, intense rainfalls, changes in the oceans' circulation and vertical distribution of nutrients (Doney et al. 2012, Fath & Fath 2014) as well as ocean acidification (Brierley & Kingsford 2009).

## 1.2 Ocean Acidification and Warming

Oceans cover about 71% of the Earth's surface (~360 600 000 $km^2$, Laffoley & Baxter (2016)) and play an essential role on the maintenance of a mild climate. Since the atmosphere and the hydrosphere are fluid bodies, there are coincident temperature between the sea surface (typically the first 75 m; Hoegh-Guldberg et al. (2014)) and the air at the troposphere (Hartmann et al. 2013), meaning that surface warming correspond to the temperature rise at the oceans' surface layer. Thus, due to the excess of GHGs released into the atmosphere, and the consequent increase in the Earth's heat content, oceans have been absorbing an excess of energy from the atmosphere as to keep the balance between these two systems, acting like a "buffer". In fact, oceans have high density and heat capacity (i.e., the ability to retain energy) and may absorb 4,000 times more energy than air (Brierley & Kingsford 2009, Laffoley & Baxter 2016), buffering atmospheric temperature changes.

The hydrosphere had taken much of the energy that has been retained in the atmosphere, absorbing more than 93% of the global warming's heat induced by human activities since 1971 from 2010 (Rhein et al. 2013). As a result, upper-ocean heat content has increased substantially in that period of time, with greater increase near the surface (>0.1 ºC per decade in the upper 75 m), decreasing with depth (0.015 ºC per decade at 700 m). From 1950 to 2009, the surface layer of the Indian Ocean suffered the highest temperature increase (0.65 ºC), followed by the Atlantic (0.41 ºC) and, lastly, the Pacific (0.31 ºC), (Hoegh-

Guldberg et al. 2014) . Nevertheless, the energy required to increase ocean temperature is higher than the necessary to the atmosphere, which means that climate change would have more noticeable effects on the air. In fact, the scale of the ocean buffer is enormous and only a small reduction in the heat uptake, or an increased flux from the ocean to the atmosphere, would have a huge impact on global air temperature. Therefore, the ocean plays a crucial role on the reduce the overall atmospheric GHGs concentration by being a sink not only for the $CO_2$ and other gases released to the atmosphere but also for the heat, which help, somewhat, to mitigate the climate impacts (Fath & Fath 2014).

Despite all that, the excess of carbon on seawater has created a critical indirect effect of climate change on the world's oceans: its acidification. Due to the existence of a balance between the atmosphere and the hydrosphere also in relation to the concentration of $CO_2$, the rise of this gas in the atmosphere automatically increases its absorption by the ocean which interrupt the seawater chemical balance and promote the chemical reaction presenting at Figure 1.1 (Dickson 2010, Rhein et al. 2013). The atmospheric $CO_2$ absorbed by the oceans (Fig. 1.1-A) easily dissolves in seawater ($H_2O$), promoting the formation of bicarbonate ($HCO_3^-$) and hydrogen ($H^+$) ions (Fig. 1.1-B). The $HCO_3^-$ suffers an acid dissociation reaction, forming carbonate ($CO_3^{-2}$) and $H^+$ ions (Fig. 1.1-C). However, the continuous increase of $CO_2$ in saltwater promotes a reaction with $CO_3^{-2}$ that originates $HCO_3^-$ ions (Fig. 1.1-D). Hereupon, the release of $CO_2$ to the atmosphere causes, in the oceans, a slight increase of dissolved inorganic carbon (DIC) ($DIC=[CO_2]+[HCO_3^-]+[CO_3^{2-}]$) and a high increase of $H^+$ ions that consequently drops the pH values of saltwater ($pH = -\lg [H^+]$), inducing *Ocean Acidification* (OA). On the opposite, the amount of carbonate ions available to associate with calcium ($Ca^{2+}$) ions decreases, affecting the formation of calcium carbonate ($CaCO_3$, calcite and aragonite) (Fig. 1.2-A) and consequently its saturation. The saturation of calcite/ aragonite ($\Omega$) is

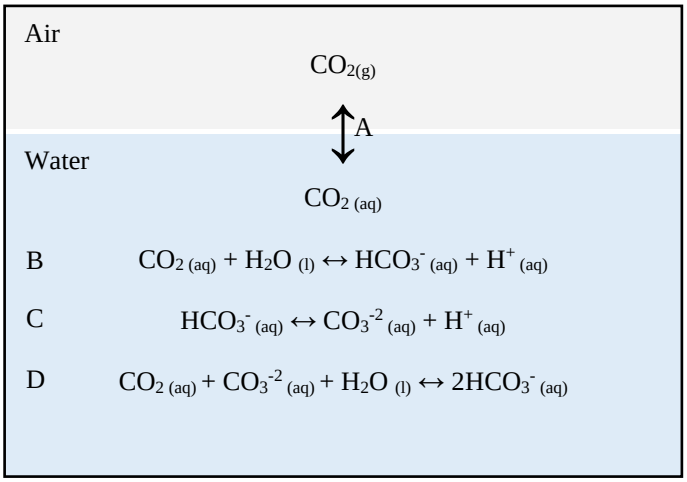


**Figure 1. 1:** The chemical equilibria reactions of the carbon dioxide system in seawater. The notations (g), (l), (aq) refer to the state of the species, i.e. a gas, a liquid, or in aqueous solution respectively. Adapted from Dickson (2010).

calculated from the product between the $Ca^{2+}$ and $CO_3^{2-}$ concentrations on saltwater, divided by the equilibrium constant ($K_{sp}$) (Fig 1.2-B) crucial to calcifying marine organisms (Fabry et al. 2008). When the solution is in equilibrium with the mineral state the saturation value of calcite / aragonite is $\Omega = 1$. A value of $\Omega > 1$ represents a supersaturated solution that favors the formation of $CaCO_3$, contrarily to a value of $\Omega < 1$ that indicates a undersaturated state and consequently a possible dissolution of calcified structures (Dickson 2010).

A $CaCO_3\,(s) = Ca^{2+}\,(aq) + CO_3^{2-}\,(aq)$

B $\Omega = [Ca^{2+}]\,[CO_3^{2-}] / K_{sp}$

**Figure 1. 2: A -** Dissolution equilibria for calcite and aragonite; **B -** Expression that define the saturation state; $K_{sp}$ represents the equilibrium constant. Adapted from Dickson (2010).

In fact, oceanic waters contain 50 times more $CO_2$ than the atmosphere and nowadays about 30% of this GHG from anthropogenic activities is being taken by our oceans (IPCC 2013c). Between the year 1000 and the eighteenth century (beginning of the industrial revolution), atmospheric $CO_2$ (measured as partial pressure – $pCO_2$) remained almost constant at about 280 μatm. However, from industrial revolution and until today, $pCO_2$ presents values 40% higher (Wolf-Gladrow & Rost 2014), which corresponds to a decrease of 0.1 pH units (IPCC 2013c). Due to its total alkalinity, seawater has a buffering capacity allowing smaller changes in pH, however, this capacity is reduced by the increase of $CO_2$ and the decrease of $CO_3^{2-}$ (Murray 2004, Ciais et al. 2013).

### 1.2.1 Near and long-term projections

The prediction of the evolution of global phenomena such as *Ocean Acidification* and *Warming* (OA-W) is extremely difficult given the enormous variability of forcing "agents" involved, and the uncertainty associated with those agents that progress in time through a multitude of different trajectories. However, the prediction of future climate conditions is of paramount importance for mankind, allowing for the early disclosure of their impacts and the development and application of potential mitigation measures.

In that sense, the Intergovernmental Panel on Climate Change (IPCC) adopted several scenarios according to which climate modeling has been performed to project climate future conditions in the near and the long-term (Kirtman et al. (2013) and Collins et al. (2013),

respectively). Those scenarios, which evolved from others initially considered (see the IPCC Special Report on Emissions Scenarios, Nakicenovic et al. (2000)), are designated *Representative Concentration Pathways* (RCPs) and were developed to better express future climate conditions and its progression (Moss et al. 2010). RCPs are based on an extensive evaluation of driving forces and data in the literature, corresponding to trajectories of GHG concentrations (and not simply of emissions) that result from different combinations of demographic, economic, technological, policy and institutional futures (Moss et al. 2010). The RCPs considered by the IPCC are mainly four and are labelled by their approximate total radiative forcing in year 2100 relative to 1750: 2.6 $W.m^{-2}$ for *RCP2.6*, 4.5 $W.m^{-2}$ for *RCP4.5*, 6.0 $W.m^{-2}$ for *RCP6.0* and 8.5 $W.m^{-2}$ for *RCP8.5* (Moss et al. 2010, IPCC 2013c).

The latest available projections of OA-W are gathered in the Fifth Assessment Report (AR5) of the IPCC (Collins et al. 2013, Kirtman et al. 2013). In the long-term (i.e., to occur by the end of the 21st century), under the RCP8.5 – worst case scenario, considering no efforts to constrain current emissions, close to the so called *business-as-usual* path (Riahi et al. 2011, Cubasch et al. 2013) – the SST might globally rise at a rate >0.1 ºC per decade (Hoegh-Guldberg et al. 2014), accounting for increases from about 1 to above 3 ºC (Collins et al. 2013, IPCC 2013a) while sea surface pH, that decreased since the Industrial Revolution at a rate of up to 0.0024 $units.yr^{-1}$ (Rhein et al. 2013), might fall off additional 0.3 to 0.5 units in the Northern hemisphere (Ciais et al. 2013).

Nevertheless, and despite the difficulty of predicting future OA-W, there are evidences that many regions and seasons have already experienced greater conditions than the actual SST and pH global averages (Ciais et al. 2013, Hoegh-Guldberg et al. 2014, Allen et al. 2018) with many impacts on marine biota being reported, particularly on molluscs' early life stages that seem to be specially prone to these phenomena and their interaction (Kroeker et al. 2013).

### 1.2.2 Effects of OA-W on marine molluscs in early life

Ocean acidification and warming (OA-W) are possibly the most environmental factors that disturbs the marine invertebrates distribution, physiological performance, morphology and behaviour (Ross et al. 2011, Doney et al. 2012, Leung et al. 2017). For this reason, it is

critical to investigate the interactive effects of the respective stressors – pH and temperature – since their simultaneous variation reflects better the reality in our changing oceans.

Ocean warming is the most serious climatic change-related stressor at some regions, including the Mediterranean, Southern North Sea, Western Antarctic Peninsula and Southeastern Australia (Hoegh-Guldberg et al. 2014). This factor has already affected the geographical distribution of aquatic animals, with enhanced risk of local extinctions or even of ecosystems (Pörtner 2008), since the increase of ocean temperature is associated with mass mortality, increased disease, hypoxia, coral bleaching, species invasions, phenological shifts in planktonic food web dynamics, physiological limitation in oxygen delivery and increased costs of metabolism (Byrne 2011, Range et al. 2011, Ko et al. 2014).

On the other hand, OA have been shown to have a significant negative effect on survival, calcification, growth, development and abundance, especially on heavily calcified organisms, including larval stages of echinoderms, calcified algae, corals, and molluscs (Kurihara 2008, Rost et al. 2008, Chan et al. 2011).

In terms of number of documented species, the Mollusca is a very large phylum of invertebrate animals. Of the total 50 000 described living molluscan species, around 30 000 species are found in marine environments (Gosling 2003) from the intertidal to deep-sea areas as well on a great latitudinal range with Class Bivalvia and Gastropoda representing the majority of the marine shelled molluscs (Gazeau et al. 2013).

In general, marine shelled molluscs present a life cycle with a planktonic larval phase that precedes the benthic adult life (Gosling 2003) and are among the most sensitive groups to OA-W (Kroeker et al. 2013). The prediction of OA-W future impacts on marine biota are extremely complex (Byrne 2011) and most of the studies concerns solely the individual effect of these stressors.

Since molluscs are poikilothermic organisms, their aerobic metabolism and the energy required to maintain cellular functions increases with the temperature rise until a thermal limit is achieved (Sokolova 2013). The energy balance is essential to the survival of organisms exposed to thermal stress conditions, since this energy is used for growth,

reproduction, activity and for somatic maintenance that, contrarily to the other components, could not be reduced beyond a certain value (Sokolova et al. 2012). Hence, a thermal stress causes a change on the aerobic metabolism since the energy that remains after the maintenance of the basal component is the one used for growth, reproduction and the other processes such as embryogenesis, the ontogenic development, swimming performance and planktonic larval phase duration (Pörtner 2010, Byrne 2011, Sokolova 2013). When the stress is extreme, the energy required could not be sufficient even to maintain the basal metabolism costs, causing a rapid deterioration of cellular processes and performance (Pörtner 2008) and, ultimately, compromising survival (Sokolova et al. 2012, Sokolova 2013).

Besides temperature, also ocean acidification is known to affect the early life stages of molluscs affecting the calcification due to a reduction on carbonate ions availability as well as a reduced carbonate saturation (Kurihara 2008, Gazeau et al. 2010, Byrne 2011) which promotes an increase on the energy required for the formation of biogenic $CaCO_3$, affecting the calcification rate of the early life stages of marine shelled molluscs (Parker et al. 2010, 2013, Byrne & Przeslawski 2013).

Moreover, the ocean acidification promotes hypercapnia i.e, the increase of $CO_2$ inside the animal (Byrne & Przeslawski 2013) which, consequently, increases the concentration of $[H^+]$ and $[HCO_3^-]$ ions affecting the acid-base balance (Parker et al. 2013). Besides the impact at the neurological level, as well as on tissues and muscles, hypercapnia also involves the spent of energy that would be necessary for physiological processes such as calcification, growth, reproduction and immune response (Pörtner 2008, Gazeau et al. 2013).

Usually, tolerances to climate-related factors are very different, not only between species but also between life stages such as larvae and adults (Pörtner 2008). Larval stages are normally associated to a higher risk of mortality and have been shown to be particularly sensitive to OA-W (Byrne 2011, Kroeker et al. 2013).

The major physiological processes affected by OA-W are calcification, development and growth (Gazeau et al. 2013, Leung et al. 2017). The reduction of calcification seems to be related with the low capacity of the animal to compensate the disturbances on the acid-base status, caused by the increase of $CO_2$ in seawater (Pörtner 2008). There are evidences

that lower marine invertebrates, including larval stages but also adults with slow or sessile modes of life and so with low metabolic rates, have less developed mechanisms that are directly involved on the reposition of the acid-base status like ion-regulatory organs and ion-exchange and non-bicarbonate buffering mechanisms (Pörtner 2008, Gazeau et al. 2013). The incapacity to compensate the extracellular pH causes a change on the metabolic rate of the early life stages of molluscs and consequently on physiological processes related to calcification rate, somatic growth, immune response, protein synthesis, reproduction and behaviour (Brierley & Kingsford 2009, Doney et al. 2012, Gazeau et al. 2013, Nagelkerken & Munday 2016).

### 1.2.3 Effects of OA-W on the behaviour of marine molluscs' early life stages

The first response of an animal to changes on the environment it inhabits is the modification of its behaviour (Tuomainen & Candolin 2011). Therefore, it is certain that climate change induces animals' behaviour alterations.

Behavioural changes in response to OA-W occurs by multiple mechanisms, including changes on physiological processes (Przeslawski et al. 2009, Byrne 2011, Clements et al. 2017). The temperature rise increases metabolic rates that, consequently, increase the activity levels, but reduced the predatory avoidance behaviour (Rosa & Seibel 2008, Zhang et al. 2014). Moreover, a higher metabolic rate, associated to a greater development, increase the burial performance (Lardies et al. 2001, Przeslawski et al. 2009), crucial to organisms with a benthic life style. On the other hand, besides temperature rise, acidic environments also disrupt the activity (Spady et al. 2014) and predator-avoidance behaviours (Watson et al. 2014, Queirós et al. 2015) with the difference that this impacts are often caused by the reduced ability to produce protective calcified structures (by altering calcification) (Bibby et al. 2007). Furthermore, OA could affect the feeding ability (Liu & He 2012, Vargas et al. 2013) as well as the byssus production, mostly on juveniles and adults bivalves (O'Donnell et al. 2013, Zhao et al. 2017). Some of impacts previously mentioned are related with a disruption at neurological level, since high $CO_2$ levels are also proved to change behavioural responses in a variety of marine organisms by affecting the function of neurotransmitters (Watson et al. 2014, Kroeker et al. 2014, Clements et al. 2017).

Surprisingly, as noticed by Parker et al. (2013) and Nagelkerken & Munday (2016), and given the important role that behaviour in early life plays in the survival of shelled molluscs, alterations of larval behaviours under OA-W have been somehow ignored, with the exception of filter-feeder molluscs' filtering rates that are commonly monitored (Vargas et al. 2015, Gray et al. 2017). However, most planktonic larvae are not passive drifters, they use active swimming to regulate their vertical positions in the water column (Chan et al. 2011) by beating the velum and generate currents to capture food (Lebour 1931, Hilbish et al. 1999). Thus, changes in the swimming behaviour under OA-W are very important and have been, to our best knowledge, neglected. Ellis et al. (2009) are the few authors revealing impacts of OA in early life stages' swimming behaviour in a mollusc: the embryos of the marine gastropod *Littorina obtusata* (Linnaeus, 1758) spent more time stationary, had slower rotation and shorter crawling periods under acidity than under control conditions.

Nevertheless, some studies had demonstrated that larval swimming patterns are controlled by morphological characteristics, such as size, shape and a velar propulsive mechanism. For instance, regarding Echinodermata larvae, reduced calcification and body size under acidified conditions induced reductions of the swimming performance, feeding success or both, with implications on survival (Chan et al. 2011). Still, the swimming behaviour in molluscs' larvae is also controlled by the larvae nervous system, which command velar muscle contractions and modulate the angle of its ciliary beating (Hadfield et al. 2000, Braubach et al. 2006, Page & Kempf 2009), that can also be affected by OA-W. Despite the evidences that warming increase metabolism (Sokolova 2013), hypercapnia is known to affect acid-base status and, consequently, influence physiological processes (Parker et al. 2013) disturbing metabolic and tissue functioning as well as the behavioural performance (Pörtner 2008).

Although very relevant for the species survival, given that the foundation of any population are the offspring that constitute recruitment cohorts, molluscs' behaviour is not only important during the larval life, at the end of which the foot evolves to assist the searching of a favorable subtract to settle and start the benthic period (Carriker 2001, Zupo & Patti 2009). Metamorphosis and settlement are critical moments for shelled molluscs survival, processes that seem to be controlled by hormones (serotonin and catecholamine) and are mediated by an increase of intracellular calcium and activation of ion mechanisms

(Braubach et al. 2006) that can also be disrupted mainly by OA (Pörtner 2005, Peng et al. 2017) . After settlement, it is also important that the physiological processes responsible for the juvenile life on the subtract are not affected impacting behaviour. Generally, the secretion of mucus at the foot epithelium is very important to a wide range of functions of this organ (e.g., adhesion to the substrate, lubrication for mobility, etc.) determining many behaviours such as burying, locomotion, food capture, mating, spawning and shell cleaning (Jung et al. 2006; Bravo et al. 2012).

Accordingly, OA-W may have multiple effects, from subtle to critical, throughout the early ontogenic development that may have implications for shelled molluscs' survival beyond those predicted for later life stages. Despite the work already done to understand how OA-W can alter animal behaviour, studies on the effects on shelled molluscs' early life stages are very scarce. This thesis is focused on filling this knowledge gap using as models the following species: the gastropod *Tritia reticulata* (Linnaeus, 1758) and the bivalve *Venerupis corrugata* (Gmelin, 1791).

## 1.3 Model species

### 1.3.1 The gastropod *Tritia reticulata* (Linnaeus, 1758)

#### *Habitat and Distribuition*

Commonly known as "netted dog whelk", *Tritia reticulata* (Linnaeus, 1758) is a marine gastropod mollusc (Phylum Mollusca: Class Gastropoda) in Family Nassariidae. The species is ubiquitous in coastal communities of the NE Atlantic (from Norway to the Canaries) and in Mediterranean, Black and Baltic Seas (Fretter & Graham 1994, Zupo & Patti 2009), (Figure 1.3).

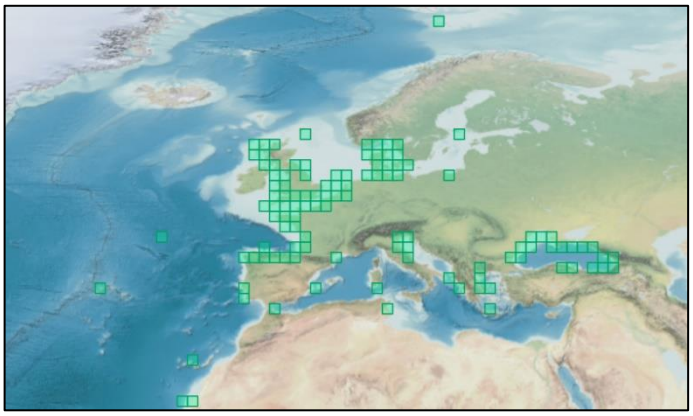

**Figure 1. 3*:*** Distribution of *Tritia reticulata*. Extracted from WoRMS-World Register of Marine Species (online available http://www.marinespecies.org/aphia.php?p=taxdetails&id=876821#distributions)

Specimens occur from the low water of spring tides to sublittoral depths down to at least 40 m (Barroso et al. 2011), along the open coast but also in the outer parts of estuaries, in both soft and hard bottoms of mud or sand where adults spend most of the time buried.

***Biology and Reproduction***

Adults netted whelks are scavangers and, thanks to a well developed olfatory system, specimens exhibit a spectacular reaction to the scent of a tiny particle of food, emerging rapidly from the sediment (Zupo & Patti 2009). The maximum size (shell height) that these animals reach is about 4.5 cm (Barroso et al. 2005) and the maximum longevity is estimated to be around 15 years (Tallmark 1980). Female sexual maturity is attained after about 4 years of life, with the males maturing one year earlier (Tallmark 1980, Zupo & Patti 2009). The species breeding period is related mostly with temperature and varies geographically but occurs, generally, during the spring and summer (March to August) and, more rarely, during the winter (January to February), (Fretter & Graham 1994, Barroso & Moreira 1998).

*Tritia reticulata* is gonochoric and its life cycle includes two distinct periods: a planktonic larval phase that precedes a benthic adult life. After internal fertilization, the adult female (Figure 1.4-A) produce an egg capsule (Figure 1.4-B) containing the embryos (Figure 1.4-C). Then, egg capsules are deposited on a favorable substratum, usually rocks or algae (Chatzinikolaou & Richardson 2010). After the attachment of a first capsule, other individuals spawn in the same area, creating a clusters of capsules (Zupo & Patti 2009). A single capsule can contain 100–120 lecithotrophic larvae (Figure 1.4-C and -D) of 160 µm diameter (Zupo & Patti 2009) floating in the intracapsular fluid (Fretter & Graham 1994).

The transparent vase-shaped capsules are generally 4.5 to 5 mm-height and 4 mm-width, (Fretter & Graham 1994); initially spherical, capsules are, then, shaped into a triangular form by the movement of the foot. This process might take several hours (Zupo & Patti 2009) but is very important to promote equal ventilation to all veligers and to allow a synchronous development (Chatzinikolaou & Richardson 2010). Furthermore, capsules protect the

embryos from the osmotic stress that could be created by salinity changes (Chatzinikolaou & Richardson 2010).

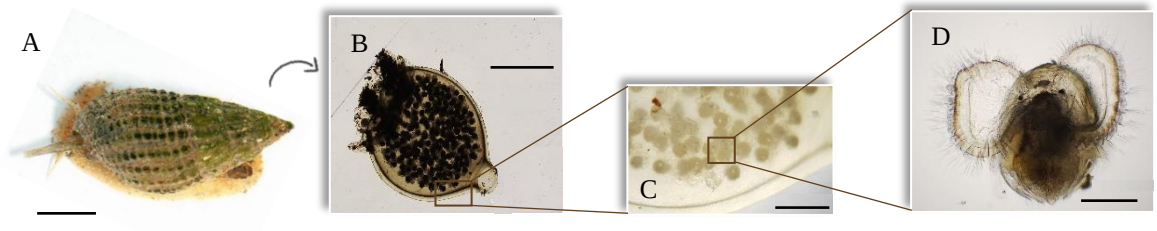


**Figure 1. 4:** *Tritia reticulata* life stages (personal photos). **A** – adult. **B** and **C** – egg capsules containing veliger larvae. **D** – recently hatched veliger. Scale bar: A- 0.5 cm; B- 2 mm; C- 500 µm; D- 250 µm. (Personal images).

After 12 (±3) days of egg capsules' laying (Zupo & Patti 2009) veligers hatch and start their free-living planktonic period on the water column, a phase full of challenges that might last 1 to 2 months relying on the performance of extremely fragile swimming larvae (Lebour 1931, Chatzinikolaou 2006, Zupo & Patti 2009).

***Ethology in early life***

The first stage of the larval phase is named the *Veliger 1* and corresponds to the newly-hatched larvae (Figure 1.5-A). This veliger has pigmented eyespots, a thin circular ciliated velum and a broad foot with a noncalcified operculum (Chatzinikolaou 2006, Zupo & Patti 2009). In general, the gastropod veliger presents a velum composed of two opposed bands of cilia: pre- and post-oral cilia (Hilbish et al. 1999) that have a preponderant role on feeding and swimming (Braubach et al. 2006, Romero et al. 2010). The pre-oral band has longer cilia and produce currents to both feeding and swimming activities, while the post-oral band are made of shorter cilia that concentrate microalgae into the food groove (Hilbish et al. 1999). Veligers in stage 1 are, thus, able to fed and swimming with their velum, and the food is retracted while animals swim (Lebour 1931). Usually, at this stage, the veliger moves vertically or whirls around itself and already exhibits positive phototaxis (Chatzinikolaou 2006).

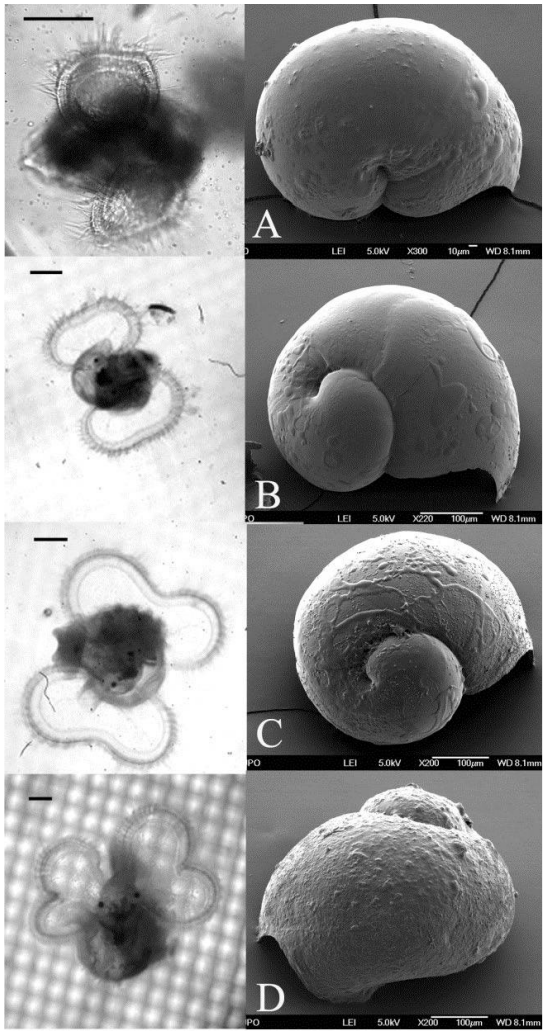


**Figure 1. 5:** *Tritia reticulata* veliger stages. **A** – Veliger 1, 2 days old (i.e., after hatching). **B** – Veliger 2, 8 days old. **C** – Veliger 3, 16 days old. **D** – Veliger 4, 30 days old and ready for settlement. Scale bars on the left: 60 μm. Extracted from Zupo & Patti (2009).

The second stage is the *Veliger 2* (Figure 1.5- B), which evolves from the first stage after 6 (±2) days of development under laboratory conditions (Zupo & Patti 2009). It is characterized by shells' torsion and a larger velum with an elongated shape (Zupo & Patti 2009). A veliger with this velum has already two types of swimming motion: a passive downward movement, when the larvae sinks in the water column, and an active upward swimming (Chatzinikolaou 2006). Once at the surface, these veligers swim horizontally, with the velum oriented upwards and the shell oriented downwards, making brief and abrupt movements (Chatzinikolaou 2006).

*Veliger 3* can be observed 2 weeks (±3 days) after hatching under laboratory conditions (Figure 1.5-C). The velum is now further thicker and larger, and exhibits a butterfly shape typical of this stage, which allows larvae to have smother and more controlled swimming patterns (Zupo & Patti 2009). The velum has still a similar structure, with the cilia directly linked to the nervous system, having the same role on swimming and feeding. At this stage the foot is more developed and presents cells with numerous purplish brown pigment granules, approaching to the adult foot structure described by Bravo et al. (2012) for *Haliotis tuberculate* (Linnaeus, 1758). It is divided in two distinct sections: the metapodium (anterior section) and the propodium (posterior) that is very important for the eminent crawling (Bickell & Chia 1979, Page & Ferguson 2013). With the foot development, this stage is also known as *Pediveliger* (Enriquez-Diaz et al. 2015). Some of its structures are similar to the larvae of *Doridella steinbergae* (Lance, 1962), now accepted as *Corambe steinbergae* (Lance, 1962), described by Bickell & Chia (1979).

The passive behaviour of sinking continues but at a shorter depth and, when on surface, the larvae are able to stabilize and swim horizontally (Chatzinikolaou 2006). In fact, such more developed larvae can swim and stabilize themselves, turning the velum in several directions at any depth, and some even start to explore the bottom (Chatzinikolaou 2006). Like the velum, the shell continues to grow being noticeably more robust.

Lastly, the *Veliger 4* is the most developed larval phase (Figure 1.5-D) and can be observed after approximately 3 weeks after hatching under laboratory conditions. This larva presents a tetra-lobated velum, and a foot that attains its maximal size, a morphological requisite for crawling (Bickell & Chia 1979, Zupo & Patti 2009). Despite being able to both swim and crawl, these larvae exhibit slow and smooth swimming upward and the ability of folding the velum to sink to the bottom when reaching the surface (Chatzinikolaou 2006). This is a very important behaviour of predator avoidance. However, these veligers are most of their time near the bottom, exploring the sediment and only waving the velum to feed on precipitated organic sediment particles (Zupo & Patti 2009).

The planktonic larval phase of *T. reticulata* ends when animals settle. The time it takes from hatching to settlement is dependent on several factors of which the feeding regime and temperature stand out; even so, it occurs, on average, in 35 days (Chatzinikolaou 2006, Zupo & Patti 2009). At this point, the organisms exhibit a well-developed foot with a pigmented propodium, similar to the described by Bravo et al. (2012) for *Haliotis tuberculata* (Linnaeus, 1758), as represented in Figure 1.6.

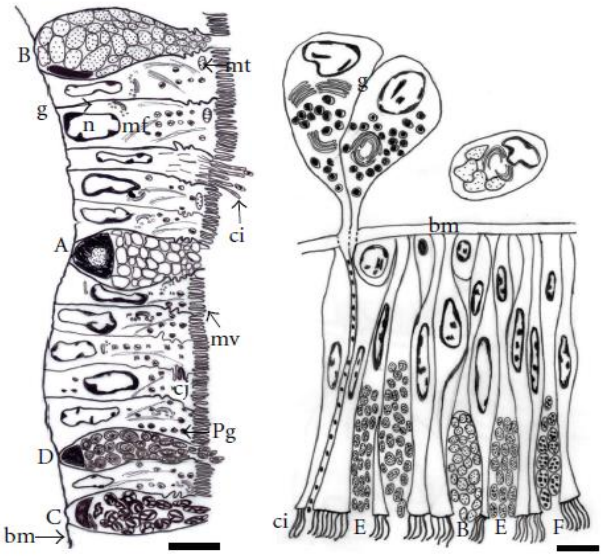


**Figure 1. 6:** Schematic drawing of the side (left scheme) and sole (right scheme) of the foot epithelia of *Haliotis tuberculata*. **A** to **F**: Secretory cells. **Abbreviations**: (bm) basal membrane; (ci) cilia; (g) Golgi complex; (cj) cell junctions; (mf) microfilaments; (mt) mitochondria; (mv) microvillus border; (n) nuclei; (pg) pigmented granules. Scale bar: A-10 μm; B- 5 μm. Extracted from Bravo et al. (2012).

The velum begins to regress and the metamorphosis to the juvenile stage occurs. At this moment the gastropod presents a shell with 2-3 whorls as well as a long siphons, and tentacles (Chatzinikolaou 2006, Zupo & Patti 2009). The juveniles have a well-developed digestive system that allows the organisms to fed, growing in sediment until reach maturity and, thus, the adult stage.

### 1.3.2 The bivalve *Venerupis corrugata* (Gmelin, 1791)

***Habitat and Distribution***

The "pullet carpet shell", *Venerupis corrugata* (Gmelin, 1791) is a marine bivalve mollusc (Phylum Mollusca: Class Bivalvia) in Family Veneridae. The species is widely distributed on the East Atlantic, from Morocco to Norway, and in the Mediterranean and Baltic Seas (Figure 1.7). It is a clam traditionally harvested at the Southwestern and Mediterranean Europe, mainly in Portugal, Spain, France and Italy (FAO 2006-2019), where specimens occur in fine sand, gravel or muddy sand subtidal banks, between the low tide line and up to 40 m-depth (Macedo et al. 1999).

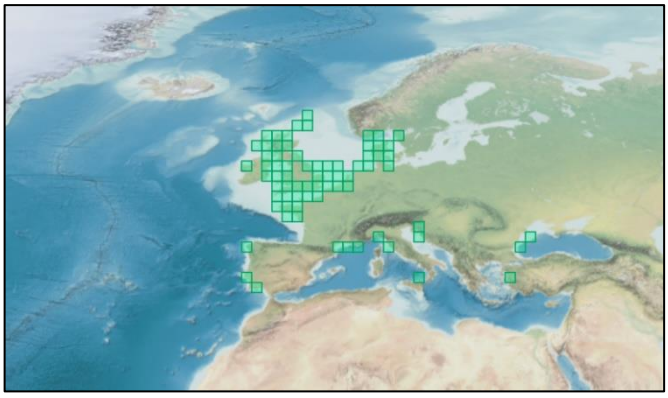

**Figure 1. 7:** Distribution of *Venerupis corrugata*. Extracted from WoRMS- World Register of Marine Species (online available http://www.marinespecies.org/aphia.php?p=taxdetails&id=181364#distributions).

***Biology and Reproduction***

*Venerupis corrugata* adults are most of the time burrowed in the sediment, feeding on organic detritus and microalgae obtained from seawater filtered through a pair of siphons (Fenchel 1991). The maximum size (shell length) specimens reach is about 5 cm (Macedo et al. 1999) and the maximum longevity is estimated to be 5-10 years (Rayment 2007). As filter-feeders, specimens play an important service to marine ecosystems contributing to their purification (Dame 1993). Moreover, the species is commercially exploited, registering a relevant economic value in Southwestern European markets (Joaquim et al. 2014).

*Venerupis corrugata* is a gonochoric species with sexual reproduction. Maturity is reached after about 1 year of development, when animals have 1-2 cm in length (Rayment 2007, Cerviño-Otero 2011). Although this species presented a long spawning period, from later winter to middle of summer (Joaquim et al. 2011), the breeding season, which is mainly dependent on environmental conditions such as temperature and food availability , usually extends from early spring to summer (February to July) with full spawning occurring between April and May (Cerviño-Otero 2011).

Mature male and female gametes are emitted to the water column (i.e., spawned) where external fertilization occurs and the indirect development of a planktonic larva follows through metamorphosis and settlement, after which starts the benthic life (Figure 1.8; Cerviño-Otero 2011).

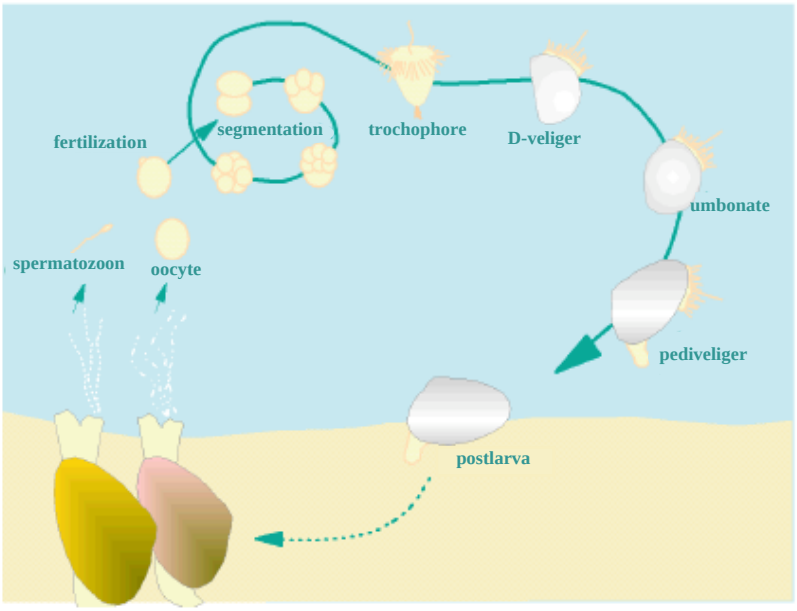


**Figure 1. 8:** *Venerupis corrugata* life cycle. Adapted from Cerviño-Otero (2011).

***Ethology in early life***

The first *V. corrugata* larval stage is the so called *Trochophore*, provided with a crown of cilia, an apical plume and a primordial shell – prodisoconch I (Cerviño-Otero 2011). This stage is about 13-14 hours of life and evolves to a second larval form that, about 48 hours after fertilization is 100-105 μm in length (Cerviño-Otero 2011). It is now completely involved by a calcified shell with a typical straight hinge which gives it a "D" shape and, hence, its name: *D-Veliger* (or *D-Larva)* (Figure 1.9-A). Besides the shell, this stage has a ciliated velum formed by three distinct bands: pre-oral, adoral and post-oral, from de interior to exterior (Cerviño-Otero 2011). In general, the bivalve' D-Veliger present its valves slightly parted and the velum is fully extended with rapidly beating cilia (Carriker 2001), allowing the capture of food particles (unicellular microalgae) during active swimming in the water column, usually near the surface and, particularly, in the dark/night (Quayle 1952).

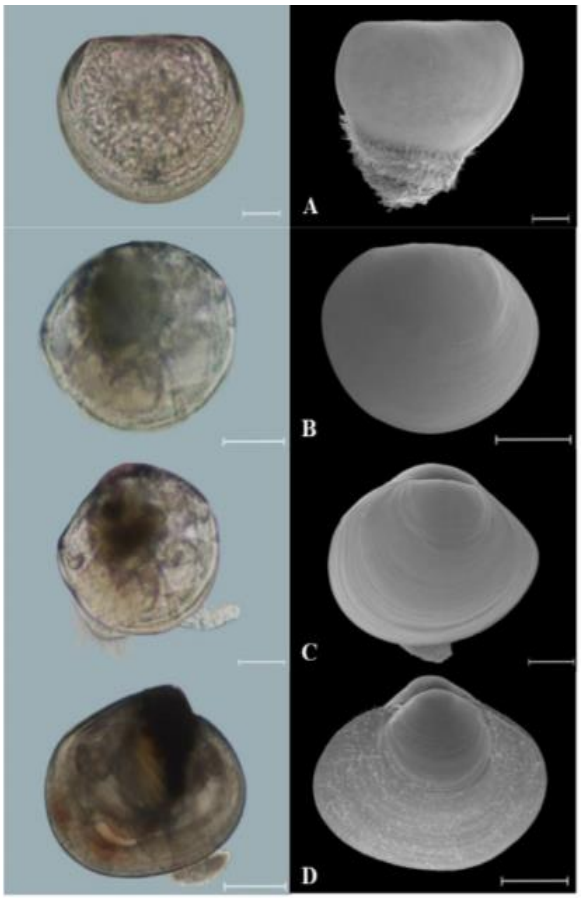


**Figure 1. 9:** *Venerupis corrugata* larval stages.
**A** – D-Veliger. **B** – Umbonate larvae. **C** – Pediveliger. **D** – Postlarva.
Scale bar: A- 20 μm; B- 50 μm; C- 50 μm; D- 50 μm. Adapted from Cerviño-Otero (2011).

The D-Veliger undergoes fast growth and as the ontogenic development progresses the shell evolves to a prodisoconch II, in which growth lines are perceived. After 8-9 days of growth after fertilization, the hinge starts to bend forming the umbo, structure to which the next larval stage owes its name: the *Umbonate* (Figure 1.9-B), that has 130-150 μm in length (Cerviño-Otero 2011). At this stage it possible to differentiate the prodisoconch I from the prodisoconch II and, generally, the swimming is slower, more steady, and larvae keep circling in many directions in graceful movements originated in their cilia (Carriker 2001).

After 16 to 18 days of growth after fertilization, larvae of 220-240 μm are still on a ciliated velum but have also developed a ciliated foot, reason why are named *Pediveligers* (Figure 1.9-C). At this stage the animal starts to search for a favorable settling surface with both the velum and the foot, which come most intimately in contact with potential substrates and are supplied with nerve endings. Pediveligers continue to be able to swim, but they can also crawl, which occurs by the extension of the foot beyond the valves and its subsequent energetic pulling movement, which promotes the animal's drag over a surface. Pediveligers crawl, moving back and forth in an apparent exploratory pattern in an attempt to choose the right substrate to settle. When surfaces are not attractive, possibly due to particle size, chemical composition, or other negative stimuli (Tallqvist 2001, de la Huz et al. 2002, Gribben et al. 2009, Morello & Yund 2016), pediveligers suddenly extend the velum, swimming away to explore a new surface. The process of "searching" is repeated until the right settling substrate is found (Carriker 2001).

Towards the end of the planktonic phase, pediveligers undergo metamorphosis becoming *Postlarva* (Figure 1.9-D). The velum retracts and the branchial filaments, gills and siphons assume their roles on the animal's feeding (Quayle 1952, Cerviño-Otero 2011). Moreover, the animal starts to adopt the adult form with the stronger spat shell – the dissoconch – which provides additional protection (Quayle 1952, Cerviño-Otero 2011). Generally, the end of the planktonic larval phase depends on the individual and, probably, on the availability of settling surfaces (Carriker 2001). Larger pediveligers do not always settle before the smaller ones; however, metamorphosis in *V. corrugata* occurs on average 21-22 days after fertilization, in animals of 250-270 μm in length. At this stage, the clam locomotion relies on the strong, highly ciliated, mobile and muscular foot that has a preponderant role on the searching and burial behaviour adopted in the juvenile and adult life (Carriker 2001). In addition, this species is also a producer of byssus threads, secreted by many bivalves that need to be firmly attached to a substrate (Quayle 1952, Waite 1992), that can somehow be related with the animal locomotor activity.

Usually, after finding a favorable substrate, the postlarva initiates the “attachment” by producing a translucent and elastic byssal thread of variable length and number of holdfasts (a distinct “lumps” of byssal secretion) well described by Carriker (2001) for *Mercenaria mercenaria* (Linnaeus, 1758) . The byssus production is extremely rapid (less than 1 minute) and is formed by byssal fluid that passes from the byssal gland through the byssal duct to the midventral base of the foot, flowing into the pedal byssal groove (see Figure 1.10; Carriker (2001)).

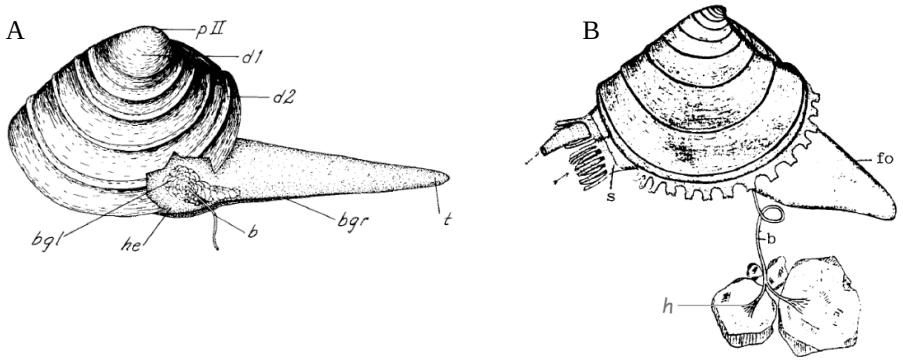


**Figure 1. 10:** *Mercenaria mercenaria*, byssal threads production. **A** – Animal with part of left valve cut away to show byssal gland in the heel (he) of the foot. **B** – Animal attached to sand grains by the byssus (b). **Abbreviations**: (bgl) byssal gland, (bgr) byssal groove; (dl) dissoconch primary shell ridges; (d2) dissoconch secondary shell ridges; (h) holdfast; (pII) prodissoconch II; (t) toe; (fo) foot; (s) siphons. Arrows indicate direction of seawater flow to and from the siphons. Adapted from Carriker (2001).

The byssal gland, also enlarge as the clam growths. After attachment of a byssal holdfast, the foot is slowly removed, leaving the byssus out behind it. Postlarvae can stay borrowed on sediment, with closed to semi-closed valves, filtering the seawater, or can keep crawling and secreting longer byssus with another (or more) holdfast. Settled clams keep feeding and growing until sexual maturity and, thus, the adult stage is reached.

## 1.4 Thesis Rationale and Objective

The present thesis arises at a time when the impacts of climate change-related phenomena in the oceans, such as *Ocean Acidification* and *Warming* (OA-W), are on the top of the World agenda and after being noticed a lack of knowledge regarding the potential negative effects of the environmental stressors involved – pH and temperature, and their interaction – on the behaviour of shelled molluscs, particularly in early life. These animals’ have been pointed as being especially prone to these phenomena, and their perpetuation relies mainly on the

fitness of their planktonic early life stages, whose behaviour under OA-W projected scenarios is, to my best knowledge, unknown.

In this sense, this work aims to assess the impacts of OA-W projected scenarios in the locomotor activity and survival of gastropod and bivalves' early life stages, disclosing if and which locomotor organs are affected, and the potential vulnerabilities of ecological and economical relevant species to future environmental conditions in our changing oceans.

The gastropod model – *Tritia reticulata* – is a ubiquitous scavenger, thus ecologically important since promotes nutrients and energy transfer across trophic levels and ecosystems, being involved on the carbon cycling, distribution and sequestration into coastal environments. The species relies on planktonic early life stages which swimming activity is preponderant for survival and which sensitivity to OA-W is unknown.

The bivalve model – *Venerupis corrugata* – is a commercially-exploited filter-feeder; thus, it is an ecosystem services provider both in an ecological point of view but also as a very important food resource for Southwestern European human populations (and markets).

This dissertation is organized into four chapters: a *General Introduction* that ends with this section (Chapter 1), two original scientific works that describe the effects of OA-W on the locomotor activity and survival of *T. reticulata* early life stages (Chapter 2) and on *V. corrugata* early life stages (Chapter 3) and, finally, the *Main conclusions and Final remarks* (Chapter 4).

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# Chapter 2: Impacts of future ocean acidification and warming scenarios on the performance of early life stages of the marine gastropod *Tritia reticulata* (Linnaeus, 1758): effects on veligers' locomotor activity and survival.

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The data included in this chapter

yield the following peer-reviewed scientific presentations:

(presenting author is underlined)

**Fonseca JG**, Freitas DB, Laranjeiro F, Oliveira IB, Rocha RJM, Domingues I, Machado J, Barroso CM, Hinzmann M, Galante-Oliveira S (2019) Assessment of the effects of ocean acidification and warming on the swimming behaviour of *Nassarius reticulatus* larvae: microscopic study of the locomotor organs. Poster presentation, IJUP'2019 Encontro de Investigação Jovem da Universidade do Porto, 13-15-Feb, Porto, Portugal. Abstract book p.429

Galante-Oliveira S, Laranjeiro F, Oliveira IB, **Fonseca JG**, Freitas DB, Domingues I, Rocha RJM, Barroso CM (2018) Evidence of altered behaviour and reduced survival in *Nassarius reticulatus* (L.) veligers exposed to marine climate change projected scenarios. SEB International Symposium on Lessons from two high $CO_2$ worlds: future oceans and intensive aquaculture, 10-12.Apr, Ponta Delgada, Azores, Portugal. Abstract book p.31

**Fonseca JG**, Freitas DB, Laranjeiro F, Oliveira IB, Rocha RJM, Domingues I, Galante-Oliveira S, Barroso CM (2018) Impacto da acidificação e aquecimento oceânico no comportamento larvar do gastrópode *Nassarius reticulatus*. Platform presentation, CNAC'2018 - Congresso Nacional sobre Alterações Climáticas, 19-20.Feb, Vila Real, Portugal. Abstracts book p.48

This chapter is being prepared

for submission as the following original scientific article:

Galante-Oliveira S, Laranjeiro F, **Fonseca JG**, Freitas DB, Oliveira IB, Domingues I, Machado J, Hinzmann M, Barroso CM (in preparation) Future ocean acidification and warming scenarios impair the swimming ability reducing survival of early life stages of the marine gastropod *Tritia reticulata* (Linnaeus, 1758).

# Abstract

The netted-whelk *Tritia reticulata* (L.) is a marine gastropod, common in intertidal communities of the coastal areas of the NE Atlantic, the Mediterranean, the Baltic and the Black Sea. Of high ecological relevance, this scavenger life cycle relies on the performance of planktonic early life stages, whose sensitivity to the climate conditions projected for the near future, specifically of ocean acidification (OA) and warming (W), is, to our best knowledge, unknown. This work investigates the effect of OA-W in the locomotor activity and survival of *T. reticulata* veligers aiming at foreseeing the species resilience to future environmental conditions it is likely to be facing in its natural habitat. Newly hatched veligers obtained in the Ria de Aveiro (NW Portugal) were exposed for 14 days to six OA-W simulated scenarios, generated by a factorial experimental design of three temperatures (18, 20 and 22 ºC) and two pH target levels (8.1 and 7.8). Mortality was assessed throughout the experiment and the swimming behaviour, characterized by the activity, the speed and the distance travelled by veligers, evaluated by analyzing video recordings acquired in ZebraBox® at the end of the exposure period. Generally, mortality increased with acidification and warming and, although more active, larvae travelled shorter distances revealing reduced swimming velocity under acidic and warmer conditions. The interaction of the tested stressors – pH and temperature – were proved to have a highly significant effect on *T. reticulata* veligers' activity, distance and speed after 14 days of exposure (activity *Pseudo-F*=32.05, *p*=0.0001; distance travelled: *Pseudo-F*=37.547, *p*=0.0001; speed: *Pseudo-F*=18.25, $p = 0.0001$). These results motivated the morpho-histological analysis of larvae preserved at the end of the exposure period, to check for the integrity of the organs involved in veligers' locomotion: the gravireceptors (i.e., the statocysts), the velum and the foot. Morphological alterations of the foot sole epithelium, namely an apparent hypertrophy and the protrusion of secretory cells, which presented less cilia and dispersed pigmented granules, indicate damage of this locomotor organ with potential functional implications. This histological impairment may be related with the detected reduction on veligers' locomotor ability of swimming and, as this epithelium corresponds to the future crawling surface, it may also compromise juvenile locomotion. Hence, this work unveils negative consequences the OA-W tested scenarios on *T. reticulata* veligers' competence, pointing towards the eminent threat that these phenomena constitute to this species survival if the projections for its occurrence become effective.

## 2.1 Introduction

Global climate change is affecting the marine ecosystems and has been progressively recognized as being a result of human activity. The increment of atmospheric $pCO_2$ released into the atmosphere from anthropogenic sources has been proved to be directly linked to the concurrent *Ocean Acidification* (OA) and *Warming* (W), (Rhein et al. 2013).

The excessive release of $CO_2$ and other "heat-trapping" greenhouse gases (GHGs) since the Industrial Revolution has been increasing the Earth's heat content causing the so called *Global Warming* and, consequently, the rise of the sea surface temperature (SST). On the other hand, higher concentration of $CO_2$ in the atmosphere also increases the amount of $CO_2$ absorbed into the oceans, inducing *Ocean Acidification* (Fath & Fath 2014).

The long-term projections (i.e. for the end of the 21$^{st}$ century) of OA-W are gathered on the Fifth Assessment Report (AR5) of the Intergovernmental Panel on Climate Change (IPCC). Taking as a reference the worst case scenario there considered (i.e. under the RCP8.5, which does not include any additional efforts to constrain current emissions, and so it is close to the so called *business-as-usual* path; (Riahi et al. 2011, Cubasch et al. 2013), it is projected that the actual SST might increase at a rate >0.1 ºC per decade (Hoegh-Guldberg et al. 2014), accounting for rises from about 1 to above 3 ºC (Collins et al. 2013, IPCC 2013), while sea surface pH, that decreased approximately 0.0024 pH units.yr$^{-1}$ since the Industrial Revolution, might decrease additional 0.3 to 0.5 units in the Northern hemisphere (Ciais et al. 2013).

Ocean Acidification and Warming (OA-W) have significant impacts across all levels of the biological organization, from gene expression to cellular and whole-organism physiology, including effects on individuals' skeletal structure and behaviour (Brierley & Kingsford 2009). There are evidences that OA-W affects molluscs' behaviour throughout life, including all ontogenic stages, with impacts on larval feeding and dispersion, substrate choice and settlement competence, predation avoidance, migration, breeding, among others (reviewed by Nagelkerken & Munday (2016)). All these responses play important roles on an individual's life and from its early beginning, notably on its normal larval development. As the foundation of any population is the offspring that constitute recruitment cohorts, larval survival is imperative for any species perpetuation, including that of molluscs that

have been pointed as being particularly sensitive to OA-W (Byrne 2011, Kroeker et al. 2013). Hence, reduced survival caused by altered behaviours in early life under OA-W might, ultimately, compromise molluscs' species perpetuation, some of them of high ecological relevance such as the scavenger *Tritia reticulata* (Linnaeus, 1758).

This is a common gastropod, extensively distributed in the Northeast Atlantic coasts and throughout the Mediterranean, Baltic and Black Seas (Fretter & Graham 1994). As scavengers, netted whelks play an important role on the environment, allowing the shift of nutrients and energy across different trophic levels, as well as on the carbon cycle, its distribution and sequestration into the oceans (Bailey et al. 2007), thus providing relevant ecosystem services.

This species' life cycle includes a planktonic phase, within which four well-defined free-swimming larval stages are described: veligers 1 to 4 (Zupo & Patti 2009). The *Veliger 1* hatch from the egg capsule spawn by the adult female, and swims freely in the water column; it exhibits a calcified and transparent shell (protoconch), with a length of ≈300 μm, a circular velum with long cilia (central role on swimming and food capture), a broad foot with a noncalcified operculum, a pair of eyespots and a pair of statocysts (i.e., the gravireceptors, organs involved on the mechanisms of balance and spatial orientation, and thus on the swimming behaviour), (Chatzinikolaou & Richardson 2007, Zupo & Patti 2009, Page & Ferguson 2013). At this initial phase, veligers swim most of the time close to the surface, moving actively, vertically or spinning around themselves while feeding (Chatzinikolaou 2006).

After 6 (±2) days under regular laboratory conditions arises the *Veliger 2*, exhibiting the first shell torsion and incremented thickness, and a large velum with an elongated shape and shorter cilia (Zupo & Patti 2009). Between this stage and up to the development of the *Veliger 3*, the metapodium (anterior part of the foot) occupies most of the operculum due to the proliferation and hypertrophy of the pedal epithelial cells (Bickell & Chia 1979, Page & Ferguson 2013).

Veligers 3 present two distinct swimming phases: the passive, characterized by the contraction of the velar lobes allowing the larvae to sink to a maximum depth of 6-7 cm from the surface; and the active, which occurs when the veliger swims to the surface in horizontal

position, with brief but abrupt movements (Chatzinikolaou 2006). These veligers are formed after 14 (±3) days from hatching and are characterized by the shell's continuous enlargement and thickness, and also the increase of the size of the velum, which exhibits a typical butterfly shape that allow a smoother and more controlled swimming. At this stage, the foot present differentiated metapodial and propodial glands that become distended by their own secretory products (Bickell & Chia 1979, Page & Ferguson 2013).

Lastly, the *Veliger 4* is generally formed after three weeks of development under laboratory regular conditions (Zupo & Patti 2009). It shows larger eyespots and a typical tetra-lobated velum with shorter cilia (Zupo & Patti 2009). These veligers are the most developed ones and have the ability of folding the velum to sink to the bottom, and even to bend the velum in many directions to stabilize their position in the water column without sinking or swaying abruptly (Chatzinikolaou 2006). Since this stage precedes settlement, these larvae spend most of the time swimming close to the bottom, waving the velum to feed on precipitated organic sediment particles (Zupo & Patti 2009). The proliferation of pedal epithelial cells allows the appearance of the propodium (posterior part of foot), providing a surface adapted to crawl (Bickell & Chia 1979, Page & Ferguson 2013).

*Tritia reticulata* planktonic life ends when larvae reaches competency to settle and undergo metamorphosis to become benthic (Tallmark 1980, Fretter & Graham 1994). At metamorphosis, the velum regresses while the foot becomes considerably more apparent, responding to the need of seeking for a favorable substrate to settle (Zupo & Patti 2009). Metamorphosis occurs about 35 days after hatching under laboratory conditions (Zupo & Patti 2009) being dependent on several abiotic factors such as temperature and pH (Scheltema 1961, Tallmark 1980). Some studies had shown that changes in seawater temperature and pH could affect the swimming behaviour of gastropods' early life stages (Manno et al. 2012, Zhang et al. 2014); however, there is still a lack of knowledge regarding the effects of OA-W on the swimming behaviour of early life stages of molluscs, particularly of gastropods.

Taking into account the facts mentioned above, it is noticeable that larval swimming capacity is dependent on the correct development and integrity of larval locomotor organs: the velum and the foot. Moreover, that behaviour is also dependent on the correct development and functioning of the statocysts, present as early as the mature, ready-to-hatch,

veliger 1 (Chatzinikolaou & Richardson 2007) as mentioned above. These paired sac-like organs are involved in the control of several behavioural reactions (e.g., geotaxis, equilibrium maintenance; (Levi et al. 2004)). Such processes are dependent on both the cellular arrangement of these organs' epithelium but also on the integrity of the non-skeletal biogenic carbonates included in their lumen –the statoliths (Chatzinikolaou & Richardson 2007, Galante-Oliveira et al. 2013).

Hence, this study is aimed at investigating if the concomitant decrease in pH (*Acidification*) and increase in temperature (*Warming*), at the levels projected by the IPCC as likely to occur in the near future, will affect *T. reticulata* swimming ability in early life, and if potentially detected effects are linked with the ultrastructural damage of the organs involved in that behaviour, affecting the species survival.

## 2.2 Materials and methods

### 2.2.1 Sampling

Egg capsules of *T. reticulata* were collected by hand in the Ria de Aveiro intertidal (NW Portugal at 40º38'34.65''N – 8º44'06.80''W; Figure 2.1) and were transported to the laboratory in local seawater. Once in the laboratory, egg capsules were acclimatized in 1 µm-filtered synthetic saltwater (Oceanfish, Prodac International, Italy) at salinity 30, 18±1 ºC and $12^{L}$:$12^{D}$ photoperiod, for 3 consecutive days.


***F*igure 2. 1:** Location and aspect of the sampling site in Ria de Aveiro (NW Portugal) at which *T. reticulata* egg capsules were collected.

Mature egg capsules (Figure 2.2-A), characterized by containing larvae with well-developed eyes (Figure 2.2-B), statocysts and a beating velum, were selected under a stereomicroscope (Leica® S8 APO, Leica Microsystems, Switzerland) and ripped following the protocol described by Génio et al. (2008) to release the veligers (Figure 2.2-C).

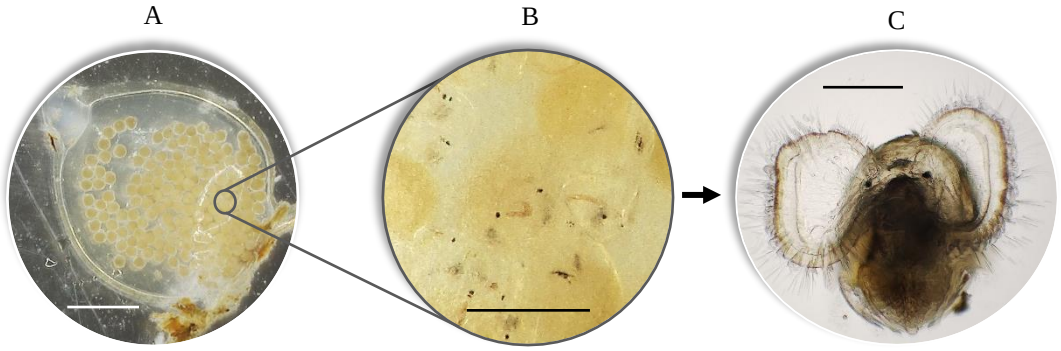



**Figure 2. 2:** *T. reticulata* early life stages. **A** – Egg capsule containing mature veligers, ready-to-hatch. **B** – detail of mature veligers, ready-to-hacth, with well-developed eyes. **C** – Recently-hatched swimming veliger. Scale bar: A- 1000 µm; B- 200 µm; C- 250 µm.

Newly hatched veligers were collected in 1 µm-filtered synthetic saltwater at salinity 30 and maintained as follows.

### 2.2.2 Maintenance of newly hatched veligers

Newly hatched veligers were kept for an additional 24 h-acclimation period, under the above mentioned conditions (salinity 30, 18±1 ºC and $12^{L}$:$12^{D}$ photoperiod) and fed an appropriate diet of the microalgae adapted from the applied by Chatzinikolaou & Richardson (2007): *Isochrysis galbana* (20 cells.µL$^{-1}$), *Rhodomonas salina* (5 cells.µL$^{-1}$), *Skeletonema costatum* (10 cells.µL$^{-1}$) and *Tetraselmis chui* (0.5 cells.µL$^{-1}$).

With the exception of the diatom *T. chui*, purchased freeze-dried (Phytobloom Prof *Tetraselmys*, Necton S.A., Olhão, Portugal) and used after reconstitution in 1 µm-filtered synthetic seawater at salinity 30 following producer's instructions, all other microalgae species were cultured at our facilities. The scheme in Figure 2.3 summarizes the procedure applied in our batch culture.

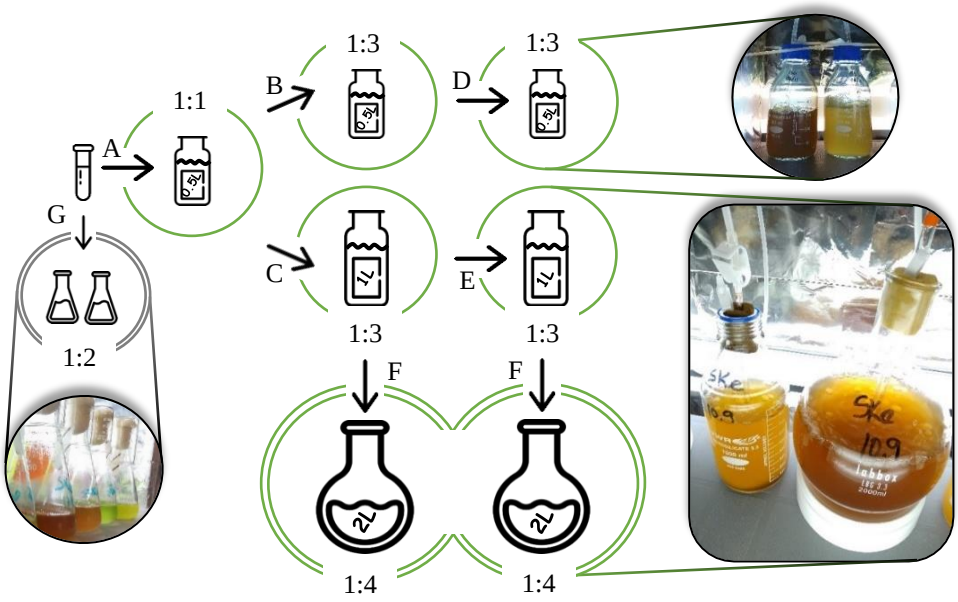



**Figure 2. 3:** Summary of the microalgae batch culture performed during the current work. The scheme here presented is the basis for the volumes required weekly to maintain each of the three species produced to feed *T. reticulata* larvae. **A** to **G** correspond to the steps followed and are referenced in the text.

Inoculants of each species were kindly provided by C-FOOD clams' farmers (Coimbra, Portugal). Cells were initially inoculated in 1:1 proportion, into sterilized Schott-Duran glass flasks containing 1 µm-filtered synthetic seawater at salinity 30, fertilized with 1 mL of Nutribloom plus culture medium (Necton S.A., Olhão, Portugal) per liter of culture (Figure 2.3-A). In addition, the diatom *S. costatum* was supplemented with Nutri Bloom Silicates Solution (Necton S.A, Olhão, Portugal) in the same proportion used for the culture medium (1 $mL.L^{-1}$). Microalgae were aerated with ambient air, filtered at 0.2 µm and at such a rate as to avoid cells' deposition at the bottom of the flasks. Flasks were kept at 18±1 ºC and under $12^{L}$:$12^{D}$ photoperiod on shelves illuminated with fluorescent tubes. After a growing period (of days), in order to increase the volume of the culture and, thus, the cells' number, the first flask was used to inoculate two other flasks: one of the same volume (Figure 2.3-B) and a larger one (Figure 2.3-C), this time at a ratio 1:3. After another growing period, at approximately weekly intervals, smaller flasks were again used to inoculate other two: one of the same volume (Figure 2.3-D) and a larger one (Figure 2.3-E), up to the point that the larger flasks were used to inoculate 2 L round bottom flasks at a proportion of 1:4 (Figure 2.3-F). Larger, 2 L-cultures were then brought to a maximum density and harvested to feed the larvae. Before each culture harvest, its concentration was assessed, through counting the

number of cells per mL using a Neubauer cell counting chamber (Figure 2.4), and the volume containing the necessary number of cells calculated. The main culture in the 2 L-flasks was maintained to be used until its end or until shows evidences of failure due to an excessive concentration. An initial inoculum of each species was kept in sterilized Erlenmeyer's as a backup (Figure 2.3-G) to be used in case of contamination. These inoculants were shaken weekly and renewed monthly.

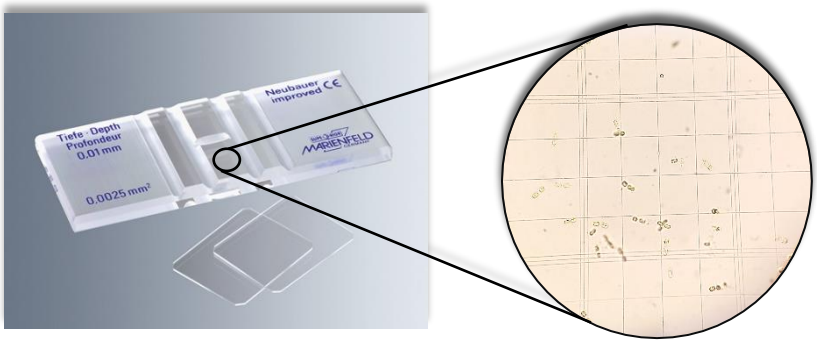



**Figure 2. 4:** Neubauer-improved glass chamber (left image) used to count microalgae cells (right image, taken under an optical microscope; the 400x times magnification was used for counting).

### 2.2.3 Experimental setup and treatments

Newly hatched veligers were exposed in duplicate to 6 experimental treatments corresponding to 6 simulated scenarios of OA-W, for 14 days. These experimental scenarios were generated in the Experimental Life Support System (ELSS) available at the Centre for Environmental and Marine Studies (CESAM), developed by Coelho et al. (2013) to address specific questions using microcosm simulation of climate change scenarios in coastal and estuarine environments, namely of *Acidification* and *Warming*.

The original version of the ELSS was modified under project *CliMaStat* (that funded the work here described) to accommodate a factorial experiment design of a maximum of 4 levels of temperature (T ºC) and 4 of pH, thus doubling ELSS's initial capacity (from 2*2 levels to 4*4 levels) and generating a maximum of 16 OA-W scenarios. In brief, the original system was composed for four autonomous saltwater reservoirs, two of them were used to prepare synthetic seawater; in the other two, the pH could be independently manipulated through $CO_2$ bubbling controlled by feedback pH control systems ($V^2$ control pH controller/monitor and $V^2$ pressure regulator pro, TMC, UK). Manipulated synthetic

saltwater in these reservoirs was, then, used to fill the experimental containers that were originally 3 L-rectangular aquaria or microcosms. However, these microcosms were not adapted for the exposure of planktonic veligers and were replaced by inverted carboys with aeration and gravity drainage by outlet pipes with 100 µm-mesh filter-protected output (at such a height to allow a maximum volume of 3 L), (Figure 2.5).

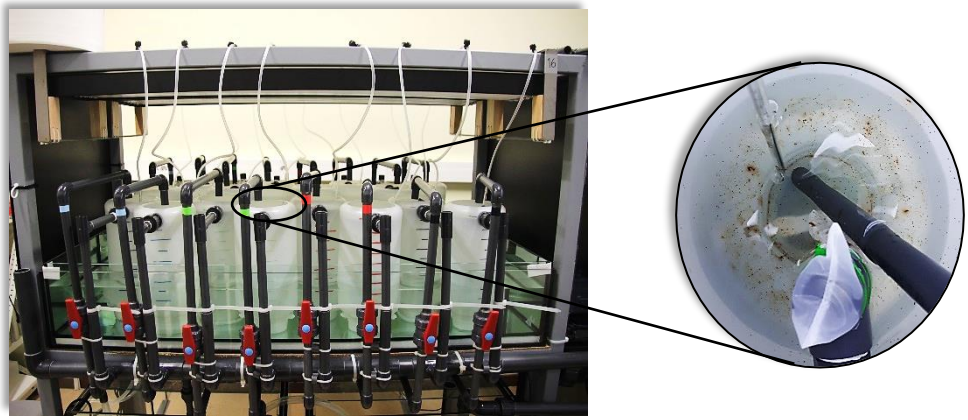


**Figure 2. 5:** Picture of half of the ELSS after installation of the new tanks (experimental containers adapted to planktonic larvae) with aeration and gravity drainage (on the left). Detail of the interior of one experimental tank (on the right).

Tanks were partially (2/3) immersed in temperature-controlled water baths equipped with temperature control systems similar to the original ones but scaled to their new dimensions (i.e., of half of the initial size). Tanks were kept illuminated by the original programmable luminaires used for diel light cycle simulation, as in Coelho et al. (2013). Using this ELSS new version (Figure 2.6-A), a perturbation experiment was designed including only 3 T ºC and 2 pH levels (3*2), generating a total of 6 OA-W scenarios that were tested in duplicate (Figure 2.6-B).

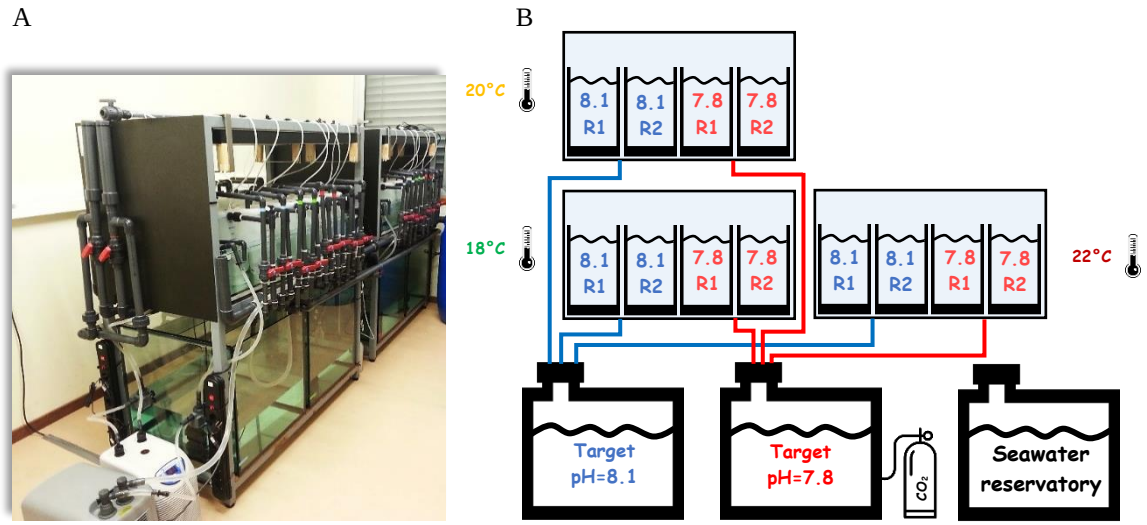



**Figure 2. 6:** Experimental setup used to expose *T. reticulata* newly hatched veligers to 6 OA-W scenarios for 14 days. **A**– Experimental Life Support System (ELSS) after the required modifications. **B**– Experimental design applied, which included 3 temperature levels (18, 20 and 22 ºC) * 2 pH levels (8.1 and 7.8).

Temperature and pH levels were selected following the latest projections of OA-W by the IPCC considering the RCP8.5 (Collins et al. 2013). Regarding T ºC, taking into account the projection of a global SST increase of up to 4 ºC by the end of the century (Collins et al. 2013, IPCC 2013), the following levels were considered: control 18 ºC, since it is the mean SST registered during the *T. reticulata* spawning season at the site where egg capsules were collected (Barroso & Moreira 1998), plus two warming conditions – 20 and 22 ºC – corresponding, respectively, to a near and a long-term projection (i.e., approximately by the years 2050 and 2100; (Kirtman et al. (2013) and Collins et al. (2013), respectively). As regards acidification, saltwater was manipulated through $CO_2$ bubbling targeting 2 distinct pH levels: control 8.1 plus an acidified condition of 7.8, corresponding to a decrease of 0.3 pH units relative to control, the minimum reduction predicted for the year 2100 by the IPCC in the northern hemisphere (Ciais et al. 2013), and in line with the available guidelines applied to OA research (Riebesell et al. 2010).

Synthetic saltwater used was produced at salinity 30 through dissolution of PRODAC Ocean Fish sea salt mixture in reverse osmosis ($V^2$ Pure 75, TMC, UK, purified freshwater at least 24 h prior to use). The saltwater was distributed by 3 of the 4 reservoirs of the ELSS. The pH was manipulated in one of the reservoirs, in order to achieve the pH target level 7.8, and unmanipulated in the remaining two: one kept the control pH saltwater and the other

was used to keep saltwater to refill the treatment ones as needed. Furthermore, to promote water mobilization and to force pH adjustment, the saltwater in all the reservoirs were recirculated for 15 min every two hours. The Ria de Aveiro semi-diurnal tidal regime and the water renewal percentage at its central area (Dias et al. 2001, Coelho et al. 2013) were simulated by 50% exposure medium exchange twice a day (manually at 9 am and 6 pm), by saltwater addition at the bottom of each tank through an inlet pipe, forcing the excess of medium to drain by gravity from the outlet pipe installed at the top. 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). A pre-assay to monitor the daily variation of the pH and T ºC generated in the ELSS under such conditions was carried out and the respective control systems' settings adjusted to avoid treatments' overlapping, allowing the predetermined scenarios to be obtained: pH controller in the acidified treatment reservoir was set to 7.95 and cooler/heater thermostats in the water baths were set to 17/18, 19/20 and 21/22 ºC.

After the 24 h-acclimation, and right before the start of the exposure period at day 0 (T0), swimming veligers were collected in a 100 µm-sieve, back-washed into a 5 L-beaker, counted and adequately aliquoted in order to get a randomly distribution and a density of 1.6 larvae.$mL^{-1}$ (i.e., an $n \approx 4800$/container) in each of the 24 tanks. Veligers were fed the microalgae blend described above, daily, after the late afternoon medium exchange (to avoid pH imbalance due to photosynthesis during the day and as larvae of marine bottom invertebrates are considerably active in the water column during dark hours favoring feeding; (Mileikovsky 1973)). The physico-chemical parameters of the exposure media and the biological endpoints were periodically assessed as describe below.

### 2.2.4 Exposure media physico-chemical analysis

Probe measurements (Multi 3430 IDS, WTW, Germany) of pH (NBS scale) and T ºC were recorded before and after both daily medium exchanges for three consecutive days from T0, to characterize the daily variations to which specimens would be exposed. After this period, those parameters were periodically monitored, at least before one of the two daily medium

exchanges until day 14 (T14). Probe measurements of salinity (SAL) and total dissolved oxygen (DO) were recorded weekly, before the morning medium exchange.

The total alkalinity (TA) was determined following the protocol of Frommlet et al. (2015) by manual volumetric titration of 20 mL samples of exposure medium, 0.2 µm-filtered, collected in the beginning of the experiment (at T0), at T2 and at T14, before and after the morning medium exchange in at least one replicate per treatment.

The $CO_2$ partial pressure ($pCO_2$), the concentration of the carbonate ion ($CO_3^{2-}$), and the saturation states of calcite ($\Omega_{Ca}$) and aragonite ($\Omega_{Ar}$), were estimated on Microsoft Excel macro CO2Sys_v2.1 (Pierrot et al. 2006), using with K1 and K2 carbonate dissociation constants from Mehrbach et al. (1973) refitted by Dickson & Millero (1987), and $KSO_4$ from Dickson (1990) and using the values of TA and of pH, T ºC and SAL measured at the time of media sampling as the inputs.

### 2.2.5 Analysis of biological endpoints

***Mortality***

Mortality was assessed throughout the exposure at day 2, 8 and 14 (T2, T8 and T14, respectively). Sampling at T2 was performed to assess the randomness of the initial distribution of the veligers, creating a baseline to compare mortality across treatments correctly. All veligers of each replicate were collected in a 100 µm-sieve, back-washed into a beaker, suspended in 3 L of the respective exposure medium and counted in three aliquots of 5 mL per replicate (i.e., 6 per treatment). At T8 and T14 survival was again determined but in 2 aliquots of 10 mL per replicate (n = 4 per treatment). Mortality was calculated from survival at all timepoints.

***Development***

At T14, twelve larvae per replicate (n = 24 per treatment) were placed on a 24-well plate, one per well, to be carefully inspected under a stereomicroscope (Leica® S8 APO, Leica Microsystems, Switzerland). The development stage of each veliger was classified into the 4 veliger stages described by Zupo & Patti (2009) and the absence or the presence of

digesting microalgae visible (at 80x magnification) inside the larvae gut was registered as an indication of the specimens' nourishment status.

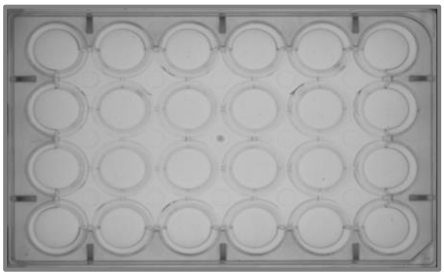
Veligers were relaxed for 15 min in a solution 1:1 $MgCl_2$ 7% in distilled water : exposure medium and primary fixed with glutaraldehyde 2,5% in Millonig's phosphate buffer, following the protocol described by Ellis & Kempf (2011). Then, samples were put together per treatment and decalcified with EDTA 5% during ≈ 10 min, a process controlled under the stereomicroscope. After washing thoroughly in Millonig's phosphate buffer, samples were kept in that solution at 4 ºC until further processing at the Abel Salazar Institute of Biomedical Sciences of the University of Porto (ICBAS-UP) aiming the histological analysis of the organs related with the swimming activity, as described below.

***Swimming behaviour***

Post-exposure swimming behaviour was assessed one day after T14 using video tracking on ZebraBox™ apparatus (Viewpoint, France). Twenty-four specimens were randomly selected per treatment (12 from each replicate) and placed in a 24-well plate (n = 24 individuals), one per well in 3 mL of the respective exposure medium.

Since *T. reticulata* veligers exhibits positive phototaxis (Chatzinikolaou 2006), each multi-well plate was placed inside the ZebraBox® and left for 5 min in the dark prior to video acquision. After that period, larvae were continuously monitored during 15 min (5 min under light, 5 min in the dark and 5 min under light) applying a background correction of 15 for enhanced detection (Figure 2.7). Videos were analyzed using the software ZebraLab® v3 (Viewpoint, France) and the following parameters determined: veligers' activity, distance travelled and speed (expressed in $s.min^{-1}$, $mm.min^{-1}$ and $mm.s^{-1}.min^{-1}$, respectively), as well as each larvae displacement angle (i.e., the number of turns on a specific angle, from -180º to +180º).

A



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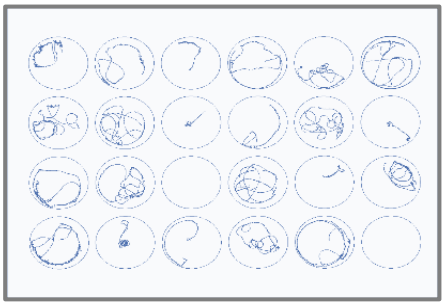

**Figure 2. 7: A**– Image of a 24-well plate placed on a ZebraBox® video tracking equipment (Viewpoint, France). **B**– Output diagram of the equipment showing the paths of the recorded veligers after 1 minute of analysis.

***Morphology and histology of the locomotor organs***

After sampling and the primary fixation at T14, 8 veligers per treatment were processed in order to obtain semithin sections of the organs related with the swimming activity for histological analysis (Figure 2.8).

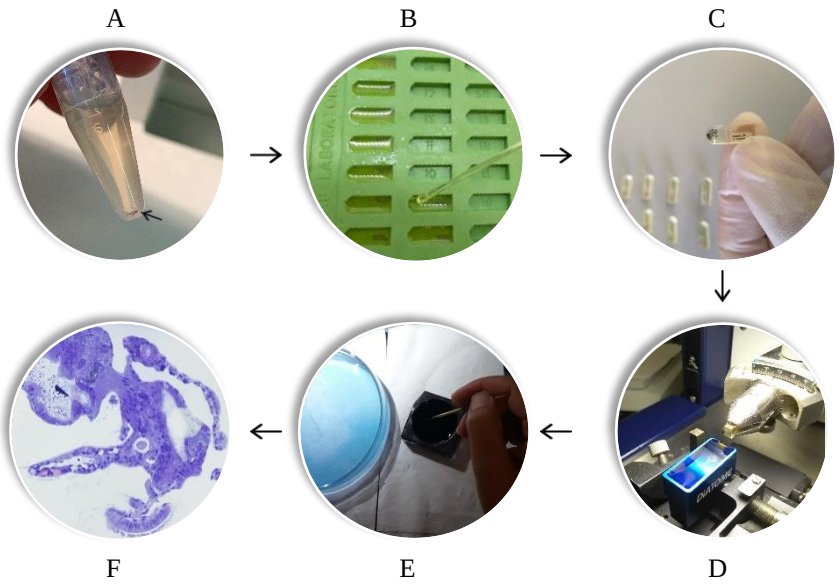


**Figure 2. 8:** Processing of *T. reticulata* veligers for the histological analysis of the locomotor organs in semithin sections. **A**– Veligers' collection on final epoxy before the inclusion (the arrow is pointing at the veligers). **B**– Inclusion of the veligers in the molds. **C**– Final blocks with veligers after cure in the oven. **D**– Cut of semithin sections with a diamond knife installed on Reichert/Leica UltraCut S ultramicrotome. **E -** Semithin sections staining in Methylene blue : Azur II (1:1) **F**– Image of a semithin section observed under the optical microscope Olympus BX41.

Prior to secondary fixation, samples were washed twice with a solution of sodium cacodylate (0.1M) and the supernatant was discarded retaining the veligers. Samples were secondarily fixed in osmium tetroxide (2%) for 1 h at 4 ºC, washed again with sodium cacodylate and kept at 4 ºC overnight. Dehydration through an alcohol series (at 30, 50, 70 and 90%, with an interval of 15 min at 4 ºC between each solution) was performed on the next day, followed by two washes in alcohol at 95% for 15 min, then other two at 100% for 20 min and the final two in propylene oxide during 10 min (these final washes were done at ambient temperature). Impregnation started by replacing the propylene oxide by a 1:1 mixture of propylene oxide with previously prepared epoxy resin (Epoxy embedding medium Kit, Sigma Aldrich, USA). On the next day, the mixture was replaced by a new mixture of propylene oxide with epoxy in the proportion 1:3. After 1 h, that mixture was

fully replaced by epoxy (only), in order to start the inclusion of veligers after 2 h (Figure 2.8-A).

During the inclusion, the veligers of each treatment were carefully placed in properly identified molds (Figure 2.8-B), which were subsequently placed inside the oven for 12 h at 45 ºC plus 24 h at 60 ºC. Finally, after some days at ambient temperature, the blocks (Figure 2.8-C) were cut, semithin sections 1-2 μm-thick on a Reichert/Leica UltraCut S ultramicrotome (Leica Microsystems, Switzerland) equipped with a diamond knife of size 4 (DiATOME, Switzerland). Cuts floating on the ultra-pure water in the knife's well (Figure 2.8-D) were selected and stained in Methylene blue : Azur II (1:1) during ≈ 1 min at 45 ºC (Figure 2.8-E). After staining, semithin sections were washed on ultra-pure water and placed on a glass slide at 45 ºC till complete drying, after which were mounted with Dibutylphthalate Polystyrene Xylene (DPX) medium under a coverslip.

Semithin sections obtained from 3 veligers per treatment (Figure 2.8-F) were analyzed under an optical microscope (Olympus BX41, Olympus Life Science Solutions, Japan) and photographed with the software Olympus cellSens standard *v*1.16.

The remaining veligers were dehydrated and, then, subject to critical point drying in order to image larvae through Scanning Electron Microscopy (SEM). The SEM exam was performed using a High resolution (Schottky) Environmental Scanning Electron Microscope with X-Ray Microanalysis and Electron Backscattered Diffraction analysis: Quanta 400 FEG ESEM / EDAX Genesis X4M. Samples were coated with an Au/Pd thin film, by sputtering, using the SPI Module Sputter Coater equipment.

### 2.2.6 Statistical Analysis

Permutational multivariate analysis of variance (PERMANOVA, Primer *v*6) was used to test for significant effects of the stressors pH, T ºC or the possible interaction between these factors at each endpoint. Regarding mortality, one value per replicate (typically the mean of the observations) was used. For the other endpoints, statistical differences were tested per individual organism. To generate the resemblance matrix Euclidean Distance was applied on the behaviour endpoints and Bray-Curtis was applied to analyze the development stage

data. A two-way crossed design (pH * T ºC) 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. In order to understand possible correlations between the variables analyzed, the routine RELATE (test of the match between resemblance matrices) was applied and the Spearman's rank correlation obtained.

## 2.3 Results

### 2.3.1 Exposure media physico-chemical characterization

The 6 OA-W scenarios generated in the ELSS are shown in Figure 2.9. All treatments were found to be significantly different (*Pseudo-F*=263.45, *p*=0.0001) and no differences between replicates were observed (*p*>0.05).

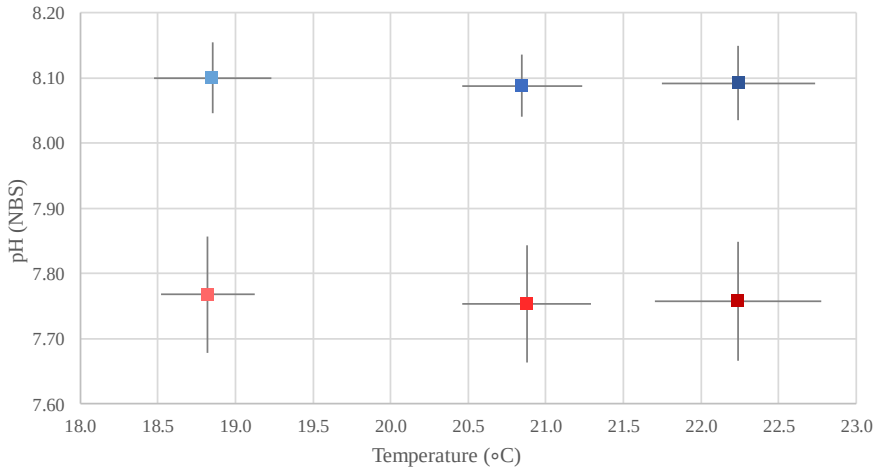


**Figure 2. 9:** OA-W experimental scenarios generated during the 14 days trial corresponding to the temperature (ºC) and pH (in the NBS scale) daily variation per treatment.

Salinity and dissolved oxygen measured before the morning exchange of 50% of the exposure medium were in average 30.1±0.2 and 7.50±0.28 mg.$L^{-1}$, respectively, and the variation of the carbonate chemistry of the exposure medium is presented on Table 2.1.

**Table 2. 1*:*** Mean carbonate system parameters of the exposure medium to which larvae were exposed during the 14 days trial, calculated from samples taken at T0, and "Before" and "After" the morning exchange of 50% of the exposure medium in one replicate per treatment at T2 and T14. The treatment column refers to target temperature ($T_{target}$) and pH ($pH_{target}$) levels selected as explained in the Materials and Methods section. Partial pressure of $CO_2$ ($pCO_2$), carbonate ion concentration ($CO_3^{2-}$) and saturation states of calcite (ΩCa) and aragonite (ΩAr) were derived from probe measurements of salinity (SAL), temperature (T) and pH, and from total alkalinity (TA) determined by volumetric titration. Values for all carbonate species "After" media exchange were estimated by CO2Sys Excel macro from $T_{target}$ levels as output parameter (since $T_{target}$ was achieved after ca. 15 min from the 50% media exchange).

| Treatment | | Probe measurements | | | | Titration | | CO2Sys Excel macro | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $T_{target}$ (ºC) | $pH_{target}$ (NBS) | SAL (psu) | T (ºC) | pH (NBS) | | TA (µmol kg-SW$^{-1}$) | | $pCO_2$ (µatm) | | $CO_3^{2-}$ (µmol kg-SW$^{-1}$) | | ΩCa | | ΩAr | |
| | | | | Before | After | Before | After | Before | After | Before | After | Before | After | Before | After |
| 18 | 8.1 | 30.1 | 18.9 | 8.11 | 8.12 | 2429 | 2582 | 527 | 539 | 151 | 162 | 3.74 | 3.99 | 2.39 | 2.55 |
| | 7.8 | 30.1 | 18.8 | 7.80 | 7.65 | 2566 | 2628 | 1245 | 1776 | 84 | 62 | 2.09 | 1.54 | 1.34 | 0.98 |
| 20 | 8.1 | 30.1 | 21.0 | 8.11 | 8.11 | 2434 | 2615 | 543 | 576 | 159 | 169 | 3.95 | 4.17 | 2.54 | 2.68 |
| | 7.8 | 30.1 | 20.9 | 7.80 | 7.64 | 2615 | 2635 | 1285 | 1863 | 92 | 65 | 2.29 | 1.61 | 1.48 | 1.03 |
| 22 | 8.1 | 30.1 | 21.7 | 8.14 | 8.09 | 2395 | 2553 | 486 | 596 | 171 | 168 | 4.25 | 4.18 | 2.74 | 2.70 |
| | 7.8 | 30.1 | 21.5 | 7.83 | 7.65 | 2620 | 2645 | 1196 | 1921 | 100 | 69 | 2.48 | 1.72 | 1.60 | 1.11 |

Carbonate system parameters shown in Table 2.1 are indicated per treatment and derived from the analysis of samples taken at T0 and before and after the morning exchange of 50% of the exposure medium (i.e., after the longest media stay during the night) from one replicate per treatment at T2 and T14. Hence, those values indicate the chemical conditions regarding the exposure media carbonate system to which larvae were exposed daily, per treatment, during the 14 days of exposure.

Mainly due to the control systems applied, temperature varied approximately within 1 ºC; however, a slightly higher variation in upper temperature treatments (22 ºC) can be perceived in Figure 2.9. Contrariwise, the pH tended to increase over time until its re-establishment to target levels upon media exchange, having higher variation at lower pH treatments (Figure 2.9, Table 2.1). Calcite and aragonite saturation states followed that pH variation pattern (Table 2.1). Carbonates' undersaturation (Ω<1) was not observed at any of the scenarios tested. However, aragonite saturation state ($\Omega_{Ar}$) at the acidified treatments (pH target level 7.8) was remarkably reduced to values closed to 1.0 after the 50% media exchange.

### 2.3.2 Larval mortality and development

The cumulative larval mortality throughout the exposure period is shown on Figure 2.10. At T2, both pH treatments at 22 ºC revealed the highest mortality (>30%); however, no significant effects of pH, T ºC or of their interaction were detected ($p$>0.05). Although, significant effects of both the T ºC and the pH on mortality at T8 were disclosed (T ºC: *Pseudo-F*=13.613, $p$=0.0107; pH: *Pseudo-F*=9.7582, $p$=0.0226), with higher mortality being registered at warmer (22 ºC) treatments (18 *vs* 22 ºC: *t*-test=3.3721, $p$=0.0384; 20 *vs* 22 ºC: *t*-test=5.2701, $p$=0.031) and under acidity (8.1 *vs* 7.8: *t*-test=3.1238, $p$=0.0228). At the end of the exposure period, at T14, T ºC was the only stressor affecting significantly the mortality (*Pseudo-F*=8.5754, $p$=0.0235), with larval survival <10% at both treatments at 22 ºC (18 *vs* 22 ºC: *t-test*=18.023, $p$=0.045).

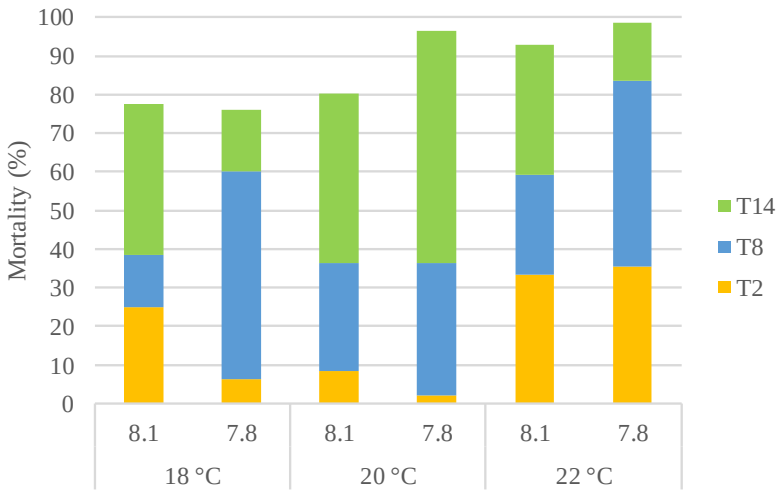


**Figure 2. 10:** Cumulative mortality of *T. reticulata* larvae calculated from the mean survival per treatment assessed throughout the experiment at 2 (T2), 8 (T8) and 14 (T14) days of exposure to 6 OA-W scenarios: 2 pH levels (8.1 and 7.8) at 3 different temperatures (18, 20 and 22 ºC).

Furthermore, T ºC was proved to significantly affect the veligers' development alone at T14 (*Pseudo-F*=8.1833, $p$=0.0006). Results on the classification of the larval development stage, and the respective nourishment status, performed at the end of the exposure period are compiled in Figure 2.11.

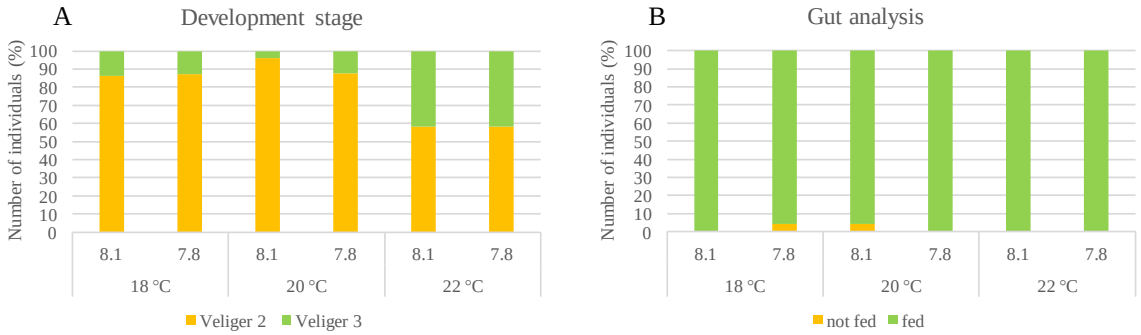


**Figure 2. 11:** Results on the classification of *T. reticulata* larval development and nourishment status at T14. **A** – Percentage of veligers at development stages 2 (Veliger 2) and 3 (Veliger 3) per treatment. **B** – Percentage of veligers with (fed) or without (not fed) microalgae inside the gut.

After 14 days of development under the 6 OA-W experimental scenarios only veligers 2 and 3 were found. A higher percentage of veligers 3 (~40%) were recorded at 22 ºC treatments when compared with the lower (~10%) registered at the other two temperatures tested (18 *vs* 22 ºC: *t*-test=2.8777, *p*=0.0057; 20 *vs* 22 ºC: *t*-test= 3.7199, *p*=0.0005; Figure 2.11-A). Moreover, >95% of the larvae were proved to be properly fed (Figure 2.11-B), and no significant effect of the stressors under study on the nourishment status was revealed (*p*>0.05).

### 2.3.3 Veligers' locomotor activity

After the statistical comparison of the locomotor parameters assessed through automated observation using ZebraBox® during the minutes recorded under light and dark conditions reveal that light had no significant effect on the veligers' behavioural response (*p*>0.05). Hence, the data obtained during the 15 min recorded was averaged in order to proceed with the analysis of the veligers locomotor behaviour.

A significant effect of the interaction between T ºC and pH was detected on both the activity (*Pseudo-F*=32.05, *p*=0.0001; Figure 2.12-A) and the distance travelled by veligers (*Pseudo-F*=37.547, *p*=0.0001; Figure 2.12-B). Also, and as expected by definition, the same significant effect of both stressors' interaction on speed was obtained (*Pseudo-F*=18.25, *p*=0.0001; Figure 2.12-C).

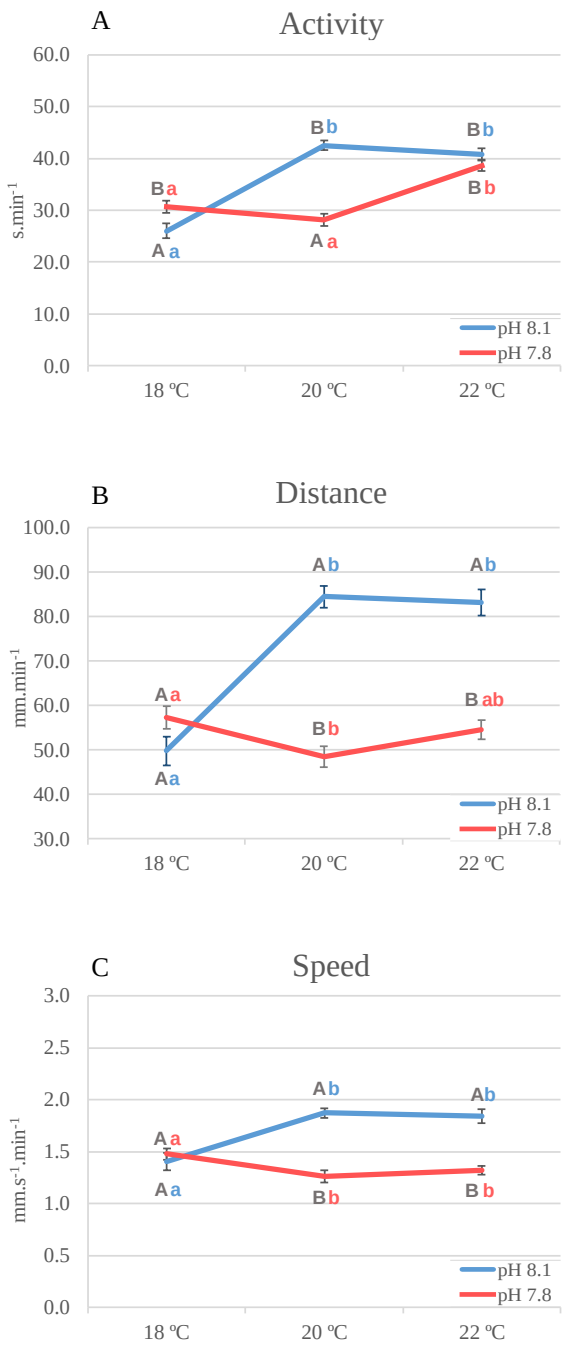


**Figure 2. 12:** Locomotor behaviour of *T. reticulata* veligers. **A** – Activity; **B** – Distance; **C** – Speed. Data are presented as the mean and the respective standard error. Lower case letters show significant differences between tested T °C within each pH treatment. Upper case letters refer to significant differences between pH treatments within a given T °C.

At pH control, larvae exposed to 20 and 22 ºC exhibited significantly increased activity (18 *vs* 20 ºC: *t*-test=9.5035, *p*=0.0001; 18 *vs* 22 ºC: *t*-test=7.9837, *p*=0.0001) and distance travelled (18 *vs* 20 ºC: *t*-test=8.4815, *p*=0.0001; 18 *vs* 22 ºC: *t*-test=7.5678, *p*=0.0001). Moreover, despite the positive correlation between the activity and the distance travelled by veligers (Spearman *r*=0.619, n=1505, $p \leq 0.01$), the larvae exposed to both pH's at 22 ºC exhibited similar activity but travelled significantly different distances (8.1 *vs* 7.8: *t*-test=7.9952, *p*=0.0001), shorter under acidity. In addition, the speed at which veligers move is positively correlated, as expected, with the distance (Spearman *r*=0.688, n=1505, $p \leq 0.01$) but also with the activity (Spearman *r*=0.475, n=1505, $p \leq 0.01$).

At control T ºC, the veligers exposed to different pH levels move at similar speed (Figure 2.12-C). However, a significant effect of the interaction between pH and T ºC was observed (*Pseudo-F*=18.25, *p*=0.0001), with the larvae under the most extreme treatment (pH 7.8 at 22 ºC) moving at reduced speed.

Regarding the displacement angle (i.e., the percentage of turns/laps veligers' take in a specific angle; Figure 2.13), the analysis statistical demonstrated that neither temperature,

acidification or both caused a significant effect ($p>0.05$), meaning that veligers exposed to different treatment presented a similar swimming pattern.

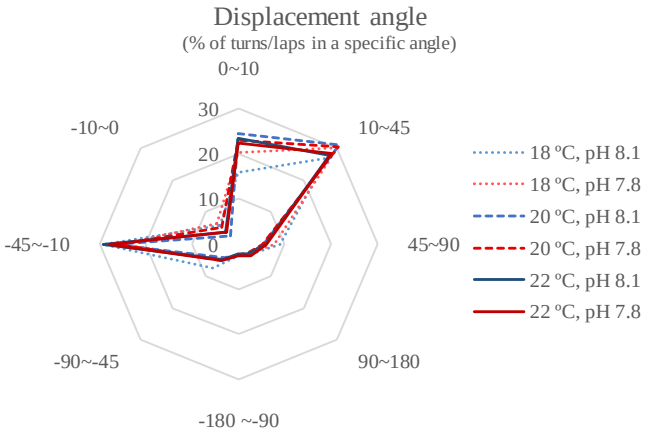


**Figure 2. 13:** Displacement angle of *T. reticulata* veligers. Mean number (%) of turns/laps each individual takes in a specific angle (from -180° to +180°) indicated per treatment.

### 2.3.4 Microscopy of the locomotor organs

The general morpho-histology of the veligers analyzed on semithin sections observed under light microscopy are summarized by each of the three temperature levels considered (18, 20 and 22 ºC) on Figures 2.14, 2.15 and 2.16, respectively. Thus, each of those figures include images of the tissues of the veligers exposed to both pH levels considered (the control and the acidified scenario) at one of the temperature levels, focusing on the organs related to veligers' locomotor capacity (the statocyst, the velum and the foot on images 1, 2 and 3, respectively). In general, larvae exposed to control temperature (18 ºC, Figure 2.14) do not reveal any alterations on the pH scenarios considered (control pH in Figure 2.14-A; 7.8 pH in Figure 2.14-B). Both the cellular arrangement of the statocyst epithelium and statolith integrity are preserved under acidity (Figure 2.14-B-1) as well as the velar and pedal morpho-histology (Figure 2.14-B-2 and -3, respectively). Contrariwise, the images in Figures 2.15 and 2.16 (of veligers exposed to 20 and 22 ºC scenarios, respectively), show damage of the larval tissues under acidity (Figures 2.15-B and 2.16-B). On veligers exposed to pH 7.8 at 20 ºC (Figure 2.15-B), it was observed the absence of statocysts' epithelia cilia (Figure 2.15-B-1). Moreover, at 20 and 22 ºC, larvae exposed to reduced pH (7.8) seem to have damaged cells in their velum and foot (Figure 2.15-B-2 and -3, and Figure 2.16-B-2 and -3, respectively).

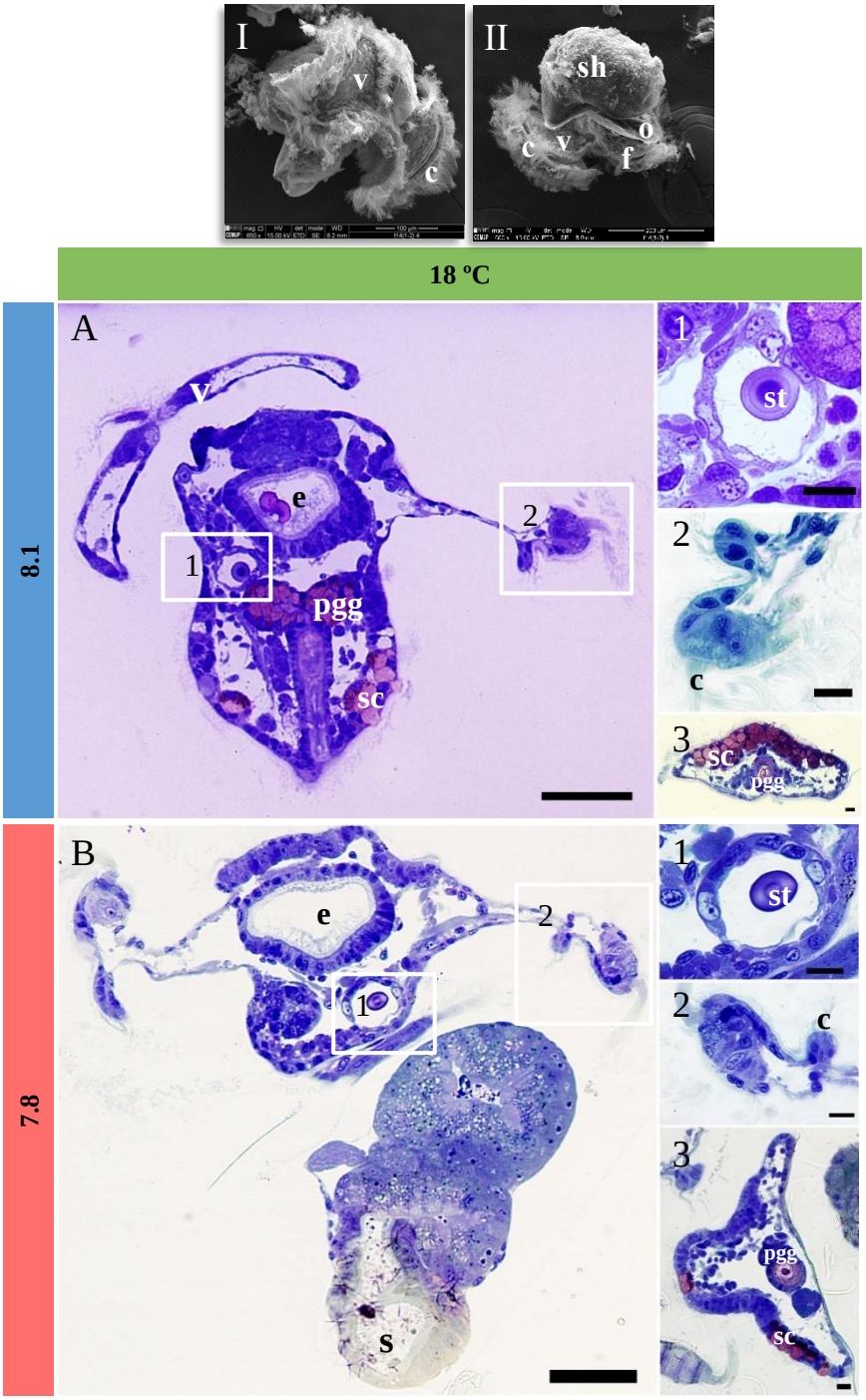


**Figure 2. 14:** Microscopic images of *T. reticulata* veligers exposed to 18 ºC and **A** – control pH (8.1) and **B** – reduced pH (7.8). I and II at the top pf the figure are Scanning Electron Microscopy (SEM) images of veligers exposed to control condition (18 ºC and pH 8.1) at T14 in which are indicated the structures observed in semithin cuts under light optical microscopy. Semithin cuts images include the tissues of larvae exposed to pH 8.1 (**A**) and pH 7.8 (**B**). Each of those cuts include some of the organs present in *T. reticulata*'s veligers. Images on the right (which may not belong to the main section shown in A or B) highlight the microstructure of the organs related to the veligers' locomotor capacity: **1** – statocyst with respective statolith; **2** – velum and respective ciliary apparatus; **3** – foot with respective operculum, pedal groove gland and secretory cells. **Abbreviations**: (c) cilia; (e) esophagus; (f) foot; (pgg) pedal groove gland; (s) stomach; (sc) secretory cells; (sh) shell; (st) statolith. Scale bars: (A and B)=50 µm; (1, 2, 3)=10 µm.

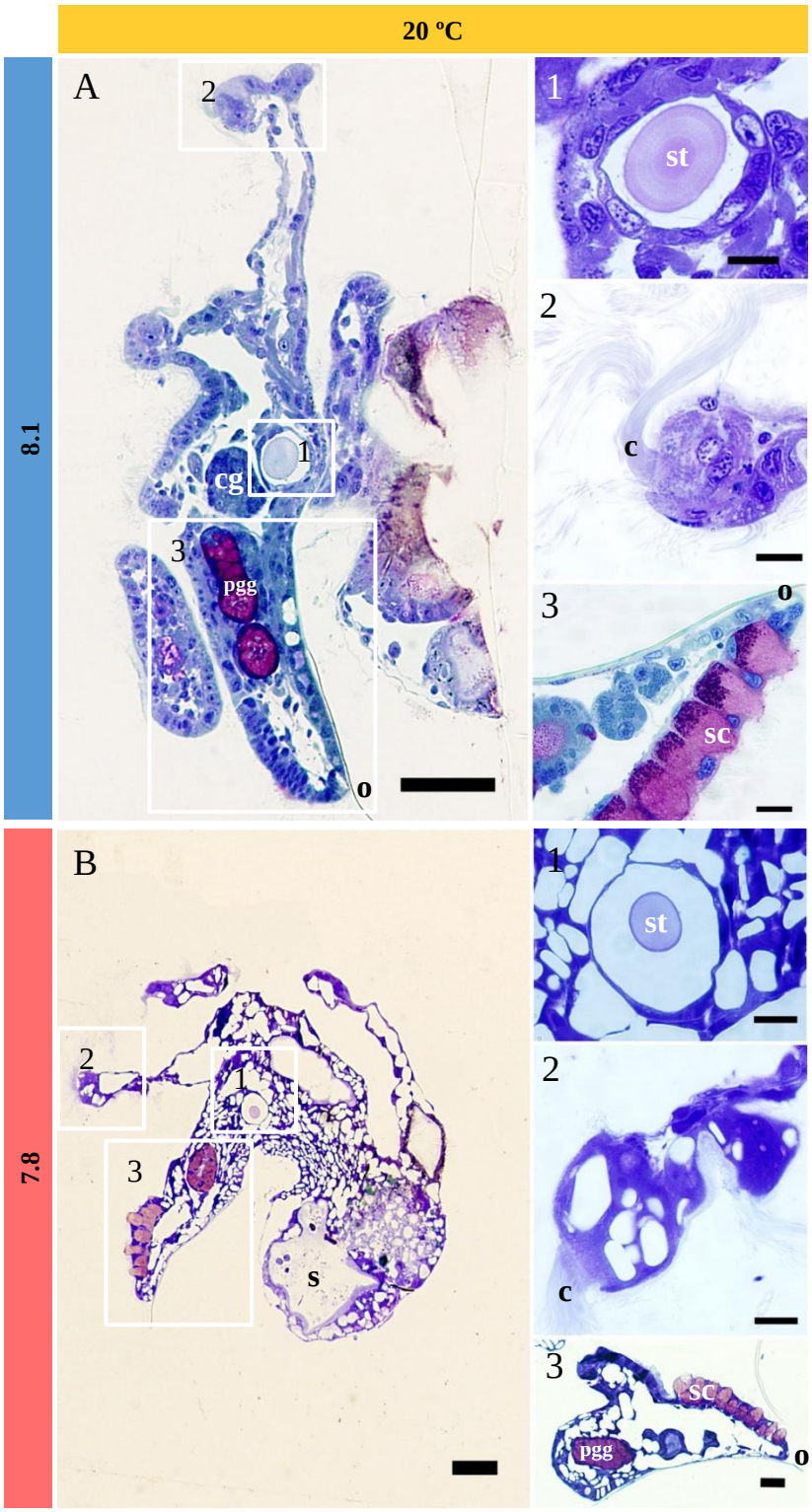


**Figure 2. 15:** Microscopic images of *T. reticulata* veligers exposed to 20 ºC. Semithin cuts include the tissues of larvae exposed to pH 8.1 (**A**) and pH 7.8 (**B**). Each of those cuts include some of the organs present in *T. reticulata*'s veligers. Images on the right (which may not belong to the main section shown in A or B) highlight the microstructure of the organs related to the veligers' locomotor capacity: **1** – statocyst with respective statolith; **2** – velum and respective ciliary apparatus; **3** – foot with respective operculum, pedal groove gland and secretory cells. **Abbreviations**: (cg) cerebral ganglion; (c) cilia; (o) operculum; (pgg) pedal groove gland; (s) stomach; (sc) secretory cells; (st) statolith. Scale bars: (A and B)=50 µm; (1 and 2)=10 µm; (3)=20 µm.

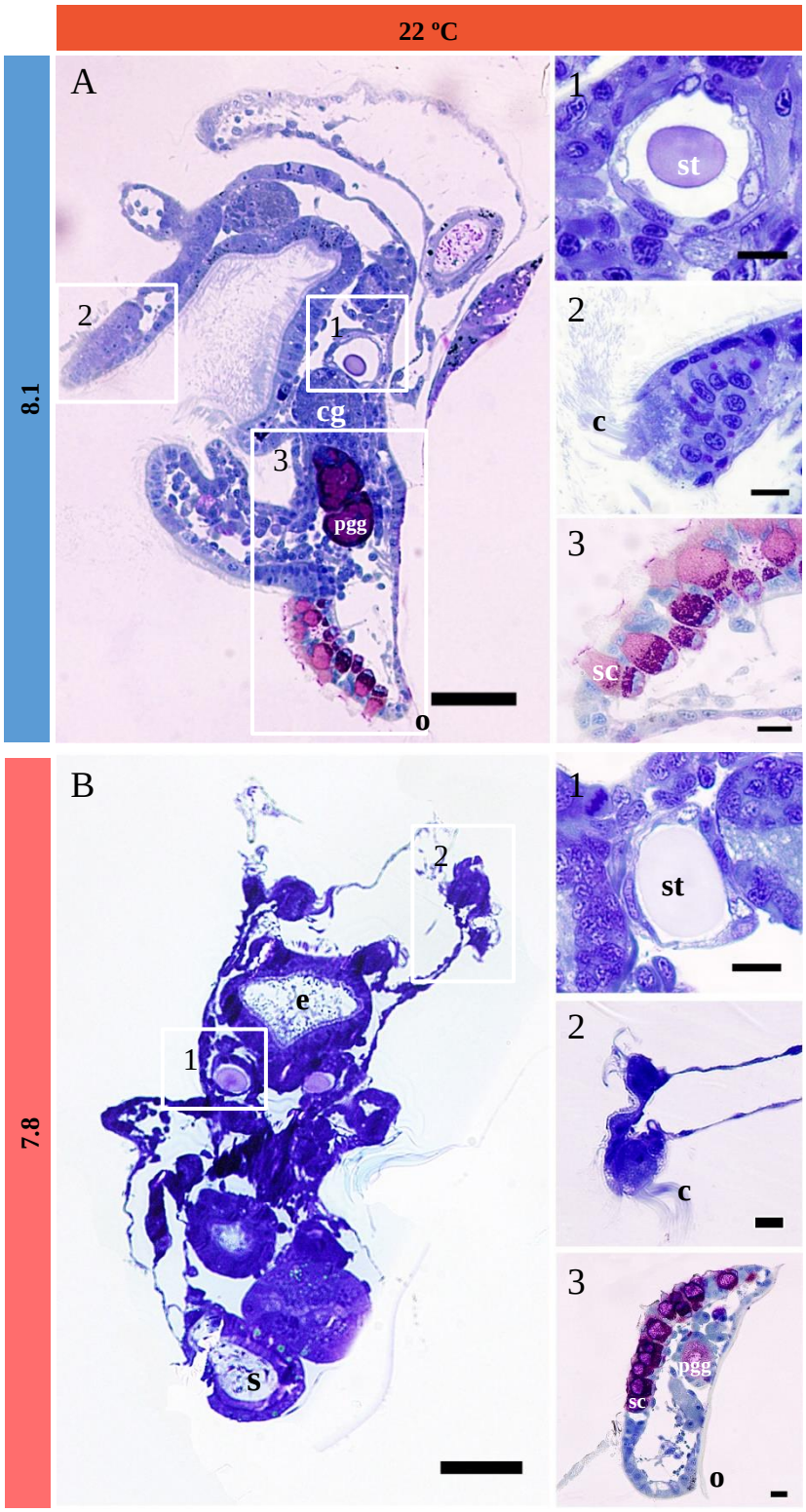


**Figure 2. 16:** Microscopic images of *T. reticulata* veligers exposed to 22 ºC. Semithin cuts include the tissues of larvae exposed to pH 8.1 (**A**) and pH 7.8 (**B**). Each of those cuts include some of the organs present in *T. reticulata*'s veligers. Images on the right (which may not belong to the main section shown in A or B) highlight the microstructure of the organs related to the veligers' locomotor capacity: **1** – statocyst with respective statolith; **2** – velum and respective ciliary apparatus; **3** – foot with respective operculum, pedal groove gland and secretory cells. **Abbreviations**: (cg) cerebral ganglion; (c) cilia; (e) esophagus;(o) operculum; (pgg) pedal groove gland; (s) stomach; (sc) secretory cells; (st) statolith. Scale bars: (A and B)=50 µm; (1,2, 3)=10 µm.

The foot seems to be the most affected organ (Figure 2.17). When compared to those of the veligers exposed to control pH (Figure 2.17-A, -B and -C), the metapodial tissues of the veligers exposed to pH 7.8 (Figure 2.17-D, -E and -F) look damaged, presenting protruded secretory cells disorganized and misaligned in the basal membrane, and with less pigmented granules and cilia.

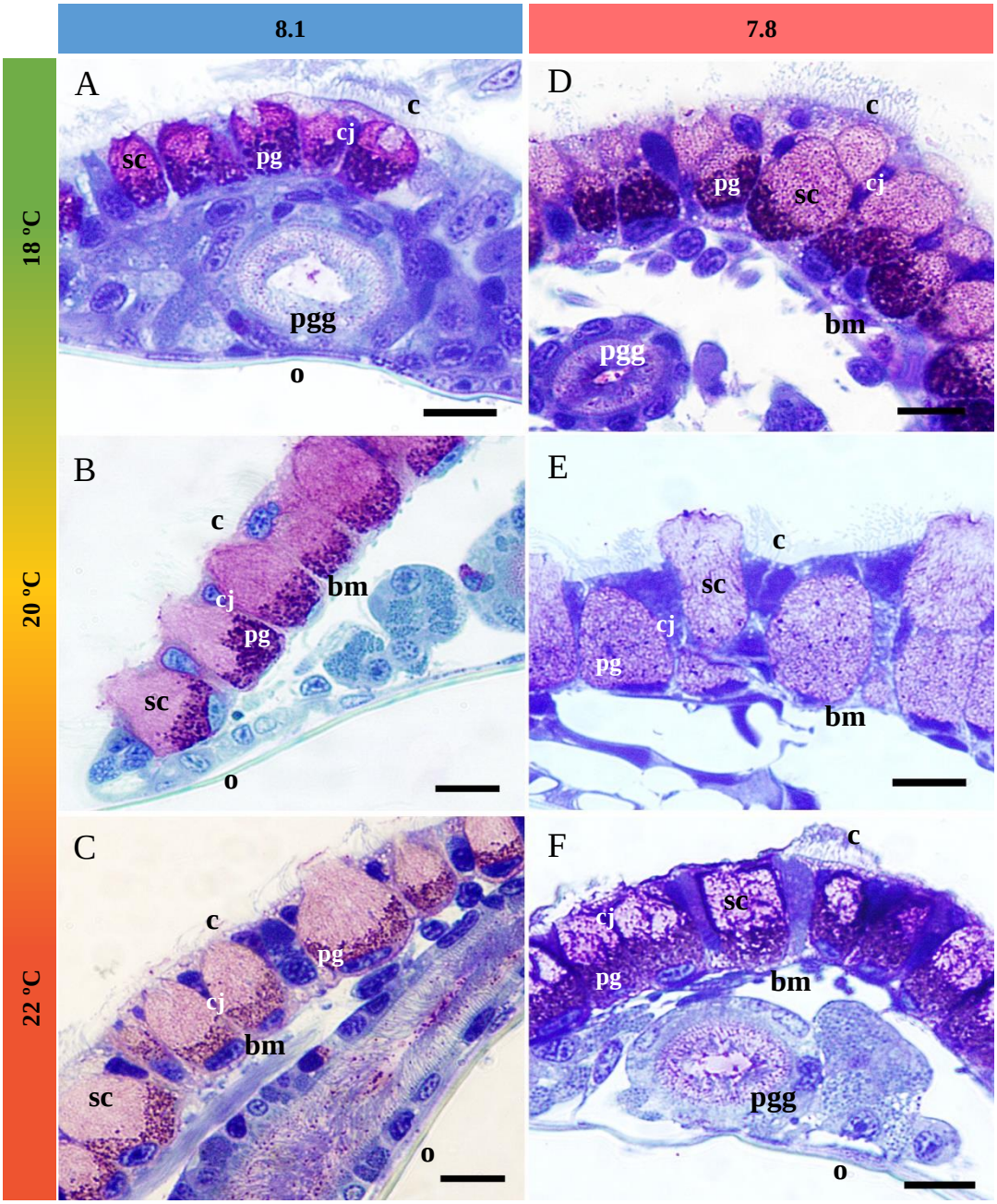


**Figure 2. 17:** Representative images of semithin section showing details of the foot of the veligers exposed to 6 OA-W experimental scenarios (from **A** – control treatment – to **F** – warmer and more acidic treatment). At T14, veligers presented a developing metapodium (anterior part of the foot) composed by a pedal groove gland (pgg) as well as secretory cells (sc) with pigmented granules (pg), gathered by cell junctions (cj) and lined on a basal membrane (bm). These images show damaged metapodial tissue in larvae exposed to acidified treatments and the misalignment of its secretory cells, with less pigmented granules and cilia. Scale bar=10 µm.

## 2.4 Discussion

Apart from the effects of future OA-W scenarios on mortality (which were somehow expected since other molluscs' larvae have been shown to be affected both *Acidification* and *Warming*; (Kurihara 2008, Pörtner 2008, Parker et al. 2013, Gazeau et al. 2013), our results demonstrate the significant effect of the interaction between the environmental stressors involved – pH and T ºC – and, thus, of the simultaneous occurrence of these phenomena, on the larval locomotor capacity after a relatively short exposure of 14 days.

Temperature is known to control the rate of fundamental processes such as enzyme reactions, diffusion, and membrane transport, due to its influence on molecular kinetic energy (Hoegh-Guldberg & Bruno 2010), increasing metabolism and development in all life stages, until a lethal threshold is reached (reviewed by Byrne & Przeslawski 2013). Thus, the increase of the activity, speed and the distance travelled by veligers under warming at control pH was expected. But besides temperature, acidity has also been proved to affect *T. reticulata* larval swimming, as in the marine gastropod *Littorina obtusata* (Linnaeus, 1758), (Ellis et al. 2009). In fact, our study reveals that the interaction between the two stressors has impact on the locomotor behaviour of this species' larvae, which in veligers 2 and 3 (the developmental stages present at T14; see Figure 2.11-A) is mostly swimming in the water column (both passively and actively, as described in section *2.1 Introduction*).

Considering the increased activity of all veligers exposed to warmer treatments (Figure 2.12-A), we expected that the distance travelled by those larvae would be proportionally incremented in both pH scenarios tested (8.1 and 7.8) which was not the case: although equally active, veligers under acidity (pH 7.8) travelled less and at lower speed. The reduction registered in the distance travelled without a simultaneous change in the activity could be an indication of a change on the swimming trajectory, like it was showed by Zhang et al. (2014) on two gastropod species' veligers –*Nassarius festivus* (Powys, 1835), now accepted as *Reticunassa festiva* (Powys, 1835), and *Nassarius conoidalis* (Deshayes, 1833)– after exposure to climate change scenarios. Our results, however, do not corroborate those observations: the displacement angle shown by our veligers, that is an indication of the trajectory followed by the veligers, was proved to the similar in all experimental the treatments (Figure 2.13). Thus, as in all the treatments veligers swam under approximately a same "trajectory", the decrease in the distance travelled can only be attributed to the

decreased swimming speed registered (Figure 2.12-C) although, as has been said, larvae were more active. In fact, during the careful inspection of the individuals at T14, it was perceived an apparent "nervousness" of the larvae exposed to the acidified treatments, more expressive in the hottest treatment, which was indeed proved by our analysis on ZebraBox®.

These alterations on the swimming behaviour described above can be attributed to multiple factors and, namely, to an abnormal development or damage on the organs with preponderant roles on the locomotor activity like the velum, the gravireceptors (statocysts) or the foot (see section *2.1 Introduction*). There are only few studies focusing on the effect of OA-W on tissues and organs in early life stages. Frommel et al. (2012) showed that ocean acidification has a negative impact on the tissues of cod fish larvae, namely in organs such as the kidney, the pancreas, the eye and the gut, damages significantly increased by $pCO_2$ concentration rise, affecting recruitment due to high mortalities. Comparing our results with histological studies performed in other gastropods (Bickell & Chia 1979, Page & Parries 2000, Page & Ferguson 2013), there is damage on velar, statocysts' and metapodium epithelia, mainly after exposure of *T. reticulata* veligers to acidity.

In addition to the consistent damage in the foot histology, registered in all acid treatments, the analysis of the semithin sections of veligers exposed to pH 7.8 at 20 ºC (Figure 2.15-B-2) revealed alterations on the tissues of the velum, which cells seem to be somehow replaced by empty spaces, and on the statocysts' epithelium, in which a lack of cilia from ciliated sensory cells (hair cells; see Chia et al. (1981)) contacting the statolith was observed (Figure 2.15-B-1). It is, somehow, speculative that these histological alterations reported in the velum and the statocyst may have been the cause for the detected reduction on speed and distance travelled by the veligers exposed to that treatment specifically. At first, it might be looked at, simply, as an artifact (e.g., an issue produced during tissues' fixation or impregnation), since the same alterations were not observed in any other acidic treatment, specially at the extreme at 22 ºC (because it is not likely that the intermediate treatment will induce such an effect, without the same or a worst effect or damage being observed in the following, extreme treatment). However, this result cannot be discarded since veligers at 22 ºC was proved to be more developed and larger and, at least in theory, more resistant. Hence, regarding the velum, as the organ has a preponderant role on both feeding and swimming (Braubach et al. 2006, Romero et al. 2010), the histological

damage reported can, in fact, have negative impacts on those behaviours. Contrary to the results presented by Chan (2012) and Chan et al. (2015) that described normal swimming capacity under altered morphology and feeding activity in an echinoderm larvae exposed to OA, our results show changes in the swimming behaviour under apparent normal feeding: larvae were apparently well fed in all treatments, an indication that the feeding ability was not affected, even though swimming was, under acidity. Veliger swimming and feeding processes are functionally interdependent (Hilbish et al. 1999) but since pre- and post-oral cilia, which compose the opposed ciliary system of velum, are differently affected by neural input (Braubach et al. 2006) one of the behaviours can be affected without implying the same to happen on the other. In the case of *T. reticulata*, changes detected in the swimming activity under acidity did not apparently implied a reduction in their feeding capacity.

As regards to the gravireceptor cellular damage, even ruling out the occurrence of technical problems in histological processing, we have to mention that serial sectioning was not the strategy adopted and, therefore, the non-observation of statocyst cilia in the larvae of this specific treatment may also be due to the fact that no cuts were taken from the area where they would be contained and visible. In this sense, further work should be done to confirm the hypothesis that cilia are not present in the veligers exposed to pH 7.8 at 20 ºC, namely through the observation of their serial sectioning. Still, if that damage is confirmed, it can be related with the changes detected in the veligers' behaviour in that treatment. The position of the statolith (biogenic calcified inert mass, which moves freely inside the fluid-filled lumen of the statocyst; see Brenzinger et al. (2013) is important to the mechanisms of balance and spatial orientation since it stimulates local sensory cells indicating the body's position in respect to gravity (Chase 2002). If those cells are affected as shown in Figure 2.15-B-1, those mechanisms can be disrupted, by the disturbance of neurosensory and muscle contraction functions (Ruthensteiner & Schaefer 2002, Croll 2009, Zhang et al. 2014), even if the mass, itself, is well formed. Although it was hypothesized that statolith calcification might be affected by acidification, as already described in other molluscs (e.g. Kaplan et al. (2013)), our work shows that *T. reticulata* veligers seem to build regular statoliths under the OA-W scenarios tested. However, if sensory cells' damage is confirmed, the mechanisms they are involved are certainly compromised. The statocyst receptor cells interact with each other and are connected to the organisms' nervous system (Levi et al. 2004, Barroso et al. 2005). All their activity is determined by the animal spatial orientation and the disruption of

this neural process could also be a possible reason justifying the decreased activity, speed and distance travelled by the veligers exposed to pH 7.8 at 20 ºC. On the other hand, the activity of velar cilia and/or musculature, which are also modulated by the reception of sensory stimuli of ampullary neurons and sensory cups (Page & Kempf 2009), may be affected if this damage detected in statocyst sensory cells is confirmed.

Nevertheless, despite all this somewhat speculative discussion, metapodial tissues were unequivocally damaged, at all acidified scenarios tested, revealing protruded secretory cells, disorganized and misaligned in the basal membrane, and less pigmented granules and cilia. Although veligers 2 and 3 do not use the foot for their locomotion, as in these development stages specimens swim either passively or actively in the water column, this structure develops gradually during the larval phase (Page & Ferguson 2013) and is very important, especially on metamorphosis. Two weeks after hatching the veligers should present a metapodium with a pair of epithelial glandular cells lined on each side of the base of foot, with the respective secretory products (Page & Ferguson 2013), as well as a reticulate material opening into the pedal groove midway along its length (Bickell & Chia 1979). Moreover, each secretory cell, which vary from species to species, should contain a variety of pigments, like carotenoid, melanin, among others, which give the appearance and characteristic color (Bravo et al. 2012). The OA-W scenarios tested appear to affect negatively this normal structure of the foot of *T. reticulata* larvae, namely causing misalignment of the secretory cells and an abnormal pedal epithelium, which possibly prevent proper mucus secretion, essential to a range of functions including lubrication, locomotion, protection and adhesion to the substrate (Bravo et al. 2012). Also, the mucus produced by the foot secretory cells contain GABA, an amino acid associated to the induction of metamorphosis in gastropods (Laimek et al. 2008, Stewart et al. 2011). Most of the research related to the mechanisms involved on behaviour disruption due to OA-W conditions is focused on effects on fish (Nilsson et al. 2012, Paula et al. 2019): when exposed to OA-W, fish revealed altered GABA function, possible due to compensatory changes in $Cl^-$ and $HCO_3^-$ ion gradients needed during acid base regulation (Watson et al. 2014). The fact that GABA receptors are presented in many invertebrates, namely in gastropods (Miller 2019), suggests that a changed behaviour is related with neurological processes (reviewed by Kroeker et al. 2014). Furthermore, the damage and abnormal formation of the metapodium (and, likely, the propodium), a morphological requisite for crawling, will affect

larval metamorphosis and settlement (Bickell & Chia 1979, Page & Ferguson 2013), and all the functions that depend on the foot itself like mobility, attachment, food capture, mating, spawning, and shell cleaning (Jung et al. 2006).

Besides the morpho-histological damage of the structures involved in locomotion, there are other possible reasons that could indirectly justify the observed decrease on *T. reticulata* larvae swimming capacity. Ocean acidification is known to affect calcification in marine calcifiers (Melzner et al. 2011, Thomsen et al. 2015, Onitsuka et al. 2018), such as *T. reticulata*, since the reduction of the carbonate saturation (Kurihara 2008, Gazeau et al. 2010, Parker et al. 2010, Byrne 2011) causes an increase on energy required for that physiological process (Byrne & Przeslawski 2013). In addition, OA causes an interruption on the acid-base balance (Parker et al. 2013): the increment of $CO_2$ in seawater induces acidosis/hypercapnia (i.e., the increase of $CO_2$ inside the animal body), promoting a higher concentration of the ions $[H^+]$ and $[HCO_3^-]$ (Byrne & Przeslawski 2013) and, consequently, impacts metabolism including thermal regulation and immunologic mechanisms (Parker et al. 2013). In order to get the acid-base balance restored, organisms have to transport and remove these ions through cells' membrane, spending energy. The energy relocation towards these processes implies that it has to be shifted from other crucial physiological mechanisms such as swimming, reducing the locomotor capacity.

This study constitutes a first attempt to disclose impacts of future OA-W on the swimming ability of *T. reticulata* early life stages. It is shown that veligers of this species will be affected if the tested conditions become effective. Since even small alterations on the larval life could negatively affect the recruitment success and, thus, future populations (Ross et al. 2011), the effects demonstrated here, namely on larval swimming and mortality, indicate the eminent threat that these phenomena constitute to this species survival if the projections for its occurrence become effective. Climate change mitigation is, thus, urgent in order to minimize its effects in an attempt to avoid a huge impact on marine life and ecosystems.

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# Chapter 3: Impacts of ocean acidification and warming scenarios on the performance of early life stages of the pullet carpet shell, *Venerupis corrugata* (Gmelin, 1791): effects on larvae and juveniles' mobility and survival

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Some of the data included in this chapter

are part of the following peer-reviewed scientific presentations:

(presenting author is 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 book p.1301

# Abstract

The pullet carpet shell, *Venerupis corrugata* (Gmelin, 1791) is a marine bivalve traditionally harvested at the Southwestern and Mediterranean Europe, mainly in Portugal, Spain, France and Italy where overfishing, indiscriminate digging and pathogen-induced mortality determined stocks' reduction, forcing aquaculture production. Intensification of *V. corrugata* aquaculture is very likely since the species has an actual high market value when compared to most shellfish species. This activity may also support stocks' recovery under high consumption and increased mortality due to disease outbreaks and extreme climatic events, predictably more frequent under the future environmental reality in a changing climate. This includes several undergoing global phenomena such as ocean acidification (OA) and warming (W), known to alter seawater chemistry impacting marine calcifiers such as clams. Both the natural stocks and aquaculture production are dependent on the performance of early life stages whose individual behaviour is critical, often determining survival. Here we assess the survival and the behaviour of *V. corrugata* in early life, specifically planktonic larvae swimming and benthic juveniles' burial and crawling, under OA-W projected scenarios. D-larvae were cultured for two months under nine OA-W experimental scenarios, generated by a factorial experimental design of three temperature (T ºC: 18, 20 and 22 ºC) and three pH levels (8.1 and 7.8 and 7.6). Mortality was assessed throughout the experiment and the mobility assessed i) after 14 days of exposure, applying a 6-stage scale developed to characterize larval behaviour, and ii) after approximately 2 months of exposure (at exactly 60 days post-fertilization), applying a sediment mobility test developed to characterize juvenile behaviours. Although significant effects of both the T ºC (*Pseudo-F*=5.1081, *p*=0.0164) and the pH (*Pseudo-F*=5.6093, *p*=0.0123) on mortality were registered, larval survival was higher under the most acidified condition (possibly due to its effect on the microbiome) and under warming (conditions under which development was potentiated). Regarding behaviour, larval mobility was significantly affected only by T ºC (*Pseudo-F*= 3.6914, *p*= 0.0042) after 14 days of exposure, with a higher percentage of specimens "searching" and "crawling" under warming, possibly looking for the ideal substrate for an early settlement as a result of a faster development. In fact, an antagonistic effect of the stressors tested was registered on larval development (*Pseudo-F*=3.5485, *p*=0.0007), which was notably accelerated by warming but relatively delayed under acidity, an effect attenuated by the concomitant rise in T ºC (specially at the hottest condition, 22ºC). In turn, juvenile clams showed highly variable behavioural responses. Even so, a readiness for burial (buried after 15 min) in individuals reared under warming was proved (significant effect of T ºC: *Pseudo-F*=5.2088, *p*=0.0096), possibly related with the clams larger size corroborating the benefits of warming (e.g. burial favors predation avoidance) in the case tested scenarios become effective. Contrarily, clams reared under higher acidity (pH 7.6) revealed lower ability (or availability) to bury, and acidity was also proved to affect byssus production significantly (*Pseudo-F*=4.603, *p*=0.0173), certainly compromising both mobility and fixation. Nevertheless, these results point towards the resilience of *V. corrugata* early life stages to projected OA-W, suggesting a beneficial effect of warming that may offset the negative effects of acidification in the case tested scenarios become effective.

## 3.1 Introduction

Temperature and pH are known to be two of the most important environmental factors controlling the distribution, physiological performance, morphology and behaviour of marine invertebrates (Byrne 2011). These are the main environmental stressors implied in the *Ocean Acidification* (OA) and *Warming* (W), ongoing phenomena that are proved to have profound impacts on marine ecosystems worldwide (see reviewed by Doney et al. 2012) and the shelled molluscs are among their most vulnerable components (Fabry et al. 2008, Branch et al. 2013). Much of the work done in this topic refer to the impacts of each of those stressors separately, while there are still few studies on their combined effects (Byrne 2011, Gazeau et al. 2013), namely on the behaviour of marine shelled molluscs and, particularly, in early life.

Embryos and larval stages of marine bottom invertebrates have specific environmental needs when compared to benthic juveniles and adults (Thorson 1950). Survival under OA-W is specially challenging in species with complex life cycles, such as the ones including planktotrophic larval stages, once these are physically, chemically and physiologically more vulnerable than the adults (Parker et al. 2013). This is the case of the marine shelled mollusc *Venerupis corrugata* (Gmelin, 1791), known as "pullet carpet shell", a bivalve that occurs in shallow waters of the East Atlantic, from the West of Africa to the South of Norway, and in the Mediterranean Sea (MolluscaBase 2019).

*Venerupis corrugata* planktonic life includes four main stages: the Trochophore, the D-Larvae, the Umbonate and the Pediveliger, which undergoes metamorphosis and becomes a Postlarva (Cerviño-Otero 2011). Generally, and under optimal/control conditions, the *D-Larvae* appears approximately 48 h after fertilization with a shell of about 100-105 μm in length and a typical straight hinge (Cerviño-Otero 2011). At this stage, the larvae swims actively in the water column, with its velum fully extended and rapidly beating velar cilia to capture food (Carriker 2001). This D-Larvae evolves to the *Umbonate* that appears, normally, 8 to 9 days after fertilization, with a shell of 130-150 μm in length and a circular hinge that forms the umbo (Cerviño-Otero 2011). At this stage swimming is slower, drawing a circling pattern in many directions (Carriker 2001). After 16 to 18 days from fertilization it is already possible to observe the *Pediveliger*, of 220-240 μm in length. Besides the velum, the pediveliger has also a developing foot (Cerviño-Otero 2011), structures that are both

essential to seek for a favorable surface by altering crawling and swimming (at the bottom) in a "searching" behaviour: when a given surface is not attractive, the pediveliger suddenly extends the velum, swimming away to a new local, repeating the searching process until a favorable settling site is found (Carriker 2001). Finally, when it happens, the animal undergoes metamorphosis to the *Postlarva* stage, in average 21-22 days after fertilization and, generally, with 250-270 μm in length. The metamorphosis includes the loss of velum and the development of branchial filaments, gills and siphons that are essential for feeding (Quayle 1952, Cerviño-Otero 2011). Furthermore, this stage present a robust foot with a fundamental role on early juvenile burial performance (Carriker 2001) which allows clams to feed by filtering water while buried, preventing predation (Tallqvist 2001, Sassa et al. 2011). Additionally, this species postlarvae also have a byssal gland for the production of byssus that is essential for early juvenile clams' dispersal and adhesion to the substrate (Waite 1992, Archambault et al. 2013). After the fixation to a favorable sediment, juvenile clams keep feeding by filtration and develop until maturity is reached and the adults reproduce, allowing the species continuity.

Therefore, all behaviours of both larvae and juveniles are crucial for this species survival, both on natural populations but also during aquaculture production that includes early life stages rearing in hatcheries and nurseries to obtain seed. France, Spain, Portugal and Italy are traditionally the main producers of the pullet carpet shell (FAO 2006-2019); however, the poor management of the captures and pathogen-induced mortality have been inducing natural stocks' reduction forcing aquaculture. In order to respond to a high market demand, and also to support stocks' recovery (Joaquim et al. 2010), the aquaculture production of *V. corrugata* has been increasing, as well as its market value (current global wholesale price over $21USD (Tridge 2019) , ≈18 € per kg) that is now higher than that of most shellfish species.

There are already evidences that OA impacts bivalves' fisheries and aquaculture, with huge monetary impacts (Branch et al. 2013), however, most of the effects described are related to defective calcification and a lack of knowledge on how concurrent OA-W could affect behaviour in early life, so important for both natural stocks' health and successful fisheries and aquaculture production of seed. Thus, the present study aims to assess the survival and the behaviour of *V. corrugata* in early life, specifically planktonic larvae

swimming and benthic juveniles' burial, crawling and byssus production, under OA-W projected scenarios, aiming at foreseeing the resilience of the earliest, most fragile, life stages to the expected future OA-W conditions.

## 3.2 Materials and Methods

### 3.2.1 Spawning induction and fertilization

About 60 adults of *V. corrugata* were obtained from a certified shellfish farmer from Ria de Aveiro (NW, Portugal) during the species natural spawning season (in May, 2019; Joaquim et al. (2011)). Animals were transported to the laboratory and were kept dry at 18±1 ºC during 48 h. After this period, clams were washed thoroughly, at first with tap water and then with 1 µm-filtered, UV-sterilized (V2 Vecton 600, TMC, UK) artificial seawater (Red Sea Salt, Red Sea Europe, France), prior to spawning by thermal induction. Spawning was achieved after two consecutive thermal shocks – alternating immersion in a hot bath at 22±1 ºC for 2 h (Figure 3.1-A) and in a cold bath at 11±1 ºC for 2 h – and further stimulation with both the suspension of mature sperm (from a male that was sacrificed for the purpose) and microalgae.

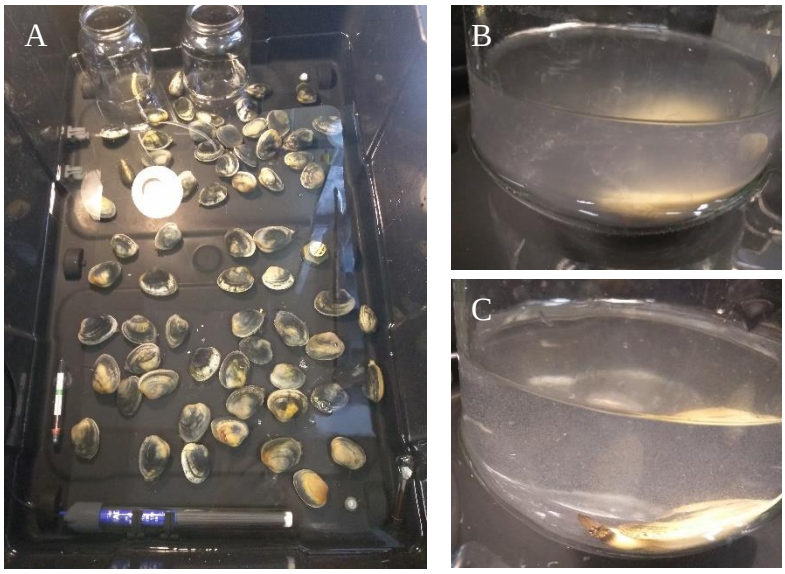


**Figure 3. 1:** *Venerupis corrugata* spawning induction. **A** – Broodstock in the hot water bath. **B** – Male and **C** – Female spawning in individualized containers.

As soon as spawning was detected, clams were individualized to emit the gametes, avoiding uncontrolled fertilization (Figure 3.1-B and -C). The oocytes obtained from 10 females were filtered through a 100 µm-mesh and resuspended in 10 L of 1 µm-filtered, UV-sterilized artificial seawater at 21 ºC and salinity 35. The sperm from 6 males was pooled and a volume of 200 mL of that mixture was used to fertilize the oocytes. After about 30 min the fertilization process was monitored under a light optical microscope (Axiolab E, Zeiss, Germany). The ratio of spermatozoa to oocytes was assessed aiming to keep it around 10:1 in order to avoid polyspermy. One hour later, the total number of fertilized eggs were determined by counting four aliquots of 1 mL of egg suspension. The eggs were then incubated in 30 L-tanks, at a density of 50 eggs.mL$^{-1}$ in 1 µm-filtered, UV-sterilized artificial seawater at salinity 35, and maintained at 18±1 ºC under $12^{L}$:$12^{D}$ photoperiod.

### 3.2.2 Incubation of fertilized eggs up to D-Larvae

Fertilized eggs were incubated without food for the first 24 h post-fertilization. Afterwards, 75 cells.µL$^{-1}$ of *Isochrysis aff. galbana* (*T-iso*) were added to the incubation medium in order to have food available as soon as D-Larvae are recorded and individuals start to feed (Cerviño-Otero 2011). *T-iso* was produced at our facilities as described in Chapter 2, as well as the diatom *Chaetoceros calcitrans* (*C. cal*) used during the exposure to OA-W scenarios, (see below), but using inoculants purchased from AQUALGAE, SL – Portugal and also the company culture medium (Gold Medium) and silicates (sodium silicate 40%). After 72 h post-fertilization, the D-Larvae were collected in a 60 µm sieve, resuspended in a volume of 4 L and counted in four aliquots of 1 mL (Figure 3.2). The D-larvae counted were, then,

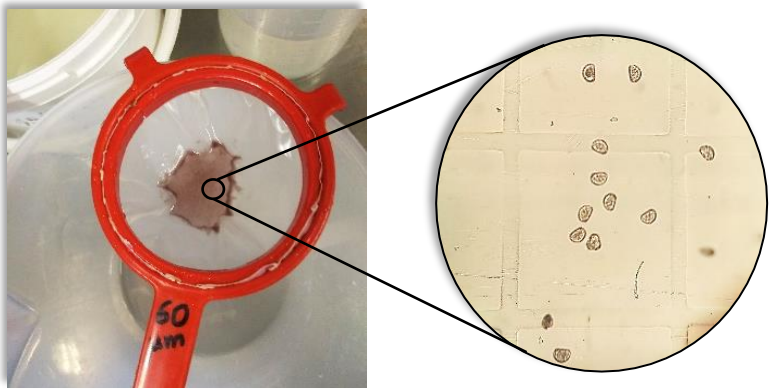


**Figure 3. 2:** *V. corrugata* D-larvae collected in a 60 µm-sieve (on the left) to be counted under the light optical microscope (on the right; the 100x times magnification was used for counting).

concentrated in 1 L of 1 μm-filtered, UV-sterilized artificial seawater at 18±1 ºC and salinity 35, and kept ready to be adequately aliquoted and distributed by the experimental containers to start the exposure to OA-W scenarios.

### 3.2.3 Exposure of D-Larvae to OA-W scenarios

***Experimental setup and treatments***

D-Larvae were exposed to 9 experimental treatments, corresponding to 9 OA-W simulated scenarios, for exactly 55 complete days. These scenarios result from a factorial experimental design of 3 temperature (T ºC) and 3 pH target levels, selected following the latest projections of OA-W by the IPCC considering the RCP8.5 (Collins et al. 2013).

Regarding T ºC, taking into account the projection of a global SST increase of up to 4 ºC by the end of the century (Collins et al. 2013, IPCC 2013), the following levels were considered: control 18 ºC, as the mean SST registered at *V. corrugata* spawning season in Ria de Aveiro (Joaquim et al. 2011), plus two warming conditions – 20 and 22 ºC – corresponding, respectively, to a near and a long-term projection (i.e., approximately by the years 2050 and 2100; (Kirtman et al. (2013) and Collins et al. (2013), respectively). As regards acidification, saltwater was manipulated through $CO_2$ bubbling, targeting 3 distinct pH levels: control 8.1 plus two acidified conditions of 7.8 and 7.6, corresponding, respectively, to decreases of -0.3 and -0.5 pH units relative to the control, the minimum and maximum reduction projected for the end of the 21st century by the IPCC in the northern hemisphere (Ciais et al. 2013), and in line with the available guidelines applied to OA research (Riebesell et al. 2010).

The 9 OA-W experimental scenarios were generated in the Experimental Life Support System (ELSS) developed by Coelho et al. (2013) and available at the Centre for Environmental and Marine Studies (CESAM), after both the modifications carried out for the work described in Chapter 2 and some additional changes required for the experiment in the current chapter (Figure 3.3-A). In brief, the expanded version of the ELSS applied in Chapter 2 (described in section 2.2.3) was also able to accommodate the factorial experimental design of 3 levels of T ºC and 3 of pH, generating the desired 9 OA-W scenarios (Figure 3.3-B). Still, larval rearing in this species is completely optimized (see

Cerviño-Otero (2011)) and involves both slow but continuous aeration and the exchange of 100% of the culture medium every two to three days. Under such conditions, it would be impossible to maintain the pH of the medium in acidified treatments at the desired target levels. Thus, the experimental containers were replaced by hermetically sealed jars, in order to avoid the exchange of $CO_2$ at the interface ambient air-exposure medium. Moreover, the saltwater reservoirs used to prepare synthetic seawater and to manipulate pH through $CO_2$ bubbling, were also replaced by hermetic ones, in which media equilibration was promoted and pH controlled and adjusted instantly (by $V^2$ control pH controller/monitor and $V^2$ pressure regulator pro, TMC, UK), forming an equilibrated atmosphere inside the reservoir that was captured and pumped (by common aquarium air pumps) to aerate the larval culture inside the experimental tanks (Figure 3.3-A). Hence, larvae were aerated by $CO_2$-enriched air from the equilibrated atmosphere whose pH treatment is concerned, keeping it at the desired level.

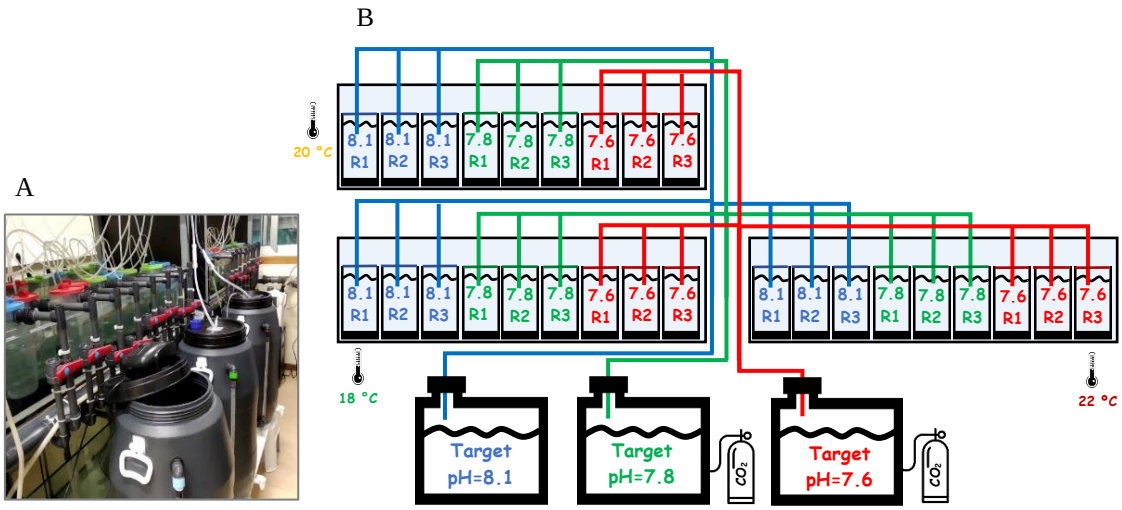


**Figure 3. 3: A** – Experimental Life Support System (ELSS) after the modifications required to exposed *V. corrugata* for 55 days to 9 OA-W scenarios. **B** – Experimental design applied in the current work including 3 T ºC and 3 pH levels.

Then, as in Chapter 2, tanks were partially (2/3) immerged in temperature-controlled water baths. Synthetic saltwater was produced at salinity 35 through dissolution of Red Sea Salt (Red Sea Europe, France) in reverse osmosis ($V^2$ Pure 75, TMC, UK, purified freshwater at least 24 h prior to use). The saltwater was distributed by three of the reservoirs and pH was manipulated to achieve the pH target levels (Figure 3.3-B), whereas the fourth

reservoir was used to keep unmanipulated saltwater to refill the remaining three as needed. Furthermore, to promote water mobilization and to force pH adjustment, the saltwater in all the reservoirs were continuously recirculated. 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 3 bubbles.$s^{-1}$. A pre-assay to monitor the diel cycles of pH and T ºC generated in the system under such conditions was carried out and the respective control systems' settings adjusted to avoid treatments' overlapping and allow the predetermined scenarios to be obtained: pH controllers were set to 7.68 and 7.30 (to allow the target pH's of 7.8 and 7.6 on the exposure medium in all replicates, respectively) and cooler/heater thermostats to 17/18, 19/20 and 21/22 ºC.

Each experimental OA-W scenario was tested in triplicate. Right before the start of the exposure period, at day 0 (T0), the D-Larvae counted and concentrated in 1 L were adequately aliquoted and randomly distributed by the 27 tanks at a density of 16 larvae.$mL^{-1}$ (≈ 26400 per replicate).

The exposure medium was completely renewed every three days, moment when the tanks were also cleaned under hatchery established protocols (Helm et al. 2004, Cerviño-Otero 2011, Joaquim et al. 2016), avoiding metabolites accumulation and bacterial proliferation. At each maintenance, tanks' location in each water bath was changed. Tanks' randomization was achieved by using the *Microsoft Exel* function "RAND()" on the code assign to each tank.

Larvae were fed an appropriate microalgae blend of equal proportions of *T-iso* and *C. cal*, based on the applied by Joaquim et al. (2014) and Fernández-Pardo et al. (2016). To avoid pH imbalance due to photosynthesis during the day, and as larvae of marine bottom invertebrates are considerably active in the water column during dark hours favoring feeding (Mileikovsky 1973), larvae were fed (preferentially and whenever possible) in the afternoon. The availability of microalgae in the exposure media was guaranteed by the adjustment of the diet in terms of feeding time (morning/afternoon) and quantity, according to the specimens' development in different treatments and the stability of the pH of the exposure medium, always allowing an *ad libitum* regime. As can be seen in Table 3.1, which summarizes the diet applied throughout the experiment, in the first 45 days of exposure

(Before T45, Table 3.1-A) microalgae quantities were adjusted only by T ºC (i.e., required adjustments were always applied to all tanks under a same temperature) as no differences between different pH treatments for a given T ºC were registered. However, after 45 days (After T45, Table 3.1-B), larvae exposed to the treatment "18 ºC pH 7.6" were notably less developed, not allowing an increase in the ration as required for the specimens exposed to all other treatments (otherwise pH would be unbalanced due to the consumption of $CO_2$ through photosynthesis during the day by an excess of microalgae in the medium). Furthermore, all veligers were fed in the late afternoon before T28 but, thereafter, food was provided always in the morning, except to treatment "18 ºC pH 7.6" that received half of the ration in the morning and the other half in the afternoon.

**Table 3. 1:** Microalgae blend used during the exposure of *V. corrugata* to OA-W scenarios. **A** – Diet provided before day 45 (T45) indicated by temperature level; **B** – Diet provided after T45 indicated by temperature, and also by pH since a different ration was applied for larvae in the most acidified treatment at 18 ºC.

A

Before T45

| Period | T (ºC) | Diet |
|---|---|---|
| T0 and T2-T4 | all | *75 cells.µL$^{-1}$ T-iso* |
| T5 and T7-T11 | all | *50 cells.µL$^{-1}$ T-iso + 30 cells.µL$^{-1}$ C.cal* |
| T12 and T14-T17 | all | *50 cells.µL$^{-1}$ T-iso + 40 cells.µL$^{-1}$ C.cal* |
| T18 -T22 | all | *50 cells.µL$^{-1}$ T-iso + 50 cells.µL$^{-1}$ C.cal* |
| T23 -T32 | 18 | *50 cells.µL$^{-1}$ T-iso + 50 cells.µL$^{-1}$ C.cal* |
| | 20 | *50 cells.µL$^{-1}$ T-iso + 50 cells.µL$^{-1}$ C.cal* |
| | 22 | *75 cells.µL$^{-1}$ T-iso + 75 cells.µL$^{-1}$ C.cal* |
| T33-T38 | 18 | *50 cells.µL$^{-1}$ T-iso + 50 cells.µL$^{-1}$ C.cal* |
| | 20 | *75 cells.µL$^{-1}$ T-iso + 75 cells.µL$^{-1}$ C.cal* |
| | 22 | *100 cells.µL$^{-1}$ T-iso + 100 cells.µL$^{-1}$ C.cal* |
| T39-T40 | 18 | *50 cells.µL$^{-1}$ T-iso + 50 cells.µL$^{-1}$ C.cal* |
| | 20 | *100 cells.µL$^{-1}$ T-iso + 100 cells.µL$^{-1}$ C.cal* |
| | 22 | *125 cells.µL$^{-1}$ T-iso + 125cells.µL$^{-1}$ C.cal* |
| T41-T44 | 18 | *50 cells.µL$^{-1}$ T-iso + 50 cells.µL$^{-1}$ C.cal* |
| | 20 | *100 cells.µL$^{-1}$ T-iso + 100 cells.µL$^{-1}$ C.cal* |
| | 22 | *150 cells.µL$^{-1}$ T-iso + 150 cells.µL$^{-1}$ C.cal* |

B

After T45

| Period | T (ºC) | pH | Diet |
|---|---|---|---|
| T45-T46 | 18 | 8.1 | *75 cells.µL$^{-1}$ T-iso + 75 cells.µL$^{-1}$ C.cal* |
| | | 7.8 | *75 cells.µL$^{-1}$ T-iso + 75 cells.µL$^{-1}$ C.cal* |
| | | 7.6 | *50 cells.µL$^{-1}$ T-iso + 50 cells.µL$^{-1}$ C.cal* |
| | 20 | all | *125 cells.µL$^{-1}$ T-iso + 125 cells.µL$^{-1}$ C.cal* |
| | 22 | all | *150 cells.µL$^{-1}$ T-iso + 150 cells.µL$^{-1}$ C.cal* |
| T47 | 18 | all | *75 cells.µL$^{-1}$ T-iso + 75 cells.µL$^{-1}$ C.cal* |
| | 20 | all | *125 cells.µL$^{-1}$ T-iso + 125 cells.µL$^{-1}$ C.cal* |
| | 22 | all | *150 cells.µL$^{-1}$ T-iso + 150 cells.µL$^{-1}$ C.cal* |
| T48 | 18 | 8.1 | *100 cells.µL$^{-1}$ T-iso + 100 cells.µL$^{-1}$ C.cal* |
| | | 7.8 | *100 cells.µL$^{-1}$ T-iso + 100 cells.µL$^{-1}$ C.cal* |
| | | 7.6 | *50 cells.µL$^{-1}$ T-iso + 50 cells.µL$^{-1}$ C.cal* |
| | 20 | all | *150 cells.µL$^{-1}$ T-iso + 150 cells.µL$^{-1}$ C.cal* |
| | 22 | all | *175 cells.µL$^{-1}$ T-iso + 175 cells.µL$^{-1}$ C.cal* |
| T49 | 18 | all | *50 cells.µL$^{-1}$ T-iso + 50 cells.µL$^{-1}$ C.cal* |
| | 20 | all | *75 cells.µL$^{-1}$ T-iso + 75 cells.µL$^{-1}$ C.cal* |
| | 22 | all | *100 cells.µL$^{-1}$ T-iso + 100 cells.µL$^{-1}$ C.cal* |
| T50-T55 | 18 | all | *100 cells.µL$^{-1}$ T-iso + 100 cells.µL$^{-1}$ C.cal* |
| | 20 | all | *150 cells.µL$^{-1}$ T-iso + 150 cells.µL$^{-1}$ C.cal* |
| | 22 | all | *150 cells.µL$^{-1}$ T-iso + 150 cells.µL$^{-1}$ C.cal* |

The physico-chemical parameters of the exposure media were periodically assessed as described below.

***Exposure media physico-chemical analysis***

Probe measurements (Multi 3430 IDS, WTW, Germany) of pH (NBS scale) and T ºC were recorded three times a day (morning, midday and afternoon) for six consecutive days from T0, to characterize the daily variations to which specimens would be exposed. After this period, those parameters were periodically monitored, generally two times a day (morning and afternoon) before feeding. Probe measurements of salinity (SAL) and of total dissolved oxygen (DO) were also recorded periodically, the SAL at least once every three days (at the time saltwater was produced) and the DO at least once a week.

Total dissolved inorganic carbon (DIC) was determined at T1 and T4, and every two weeks from T8 to T55, in 15 mL samples of 0.2 µm-filtered exposure medium collected in two of the three replicates per treatment. Immediately after collection, samples were analyzed for DIC quantification in a Carbon analyzer (Multi N/C 3100, Analytik Jena, Germany) following the manufacturer instructions and standard protocols applied on OA research (Goyet & Snover 1993, Dickson 2010). The values of DIC and of pH, T ºC and SAL read at the time of media sampling, were the inputs to calculate $CO_2$ partial pressure ($pCO_2$), carbonate ion ($CO_3^{2-}$) concentration and the saturation states of calcite ($\Omega_{Ca}$) and aragonite ($\Omega_{Ar}$) 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 & Millero (1987), and $KSO_4$ from Dickson (1990).

### 3.2.4 Mortality monitoring

The mortality of *V. corrugata*' larvae was calculated from survival, which was assessed throughout the experiment at day 3, 8, 10, 14, 18, 22, 25, 28, 43 and 49, and at the end of the exposure period (i.e., two times a week till T28 and once a week from T28 onwards). Sampling at T3 was performed to assess the randomness of the initial distribution of the D-Larvae, creating a baseline to compare mortality across treatments. At this time, all larvae of each replicate (i.e., tank) were collected in a 60 µm-sieve, back-washed into a beaker, suspended in 400 mL of the respective exposure medium and counted in at least two aliquots of 1 mL per replicate (i.e., 6 per treatment). In the remaining timepoints, the protocol was similar to the described for T3, although the volume in which the larvae were suspended was adjusted to the number of larvae available, and the mesh of the sieve used took into account the larval size: the 60 µm-sieve was used until T18, the 80 µm-sieve from then until T25,

the 100 µm-sieve from then until T43, time at which larvae exposed to 18 ºC and pH 7.6 were collected on a 200 µm-sieve, while in the remaining treatments a 300 µm-sieve was used; from T49 onwards the 300 µm-sieve was used for all treatments. Until T28, larvae were counted under the light optical microscope (Figure 3.4-A). Complete settlement (100% settlement in all treatments) was recorded at T28 and differences in the density of individuals between treatments (and even replicates) were registered, inducing undesired variations of the pH of the exposure media. In order to maintain the experimental conditions, particularly regarding acidification, it was necessary to adjust the density, normalizing it throughout replicates. Postlarvae were collected and counted to determine mortality, and only 600 individuals were returned to the respective tank. From this time to the end of exposure it was possible to count all live and dead individuals, or under a stereomicroscope (Leica® S8 APO, Germany) or even by eye (Figure 3.4-B). At the end of the 55 days exposure, all animals were collected per replicate at T56. Seventy live juveniles were selected for a "Sediment mobility test" described below and the remaining were preserved in absolute ethanol. Mortality was then calculated after counting the number of preserved specimens adding the 70 that were separated at T56 for the mobility test.

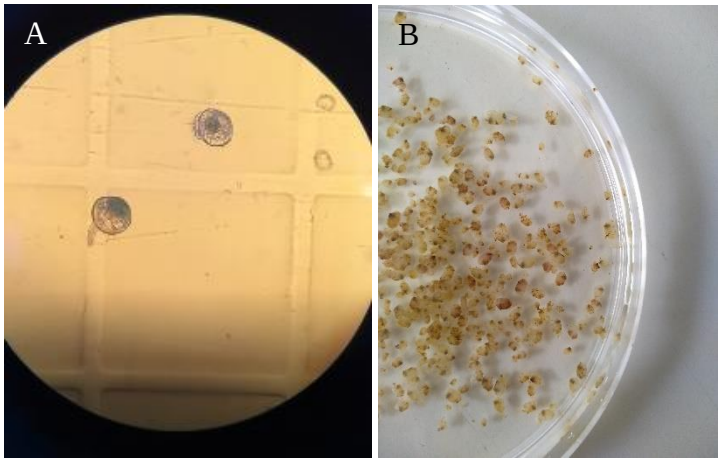


**Figure 3. 4: A** – Observation and counting of larvae and postlarvae under the light optical microscope. **B** – Early juveniles counted by eye.

### 3.2.5 Larval behaviour analysis

Larval mobility was characterized after 14 days from the beginning of the exposure (at T14). Eight specimens of each replicate were randomly collected with a pipette and individualized on an eight-well microscope glass slide (Figure 3.5), (n=24 individuals per treatment). Three

drops of exposure media were used per well. After a period of 5 min of acclimation, each specimen was observed and its behaviour characterized under the optical microscope, according to the scale developed here to classify larval mobility, from less active to more active: 1- at the bottom with the valves closed; 2- at the bottom with the velum or the foot extended; 3- at the bottom spinning or circling; 4- swimming; 5- searching; 6- crawling (see Figure 3.5).

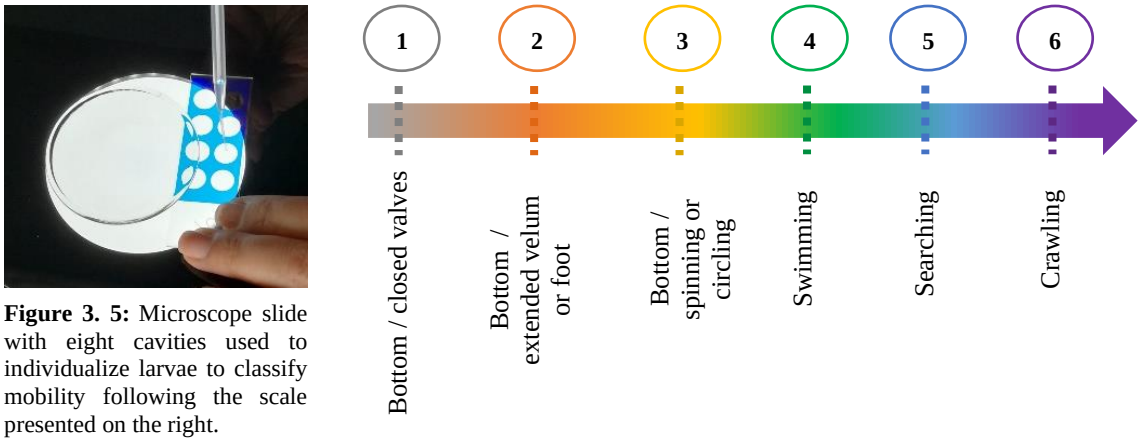


**Figure 3. 5:** Microscope slide with eight cavities used to individualize larvae to classify mobility following the scale presented on the right.

After the observation of the larvae to assess their behaviour, the development stage of each specimen was also classified into the 4 different stages described by Cerviño-Otero (2011): D-Larvae, Umbonate, Pediveliger and Postlarva (Figure 3.6). Also, the absence or the presence of digesting microalgae visible inside the gut was registered as an indication of the specimens' nourishment status.

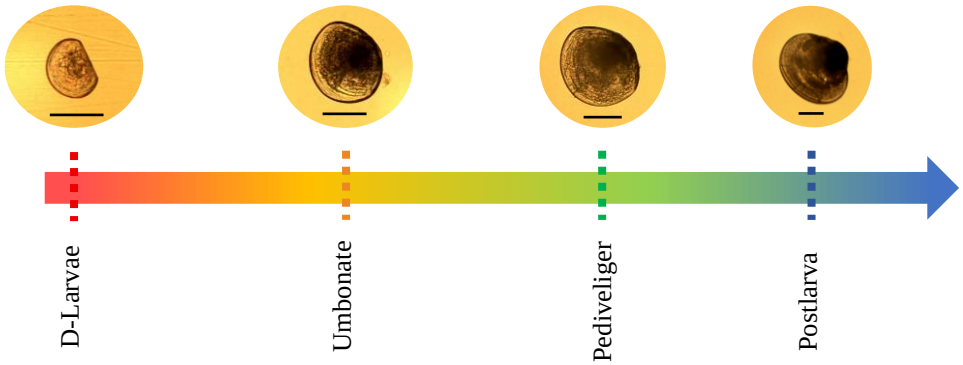


**Figure 3. 6:** Schematic representation of the scale used to classify the development stage of *V. corrugata* individuals following Cerviño-Otero (2011): from the less developed D-Larvae to the most developed Postlarva. Scale bar=100 µm.

### 3.2.6 Juveniles' behaviour analysis

***Sediment mobility test***

To assess potential effects of the OA-W experimental scenarios on the mobility of juvenile clams, it was necessary to develop a test that included the evaluation of the different behaviours involved in this life phase, namely burial and crawling activities, and also byssus production.

After the 55 days of exposure (at T56) the animals were collected, counted to assess mortality, and 70 individuals from each replicate were selected using different sieves per treatment, depending on the specimens size, as follows: for the juveniles reared at 18 ºC were used sieves of 1.0 mm-mesh (for pH 7.6) and 1.2 mm (for pH 7.8 and 8.1); at 20 ºC the sieve 1.2 mm-mesh was used for all pH treatments; at 22 ºC the sieve 1.7 mm-mesh was used also for all pH treatments. The juveniles were kept ready for the experiment on the next day (T57), at 60 days post-fertilization, inside a 500 µm-mesh bag under their respective test conditions (Figure 3.7).

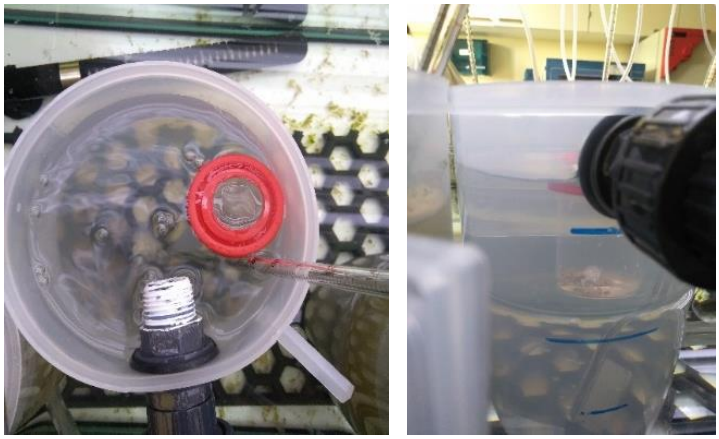

**Figure 3. 7:** Picture of the 500 µm-mesh bag containing the 70 juveniles selected at T56 for the "Sediment mobility test" performed at T57.

Surface sandy sediment (first 5 cm layer) was collected in Ria de Aveiro (NW Portugal) at a location (40º38'34.65''N - 8º44'06.80''W) where *V. corrugata* is known to occur (Figure 3.8).

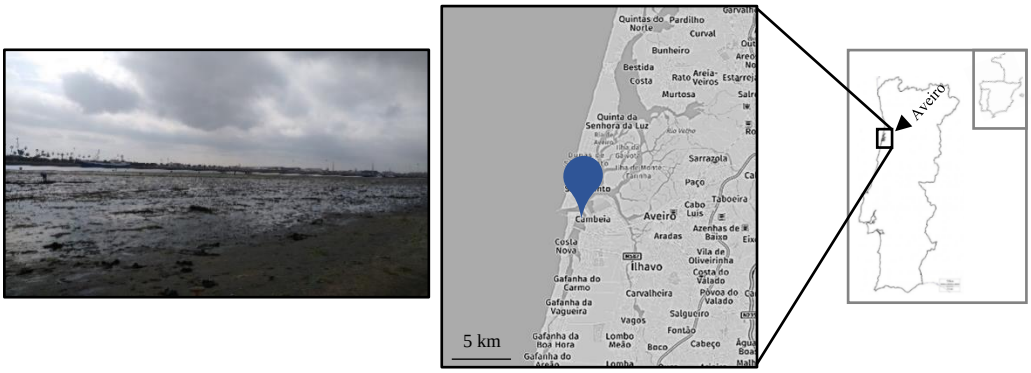


**Figure 3. 8:** Location of the site where surface sandy sediment for the "Sediment mobility test" was collected: Ria de Aveiro intertidal (NW Portugal, 40°38'34.65''N - 8°44'06.80''W).

Due to the reduced size of the juveniles being tested (1-3 mm), sediment was wet sieved to retain only fine to medium sand (i.e., the fraction ≥0.125 to <0.500 mm). A biologically inert substrate for the trial was achieved by applying the protocol described by Otte et al. (2018) for coastal marine sediments sterilization by autoclaving was followed (20 min at 121 ºC in beakers covered with aluminum foil for two consecutive cycles, with 48 h-storage at room temperature in between). The protocol applied by Cunha & Ravara (2003) to characterize sandy sediments, 6 sub-samples of the cleaned sediment were dried at 60 ºC for 72 h in a convection oven in pre-weighted porcelain crucibles, placed in a desiccator to cool to room temperature and re-weighted. The water content (moisture) was determined by subtracting the dry to the wet weight. Samples were then incinerated at 450 ºC for 5 h in a muffle furnace, placed in a desiccator to cool to room temperature and re-weighted to determine the organic content by subtracting the incinerated to the dry weight. Sub-samples were then mixed together, weighted and dry-sieved through a series of sieves (0.125, 0.250 and 0.500 mm) in a shaker for 15 min. The granulometric composition was determined re-weighting the fractions retained in each sieve and calculating their relative proportions expressed as a percentage of their mass.

Sediment was divided into 9 fractions and each of them overlain by one of the 9 exposure media under study. Sediments were kept equilibrating in that respective exposure medium, under continuous aeration, in parallel with the animals growing under the same condition, for 24h prior to the crawling, burial and byssus production assessment. After the 24 h-period for sediment equilibration, the overlying medium was discarded.

The “Sediment mobility test” was conducted in the ELSS controlled-temperature water baths (Figure 3.9-A), in 95 mm-diameter and 200 ml-volume covered glass crystallizers. A Petri dish of 40 mm-diameter was placed centered inside each crystallizer, creating two distinct areas/rings (center vs. margin, see Figure 3.9-B). A layer of about 1 cm-high of treated sediment was placed on the experimental container (Figure 3.9-C), covering all the crystallizer bottom, including the smaller Petri dish in its center (~200 g of sediment per container). Immediately prior to the beginning of the trial, sediment was overlain with 100-150 mL of the respective exposure medium. Probe measurements (Multi 3430 IDS, WTW, Germany) of pH (NBS scale), T ºC and SAL were performed immediately before the filling of crystallizers on the respective exposure medium; the pH of the sediment was also measured.

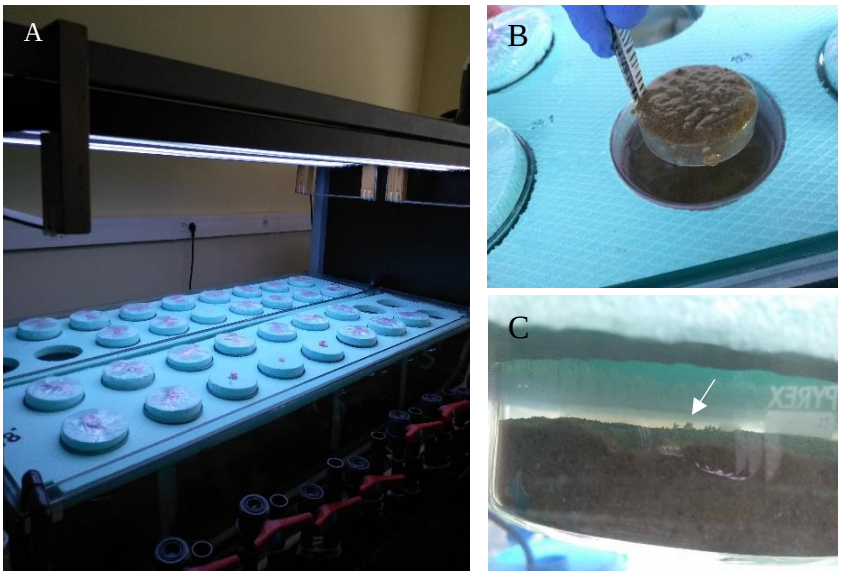


**Figure 3. 9: A** – ELSS supporting the apparatus for the “Sediment mobility trial”, created to assess crawling and burial activities, and byssus production, of *V. corrugata* juveniles, showing the experimental containers – crystallizers – covered to avoid $CO_2$ exchanges at the medium-air interface. **B** – Petri dish of 40 mm-diameter placed centered inside each crystallizer, creating two distinct areas/rings (center vs. margin); **C** – Picture of the profile of one crystallizer showing the layer of about 1 cm-high of treated sediment with all the bottom covered (including the smaller Petri dish in its center), with the clams siphoning at its surface (arrow).

The test started by pipetting 20 individuals of the previously separated to the sediment surface, at the center of the experimental container (i.e. 20 animals per crystallizer). Three replicates (i.e. crystallizers) were prepared per each original replicate (of the OA-W scenarios exposure) and so 60 juveniles per each replicate of the initial exposure were assessed. The behaviour of the clams was evaluated as follows.

***Burial activity***

Juveniles' burial rate was assessed at T57 by counting the number of specimens visible on the sediment surface every 15 min from the beginning of the trial, and for 1 hour. Clams were considered not buried if lying flat, or relatively flat, on the sediment surface and if no attempt at burrowing was apparent, as considered by Archambault et al. (2013). Those that were buried in the siphoning position (as described by Carriker 2001) were recorded as buried. After 24 h, at T58, the number of specimens visible on the sediment surface were again counted and probe measurements of pH, T ºC and SAL were also taken to assess the exposure medium conditions at the end of the trial.

***Crawling activity***

Juveniles' crawling distance was assessed after completion of 24 h from the beginning of the trial, at T58, by recovering the specimens in the sediment inside the smaller-40 mm Petri dish centered in the experimental container and the specimens in the remainder sediment (on the crystallizer peripheral ring). Clams were separately recovered in a 1.0 mm-sieve, washed on distilled water and preserved in 70% ethanol. After approximately 24 h, the specimens were moved to absolute alcohol and kept preserved for posterior analysis. The animals were then counted and the number of specimens with "short crawling distance" (recovered from the central 40 mm) and "long crawling distance" (recovered from the margin) were determined.

***Byssus production***

Byssus production was assessed in the specimens preserved in absolute ethanol through the analysis of the presence vs. absence of byssal threads under the stereomicroscope, using the dichotomous dependent variable index of Archambault et al. (2013): 0- "byssus not detected" and 1- "byssus detected" (Figure 3.10).

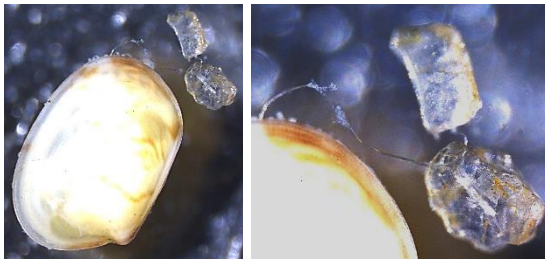

**Figure 3. 10:** Juvenile clam, showing the presence of byssus adherent to two grains of sediment.

### 3.2.7 Statistical Analysis

Permutational multivariate analysis of variance (PERMANOVA, Primer v6) was used to test for significant effects of pH, T ºC and the possible interaction between these factors for each endpoint analyzed. Regarding mortality, one value per replicate (typically the mean of the observations) was used and Euclidean distance was applied to generate the resemblance matrix. At T14, the larval behaviour and the development stage were tested per individual and Bray-Curtis similarity was used to generate the resemblance matrix. At the end of the exposure, three values per replicate were used for each of the behaviours analyzed (crawling and burial) and the Euclidean Distance was applied. After obtaining the resemblance matrix for each of the endpoints assessed, a two-way crossed design (pH * T ºC) 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.

## 3.3 Results

### 3.3.1 Exposure media physico-chemical characterization

Figure 3.11 shows the 9 OA-W scenarios generated in the ELSS and the data on the exposure media carbonate chemistry are compiled in Table 3.2. All treatments were found to be significantly different (*Pseudo-F*=2823.8, *p*=0.0001) however significant differences between replicates from the same treatment were observed (*Pseudo-F*=2.5911, *p*=0.0001), which can be attributed to the differences in the density registered between replicates mainly until T28.

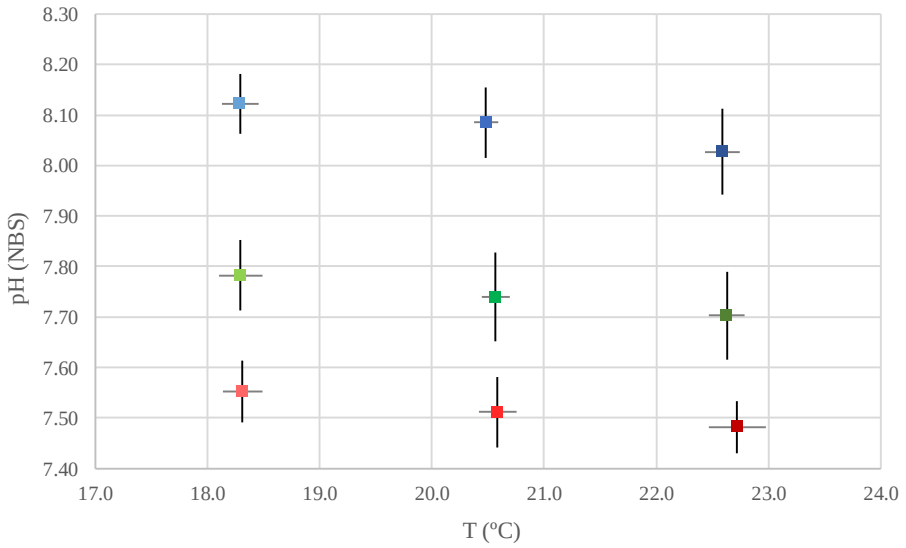


**Figure 3. 11:** OA-W experimental scenarios generated during the 2 months exposure, corresponding to the temperature (T °C) and pH daily variations per treatment. Mean values and respective standard deviation of probe measurements of T °C and pH (measured three times a day from T0 to T6 and once to twice a day thereafter, until T55).

Salinity and total dissolved oxygen registered throughout the experiment were, in average, 34.3±0.6 and 9.05±0.69 mg.L$^{-1}$, respectively. Since the exposure medium was changed completely every three days, the measurements of T ºC and pH performed three times per day during the first week, and two times per day till the end of exposure, characterize the variation of the system parameters to which the organisms were exposed during three consecutive days. Temperature revealed a maximum variation of ±0.3 ºC and the pH tended to decrease over time, being re-established to the target levels upon media exchange, with higher amplitudes being registered at higher T ºC.

Carbonates' undersaturation (Ω<1) was not observed at any of the acidified scenarios tested (Table 3.2). However, aragonite saturation state ($\Omega_{Ar}$) presented values close to 1.0 at the most acidified treatments (pH target level 7.6).

**Table 3. 2:** Mean carbonate system parameters calculated from samples taken at T1 and T4 and every two weeks from T8 to T55 on the day after the medium exchange and in two replicates per treatment. The treatment column refers to target temperature ($T_{target}$) and pH ($pH_{target}$) levels selected as explained in the Materials and Methods section. Partial pressure of $CO_2$ ($pCO_2$), carbonate ion concentration ($CO_3^{2-}$) and saturation states of calcite (ΩCa) and aragonite (ΩAr) were derived from probe measurements of salinity (SAL), temperature (T) and pH, and from total dissolved inorganic carbon (DIC) quantified by acidification, gas stripping and infrared detection in a Carbon analyzer. Values for all carbonate species were estimated by CO2Sys Excel macro from $T_{target}$ levels as output parameter.

| Treatment | | Probe measurements | | | Titration | CO2SysExel macro | | | |
|---|---|---|---|---|---|---|---|---|---|
| $T_{target}$ (°C) | $pH_{target}$ (NBS) | SAL (psu) | T (°C) | pH (NBS) | DIC (μmol kg-SW$^{-1}$) | $pCO_2$ (μatm) | $CO_3^{2-}$ (μmol kg-SW$^{-1}$) | ΩCa | ΩAr |
| 18 | 8.1 | 34.3 | 18.4 | 8.15 | 3112 | 634 | 255 | 6.11 | 3.95 |
| | 7.8 | 34.1 | 18.5 | 7.76 | 3220 | 1696 | 110 | 2.63 | 1.70 |
| | 7.6 | 34.1 | 18.5 | 7.53 | 3449 | 3079 | 69 | 1.66 | 1.07 |
| 20 | 8.1 | 34.7 | 20.4 | 8.12 | 3020 | 693 | 249 | 5.97 | 3.88 |
| | 7.8 | 34.2 | 20.4 | 7.74 | 3166 | 1776 | 112 | 2.69 | 1.75 |
| | 7.6 | 34.2 | 20.6 | 7.51 | 3312 | 3173 | 70 | 1.68 | 1.09 |
| 22 | 8.1 | 34.7 | 22.3 | 8.07 | 2955 | 766 | 239 | 5.75 | 3.76 |
| | 7.8 | 34.3 | 22.4 | 7.72 | 3147 | 1892 | 116 | 2.79 | 1.82 |
| | 7.6 | 34.3 | 22.4 | 7.50 | 3262 | 3244 | 73 | 1.77 | 1.16 |

### 3.3.2 Mortality

Figure 3.12 show the mortality determined throughout the 2-months exposure to OA-W experimental scenarios. After 28 days of exposure, a significant effect of T ºC (*Pseudo-F*=5.1081, *p*=0.0164) and pH (*Pseudo-F*=5.6093, *p*=0.0123) on mortality was registered. Regarding T ºC, larvae exposed to warmer conditions (20 and 22 ºC) presented lower mortalities than larvae on control T ºC (18 *vs* 20 ºC: *t*-test=2.8645, *p*=0.0143; 18 *vs* 22 ºC: *t*-test=2.5952, *p*=0.0269). Moreover, a higher resilience of the larvae exposed to the most acidified treatment (pH 7.6) was observed, showing lower mortality than the exposed to pH 7.8 and 8.1 (7.6 *vs* 7.9: *t*-test=2.6692, *p*=0.0242; 7.6 *vs* 8.1: *t*-test=2.6658, *p*=0.0245). No further effects of any of the stressors involved in this experiment on mortality were registered from T28 onwards.

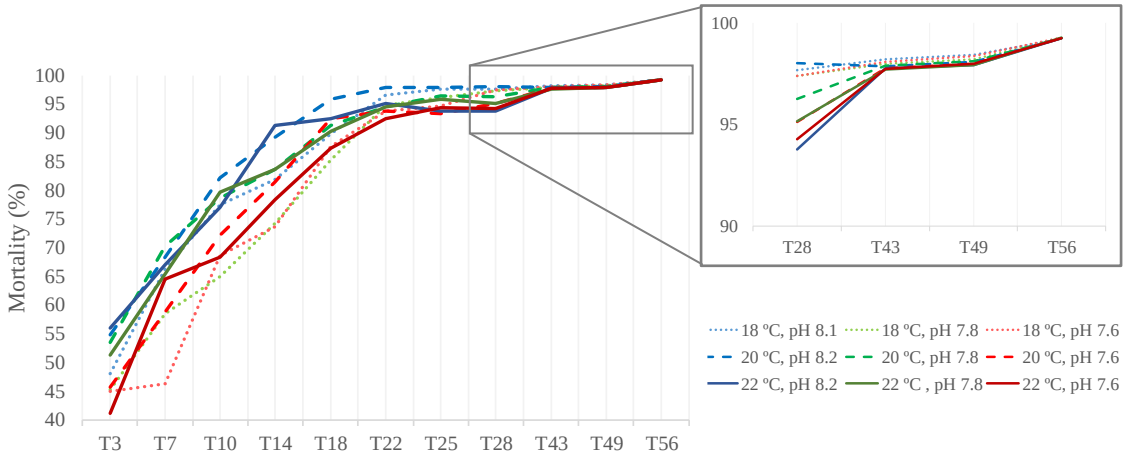


**Figure 3. 12:** Mortality of *V. corrugata* calculated from the mean survival per treatment throughout the experiment two times a week till T28 and once a week from T28 to T55 days of exposure to the 9 OA-W scenarios: 3 pH levels (8.1, 7.8 and 7.6) at 3 different temperatures (18, 20 and 22 °C).

### 3.3.3 Larval mobility

All the individuals observed at T14 were well nourished: 100% of the specimens observed presented microalgae inside the gut. Results on the larval behaviour analysis are showed on Figure 3.13, together with the classification of the development stage of the individuals analyzed.

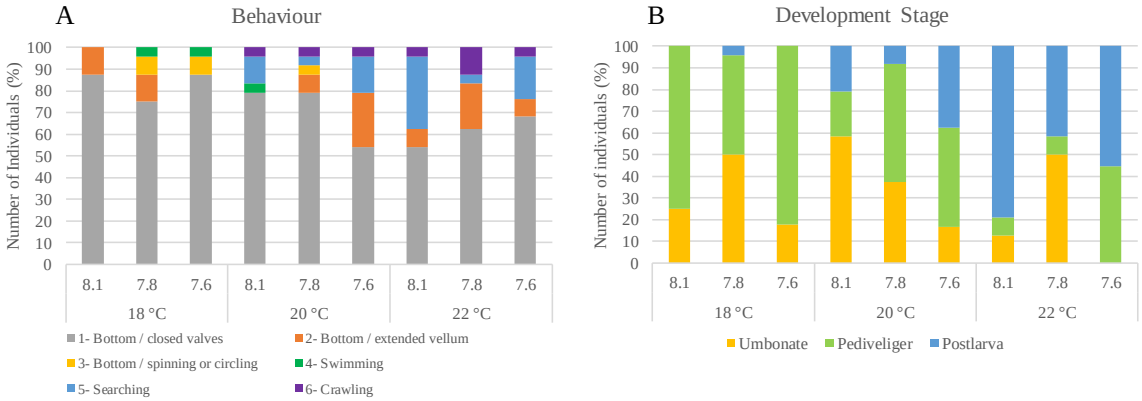


**Figure 3. 13:** *V. corrugata* larval behaviour. **A** – Results on the classification of the behaviour exhibited at T14, presented as the percentage of individuals exhibiting a particular behaviour (from 1 to 6) per treatment. **B** – Percentage of individuals on a given development stage (Umbonate, Pediveliger or Postlarvae) per treatment.

The acidified treatments were the only ones at which larva were spinning or circling at the bottom; however, the only significant effect on larval behaviour registered was of T ºC

(*Pseudo-F*=3.6914, *p*=0.0042), with warmer treatments (20 and 22 ºC) presenting a higher number of specimens "searching" and "crawling". Moreover, significant effect of the interaction between T ºC and pH (*Pseudo-F*=3.5485, *p*=0.0007) on the development stage was registered. The larvae reared at control pH (8.1) were significantly more developed under warming ($p<0.05$ at 20 and 22 ºC). However, at intermediate pH (7.8) a significant increase in larval development was only registered at the highest T ºC tested (18 *vs* 22 ºC: *t*-test=2.4166, *p*=0.0032; 20 *vs* 22 ºC: *t*-test=2.6200, *p*=0.0016) with a higher number of postlarva being observed (see Figure 3.13-B). Additionally, the larvae exposed to the most acidified condition (7.6) were less developed at the lowest, control T ºC (18 *vs* 20 ºC: *t*-test=2.6028, *p*=0.0035; 18 *vs* 22 ºC: *t*-test=1.0544, *p*=0.0003). Furthermore, differences between the development stage of larvae exposed to intermediate and more acidic media were registered at control T ºC (18 ºC): a higher number of postlarvae were recorded at pH 7.8 while at pH 7.6, despite the reduced number of umbonates and a higher number of pediveligers, no postlarvae were registered (7.6 *vs* 7.8: *t*-test=2.5663, *p*=0.0135). Additionally, at 20 ºC, development was significantly favored in the acidified treatments (8.1 *vs* 7.8: *t*-test=1.8593, *p*=0.0465; 8.1 *vs* 7.6: *t*-test=2.2543, *p(MC)*=0.0081) and the warmest treatment (22 ºC) induced some contradicting significant differences between different pH levels ($p<0.05$ for all comparations between pHs): a higher number of postlarvae was effectively registered at control pH but around 50% of the larvae analyzed from pH 7.8 were umbonates, while the majority at pH 7.6 were pediveligers.

### 3.3.4 Juveniles' mobility

The Table 3.3 show the characteristics of the sediment applied to analyze the behaviour of the juveniles exposed to OA-W scenarios for 2 months and in Table 3.4 are summarized the initial conditions to which the animals were exposed at T57 and the final, at the end of the trial, at T58.

**Table 3. 3:** Characteristics of the sand used to the "Sediment mobility test"performed at T57. The mean values (AVR) and respective standard deviation (SD) of their relative proportions of water, organic matter and each fraction of the sediment are expressed as a percentage of their mass.

| Water content | | Organic content | | Granulometric composition | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | <125 µm | | 125-250 µm | | 250-500 µm | | > 500 µm | |
| AVR (%) | SD | AVR (%) | SD | AVR (%) | SD | AVR (%) | SD | AVR (%) | SD | AVR (%) | SD |
| 10.62 | 0.72 | 0.24 | 0.02 | 0.18 | 0.02 | 11.56 | 0.36 | 87.50 | 0.72 | 0.17 | 0.04 |

**Table 3. 4:** Mean values and respective standard deviation of probe measurements of pH (NBS scale), temperature (ºC) and salinity (SAL) taken immediately prior to the beginning (T0) and at the end (T24 h) of the trial. At T0, pH was measured on saltwater ($pH_{water}$) (used to fill the respective crystallizers) and also on the sediment layer ($pH_{sed}$). At T24h, pH was measured at the interface between the sediment and the exposure medium ($pH_{int}$) in all replicates.

| Treatment | | Probe measurements | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | T0 | | | | T24 h | | | | | |
| $T_{target}$ (°C) | $pH_{target}$ (NBS) | $pH_{water}$ (NBS) | $pH_{sed}$ (NBS) | T (°C) | SAL (psu) | $pH_{int}$ (NBS) | | T (°C) | | SAL (psu) | |
| | | | | | | AVR | SD | AVR | SD | AVR | SD |
| 18 | 8.1 | 8.12 | 7.40 | 22.6 | 33.3 | 7.27 | 0.18 | 19.0 | 0.1 | 33.3 | 0.2 |
| | 7.8 | 7.77 | 6.95 | 21.9 | 33.2 | 7.19 | 0.11 | 18.8 | 0.5 | 33.1 | 0.2 |
| | 7.6 | 7.59 | 7.03 | 22.8 | 33.2 | 7.34 | 0.15 | 18.5 | 0.5 | 33.1 | 0.2 |
| 20 | 8.1 | 8.11 | 7.45 | 23.1 | 33.3 | 7.26 | 0.14 | 21.5 | 0.1 | 33.9 | 0.1 |
| | 7.8 | 7.76 | 7.24 | 22.0 | 33.2 | 7.01 | 0.07 | 21.6 | 0.1 | 33.5 | 0.1 |
| | 7.6 | 7.41 | 6.99 | 23.0 | 33.1 | 7.11 | 0.15 | 21.4 | 0.3 | 33.5 | 0.1 |
| 22 | 8.1 | 8.11 | 7.26 | 22.3 | 33.3 | 7.33 | 0.18 | 23.6 | 0.5 | 33.6 | 0.2 |
| | 7.8 | 7.82 | 7.02 | 21.7 | 33.2 | 7.00 | 0.18 | 23.7 | 0.8 | 33.6 | 0.2 |
| | 7.6 | 7.46 | 7.08 | 22.5 | 33.2 | 7.03 | 0.17 | 23.5 | 0.8 | 33.6 | 0.2 |

Despite the previous incubation of the sediment in $CO_2$-equilibrated saltwater, and as expected, the physico-chemical characteristics of the exposure medium (at its interface with the sediment) to which the specimens were exposed to test juvenile behavioural responses were not exactly correspondent to the OA-W scenarios under which animals were reared for 2 months. In addition, all the sediments were more acidic than the exposure medium at the beginning of the trial. However, natural surface sediments in which this species' juveniles live buried, namely in Ria de Aveiro, have pH conditions similar to lower to the showed in Table 3.2 (see Cunha et al. (2005)). Accordingly, the sediment mobility test should be taken as a test to assess if the burial and crawling capacities of the juveniles were affected by the

larval development in a water column with physico-chemical characteristics of the OA-W scenarios tested.

***Burial activity***

Burial capacity varied over time and is shown in Figure 3.14. After 15 min (Figure 3.14-A), a significant effect of T ºC occurred (*Pseudo-F*=5.2088, *p*=0.0096) with >90% of the clams buried at 22 ºC.

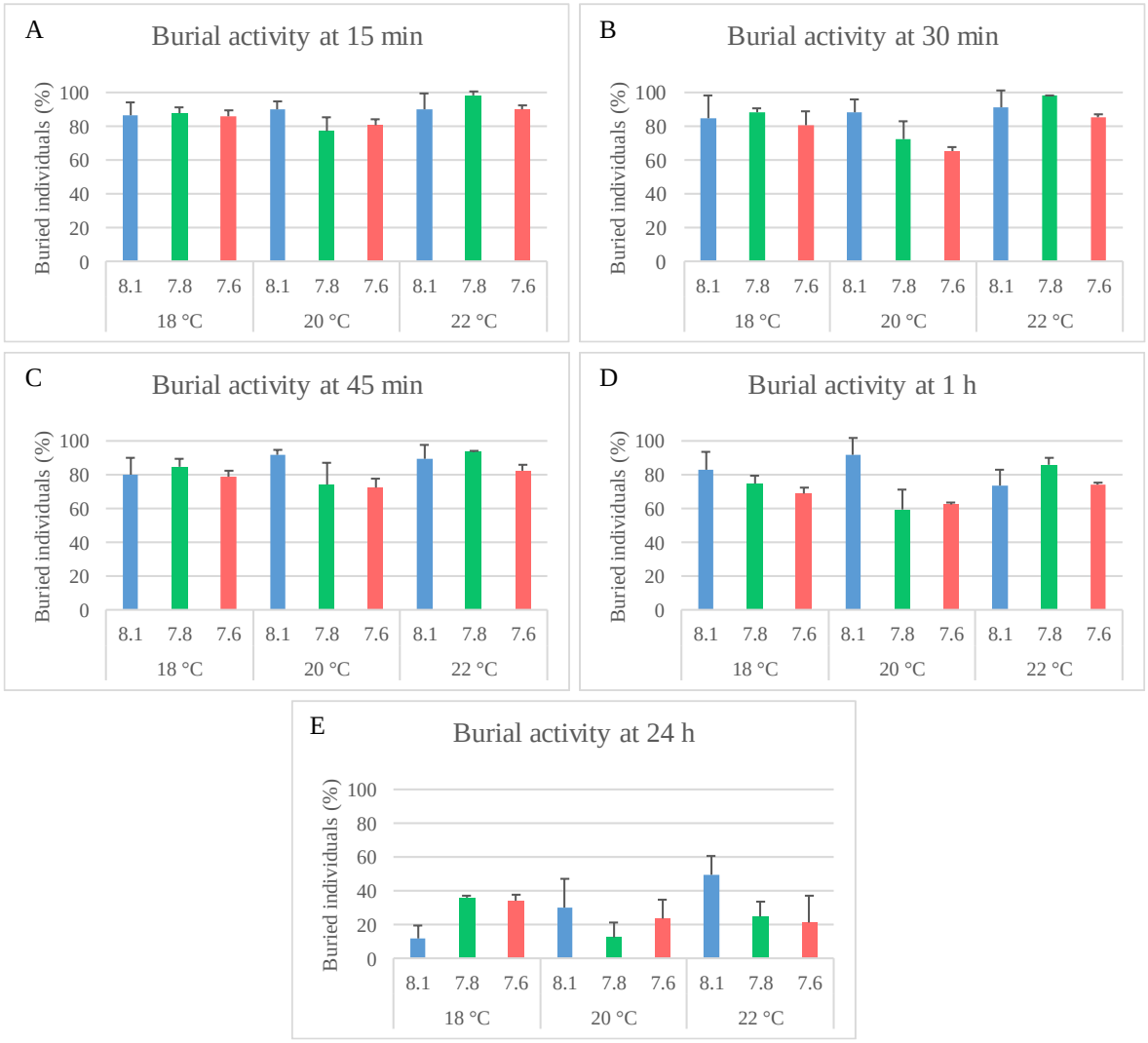


**Figure 3. 14:** *V. corrugata* burial activity expressed as the percentage of buried individuals per treatment at the different timepoints: **A** – after 15 min; **B** – after 30 min; **C** – after 45 min; **D** – after 1 h; **E** – after 24 h.

After 30 min (Figure 3.14-B), beyond the effect of T ºC (*Pseudo-F*=12.968, *p*=0.0002) a significant effect of pH was also registered (*Pseudo-F*=7.025, *p*=0.0024). The percentage

of animals buried increased significantly with T ºC but, contrarily, clams reared under extreme acidity (pH 7.6) were buried in a lower percentage (8.1 *vs* 7.6: *t*-test=3.3736, *p*=0.0024; 7.8 *vs* 7.6: *t*-test=3.1951, *p*=0.0039).

The separate effect of T ºC and pH continued to be observed after 45 min (Figure 3.14-C), with the warmer treatment (22 ºC) presenting a significant higher number of buried clams, as well as the pH control in which a higher burial was registered when compared to the animals reared under the most acidic condition (8.1 *vs* 7.6: *t*-test=2.3978, *p*=0.0225).

After 1 h (Figure 3.14-D), an interaction of both stressors was verified (*Pseudo-F*=5.1653, *p*=0.0011). At control pH, the clams exposed to 20 ºC showed a significantly higher burial capacity when compared to the exposed to 22 ºC (20 *vs* 22 ºC: *t*-test=2.8239, *p*=0.0496). On the opposite, those reared at pH 7.8 revealed lower burial activity at 20 ºC than the clams at 18 ºC and 22 ºC (18 *vs* 20 ºC: *t*-test=2.6204, *p*=0.0325; 20 *vs* 22 ºC: *t*-test=3.7454, *p*=0.0071). The clams reared under the most acidic condition (pH 7.6) did not show differences between temperatures. On the other hand, despite no differences were observed between different acidification levels at the control T ºC (*p*>0.05), the clams exposed to warmer conditions revealed significant differences between the three pH levels. The burial activity at 20 ºC was significantly higher in animals reared under pH control than in those grown under pH 7.8 (8.1 *vs* 7.8: *t*-test=3.6327, *p*=0.011). Furthermore, the burial capacity of the juveniles reared under pH 7.6 at 22 ºC was significantly affected since fewer animals were buried when compared to the reared under pH 7.8 at the same T ºC (7.8 *vs* 7.6: *t*-test=2.4551, *p*=0.039).

In general, after 24 h, the number of animals buried in most treatments decreased, and an interactive effect of T ºC and pH was evident (*Pseudo-F*=3.1086, p=0.0247). Regarding pH, significant differences occurred at 18 ºC with burial activity being higher on clams reared under acidity (8.1 *vs* 7.8: *t*-test=2.4725, *p*(MC)=0.0451; 8.1 *vs* 7.6: *t*-test=2.4962, *p*(MC)=0.0365). No differences were found on the other temperatures. At control pH, the hottest treatment (22 ºC) showed a highest number of buried clams (18 *vs* 22 ºC: *t*-test=3.2947, *p*=0.0234). Contrarily, the specimens reared under pH 7.8 revealed a significant higher burial activity at control T ºC than at 20 ºC (18 *vs* 20 ºC: *t*-test=3.196, *p*=0.0111).

***Crawling activity***

Results on the crawling assessment performed after 24 h from the beginning of the sediment mobility test are shown in Figure 3.15. Significant differences between replicates of a same treatment were achieved (*Pseudo-F*=5.0038, *p*=0.004), an indication of the high variance within groups, and so of the high variability of the response measured as shown on Figure 3.15. Nevertheless, the results of statistical analyses has showed that neither temperature, pH or both has been affect the crawling activity (p>0.05). The fact that crawling distance measured as the percentage of specimens in the center *vs* in the margin is indeed highly variable, with no pattern between treatments, points towards the possible inadequacy of the method used to measure the crawling activity, generating inconclusive results.

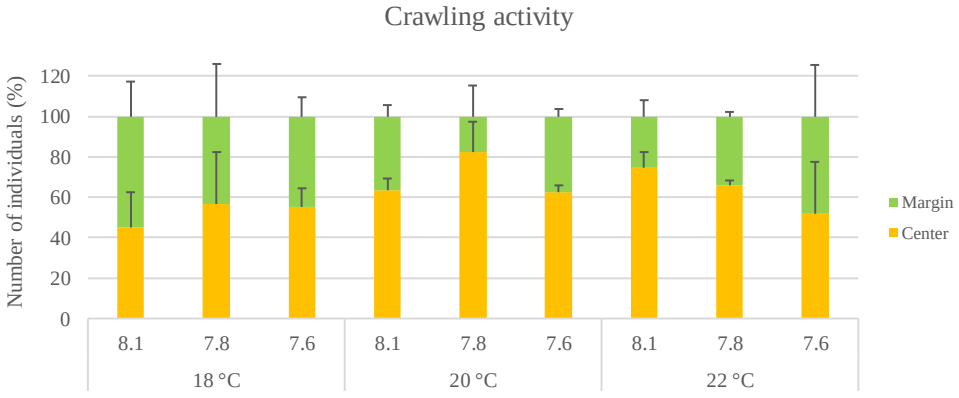


**Figure 3. 15:** *V. corrugata* crawling activity expressed as the percentage of clams accounted after 24 h from the beginning of the trial on the 40 mm "Center" and on the "Margin" of the experimental container (i.e., crystallizer).

***Byssus production***

The percentage of clams presenting byssus threads after 24 h from the beginning of the sediment mobility test is showed in Figure 3.16. A significant effect of pH was detected (*Pseudo-F*=4.603, *p*=0.0173). Clams reared under acidity (pH 7.8 and 7.6) revealed significantly reduced byssus production (8.1 *vs* 7.8: *t*-test=2.4509, *p*=0.0219; 8.1 *vs* 7.6: *t*-test=3.0878, *p*(MC)=0.0061).

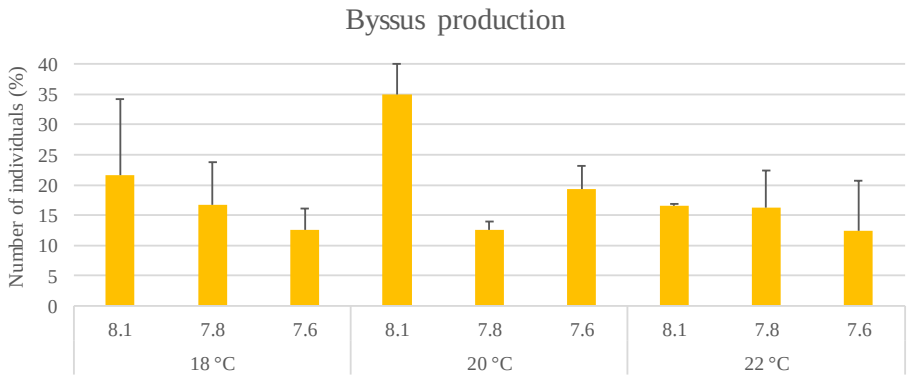


**Figure 3. 16:** *V. corrugata* byssus production expressed as the percentage of clams with byssus threads after 24 h from the beginning of the trial indicated per treatment.

## 3.4 Discussion

The analysis of the survival and the behaviour of *V. corrugata* throughout the early ontogenic development under the generated OA-W scenarios revealed interesting results that point towards the species resilience, somehow unexpected.

Going deeper into the chemistry of the medium to which animals were exposed, it is important to refer that it was never undersatured as regards both calcite and aragonite, even in the most acidified treatment – pH 7.6. Carbonate saturation (i.e., $\Omega>1$ and $\Omega = [Ca^{2+}] \times [CO_3^{2-}]$ ) / $[CaCO_3]$) is fundamental for calcification, a process of upmost importance in shelled molluscs which survival depend, among other things, on their protective and supportive carbonate shells. There are many evidences that OA promote carbonate undersaturation, affecting calcification (Kurihara et al. 2008, Dickinson et al. 2012, Thiyagarajan & Ko 2012) and, consequently, key processes such the predatory defense, swimming, feeding, among others that are crucial for survival (Chan et al. 2011, Gazeau et al. 2013). For instance, negative impacts of OA on oysters calcification are widely described (e.g., Ko et al. 2014) but there are also evidences of severe effects on fertilization, hatching, larval growth and survival, even under high alkalinity and calcite and aragonite saturation (Barros et al. 2013). Moreover, our pH target levels were achieved and higher concentrations of $CO_2$ and $H^+$ were available in the exposure media in the acidified scenarios. After all, carbonate saturation was reduced in our acidified conditions, and very close to 1 regarding aragonite (see Table 3.2) in the most acidic treatments (target pH 7.6). Hence, even knowing that carbonate saturation was not achieved, our experimental treatments can effectively be

regarded as potential OA-W scenarios projected for the near to the long-term future. In this sense, the results here obtained might be looked at as a picture of the combined effects of OA-W on the survival and the behaviour of *V. corrugata* in early life.

### 3.4.1 Larval resilience under future OA-W

The high mortality until settlement (>95% at T28) was expected since, as in many marine bottom invertebrates, *V. corrugata* reproductive strategy include the so called 'epidemic spawning' (Thorson 1950) that results in large numbers of planktotrophic larvae which suffer high mortality during their stay in the water column (e.g. Cerviño-Otero (2011) and Joaquim et al. (2014)). Moreover, all the handling for the constant medium exchanges and cleaning of the very small tanks used in this experiment (1.6 L) may have caused higher animal losses than it normally occurs, for instance, in a hatchery environment. Even so, a significant effect of both stressors under study on larval mortality after 28 days of exposure to OA-W scenarios was proved, without its simultaneous occurrence having a more relevant effect (no significant interaction).

Temperature is known to be one of the main factors that influence the metabolic rate, promoting the development until thermal limits are reached (Pörtner 2008, Byrne 2011). In this work, the tested T ºC of 20 and 22 ºC should be within the thermal range of *V. corrugata* larvae that, consequently, developed more faster, shortening the planktonic phase associated to higher mortalities (Cerviño-Otero 2011). Likewise, larvae exposed to more acidic conditions (pH 7.6) suffered lower mortalities until T28. Similar results were obtained by Thiyagarajan and Ko (2012) with *Crassostrea angulata* (Lamarck, 1819) larvae, looked at as unexpected since OA has been described as a promotor of higher mortalities and slower development in marine invertebrates (Kurihara et al. 2008, Gazeau et al. 2010, 2013, Kroeker et al. 2013). However, we speculate that changes in the microbiome might be involved in this increased survival under acidity, as suggested by other authors (O'Brien et al. 2016, Kandler et al. 2018); even so, this hypothesis needs to be confirmed by further studies. Yet, larvae resilience to lower pH can also be achieved if breeders are grown under a similar environment. An example of that is the study performed by Thomsen et al. (2017) in which veligers of *Mytilus edulis* (Linnaeus, 1758) born from adult mussels from the Baltic Sea (known to have actual lower pH and high $pCO_2$ values during the species spawning

season) revealed higher adaptation to OA conditions than veligers obtained from North Sea breeders (under more stable pH and higher alkalinity) that present slower development, lower settlement and survival rates. In the current study, the broodstock comes from the Ria de Aveiro, a typical estuarine system in the NW Portuguese coast. Estuaries are usually associated to a higher variability of pH, and many can reach extreme ranges like 7.0 to 8.7, depending on the distance to the sea, and on the geographical location (Ringwood & Keppler 2002, Velez et al. 2014). In Ria de Aveiro, the pH of the sediments' surface layer where this species adults can be found might experience pH values from 6.6 to 6.9 (Cunha et al. 2005), and also vary regarding other abiotic factors, such as salinity and temperature. Hence, it is also plausible that the broodstock may have been exposed to lower pH's being, somehow, more adapted, giving rise to larvae more resistant, resilient to the tested OA-W scenarios, supporting the improved survival registered during the planktonic phase. These results can also be indicative that the *V. corrugata* populations from Ria de Aveiro may be useful as preferred breeders to avoid or reduce negative impacts of future OA-W like lower development and higher mortality rates (Parker et al. 2012).

In addition, another unexpected result was that neither temperature nor pH were proved to affect larval feeding. Contrarily to some studies demonstrating that larvae exposed to OA conditions reveal reduced fitness due to impairment of the ciliary system inducing a decrease in the food intake ability (Kurihara et al. 2007, Gazeau et al. 2013), 100% of the larvae analyzed at T14 seemed to be well fed (or were, at least, fed). Even so, it cannot be ruled out the possibility that the filtration rate was reduced, at least in some of them, since our assessment was only qualitative (presence vs. absence) and once effects of acidification on larval mobility after 14 days of exposure were registered.

Larvae circling at the bottom were only observed in the acidified treatments, which could be indicative of some swimming instability under reduced pH. Impacts of OA on larval swimming were already reported in Chapter 2 for the gastropod *Tritia reticulata*, and have been described by other authors for other species (e.g. Ellis et al. (2009)), being attributed to shells' abnormal calcification and damage of structures involved on that behaviour like the velum, under increased $pCO_2$ (Kurihara 2008, Wheeler et al. 2015). However, the only significant effect on *V. corrugata* larval mobility is that of the T ºC (i.e., the effect of pH on larval behaviour at T14 is not significant). The fact that a high frequency of animals "at the

bottom with the valves closed" was recorded (see Figure 3.13-A), a behaviour associated with all the development stages, and well represented among all treatments, somehow justify the statistical result of a significance of the single, stronger factor (T ºC). This strong effect is definitely related with the natural change in behaviour throughout the different development stages assumed in planktonic life culminating in settlement (Quayle 1952, Carriker 2001) that occurred sooner in warmer treatments (as a higher percentage of postlarvae were registered at 22 ºC). This is the reason why "crawling" and "searching" were more frequent under warming. These are important behaviours on the search for a favorable subtract to settle and initiate the benthic life (Carriker 2001). Therefore, since more developed specimens were registered under warming, a significant effect of the T ºC was detected.

Nevertheless, and looking for the development itself, a significant effect of the interaction between both stressors under study was registered: development was notably accelerated by warming but relatively delayed under acidity. Moreover, this effect of the acidification was attenuated by the concomitant rise in T ºC, specially at the hottest condition (22 ºC) in which animals were more developed even under extreme acidity (pH 7.6). Thus, in this work we prove that OA-W have antagonistic effects on *V. corrugata* development in early life. Even so, these results emphasize the possible resilience of *V. corrugata* to future OA-W since the simultaneous occurrence of warming somehow reduces the negative effects of acidification alone (under control T ºC), contrarily to what is described by other authors (Byrne 2011, Kroeker et al. 2013). At a first glance, a faster development (and its associated behaviours) is beneficial for the species survival since promotes an earlier metamorphosis and settlement to start the benthic life, reducing the planktonic phase, a period of high fragility and associated mortality (Parker et al. 2013). Moreover, this faster development is also very welcome in a hatchery scenario, reducing the costs associated with seed production.

### 3.4.2 Potential fragilities of the early benthic life under acidity

After revealing an apparent resilience to OA-W projected scenarios during the larval phase, settled juveniles were placed in the presence of sediment 60 days after fertilization to assess their burial and crawling performance, in a situation closer to the available in their natural habitat. A "Sediment mobility test" was developed for that purpose. It proved to be useful to

study the burial ability, particularly in the first hour from the beginning of the trial (since the media conditions were far from the target of our OA-W experimental scenarios after 24 h; see Table 3.4), but was inadequate for the assessment of the crawling activity, rendering inconclusive results. For this reason, here we will only discuss the results on the burial ability and byssus production, after some considerations on the test itself.

It is known that bivalves' burial rate is affected by the sediment grain size (de la Huz et al. 2002, Sassa et al. 2011). Thus, in order to avoid an impact on the burial performance, *V. corrugata* early juveniles were exposed to fine and medium sand collected at Ria de Aveiro, from where broodstock was obtained. In addition, in an attempt to standardize the size of the juveniles, which was extremely variable after 2 months of development under OA-W conditions, the criterion was the random selection of the animals among the largest obtained after calibration with pre-determined mesh screens. After the discussed above on the fast development under warming and reduced under acidity, it is obvious that it was not possible to apply the same mesh to all treatments. However, the more adequate were chosen in order to render sufficient number of animals for the trial. In the most acidic treatment at 18 ºC, the clams were reportedly smaller and had to be collected in a smaller sieve (1.0 mm-mesh) than all the other treatments. In its turn, clams reared at 22 ºC were much larger, reason why where collected in a larger mesh (1.7 mm).

The first fact is that a higher number of buried clams were observed at 22 ºC after 15 min from the beginning of the trial and this was, somehow, expected since these juveniles were larger and size is crucial for the burial capacity (Lardies et al. 2001). Many studies have shown a positive relation between the locomotor performance and the increase in temperature: Block et al. (2013) have shown that T ºC stimulates the extension of the foot in the freshwater mussel *Potamilus alatus* (Say, 1817), and its muscles' contraction were directly linked to searching and burial behaviours. Moreover, temperature is known to control many physiological and biochemical processes, increasing the metabolism and the activity of invertebrates, which promotes the ability to bury at greater depths as shown by Przeslawski et al. (2009). However, these results are related to the species thermal limits that, once reached/exceeded, have negative impacts on the burial performance (Archambault et al. 2013, Block et al. 2013). So, the results here presented demonstrate that the 22 ºC is

within the thermal limits of our model, meaning that *V. corrugata* could experience better burial performance being reared under warming conditions.

Nevertheless, although T ºC continues to have a significant (positive) effect on the burial activity after 30 and 45 min from the beginning of the trial, the clams reared under acidity showed a significant reduced burial performance. For now, we can only speculate that this can be related with the clams reduced growth, and so size, under acidity. However, without further studies, we cannot rule out the possibility of any damage on the foot as described in Chapter 2 for the gastropod *Tritia reticulata*, since this organ is essential for the bivalve "dual-anchor system": the alternating repetitive motion of the foot (that pulls the shell down into the sediment) and of the shell (that stays fixed in the sediment while the foot extends further down) that allows the animal to bury (Carriker 2001, Dorgan 2015). Another hypothesis for a decrease on the burial performance under acidity is a reduction on the metabolism and on the activity of $Ca^{2+}/Mg^{2+}$-ATPase as shown for other bivalves (Clements and Hunt 2014; Peng et al. 2017).

Even though an interaction between the stressors under study was significant after 1 and 24 h from the beginning of the trial, no evident pattern is perceptible, and results are difficult to explain. However, a general reduction of the number of buried clams was observed, being quite evident at the end of the trial. As no food was supplied we suppose that buried individuals may have emerged from the sediment in the search for food (Lardies et al. 2001). Although this seems plausible, we cannot ignore the fact that the pH of the exposure medium was very far from the target levels at the end of the trial: it was much more acid and in all treatments. Hence, as those were not the conditions to be tested and had certainly impacted juveniles behavioural response, we should not consider these results as unscathed and a new way of assessing the burial activity after 24 h of exposure should be considered.

Nevertheless, although it was evaluated in individuals exposed to those test conditions for 24 h, a consistent effect of the acidification on the byssus production was registered. It is known that byssus production is influenced by some abiotic factors such as temperature and water flow (Selin & Vekhova 2004, Alfaro 2006). However, contrarily to the results of Dickey et al. (2018), this study demonstrates that, regardless of temperature, acidity induce a reduction of the number of *V. corrugata* juveniles producing byssus. The byssus threads

are formed by byssal fluid that passes on byssal glands on bivalves' foot (Carriker 2001). This fluid consists mainly on proteins that confer the elasticity and robustness to the byssal thread, crucial to the adhesion to the subtract, being also involve in the dispersal behaviour (Sigurdsson et al. 1976, Waite 1992). There were already evidences that OA affects the byssus of bivalves, creating weaker and less elastic byssus, mainly due to the decrease of pH, that is directly related to the function of DOPA (O'Donnell et al. 2013) – an amino acid crucial for the adhesion to the substratum (Waite 1992, Wei et al. 2013). Despite Zhao et al. (2017) had demonstrated that the mussel *Mytilus coruscus* (Gould, 1861) revealed up-regulation of some byssal proteins as a possible adaptation to OA, the most specific byssal proteins were down regulated, resulting on less resistant byssus as well as on the reduction of the number of byssus threads. Hence, despite the apparent readiness of *V. corrugata* juveniles to burry after being reared for 60 days under warming, smaller animals reared under acidity might be less able (because of their reduced size or of any potential damage on their foot), or less available (due to potential metabolic compromise), to bury. In addition, acidification might also affect byssus production compromising juveniles' dispersal and the adhesion to the subtract.

Despite these potential fragilities of the early juvenile life under projected acidification scenarios, survival was proved to be favored and larval development notably accelerated under warming, a process that might attenuate the negative effects of the acidity if occurring simultaneously. Hence, our results reveal the resilience of the pullet carpet shell *V. corrugata* to future OA-W and point towards its productive potential if the tested scenarios, as predicted, become effective.

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# Chapter 4- Main conclusions and Final remarks

Marine shelled molluscs are of high ecological and economic importance and are being affected by the ongoing change of the World climate that is inducing profound modifications of their habitat chemical and physical properties, namely its *Acidification* and *Warming*.

This study came to contribute to a gap of knowledge identified by some authors regarding the interactive effects of the environmental stressors involved on those simultaneous phenomena – pH and temperature – on the behaviour of early life stages of gastropods and bivalves, using *Tritia reticulata* and *Venerupis corrugata* as models. Although their sensitivities cannot be directly compared, since the experimental conditions to which both species were exposed were not exactly the same, the results presented in this thesis are unequivocal, showing different effects of *Ocean acidification* and *Warming* (OA-W) will possibly have on these species if the Intergovernmental Panel on Climate Change (IPCC) predictions become effective.

The netted whelk, *T. reticulata* will suffer increased mortalities and altered swimming patterns under the OA-W scenarios projected for the end of the century. Concurrent OA-W was proved to induce increased larval activity, while reducing the speed and the distance travelled by the veligers, which may impact larval feeding, dispersal and also increase the vulnerability to predation. Also, a clear histological damage of the foot surface under OA-W was detected, with potential functional implications: as the foot is important not only for the larvae locomotor ability, future juvenile locomotion can also be compromised as its epithelium corresponds to the future crawling surface. Hence, not only larval feeding and dispersal might be affected but also the ability of searching for a favorable settlement site. Together with increased mortalities, these potential impacts might result in harmful consequences for the early life of this species under the future OA-W, specifically on the veligers' competence, pointing towards the eminent threat that these phenomena constitute to its survival if the projections for its occurrence by the IPCC become effective.

On the other hand, the exposure of the pullet carpet shell, *V. corrugata* to OA-W projected scenarios rendered completely different results, pointing to a relative larval resilience in the case of a simultaneous occurrence of the phenomena under study. At first larval mortalities were lower under OA-W. In addition, development was accelerated under warming and, although it was relatively delayed under acidity, an antagonistic effect of both stressors was evident and the concomitant increase in the temperature was able to attenuate

the negative effects of the reduced pH. Moreover, larval development was faster under warming and individuals' behaviour was proved to follow development at least until settlement. However, some fragilities of the juvenile life under acidity are also reported, namely a reduced capacity to bury and to produce byssus, which might compromise the survival of juvenile clams in both their natural habitat and also in a nursery, under aquaculture environment. Nevertheless, as OA-W are predicted to occur simultaneously and temperature rise was proved to have an antagonistic effect of that of the pH decrease, it is encouraging that the development of this species of high ecological and economic value might be guaranteed under future OA-W. Clams reared under higher temperatures, even under some acidity, were proved to grow better and faster, and have also demonstrated an increased ability to bury and to produce byssus.

In short, both experiments here reported prove that projected OA-W will affect the survival and the behaviour of both species used as models, although differently at different stages of their life cycle. This is, to our best knowledge, the first report of the effects of projected OA-W scenarios on these species' early life stages survival and behaviour. While the gastropod revealed a high vulnerability, the bivalve showed resilience, clearly demonstrating the need for further studies, in different species that, even close in terms of the biological hierarchy, have specificities that determine their future in a changing ocean.