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Role of environmental variability on the response of Mediterranean corals to global change

Chloé Carbonne

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Sorbonne Université

École doctorale des Sciences et de l'Environnement d'Ile de France (ED 129)

Laboratoire d'Océanographie de Villefranche UMR 7093

**Role of environmental variability on the response of
Mediterranean corals to global change****Par Chloé Carbonne**

Thèse de doctorat en Biologie et Ecologie Marine

Dirigée par Jean-Pierre GATTUSO (Directeur),
Nuria TEIXIDO (Co-directrice)
et Steeve COMEAU (Co-encadrant)Présentée et soutenue publiquement le 21 octobre 2021
au Laboratoire d'Océanographie de Villefranche

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Nine-month old recruits of *Astroides calycularis*

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- Carbonne, C., Comeau, S., Chan, P., Plichon, K., Gattuso, J-P., Teixidó, N. (2022) Early life stages of a Mediterranean coral are vulnerable to ocean warming and acidification. *EGUsphere*, 1-16. doi: 10.5194/egusphere-2022-240 (Biogeosciences, in press)
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- Carbonne, C., Meynadier, M., Comeau, S., Copley, R.R., Ramon-Mateu, J., Plichon, K., Gattuso, J-P., Teixidó, N. (*in prep.*) Parental exposure to natural CO₂ vents alters the response to low pH of a temperate coral early life stages
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- Carbonne C., Comeau S., Plichon K., Gattuso J-P., Teixidó N., Physiological response of two temperate scleractinian corals to ocean warming and heatwaves. ASLO 2022 Ocean Sciences Meeting : Session IS01 Legacy effects of marine heatwaves on coral reef ecosystems 02, 24 February- 4 March 2022. Talk presentation.
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- Carbonne C., Comeau S. , Moore B., Plichon K., Mirasole A., Gutierrez T., Gattuso J-P., Teixidó N., Early life stages of Mediterranean corals are more sensitive to ocean acidification than adults, The 5th Symposium on the Ocean in a High-CO₂ World 2022 : Session Insight from natural Ocean Acidification analogs, CO₂ vents, 13-16 September 2022, Lima, Peru, Talk presentation

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General Introduction

I will first introduce key concepts on environmental changes in the ocean and on corals, before listing the objectives of this work and introducing the experimental work performed.

Global ocean change

Anthropogenic emissions of greenhouse gasses such as carbon dioxide (CO₂) have been increasing since the beginning of the Industrial Revolution, mainly as a result of fossil fuel burning. Atmospheric CO₂ concentration increased from 277 parts per million (ppm) in 1750 (Joos and Spahni 2008) to 416 ppm nowadays (Archive 2022-06-01 updated from Keeling and Keeling 2017). Future projections were modeled by the Climate Model Intercomparison Projects (CMIP). CMIP5 (Taylor *et al.* 2011) in the Fifth Assessment Report (AR5) of the Intergovernmental Panel on Climate Change (IPCC) was based on Representative Concentration Pathways (RCPs) which are atmospheric concentration trajectories as baseline scenarios. RCP2.6 (stringent mitigation), RCP4.5 (intermediate), RCP6.0 (intermediate) and RCP8.5 (very high emissions) are labeled after a range of radiative forcing values at the end of the century (2.6, 4.5, 6, and 8.5 W/m², respectively). The new CMIP6 (O'Neill *et al.* 2016) of IPCC AR6 differs from CMIP5 by being based on the Shared Socio-economic Pathways, which provide revised emission scenarios through socio-economic trends compared to the RCPs (Kwiatkowski *et al.* 2020)¹. The concentration of CO₂ in the atmosphere under these scenarios is projected to increase to ~ 600 ppm under SSP2-4.5 and RCP4.5 to ~ 1000 ppm under RCP8.5 and ~ 1150 ppm under SSP5-8.5 at the end of the century, depending on anthropogenic CO₂ emissions. Partial pressure of CO₂ (*p*CO₂) at the ocean surface tends to equilibrate with the partial pressure of the atmosphere, thus CO₂ dissolves in the ocean when atmospheric CO₂ increases, making the ocean a sink for atmospheric CO₂. For the decade 2011-2020, the input of CO₂ to the atmosphere by emissions from human activities, was partitioned among the atmosphere by 48%, the land by 29% and the ocean by 26 %, and unattributed budget imbalance of 3% (Friedlingstein *et al.* 2022).

¹ The RCPs are used in this thesis as the new SSP scenarios were not released at the beginning of the experiments

The increase of atmospheric CO₂ is driving changes in the ocean’s physical and chemical properties, with important consequences for its ecosystems and the services they provide to humans (Gattuso *et al.* 2015). The ocean is a critical component of Earth’s climate system, acting as a climate regulator by (1) absorbing Earth’s additional heat while keeping the atmosphere cooler; and (2) capturing anthropogenic CO₂ emissions, leading to ocean acidification (OA).

Ocean warming and marine heatwaves

Rising concentrations of greenhouse gases in the atmosphere, such as CO₂, warms the Earth surface (Hegerl and Bindoff 2005) but about 90% of this heat accumulates in the ocean, leading to ocean warming (Cheng *et al.* 2022). Between 1993-2003, the ocean heat content increased by $8.11 \cdot 10^{22}$ J, corresponding to a warming of 0.1°C per decade (Bindoff *et al.* 2007). Projections under the RCP8.5, report a potential increase of 3.04°C of sea surface temperature by the end of 2100 (Kwiatkowski *et al.* 2020, Figure 0.1). The new scenario SSP5-8.5, more pessimistic than the RCP8.5, projects an increase of sea surface temperature by 3.47°C at the end of the century (Kwiatkowski *et al.* 2020, Figure 0.1).

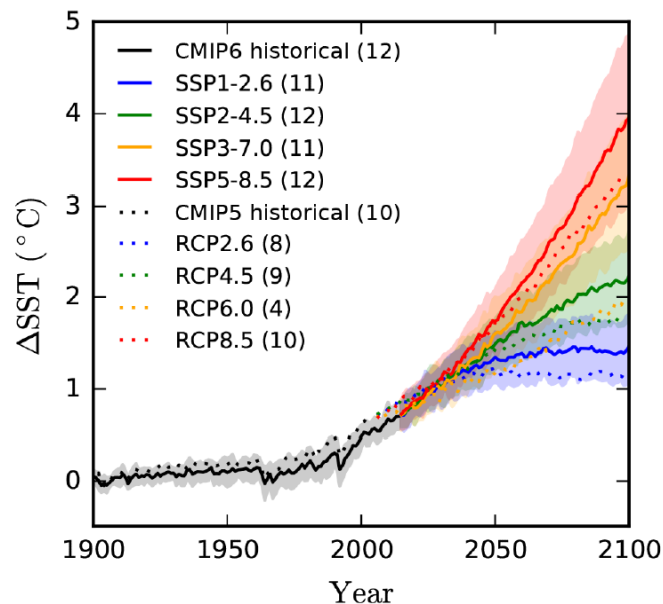


Figure 0.1 : Global average surface temperature change for the 1900–2100 period relative to 1870-1899 from CMIP5 and CMIP6 concentration-driven experiments. CMIP6 mean anomalies for the historical and SSP simulations are shown as solid lines with shading representing the inter-model SD. CMIP5 projections are shown as dashed lines. The model ensemble size for each scenario is given in parentheses. Figure adapted from Figure 1 Kwiatkowski *et al.* 2020).

In addition to the long-term and gradual warming of the ocean, there has been a significant rise in the frequency and intensity of extreme climatic events such as marine heatwaves (MHWs, Oliver *et al.* 2021). These exceptional warm water events are defined as seawater temperature exceeding a threshold, usually defined as the 90th percentile of the climatological mean, for at least five consecutive days (Hobday *et al.* 2016, Figure 0.2).

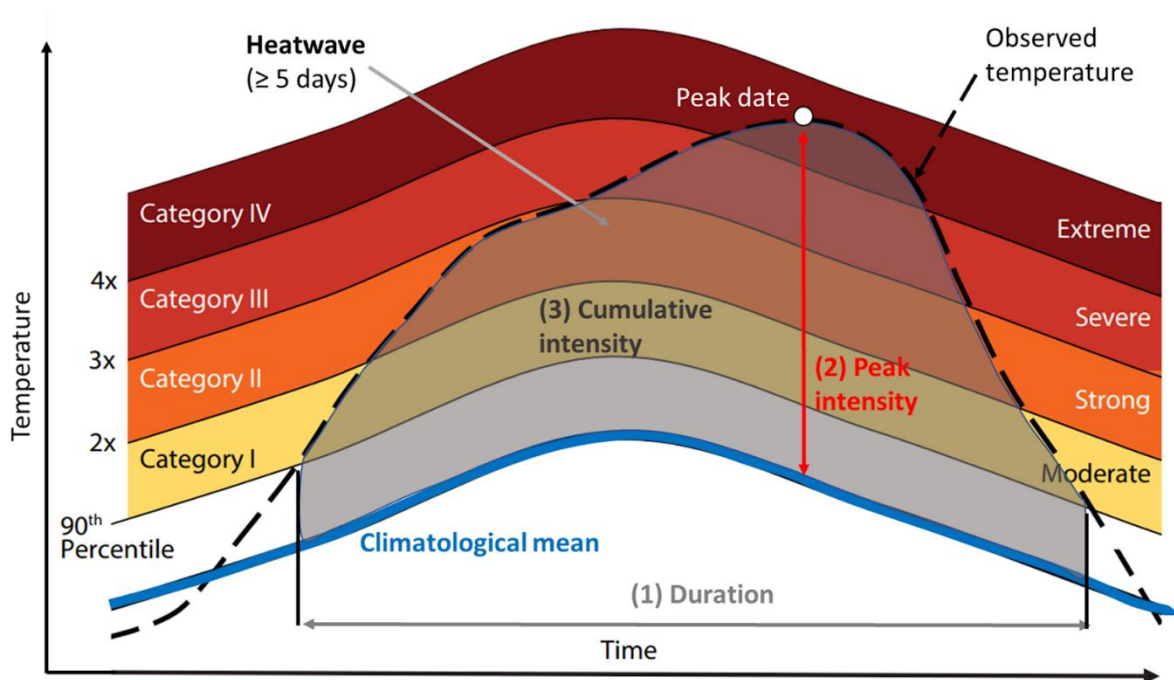


Figure 0.2 : **Schematic of metrics and categorization used to define a marine heatwave (MHW)** showing the observed temperature time series (dashed line), the long-term regional climatology (blue line), and the 90th percentile climatology (thin line). The three properties of MHW are: (1) the duration, the consecutive days of the event, (2) the maximum intensity (peak intensity), the highest temperature anomaly in °C, and (3) the cumulative intensity, the sum of the daily intensities during the event in °C days (Hobday *et al.* 2016). Four categories of MHWs are defined based on the 90th threshold to better assess the characteristics of a MHW and help in describing its evolution. The threshold for each category is defined as being multiple of the 90th percentile: Category I (moderate, 1-2x the 90th percentile; Category II (strong, 2-3x); Category III (severe, 3-4x), and Category IV (extreme, >4x). This example peaked as a Category IV (extreme) MHW. Adapted from Hobday *et al.* 2018

To compare MHW between each other they are characterized by their duration, maximum intensity, cumulative intensity and the proportions of time spent in each of the four categories (Hobday *et al.* 2018). The Mediterranean Sea had two of the first MHW described in literature; they happened in 1999 and 2003 reaching 3-5°C above the climatological mean (Garrabou *et al.* 2009). In 2011, the coast of Western Australia suffered a similar MHW of more than 10-week long (Pearce and Feng 2013). The widest MHW and related to El Niño 2015-2016 was recorded in the Northeast Pacific Ocean with anomalies of up to 6°C in

Southern California (Jacox *et al.* 2016). MHWs have devastating impacts on species and ecosystems, leading to major ecological and physiological changes (Smith *et al.* 2021b). These wide-range effects of MHWs on species' physiological process can result in mass mortality events and local extinctions if the upper thermal threshold and acclimation potential of a species is beyond its limits (Garrahou *et al.* 2022).

Ocean acidification

Ocean acidification is caused by the uptake of atmospheric CO₂ by the ocean. When CO₂ dissolves in seawater it modifies the carbonate chemistry and causes a decrease in pH, in carbonate ion (CO₃²⁻) concentration, and saturation states of calcium carbonate (see Box.1 Seawater carbonate chemistry). All of these parameters play key physiological roles and are notably fundamental to control the formation of the skeleton and shells of calcifying marine organisms (Gattuso and Hansson 2011a). Surface pH has already decreased by 0.1 units since the beginning of the industrial era (Caldeira and Wickett 2003; Orr *et al.* 2005), at a rate of 0.0018 units yr⁻¹ over the last 25 years (Bates 2007; Dore *et al.* 2009). As CO₂ emissions increase, projections under RCP8.5 suggest that an additional decrease of 0.38 units of pH will be observed at the end of the century, while new projections under SSP5-8.5 suggest a decrease of 0.44 units (AR5 of IPCC, Kwiatkowski *et al.* 2020, Figure 0.3). As pH is defined as the negative logarithm of proton (H⁺) concentration (see Box.1), this projection represents an increase in [H⁺] concentration of 2.5 times higher than the value estimated when the industrial era began (Feely *et al.* 2009).

The studies assessing the impact of ocean acidification on calcifying species suggest that low-pH results in a decrease in some benthic organisms' calcification such as corals, calcifying algae and bivalves (Andersson *et al.* 2011; Leung *et al.* 2022). Experiments have demonstrated that the impact of high CO₂ on calcifying species can be complex and species-specific, particularly for photosynthetic species such as calcifying coralline algae, however, even if the impact is not negative, deposition of CaCO₃ under lower Ω and pH is thermodynamically less favorable, and high energy-consuming for the organism (Wood *et al.* 2008). On the other hand, non-calcifying photosynthetic organisms such as seagrasses or fleshy seaweeds can benefit from increasing seawater CO₂ for photosynthesis and due to a decrease in calcifying epiphytes that reduce their photosynthetic surface (Koch *et al.* 2013; Harvey *et al.* 2013). It is thus likely that benthic ecosystems might transition to non-calcifying species favored over calcifying species whose growth and recruitment are negatively affected

by OA, such as observed in natural gradients of pH (Hall-Spencer *et al.* 2008; Andersson *et al.* 2011).

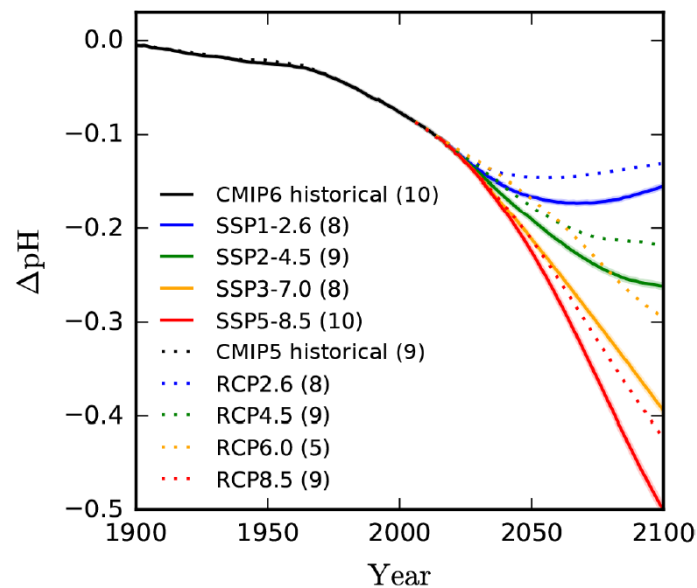


Figure 0.3 : Global average surface pH change for the 1900–2100 period relative to 1870-1899 from CMIP5 and CMIP6 concentration-driven experiments. CMIP6 mean anomalies for the historical and SSP simulations are shown as solid lines with shading representing the inter-model SD. CMIP5 projections are shown as dashed lines. The model ensemble size for each scenario is given in parentheses. Figure adapted from Figure 1 Kwiatkowski *et al.* 2020).

To date the majority of what is known concerning the impacts of OA on benthic marine organisms comes from laboratory-controlled experimental studies focused on the direct responses and sensitivities of selected species or artificial communities (Boyd *et al.* 2018). Whereas experiments are essential to isolate stressors, identify physiological responses, tolerance curves and thresholds to OA, they are limited to predict the long-term consequences for communities and ecosystems as well as the potential of marine species to adapt to OA. Naturally acidified systems, such as CO₂ vents, capture the emergent effects of OA with lower pH and lower saturation of aragonite and calcite on entire ecosystem over long time scales (10 to 18,000 years), thereby providing crucial information on the effects of OA on biodiversity and the potential for species acclimatization or adaptation by combining field work with laboratory experiment or *in situ* cross-transplantation (Rodolfo-Metalpa *et al.* 2011; Calosi *et al.* 2013; González-Delgado and Hernández 2018). Volcanic CO₂ vent systems cause a local acidification of seawater by bubbling gas with 90% to 99% CO₂ from the seafloor and affects the surrounding ecosystem (Hall-Spencer *et al.* 2008; González-Delgado and Hernández

2018). To date, twenty-three CO₂ vent systems have been studied across the globe (Figure 0.4, González-Delgado and Hernández 2018; Aiuppa *et al.* 2021), with a particular focus on the Mediterranean Sea where pioneer work has been conducted.

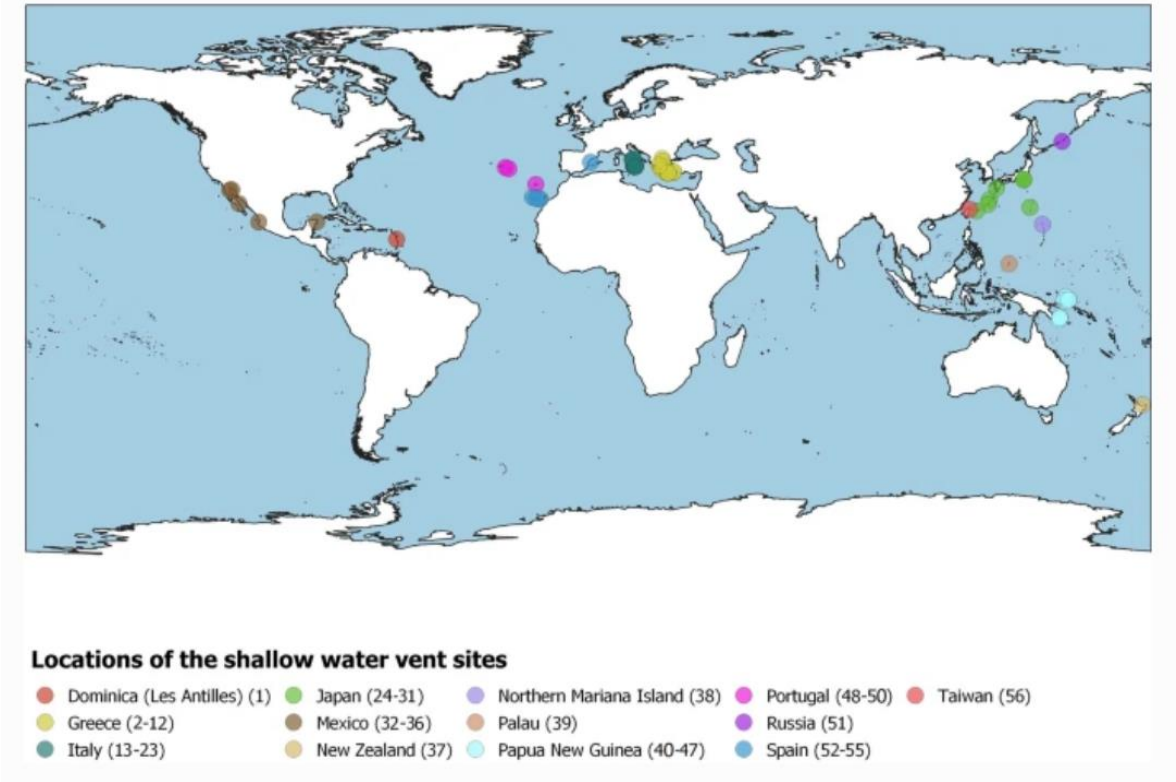


Figure 0.4 : **Locations of shallow water CO₂ vent sites** (The number in brackets refers to the ID code of each location, see Table S1 of Aiuppa *et al.* 2021 for further details)

Box.1: Seawater carbonate chemistry

When atmospheric carbon dioxide ($CO_2(g)$) dissolves in seawater a series of reactions of equilibria happens (Figure 0.5) between the three main dissolved inorganic carbon forms in seawater: free aqueous carbon dioxide ($CO_2(aq)$), bicarbonate ion (HCO_3^-), and carbonate ion (CO_3^{2-}), Figure 0.6). Carbonic acid (H_2CO_3) is also present but at very low concentration. HCO_3^- represents 90% of the DIC (Figure 0.6).

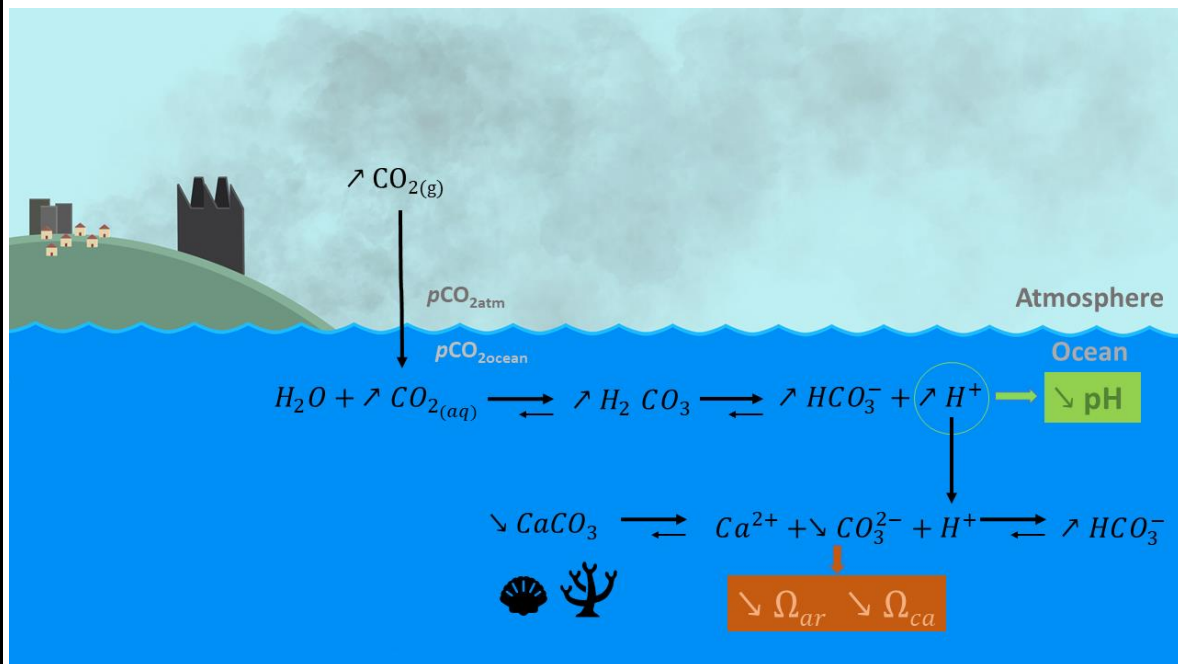
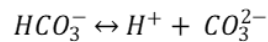
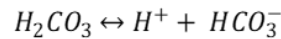
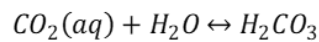
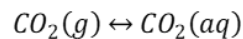


Figure 0.5 Seawater carbonate chemistry shifts caused by the dissolution of CO_2 in seawater.

OA is defined as a reduction in pH after a suite of changes in seawater chemistry. The uptake of excess anthropogenic CO_2 ($CO_2(g)$) by the ocean increases the partial pressure of carbon dioxide (pCO_{2atm}) and dissolved inorganic carbon ($CO_2(aq)$) while decreasing pH and saturation states of the biologically important $CaCO_3$ minerals calcite (Ω_{ca}) and aragonite (Ω_{ar}), both being critical compounds of shells and skeletons of marine organisms

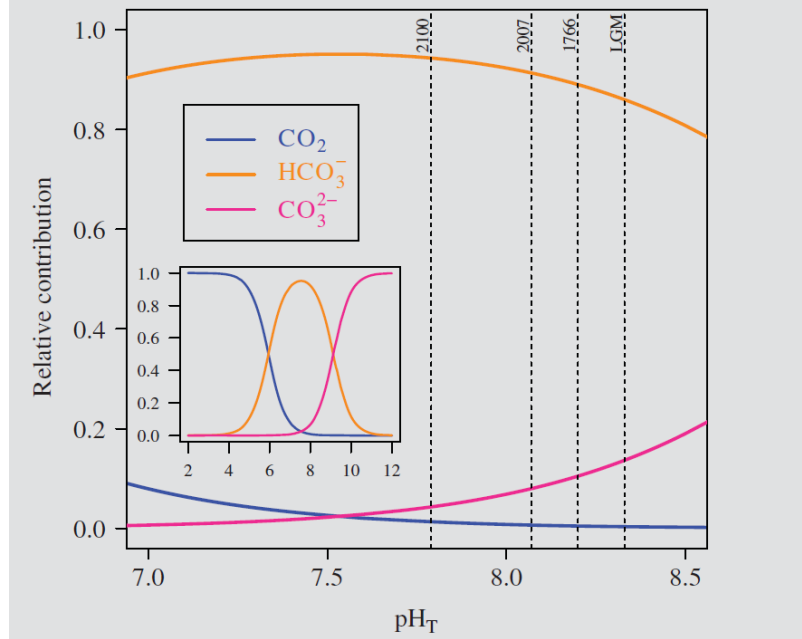


Figure 0.6 : Bjerrum plot showing the relative contributions of carbon dioxide (CO₂), bicarbonate (HCO₃⁻), and carbonate (CO₃²⁻) to the dissolved inorganic carbon as a function of pH_T at 15°C and a salinity of 35. The dashed vertical lines indicate the average open-ocean surface pH T during the Last Glacial Maximum (LGM), 1766, 2007, and 2100. (Gattuso and Hansson 2011)

The sum of the concentration of the three dissolved carbonate forms is the total dissolved inorganic carbon C_T :

$$C_T = [CO_2] + [HCO_3^-] + [CO_3^{2-}]$$

pH

pH measures the activity of protons and represents the ocean acidity, it is expressed as:

$$pH = -\log_{10}[H^+]$$

As the pH is a logarithmic measure, the decrease of one unit of pH means an increase by 10 of the proton concentration. In seawater, it is recommended to use the total scale pH (pH_T, Dickson *et al.* 2007), which takes into account sulfate protons. pH_T is used whenever possible in this thesis.

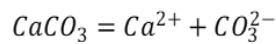
Total alkalinity

Total alkalinity (A_T) is related to the number of hydrogen ion moles corresponding to the excess protons acceptors (bases formed by weak acids) over donors (acids) in 1 kg of seawater (Dickson *et al.* 2007).

$$A_T = [HCO_3^-] + 2[CO_3^{2-}] + [B(OH)_4^-] + [OH^-] - [H^+] + \text{minor compounds}$$

Calcium carbonate

The calcium carbonate precipitated by marine organisms to construct shells and skeletons, exists in three main crystal forms: calcite, aragonite and magnesian calcite. Aragonite, which is the mineral form mostly used by corals to build their skeleton, is 1.5 times more soluble than calcite (Erez *et al.* 2011). The dissolution equilibrium of calcium carbonate is:



At equilibrium, a constant called the apparent solubility product of calcite and aragonite (K'_{sp}) can be assessed and is defined in where $[CO_3^{2-}]_{sat}$ and $[Ca^{2+}]_{sat}$ are the concentrations of the carbonate ions and dissolved calcium in equilibrium with calcium carbonate.

$$K'_{sp} = [Ca^{2+}]_{sat} [CO_3^{2-}]_{sat}$$

The precipitation and dissolution rates of calcium carbonate depend on the saturation state of calcite or aragonite (Ω) which is defined at equilibrium as:

$$\Omega = [Ca^{2+}][CO_3^{2-}]/K'_{sp}$$

Under ocean acidification, Ω decreases with $[CO_3^{2-}]$ (as $[Ca^{2+}]$ is almost constant. When $\Omega=1$, the seawater is in equilibrium with calcium carbonate, when $\Omega>1$, it is supersaturated and the precipitation of $CaCO_3$ is thermodynamically favored and when $\Omega<1$, it is undersaturated and dissolution of calcium carbonate is favored

The Mediterranean Sea: a hot-spot of global change

The Mediterranean Sea is semi-enclosed, surrounded by mountains and highly urbanized littorals which affect the atmospheric, oceanic and hydrological factors (Ducrocq 2016). Due to this geography, future warming in the Mediterranean Sea is projected to be 20% (annual) and 50% (summer) above the global mean projections (Lionello and Scarascia 2018). Sea surface temperature has already increased by 0.29 to 0.44°C (according to regions), and a warming of 0.8 to 3.8°C is projected for the end of the century due to regional variability (Darmaraki *et al.* 2019). Marine heatwaves in the Mediterranean Sea under the RCP8.5 will consist of one long-lasting marine heatwave every year, projected to be three months longer in duration and four times higher in intensity (WGII AR6 Chapter 9 CCP4). For example, between 2015-2019 the Mediterranean experienced a series of MHWs that affected all the regions of the basin, with a mean duration of 75 days, and where 99.99% of the seawater surface was affected by category II, 55% by category III, and 3% by the highest category IV on an annual basis ((Garrabou *et al.* 2022, Figure 0.7).

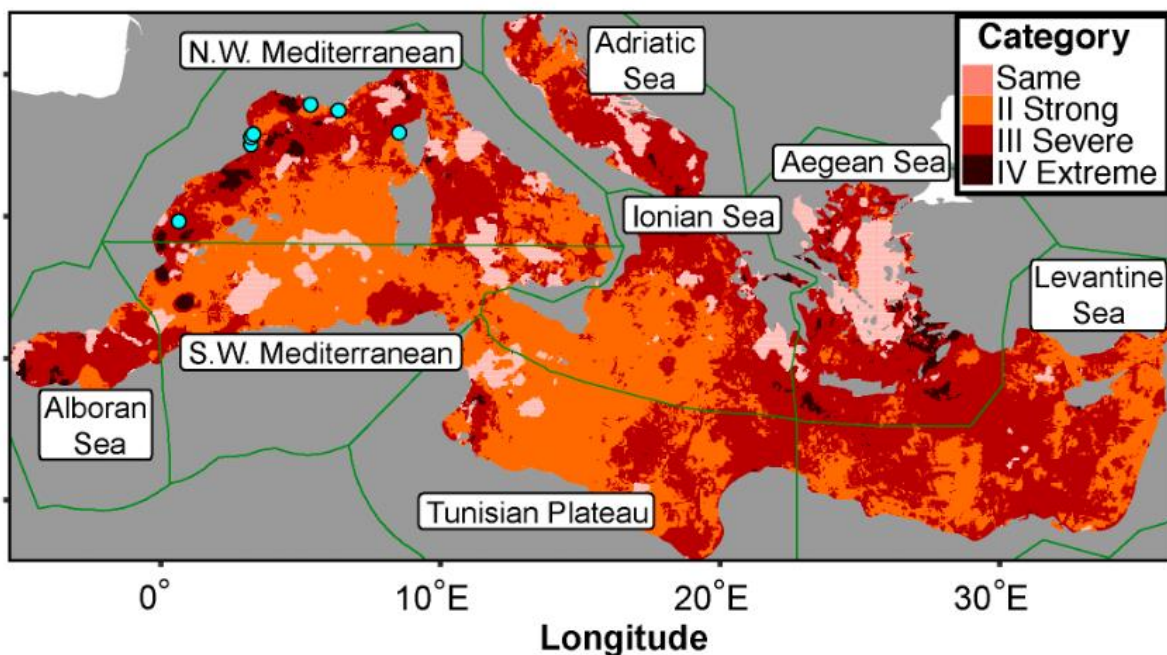


Figure 0.7 : **The highest category of MHW experienced from 2015 to 2019 at locations where only a category I MHW was experienced in 1982 to 1986.** Light pink areas show when a category II or greater MHW occurred in the earlier period. Green lines and blue dots have been added to visualize the Mediterranean ecoregions and the monitoring areas with in- situ (along depth) temperature monitoring, respectively. From Figure 1b Garrabou *et al.* 2022.

These extreme events resulted in mass mortality of 50 species across habitats and depths (from the surface to 45 m depth), where the most impacted phylum was the cnidarian (55%), including six species of scleractinian corals (Garrabou *et al.* 2022). The study showed that the 2015-2019 period was the warmest since the beginning of satellite records in 1982, the Levantine and Aegean Seas were the warmest regions, and marine heatwaves are becoming common, intensifying rapidly, and extending over larger areas. The MHWs are an unprecedented threat to the health and functioning of the Mediterranean ecosystem and that the dynamics of these extreme events need a better understanding to predict responses and spatial refuges for management (Smith *et al.* 2021a; Garrabou *et al.* 2022). Interestingly and worrying, in summer 2022 the Mediterranean Sea appears to be affected again by some of the most extreme temperatures with sea surface temperature anomaly of + 5°C recorded along the coast of France and Italy (<http://www.ceam.es/ceamet/SST/index.html>, <https://t-mednet.org/t-resources/2022-marine-heatwaves>).

As for warming, acidification in the Mediterranean Sea seems more intense than in the global ocean because of faster ventilation times (Palmiéri *et al.* 2015). In the Bay of Villefranche, in the northwestern Mediterranean Sea, the rate of surface ocean acidification is -0.0028 +/- 0.0003 units pH_T yr⁻¹ (Kapsenberg *et al.* 2016). pH is projected to decrease by an additional 0.46 units by the end of the century under the RCP 8.5 in the Mediterranean Sea (Goyet *et al.* 2016).

Corals

This thesis is mostly focused on corals of the order Scleractinia. Most of these corals from shallow tropical reefs are zooxanthellate, i.e. making an intracellular symbiosis with dinoflagellate algae from the genus *Symbiodinium*, also called zooxanthellae (Davy *et al.* 2012). A high density of this unicellular symbiont, usually at least one million of cells per cm² of coral tissue, is hosted in the cells of the coral (Oakley and Davy 2018). The foundation of this symbiosis is the mutualistic intercellular nutrient exchange, by symbiont translocating fixed carbon products of photosynthesis to the host, in exchange for inorganic nitrogen, phosphorus and carbon, while being protected from herbivory by the host and exposed to high light environment (Weis 2008; Davy *et al.* 2012). Some corals are azooxanthellate (non-symbiotic), thus heterotrophy is the only trophic strategy, by eating zooplankton and organic debris that they capture with their tentacles.

Coral reproduction

Corals have a life cycle composed by a dominant benthic polyp phase predominated by growth of tissue and skeleton, regular cycles of sexual reproduction and occasionally asexual reproduction, and a short planktonic larval phase (Harrison 2011, Figure 0.8). Coral sexual reproduction is highly important for population connectivity and genetic diversity as coral larvae are the only pelagic phase of the coral life cycle, when dispersal potential is maximized (Bay and Palumbi 2014). An effective sexual reproduction is characterized by: (1) a good production of gametes, fertilization, and embryonic development for a successful pelagic larval dispersal, (2) a high recruitment by the recognition of appropriate substrate for settlement and metamorphosis, (3) and post-settlement survival and growth success (Ritson-Williams *et al.* 2009, Figure 0.8).

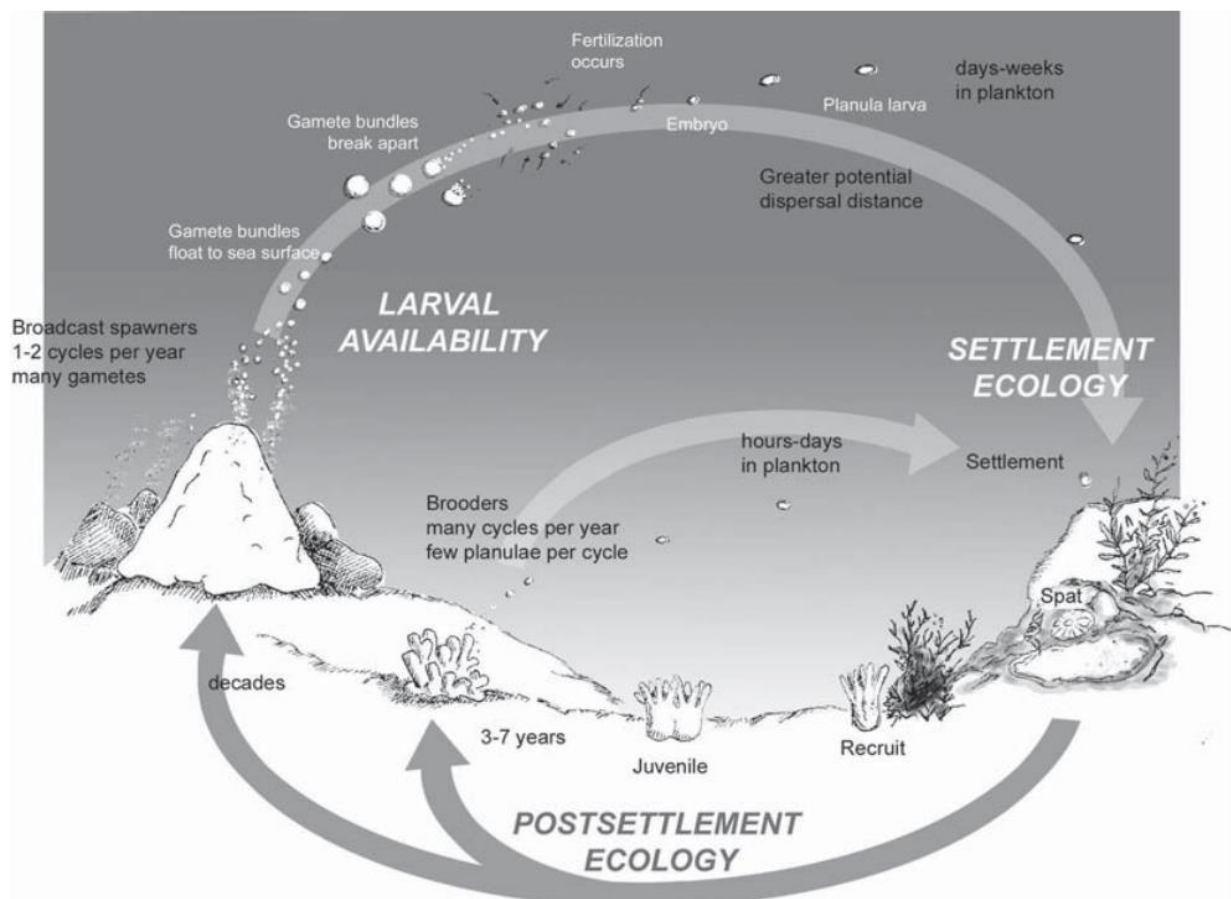


Figure 0.8 **The three stages of the corals' life cycle for a successful coral recruitment, from larval availability, settlement ecology and post-settlement ecology** (Ritson-Williams *et al.* 2009)

Two sexuality modes can be observed on scleractinian corals, gonochoric- where colonies are either female or male- and hermaphroditic- where colonies or polyps produce both gamete

types, sperm and oocytes. A study led by Baird *et al.* (2009) observed that 31% out of 393 analyzed tropical coral species were gonochoric. However, exceptions from this binary reproductive style reveal that at least 27 coral species present mixt modes (Neely *et al.* 2018). Gametes, commonly develop in the mesogleal linings of the mesenteries (Fadlallah 1983). As gametogenesis proceeds, the gonads, ovaries (female) and testes (male), expand with the development of oocytes and spermaries, respectively (Glynn *et al.* 2017). When the gametes mature, two developmental modes can be observed in corals, brooding and broadcast spawning. In brooding corals, the fertilization of the oocytes and embryonic development occur within the parental polyps. Sperm are usually released on a lunar cycle over several months and enter into polyps to fertilize the oocyte. After fertilization, embryos develop for weeks in the gastrovascular cavity before being released by the parent as mature and natant larvae (Szmant 1986). The brooded larvae are competent to settle and metamorphose as soon as it is released and might attach close to the parent population (Costantini *et al.* 2007). In contrast, broadcast spawners have a synchronous release of female and male gametes, which by buoyancy will float at the surface where fertilization occurs (Harrison 2011). The embryos obtained require a planktonic period of 4 to 7 days before to gain competency and look for a benthic substrate to settle (Szmant 1986). Broadcast spawners are by far the most common reproductive strategy of corals with 354 species spawning their gametes among 444 studied tropical coral species (Harrison 2011).

After dispersal, settlement happens when larvae select their substrate for recruitment and attach by the aboral pole on the substrate (Ritson-Williams *et al.* 2016). When settled, it loses its larval characteristics through metamorphosis, and emerges in a functioning polyp recruit (Hadfield *et al.* 2001). Recruits will then start growing tissue and skeleton. Growth is based on budding polyps and creates a colony being genetically identical, and are meant to lengthen the survival of the genotype (Harrison 2011). Different asexual modes are: 1) colony fragmentation (due to mechanical division during a storm, by wave impacts or corallivory (Lirman 2000); 2) polyp “bail-out”, the expulsion of a free skeletonless polyp from its calyx (phenomenon that occurs during environmental stress (Sammarco 1982); and 3) is asexual production of brooded larvae, produced by parthenogenesis (Yeoh and Dai 2010). Coral species are thus able to promote or hinder evolutionary adaptation through genetic recombination and maintain an interconnectivity between populations by sexual reproduction and to survive and quickly recolonize the environment after a disturbance by asexual reproduction.

Impact of warming and acidification on corals

For the last 30 years, warming of sea surface temperature has triggered an increasing number of coral mass bleaching, including pan-tropical events in 1998, 2010 and 2015/(Heron *et al.* 2016; Hughes *et al.* 2017). Bleaching is the breakdown of symbiotic relationship between the coral (host) and its endosymbiotic zooxanthellae or a loss of photosynthetic pigments of these microalgae symbionts (Davy *et al.* 2012). Bleached zooxanthellate coral have a reduced ability to fulfill metabolic needs as most of the carbon requirements are obtained from the photosynthetic products translocated from the symbionts (Goldberg 2018). Corals are constrained to draw from their energy reserves (lipids and carbohydrates) and make a tradeoff between survival, growth and reproduction (Shore-Maggio *et al.* 2018).

Bleaching in corals usually occurs under an oxidative stress (Lesser 1997), which disrupts calcium homeostasis and decreases calcification (Desalvo *et al.* 2008). Besides this possible decrease in growth rate, warming and adult bleaching also has an impact on coral populations vulnerability by disrupting reproduction (Hagedorn *et al.* 2016) and decreasing connectivity between populations by reducing the larval dispersion, thus decreasing genetic diversity (Figueiredo *et al.* 2022). As matter of fact, larvae under high temperatures present an accelerated development, leading to abnormality (Woolsey *et al.* 2013), lower survival (Baria *et al.* 2015; Bahr *et al.* 2020), and faster metamorphosis and settlement (Nozawa and Harrison 2007; Chua *et al.* 2013), which increase the larval retention to the native population.

Most of the knowledge accumulated on the impact of warming has been gathered on tropical corals. The response of zooxanthellate and azooxanthellate temperate Mediterranean corals to repetitive mass mortality events due to MHWs (*e.g.* Kersting *et al.* 2013; Kružić *et al.* 2016; Bisanti *et al.* 2022) is comparatively much less studied. Mediterranean azooxanthellate corals have been found to be more resistant to increased temperature than zooxanthellate corals. *Leptopsammia pruvoti* and *Caryophyllia inornata* presented no difference in growth rate and reproductive efficiency along a gradient of increasing temperatures (from 16.74 to 19.15°C in annual mean depending on the sites) along the 8° coast of Italy (Caroselli *et al.* 2012; Marchini *et al.* 2020), while the zooxanthellate species *Balanophyllia europaea* showed a decrease in reproduction and an inhibition of calcification possibly due to inhibition of photosynthesis at elevated temperature (Goffredo *et al.* 2007; Airi *et al.* 2014). Mediterranean zooxanthellate corals also show a thermal-induced bleaching as seen in *Oculina patagonica*,

Madracis pharensis and *Cladocora caespitosa* (Shenkar *et al.* 2005; Kružić and Popijač 2015; Jiménez *et al.* 2016). Warming has also a role in the increase of the abundance of some bacteria pathogens belonging to the genera *Vibrio* and which might be responsible for necrosis on *Cladocora caespitosa* or bleaching on *Oculina patagonica* (Kushmaro *et al.* 1997; Vezzulli *et al.* 2012; Rubio-Portillo *et al.* 2018). However, warming and its intimate link with an increase in the number of MHWs (Smale *et al.* 2019) is not the only threat to corals.

Ocean acidification is responsible for a decrease in coral calcification by on average 15% under pH values expected by the end of the century under the RCP 8.5 scenario (Cornwall *et al.* 2021). The calcification in scleractinian corals takes place in the calcifying fluid, a semi enclosed environment between the skeleton and the calicoblastic cell layers (Wall *et al.* 2019). The calcifying fluid carbonate chemistry is physiologically regulated through cellular mechanisms such as transport of HCO_3^- to the calcifying fluid (Figure 0.9 (3)), and the active removal of H^+ from the calcifying fluid (Figure 0.9 (4)). This allows the pH and the saturation state in the calcifying fluid to level much higher than the surrounding seawater, which in turn facilitate the precipitation of calcium carbonate (Figure 0.9, Comeau *et al.* 2017). However, ocean acidification can decrease the efficiency of these mechanisms which explains the decrease in calcification with OA observed in many corals. As described by Wall *et al.* (2019), in *Balanophyllia europaea*, in an attempt to maintain calcification by deploying more energy for homeostasis of the chemistry in the calcifying fluid, it results in the inactivation of some calicoblastic cells making the new skeleton less dense and more porous.

Under acidification conditions, gametogenesis is usually not impacted, however, it can induce an asynchrony of spawning (Olischläger and Wild 2020; Liberman *et al.* 2021). Moreover, fertilization can be decreased under acidification (Albright *et al.* 2010), due to a reduction in flagellar motility (Morita *et al.* 2010). Low pH can also decrease larval metabolism (Albright 2011), and a decrease their settlement by disrupting the microbial film in the crustose coralline algae (Webster *et al.* 2011; Doropoulos and Diaz-Pulido 2013) The recruits and juveniles have also been reported to be impacted by acidification, with a decrease of 50% of growth under low pH (Albright and Langdon 2011) or deformed and porous skeleton (Foster *et al.*, 2016). However, temperate corals seem to be more tolerant to ocean acidification than tropical corals as their calcification is rarely affected by low pH (Rodolfo-Metalpa *et al.* 2011; Carbonne *et al.* 2021).

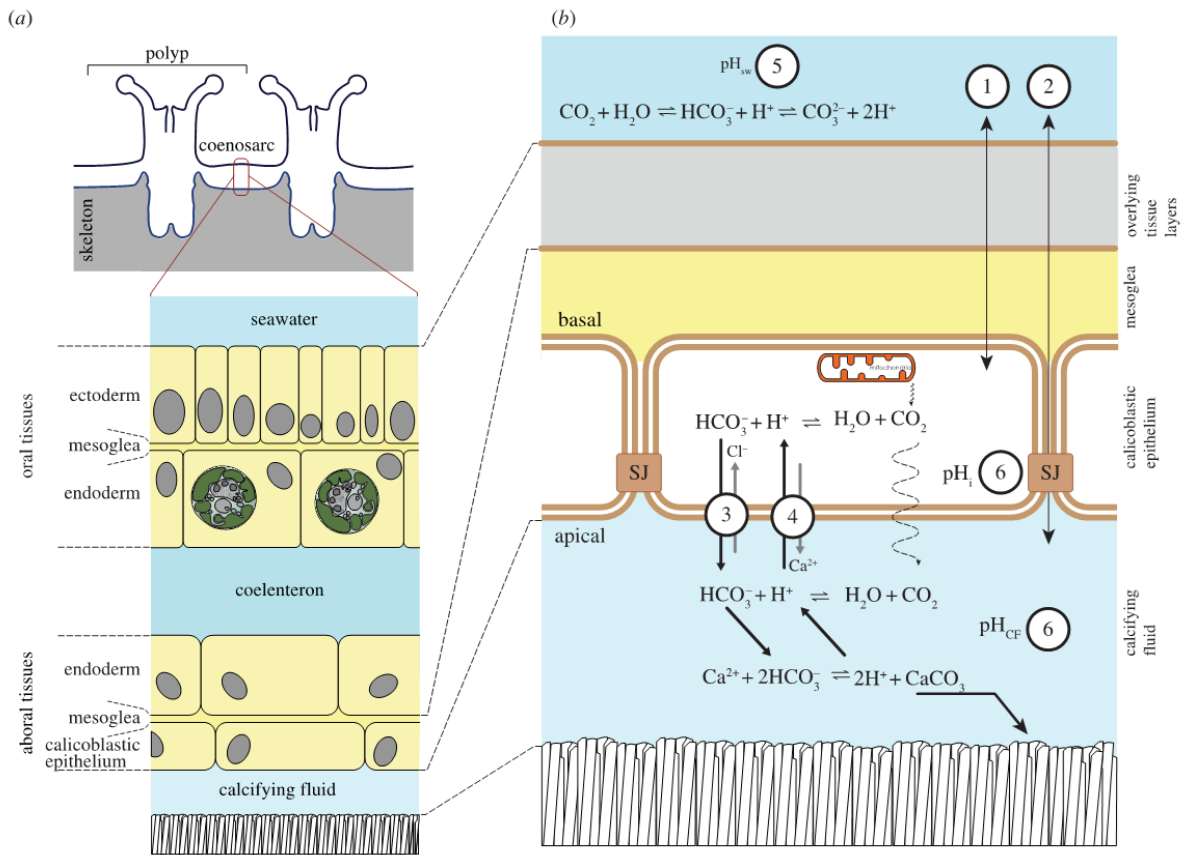


Figure 0.9 : (a) Schematic of cross section through the coral tissues at the coenosarc (tissue between the polyps). (b) Schematic of ion transport processes influencing calcifying fluid (CF) pH in *Stylophora pistillata*. (1–4) Ion exchange between seawater, the CF and calcicoblastic cells, (5 and 6) Changes in seawater carbonate chemistry associated with shifts in seawater pH, affect the internal pH in the calcicoblastic cells (pHi) and pH_{CF}. SJ, septate junction. (From Comeau *et al.* 2017)

Ocean warming is projected to increase along with ocean acidification (Kwiatkowski *et al.* 2020) and could, synergistically, reduce carbonate accretion by 33% by the end of the century and increase coral mortality (Prada *et al.* 2017; Cornwall *et al.* 2021).

Phenotypic plasticity and local adaptation to environmental change

How populations and species respond to environmental conditions is critical to their persistence under the present and future climate conditions. Species can adjust to these novel environmental conditions through phenotypic plasticity, adapt through natural selection or migrate to more favorable conditions. Phenotypic plasticity is the ability of an individual organism to modify its physiological, developmental, behavioral, or morphological phenotypic with its existing genotype in response to a variable environment generating rapid variability in traits usually reversible (Putnam *et al.* 2016). Adaptation is a genetic change in a

population due to natural selection to improve functions in a given environment, and where these changes are heritable and passed on to the next generation (Edmunds and Gates 2008)

Among the phenotypic responses, acclimatization refers to the changes within an organism in response to their natural environment, whereas acclimation differs from acclimatization in that the environmental parameters is artificially induced, typically under laboratory conditions, and over short time (Coles and Brown 2003). Phenotypic plasticity is important as it may be a rapid response to a sudden environmental change (Chevin *et al.* 2010). For example, coral bleaching usually occurs in a few weeks during a MHW, corals have shown that they were able to gain thermal tolerance within 48h to a week of pre-conditioning (Middlebrook *et al.* 2008; Bay and Palumbi 2014). For long-lived organisms such as corals, with a low frequency of new generations, acclimatization is necessary as it intervenes in a single lifespan, and does not require shift in genetic information over generations such as adaptation (Thomas *et al.* 2018). In corals, acclimatization to changing environment can involve a change in symbiont community with the thermotolerant Clade D under high temperature (*Symbiodinium thermophilum* sp., (Hume *et al.* 2015), an increase in heterotrophy when bleached (Grottoli *et al.* 2006), and under low pH, a maintain of the skeletal linear extension by increasing porosity (Tambutté *et al.* 2015; Wall *et al.* 2019). This plasticity provides a better phenotype-environment match; however, it is maintained at a cost, it is highly energy-consuming for the regulation and might result in a tradeoff between energy reserves, reproduction, and survival (Jones and Berkelmans 2011). Natural selection reduces these costs over evolutionary time (DeWitt *et al.* 1998) and fixes genetic differences between populations with different environmental tolerances (Somero 2010). Local adaptation is a balance between selection and gene flow (gene migration), where genetic differentiation between populations occurs when selection is strong enough to counteract the replacement of locally-adapted alleles by non-adapted outsiders (Savolainen *et al.* 2013). Adaptation creates a high fitness of the population to its native site with fixed genetic factors. In order to distinguish effects due to phenotypic plasticity to local adaptation, reciprocal transplant experiments are a particularly relevant method as it compares the fitness of the native population to the novel one (Savolainen *et al.* 2013). As an example, Kenkel *et al.* (2013) have observed that *Porites astreoides* colonies from thermally variable reefs in Florida have greater thermal tolerance of the offshore populations and a reciprocal transplantation confirmed a local adaptation (Kenkel *et al.* 2015). Moreover, a high thermal variability environment increases the capacity for coping with rising temperatures (Schoepf *et al.* 2015).

The Mediterranean Sea with high thermal seasonality between winter and summer seasons, might thus present more tolerant and/or plastic species and this may favor survival and increased heat tolerance, besides the fact that it is a hot-spot of climate change. Studying natural populations that occur in marginal and extreme environments such as highly variable seasonal temperatures, extreme MHWs, and natural CO₂ vents allow to better understand how organisms respond to, and cope with, environmental stress and help to get new insights into how corals may acclimatize or adapt to future environmental conditions.

Mediterranean scleractinian corals

The Mediterranean Sea has a long history with scleractinian corals revealed in the fossil records (Zibrowius 1995; Vertino *et al.* 2014). In the Early-Middle Miocene, over a 100 of scleractinian coral species (>80 genera) were forming coral reefs in the Mediterranean Sea (Vertino *et al.* 2014). However, at the end of the Miocene, the Messinian salinity crisis (nearly complete desiccation of the Mediterranean Sea from 5.96 to 5.33 million years ago) led to the disappearance of coral reefs and almost all the zooxanthellate corals (Perrin and Bosellini 2012). Nowadays, only 33 species (25 genera) are remaining in the Mediterranean Sea (Zibrowius 1980). The banks formed by the shallow water corals *Cladocora caespitosa* and eventually *Astroides calycularis* are, with coralligenous formations, deep-sea or cold corals, and vermetid reefs one of the main bioconstructions in the Mediterranean Sea (Ingrosso *et al.* 2018). This Thesis focuses on two Mediterranean long-lived corals of key relevance for conservation, the zooxanthellate species *C. caespitosa* (Linnaeus, 1767, Box.2 *Cladocora caespitosa*) and the azooxanthellate species *A. calycularis* (Pallas, 1766, Box.3 *Astroides calycularis*).

Box.2 *Cladocora caespitosa*

Cladocora caespitosa (Linnaeus 1767) is a unique zooxanthellate reef-building coral, endemic to the Mediterranean Sea (Zibrowius 1980). Fossil records reveal *C. caespitosa* in the Mediterranean Sea from the late Pliocene, when it was forming monospecific reefs (Aguirre and Jiménez 1998). *C. caespitosa* is nowadays, the only Mediterranean coral which build reef-like constructions, banks, large formations covering several square meters of surface, as seen in Columbretes Island (Spain, Kersting and Linares 2012) and Mljet National Park (Croatia, Kružić and Benković 2008). However, it generally forms separate and dispersed colonies, beds, and size of semi-spherical colonies from 10 to 30 cm in diameter (Rodolfo-Metalpa *et al.* 2005). *C. caespitosa* have been repeatedly highly affected by marine heatwaves of the last two decades, presenting direct necrosis (Rodolfo-Metalpa *et al.* 2005; Kersting *et al.* 2013b) or bleaching (Jiménez *et al.* 2016; Kružić *et al.* 2016; Mačić *et al.* 2019).



Figure 0.10 Picture of a colony of *Cladocora caespitosa in situ* (10m, Ischia, Italy) with polyps presenting a loss of pigmentation and open tentacles (Colony of ~20 cm in diameter).

Little is known about *C. caespitosa* reproduction. Few broadcast spawning events have been reported in the Adriatic Sea at the beginning of summer (Schiller 1993; Kružić *et al.* 2008) and the species has been identified as hermaphroditic during these events. However, histological analysis of colonies from the Columbretes Island suggest that they are gonochoric and spawning occurs at the end of summer (Kersting *et al.* 2013c). For its ecological and conservation importance as a reef-forming coral and its sensitivity to global change, *C. caespitosa* have been included on the IUCN Red list of Threatened Species, the Appendix II of CITES and the List of Threatened Species of the Council of Europe Bern Convention besides other list at European, Mediterranean and Spanish levels (Kersting *et al.* 2013b).

Box.3 *Astroides calycularis*

Astroides calycularis (Pallas, 1766) is an azooxanthellate scleractinian colonial coral endemic to the Mediterranean, characterized by the bright orange color of its coenosarc and polyps (Zibrowius 1995). It is considered a long-lived species and commonly found in dimly lit, shallow rocky habitats (vertical walls, cave entrances, overhangs, from the surface to 50 m depths, Zibrowius 1995).

It has a limited geographic distribution, with a southwestern distribution in the Mediterranean Sea (Zibrowius 1995). This coral is considered a thermophilic species with a narrow temperature tolerance confined to minimum 14°C during the winter (Bianchi 2007). Mortality events of *A. calycularis* during late summer have been reported during the last years in the coast of Ischia and Pelagie Islands (Bisanti *et al.* 2022; Garrabou *et al.* 2022) with the summer 2020 temperature causing a widespread mortality event in the Pelagie Islands (Strait of Sicily).



Figure 0.11 **Picture of a colony of *Astroides calycularis in situ*** (2 m, Ischia, Italy), characterized by the bright orange color of its coenosarc and polyps (colony of ~15 cm in diameter).

A. calycularis is a gonochoric internal brooder (Goffredo *et al.* 2011). Male spawning happens between April to May, and after fertilization and embryonic development inside the female, planulation takes place at the end of June, when seawater temperature reaches around 23°C (Goffredo *et al.* 2011; Casado-Amezua *et al.* 2013). The released bright orange larvae of approximately 2 mm are mature and ready to settle (Goffredo *et al.* 2010). Due to its limited geographic distribution and current regression caused by human activities, *A. calycularis* is classified as vulnerable on the IUCN Red List, protected by the Berna and Barcelona Conventions, as well as included in the Appendix II of CITES.

Aims and scientific approaches of the thesis

The main goal of this thesis is to determine the effects of present and future coastal environmental conditions and variability on Mediterranean corals. This thesis uses corals from two newly discovered volcanic CO₂ vents in Ischia, (Italy) which cause local acidification of seawater with pH_T close to values expected for the end of the century (See Box Ischia)- as natural analogs for future ocean acidification conditions. This research is focused on natural populations of two Mediterranean long-lived corals living in the CO₂ sites that have key relevance for conservation as habitat-forming species: the zooxanthellate species *Cladocora caespitosa* (Linnaeus, 1767, see Box *Cladocora caespitosa*) and the azooxanthellate species *Astroides calycularis* (Pallas, 1766, see Box *Astroides calycularis*). In particular, I aim to integrate environmental data, ecological field surveys at the CO₂ vent sites and reference areas with Ambient pH, and laboratory experiments with controlled temperature and pH. How Mediterranean corals respond to ocean warming, acidification and to their combined effects under present-day and future climatic conditions remains little explored and is the focal point of this PhD Thesis.

The questions that lead this thesis are:

- How present-day and future temperature and/or pH affect the physiology of *Cladocora caespitosa* and *Astroides calycularis* at every life stage (adult, gametogenesis, larvae, and recruits)?
- Are the different life stages equally impacted by future environmental conditions?
- Does exposure to low pH at CO₂ vents enhance the tolerance of corals to future acidification?

Box.4 Ischia

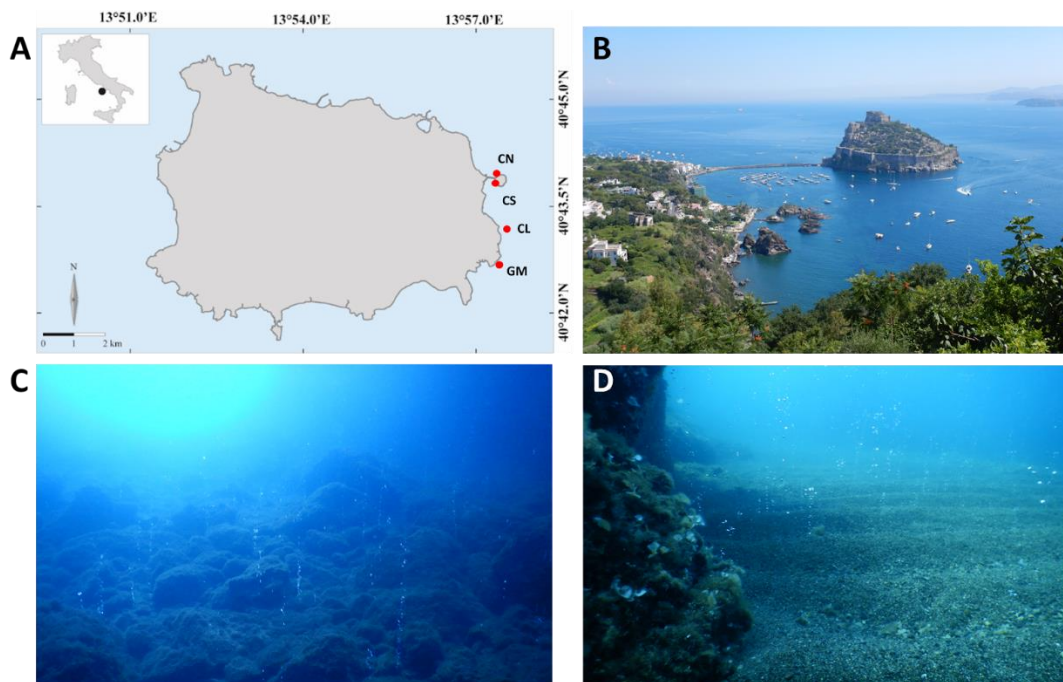


Figure 0.12 **Map and pictures of the CO₂ vent sites along the coast of Ischia studied in this thesis.** The site Castello Aragonese is indicated as reference. (A) Map of Ischia with the CO₂ vent sites: CN: Castello North, CS: Castello South, CL: Chiane del Lume, GM: Grotta del Mago, (B) Picture of the Castello Aragonese, (C) CO₂ vents in the Grotta del Mago (5 m depth) and (D) Chiane del Lume (11 m depth).

The study sites are located along the coast of the island of Ischia, Italy (Figure 0.12). Ischia, together with the Phlegrean Fields, is part of the volcanic system lying west to the Bay of Naples in the Tyrrhenian Sea (Italy) (Foo *et al.* 2018). Subterranean faults are gateways for CO₂ from geochemical magmatic processes to the seafloor (Foo *et al.* 2018) that create CO₂ vents (Figure 0.12.C and 0.12.D). Two newly discovered CO₂ sites have been the main study sites for this PhD thesis: the Grotta del Mago, a shallow semi-submerged cave (depth 5 m) with a pH_T between 7.88 and 7.60 (from 2 to 4m depth, Teixidó *et al.* 2020) and Chiane del Lume, a rocky reef at 10 m depth, where pH_T is 7.99 to 7.89 (Carbonne *et al.* 2021). These two CO₂ sites have been monitored with high-resolution sensors of pH (SeaFET™ Ocean pH sensors, Satlantic), temperature (HOBO Water Temperature Pro v2 Data Logger, Onset) and P.A.R. (P.A.R. loggers, Odyssey). In addition, temperature data in Ischia has been acquired since 2012 in order to characterize temperature patterns from 5 to 40 m depth, which belong to the temperature network t-MEDNet (<https://t-mednet.org>). Interestingly, populations of scleractinian corals naturally occur in these CO₂ vent sites: the zooxanthellate coral *Cladocora caespitosa* at Chiane del Lume (10 m depth) and the azooxanthellate coral *Astroides calycularis* in the Grotta del Mago (5 m). These CO₂ vent systems and their populations provide a great opportunity to assess how corals may survive under future ocean acidification conditions and the mechanisms of potential acclimatization or adaptation to ocean acidification.

Structure and chapters of this PhD

Chapter 1 Response of two temperate scleractinian corals to projected warming and heatwaves (Proceedings B, to be submitted)

Recurrent and more frequent MHWs lead to mass mortality of corals, after summer. To have a better understanding of the present and future consequences of MHWs on corals, it is important to better understand the physiological responses and potential recovery to MHWs and future warming conditions. In this chapter, we exposed for 5 months, the zooxanthellate coral *Cladocora caespitosa* and the azooxanthellate coral *Astroides calycularis*, to different warming scenarios and MHWs in laboratory conditions, based on nine years of field data collected at the collection site. We provide new insights into their responses and recovery to warming and extreme temperatures.

Chapter 2 Two temperate corals are tolerant to low pH regardless of previous exposure to natural CO₂ vents (Limnology & Oceanography, published September 2021)

While ocean acidification is one of the main threats for corals, only few studies have tested the impact of previous long-term exposition to low and variable pH on Mediterranean corals. In this Chapter, we assess physiological response of adult colonies of *Cladocora caespitosa* and *Astroides calycularis*, from CO₂ vent and ambient pH sites, maintained in a laboratory under present-day (pH on the total scale, pH_T 8.07) and low pH conditions (pH_T 7.70). Interestingly, neither species showed any change in the different parameters analyzed regardless of their environmental history. The results indicate that *C. caespitosa* and *A. calycularis* could tolerate future ocean acidification conditions.

Chapter 3 Early life stages of a Mediterranean coral are vulnerable to ocean warming and acidification (Biogeosciences, in press)

Early life stages are one of the most important stages for the maintenance and recovery from disturbance of natural populations. However, early life stages are generally more sensitive to global environmental change than adult colonies and very few studies have been assessing the impact of warming and acidification on larvae and recruits. In this chapter we tested whether ocean warming and acidification can affect larval development and survival, and growth of

recruits of *Astroides calycularis*. For this purpose, larvae from an ambient pH site were raised under two different temperatures and pH in the laboratory for 9 months. This study provides new evidence of the threat that represents ocean warming and acidification for the larval recruitment and the growth of recruits of corals.

The following two studies are not presented as scientific articles but as abstract, material and methods and results because of the need for complementary acquisition of data and further analysis of the results. They are consigned in the appendix section and their results are discussed in the discussion section.

Appendix IV Parental exposure to natural CO₂ vents alters the response to low pH of a temperate coral's early life stages

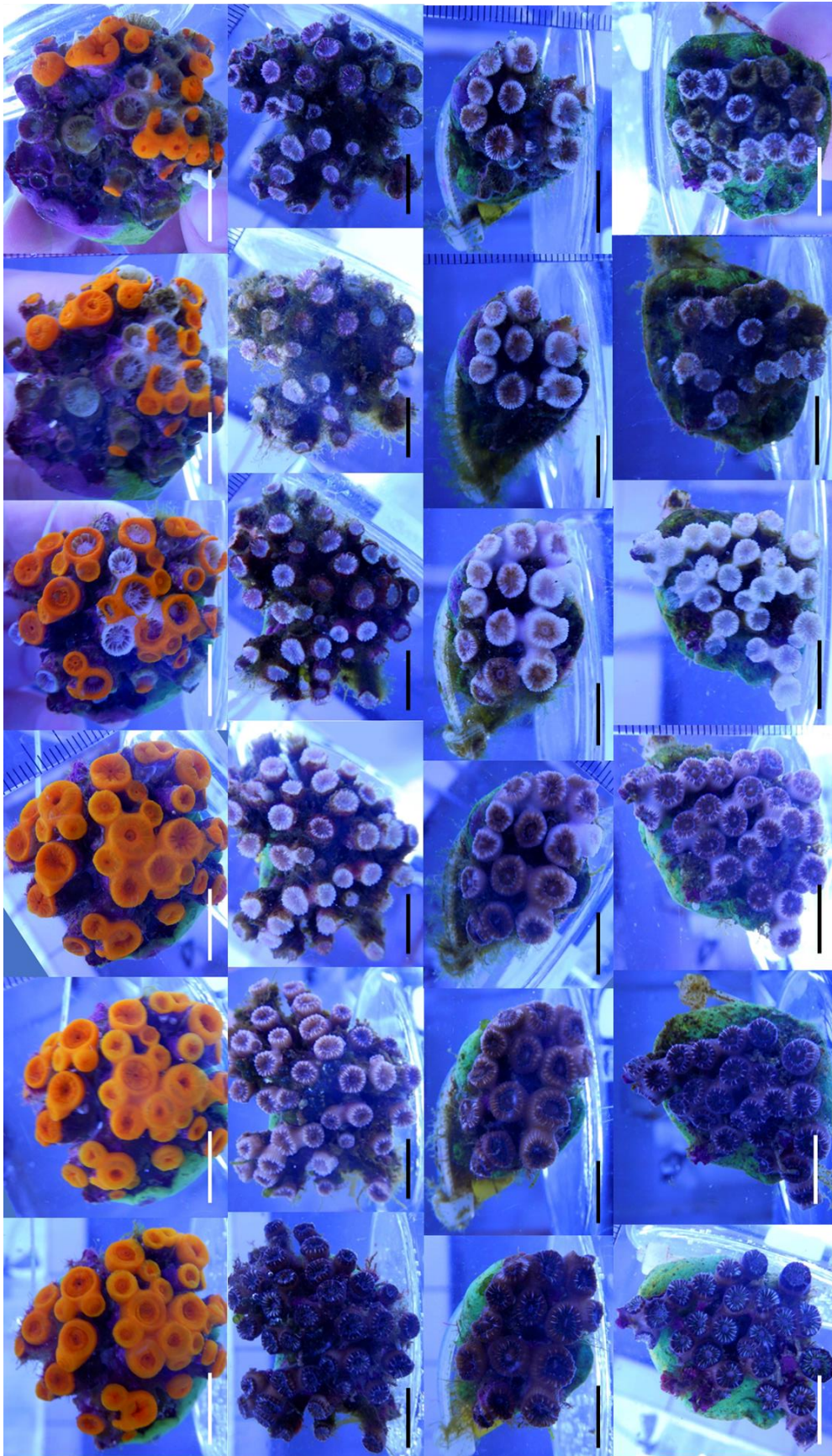
As reproduction and recruitment of *A. calycularis* was occurring in the CO₂ vent site, we were interested in knowing if acclimation and differential gene expression to low pH was acquired by early life stages. In this study, we obtained larvae from a CO₂ vent and an ambient pH site and we maintained them in different pH conditions in the laboratory, ambient (~8.05), low (~7.7) and extreme low pH (~7.5). We quantified their development into recruits, mortality, growth, and extracted the RNA to analyze differences in gene expression between the populations and pH treatments. This study provides new insights into differentially expressed genes and the difference of larval development response between origins regardless of the pH treatment in the laboratory.

Appendix V Exposure to natural CO₂ vents delays the spawning of a temperate coral

As sexual reproduction is one of the mechanisms that can replenish a population after a disturbance, it is important to evaluate whether reproduction can be impaired under acidification. As very little is known about sexual reproduction of *Cladocora caespitosa* and about the impact of acidification on it, we studied the gametogenesis of colonies from a CO₂ vent and from an ambient pH sites by histological cuts. For this purpose, samples of the same colonies were obtained in July and September 2020 to analyze the progression of gametogenesis and to try identifying the moment of the spawning. In this study we expose an

asynchrony of spawning of the colonies from the CO₂ vents in September while the size of the gametes was not different from the ambient pH site.

This PhD has been conceived as a whole; however, each chapter has been submitted as separate papers for publication in scientific journals. Chapter 2, have already been published and Chapter 3 is in print, but format has been unified in order to keep it consistent throughout the document. Original text, figures and tables that appear in the published papers have been preserved. References of the General Introduction and General Discussion have been unified in a single reference list at the end of the document, however the reference lists of Chapters 1, 2 and 3 are presented at the end of their corresponding chapter.



Impact of temperature on colonies of *Cladocora caespitosa* and *Astroides calycularis*: bleaching and necrosis

Chapter 1: Response of two temperate scleractinian corals to projected warming and heatwaves

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Keywords: Ocean warming, marine heatwaves, Mediterranean Sea, coral, bleaching

Abstract:

The Mediterranean Sea is a hot-spot of Global change with warming sea surface temperature and an increasing occurrence of marine heatwaves. Here, we investigate whether colonies of the zooxanthellate coral *Cladocora caespitosa* and the azooxanthellate coral *Astroides calycularis* show physiological impact and recovery, under four present and future temperatures treatment with warming and extreme temperature events based on field data. For five months in the laboratory, colonies of both coral species presented an increase of relative number of polyps per colony and respiration during the summer peak of temperature. Some *A. calycularis* colonies also presented high loss of polyps by necrosis under the warmest treatment. *C. caespitosa* symbiosis was impaired by temperature with a decrease in effective quantum yield, density of zooxanthellae and an increase in percent whiteness in all the treatments except for the coolest treatment. Under the warmest treatment, *C. caespitosa* showed some severe bleached colonies. However, both species recovered with respect to respiration, regrowth of polyps of *A. calycularis*, and regain of pigmentation and zooxanthellae after bleaching for *C. caespitosa*. Combined, these results suggest that under the present and predicted warming trend, these Mediterranean coral species will be able to recover after the summer heat.

1. Introduction

Climate change severely impacts marine biodiversity and ecosystem functioning globally (Smale *et al.* 2019). The upper layers of the ocean store over 90% of the excess of heat caused by global warming, resulting in a warming trend of 0.15 °C per decade over the past 40 years (Cheng *et al.* 2022). In addition to gradual ocean warming, the frequency and severity of extreme climatic events such as marine heatwaves (hereafter MHWs) have increased considerably (Smale *et al.* 2019). These exceptional warm water events are defined as seawater temperature exceeding a threshold, usually defined as the 90th percentile of the climatological mean, for at least five consecutive days (Hobday *et al.* 2016). Three properties characterize MHW: (1) their duration, the consecutive days of the event, (2) the maximum intensity, the highest temperature anomaly in °C, and (3) the cumulative intensity, the sum of the daily intensities during the event in °C days (Hobday *et al.* 2016). MHWs have significant impacts on species and ecosystems, driving major ecological and physiological changes (Arafeh-Dalmau *et al.* 2019, Smale *et al.* 2019). These wide-range effects of MHWs on species' physiological process can result in mortality and local extinctions if the upper

thermal threshold and acclimation potential of a species is beyond its limits (Hughes et al 2017, Gomez-Grass et al 2019). Impacts of MHWs have been described across many regions where habitat-forming species, which support a large number of associate species, have been affected, including the loss of kelp forests (Arafeh-Dalmau et al 2019), coral bleaching (Hughes et al 2017), and widespread mortality of habitat forming macrophytes and invertebrates in the Mediterranean Sea (Cerrano *et al.* 2000, Garrabou *et al.* 2022).

The Mediterranean Sea is considered a hotspot of climate change where important ecological effects on marine biodiversity are projected (Giorgi *et al.* 2006, Cramer et al 2018). It is estimated that the increase in sea surface temperature (SST) will be 20% higher than in the global ocean (Lionello and Scarascia, 2018). The global SST is projected to increase by 3.2°C at the end of this century under the RCP8.5 scenario (Kwiatkowski *et al.* 2020). From 1993 to 2017, SST has increased by $0.04 \pm 0.002^\circ\text{C}$ per year in the Mediterranean Sea (von Schuckmann *et al.* 2019). One of the most extreme MHW happened in 1999, with temperatures remaining above the mean by 1-4°C for more than a month from the surface down to 32 m depth (Cerrano *et al.* 2000). MHW causes extensive tissue necrosis and benthic species suffer mortality which causes long-term population declines of habitat-forming species including corals and gorgonians that are the most affected (Garrabou et al 2022). It is therefore necessary to identify and understand the response of Mediterranean corals to gradual warming as well as to extreme events such as MHWs and assess the extent of recovery, in order to obtain accurate predictions of physiological responses under future temperature conditions.

Zooxanthellate corals are threatened by the increase in seawater temperature leading to heat stress that causes bleaching (Hughes *et al.* 2017). Bleaching is a breakdown of symbiosis between the coral host and its endosymbiotic zooxanthellae or a loss of photosynthetic pigments of these microalgae symbionts (Davy *et al.* 2012). Most carbon requirements of corals are obtained from photosynthetic products translocated from their symbionts (Goldberg 2018). Thus, bleaching leads to a reduced ability to fulfill metabolic needs, a loss of reserves, a decrease in growth rate, shifts in the microbiome, higher disease susceptibility, and an increase in mortality (McLachlan *et al.* 2020). Warming also makes coral populations vulnerable by disrupting reproduction (Airi *et al.* 2014) and decreasing connectivity between populations by reducing the larval dispersion, thus decreasing genetic diversity (Figuereido *et al.* 2022). Most of the knowledge accumulated of this issue has been gathered on tropical corals. The response of temperate Mediterranean corals to repetitive mass mortality events

due to MHWs (*e.g.* Kersting *et al.* 2013, Kruzic *et al.* 2016, Bisanti *et al.* 2022) is comparatively much less understood.

The present study focuses on two Mediterranean habitat-forming corals of key relevance for conservation, the zooxanthellate species *Cladocora caespitosa* and the azooxanthellate species *Astroides calycularis*. *C. caespitosa* is one of the few zooxanthellate corals of the Mediterranean Sea (hosting endosymbionts) and the only one forming extensive bioconstruction comparable to coral reefs in the tropics (Kruzic and Benkovic, 2008, Kersting and Linares, 2012). Field studies have investigated its sensitivity to MHWs across the Mediterranean and laboratory experiments looked at its thermal tolerance (*e.g.* Kersting *et al.* 2013, Rodolfo-Metalpa *et al.* 2006, Jimenez *et al.* 2014). In the laboratory, the increase in temperature during summer led to an increase in growth rate, calcification, respiration, photosynthesis, and asexual reproduction (Rodolfo-Metalpa *et al.* 2008, 2010). The thermal threshold measured was 24°C (Rodolfo-Metalpa *et al.* 2006), which is below current mean summer temperature, possibly due to the inhibition of photosynthesis above this threshold. Elevated summer temperature lead to bleaching in Croatia, Montenegro and Cyprus (Kruzic *et al.* 2014, Jimenez *et al.* 2016, Mačić *et al.* 2019) or to direct necrosis in the Ligurian Sea and Columbretes Islands (Rodolfo-Metalpa *et al.* 2005, Kersting *et al.* 2013). *A. calycularis* is commonly found in low light, rocky habitats, from surface to 50 m depths (Zibrowius 1995). It has a limited geographic distribution, with a southwestern distribution in the Mediterranean Sea (Zibrowius, 1995). This coral is considered a warm-water species with a narrow temperature tolerance confined to minimum 14°C during the winter (Bianchi, 2007). In laboratory experiments, *A. calycularis* showed a decrease in calcification by 25% and an increase of porosity of the skeleton when exposed to an increase of 3 °C compared to ambient temperature (Movilla *et al.* 2016). Mortality events of *A. calycularis* during late summer have been reported during the last years in the coast of Ischia and Pelagie Islands (Bisanti *et al.* 2022, Garrabou *et al.* 2022) with the summer 2020 temperature causing a widespread mortality event in the Pelagie Islands (Strait of Sicily).

We studied the physiological performance and recovery of the two habitat-forming species *C. caespitosa* and *A. calycularis* at present and future summer temperatures. Specifically, the aim was to (1) investigate the physiological impacts on these corals of present and future temperatures based on nine years of field data, (2) to determine whether the symbiosis amplifies the susceptibility to elevated temperature in *C. caespitosa*, and (3) to test the recovery potential of the two species to a range of summer temperatures.

2. Materials and methods

2.1. Sampling site

Forty colonies of *Cladocora caespitosa* and forty colonies of *Astroides calycularis* (~5 cm of diameter) were collected at 10 m deep, on 1st July 2020, by scuba diving in Ischia, Italy (Sant'Angelo site for *A. calycularis*, 40.691972, 13.893056), and Chiane del Lume site for *C. caespitosa*, 40.717444, 13.967278). They were maintained in a 30-liter tank filled with ambient seawater and with water motion provided by a NEWA mini 606 pump for 24 h prior to an 11 h-long transportation to the aquarium facilities of the *Laboratoire d'Océanographie de Villefranche*, France. Colonies were cleaned, their bases covered with water-resistant epoxy, tagged, and maintained at ambient temperature of 23°C for 2 weeks. For *C. caespitosa*, eight additional colonies were sampled on 1st July and 18th September 2020 at the same depth and site for zooxanthellae density measurements as reference samples (hereafter called “*In situ*” samples). The samples were snap frozen after sampling and stored at -80 °C pending analysis.

2.2. Seawater temperature in Ischia

Seawater temperature at the two collection sites was recorded hourly from June 2013 to June 2021 at 10 and 15 m depth with HOBO Water Temperature Pro v2 Data Loggers (Onset). Over 8 years, the monthly mean maximum occurred in August at 10 m depth with a temperature of 26.3°C and in September at 15 m depth with 24.1°C (Figure S1-Appendix I). The monthly mean minimum was 14.6 and 14.5°C in March, for 10 and 15 m depth, respectively. The maximum temperature was 28.3°C at 10 m in summer 2018, and 27°C at 15 m in summer 2015. From 2013 to 2020, mean summer temperatures increased by 0.032°C per year at 10 m depth and 0.051°C per year at 15 m (Figure S2-Appendix I). Summer 2018 was the summer presenting the highest number of marine heatwaves (2 at 10 m and 3 at 15 m) and cumulative intensities (> 40°C x days at 10 m and >60°C x days at 15°C, Figure S3-Appendix I). The number of MHWs and cumulative intensities were analyzed with the HeatwaveR package (Schlegel and Smit, 2018). MHWs events, being periods of at least five days over the 90th percentile, and their metrics such as cumulative intensity (the sum of the daily intensities during the event) were determined with the detect_event function applied to a daily data of the climatology period of Ischia created with the ts2clm function with temperature data from 2013 to 2020.

2.3. Experimental set-up and treatments

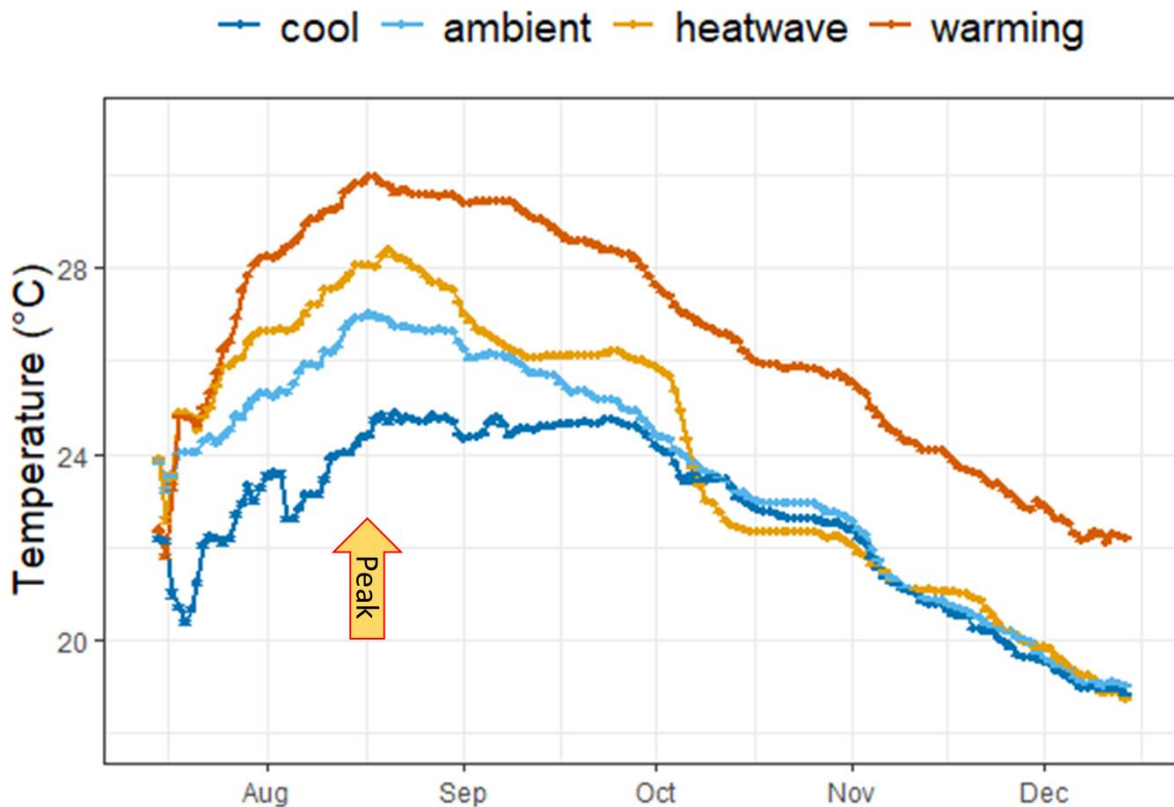


Figure 1.1 **Temperature treatments applied during the 5-month experiment.** Values are daily means. “Ambient” is the daily mean temperature from 2012 to 2019 at 10 m deep, “warming” is the mean temperature at 10 m deep +3.2°C, “heatwave” the temperature of the year 2018 presenting the highest number of heatwaves recorded from 2012 to 2019, and “cool” the daily mean temperature from 2012 to 2019 at 15 m deep. The color of the dots indicates the temperature treatment. The “peak” arrow indicates the peak of temperature of the experiment (16 August 2020).

Colonies of *C. caespitosa* and *A. calycularis* were maintained in four temperature treatments for 146 days (Figure 1.1). The “ambient” treatment mimicked the daily mean temperature from 2012 to 2020 at 10 m depth. The “warming” treatment was ambient +3.2 °C, which is the mean increase in global SST projected at the end of the century under the RCP 8.5 scenario (Kwiatkowski *et al.* 2020). The “heatwave” treatment mimicked the daily mean temperature of 2018 which was the year presenting the highest number of MHWs at 10 and 15 m depth (Figure S2-Appendix I). Finally, the “cool” treatment replicated the daily mean temperature from 2012 to 2020 at 15 m depth. Eight colonies of each species were randomly distributed in separate 5 L experimental tanks for each of the four temperature treatments, for a total of 32 experimental tanks. Seawater pumped from Villefranche Bay at 5 m depth was continuously flowing into eight 25 L header tanks that overflowed into four experimental tanks, each at a rate of 100 ml min⁻¹. The experimental tanks were maintained in water baths

to control their temperature using temperature controllers (APEX, Neptune Systems) which controlled 300 watt heaters (Thermocontrol, Eheim), allowing to control temperature with a difference of less than 0.1°C between tanks of the same treatment. Temperature was adjusted every day, to mimick the natural daily change in temperature. Light was provided by 89 W LED light bars (Aquaristik, Aqualumix). Irradiance gradually increased from 0 at 06:50 to a maximum irradiance of 150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 12:30, and gradually decreased to 0 at 19:00. Irradiance was measured with a LI-1400 datalogger mounted with an underwater quantum sensor LI-192 UWQ8268 (LI-COR). Dark plastic bags covered one side of each experimental tank to shade colonies of *A. calycularis* from direct light. MINI 404 submersible pumps (420 L h⁻¹, NEWA) provided water motion in each experimental tank. Twice a week, one scoop of Marine Power coral food LPS pellets (Tropical) was added to each tank for feeding, and polyps of *A. calycularis* were hand-fed once a week with small pieces of fish filet.

2.4. Changes in polyp numbers and calcification

Pictures of the colonies of both species in seawater were taken every month into a glass aquarium with a Coolpix W300 camera (Nikon) from each side of the colony. They were used to count the number of polyps per colony. The relative number of polyps per colony (ρ) was defined as :

$$\rho(m) = \frac{N(m)}{N(m-1)} - 1$$

where m is the month and N is the number of polyps

Calcification rates were assessed at the beginning and after 3 months (before sampling polyps from each colony) using the buoyant weight technique (Davies 1989). The change in weight was normalized by the surface of living tissue and the number of days (88 d). The surface area was determined using the aluminum foil technique (Marsh 1970).

2.5. Physiological measurements

Dark respiration and net photosynthesis were measured by incubating each colony separately in 500-mL transparent perspex chambers at the beginning of the experiment, at the peak temperature (16 August 2020) and about one month after the peak temperature (30 September 2020), when temperature was the same than during the initial incubations. The chambers were placed in a temperature-controlled water bath to maintain the corals at temperatures similar to

the ones in the experimental tanks. Incubations lasted 30 to 60 min depending on the metabolic activity. Colonies were acclimated in the dark for 10-min prior to the start of dark respiration measurements. Net photosynthesis was measured on *C. caespitosa* under an irradiance of 150 $\mu\text{mol photons m}^2 \text{ s}^{-1}$. Magnetic stirrers provided water motion during the incubation. An incubation with no coral was used as a blank for each temperature treatment. Oxygen saturation within the chambers was measured every 5 s using a fiber optic oxygen sensor (PreSens, OXY-4 mini), which was calibrated at 0% and 100% saturation. The difference in oxygen saturation between the beginning and the end of the incubation was converted to $\text{O}_2 \text{ mg L}^{-1}$ using the `gas_O2sat` function of the `marelac R` package (Soetaert *et al.* 2020). Oxygen consumption was then normalized by the coral surface area, incubation time, and chamber volume to obtain dark respiration and net photosynthesis. Gross photosynthesis was calculated assuming that respiration is the same in the light and dark.

2.6. Response of zooxanthellae in symbiosis with *Cladocora caespitosa*

Quantification of *C. caespitosa* bleaching by means of grey scale was done as described by McLachlan and Grottoli (2021). Photographs were taken monthly using a Coolpix Nikon W300 camera in seawater into a glass aquarium with the same light and parameters. Every polyp of the colonies was manually selected on greyscale pictures and a mean greyscale was obtained for each polyp (from 0, pure black, to 255, pure white). A measure of percent whiteness was obtained for each colony from the mean of greyscales of its polyps corrected by a white standard.

Photophysiological responses of zooxanthellae in *C. caespitosa* were assessed using a pulse amplitude-modulated fluorometer (Diving-PAM, Walz) every Mondays, Wednesdays and Fridays with a break between 12 October and 11 November 2020 due to logistic constraints. The effective quantum yield ($\Delta F/F_m'$) was measured at midday, when the corals were exposed to the maximum irradiance (150 $\mu\text{mol photons m}^2 \text{ s}^{-1}$). Point measurements were done with a 0.8 s period saturating light pulse with the optical fiber at a distance of 1 cm and at a 90° angle to the top center of the coral colonies. The Diving-PAM settings of the “measuring light intensity” and “gain” were 6 and 4, respectively. When corals were severely bleached, the signal was too low to be measured. Then, the number of colonies with no data was reported on the graphic.

The symbiont density for *C. caespitosa* was measured on one polyp of each of the eight reference colonies sampled *in situ* and from one polyp of each of the experimental colony

removed with clamps after three months (12 October 2020) and at the end of the experiment (15 December 2020). Tissues were removed with an air-brush and homogenized with an ultra-turrax (IKA) in 0.5 ml of MilliQ water. Samples were then centrifuged at 800 g for 2 min (Centrifuge 5417C, Eppendorf®). The supernatant was removed, the pellet being symbiont material which was stored at -80°C in 1 mL of MilliQ water. Four 200 µL replicates of each sample were disposed in an haemocytometer (Malassez cell, Marienfeld) and images were collected on an inverted ZEISS Observer microscope (Zeiss, Jena, Germany) equipped with a 10X/0.3 EC Plan-Neofluar objective lens using a monochrome Flash 4 (Hamamatsu photonics, Japan). Chlorophyll fluorescence was acquired with 405 nm excitation and 659-701 nm emission. Mosaics of 9 mm² were typically acquired for brightfield and chlorophyll fluorescence using motorized stage driven under Zen software. We developed under ImageJ a set of macros to easily settle the parameters and analyze automatically in batch all the images providing a datasheet for statistics with among other measures, the absolute volumetric concentration of zooxanthellae. The macros are available on Github with tutorial and example : <https://github.com/SebastienSchaub/CounZoox/> (and Method S1-Appendix I, for more information). Zooxanthellae density was normalized by the volume of the sample and the polyp photosynthetic surface. The photosynthetic surface (PS) was determined as described by Rodolfo-Metalpa *et al.* (2006).

2.7. Data analysis

Linear mixed-models with a hierarchical (HLM) structure were used to evaluate the temperature treatments effects through time on the relative number of polyps per colony, the rates of respiration and net and gross photosynthesis as well as the percent whiteness and the effective quantum yield. Hierarchical linear models were used since data were compiled from repeated measures of the same pool of colonies over time. The models were fitted using the lmer function of the R package lme4 (Bates *et al.*, 2015). The fixed factors of the models were temperature and time, and experimental tank and header tank were assigned as random effects. The structure of the random term was selected by comparing models with different error structures using the Akaike information criterion (Table S1-Appendix I). Tukey's post-hoc tests were conducted when significant differences were detected.

Permutational Multivariate Analysis of Variance (PERMANOVA) with the adonis2 function of the vegan R package were used to test for the effects of the treatments on calcification rates, effective quantum yield and zooxanthellae density because these parameters are not

time dependent or based on repetitive measurements on the same organisms. Temperature and time factors were used as fixed factors.

3. Results

3.1. Change in number of polyps and calcification

During the month following the peak temperature, colonies of *C. caespitosa* under the Warming and the Heatwave treatments exhibited an increase in the number of polyps by budding (ρ) up to 6 times higher than before the peak and two months after, under the Warming and the Heatwave treatments during the peak was also 3 times higher than under the Ambient and Cool temperature treatments. Temperature and time significantly affected the relative number of polyps per colony (HLM, $F_{9,110}=41.22$, $p<0.001$, Figure 2.A, Table. S2-Appendix I) as colonies presented more new polyps or buds after the peak when maintained under higher temperatures (Cool < Ambient < Heatwave < Warming). Only one colony from the Warming treatment presented necrosis, with loss of six polyps during the second month after the peak.

A. calycularis presented very little to no change in number of polyps per colony (from -0.265 to 0.125, with 85% of colonies showing no change) and no difference between treatments and/or over time were found (HLM, $F_{9,108}=14.10$, $p=0.12$, Figure 1.2.B, Table. S2-Appendix I). However, colonies of *A. calycularis* under the Warming treatment presented very variable responses a month after the peak temperature, with some colonies showing growth with new polyps by budding while others lost polyps, showing denuded skeleton due to necrosis. The second month after the temperature peak, *A. calycularis* in the Warming treatment had four colonies with necrosis (ranging from 1 to 14 polyps loss).

Here, the observed necrosis in both species was the loss of tissue in the center of the calyx along with the death of the polyp, with occasionally remaining tissue in the external outline of the skeleton.

Calcification rate of both species was not significantly affected by temperature (PERMANOVA, $F_{3,28}=0.42$, $p=0.74$ for *C. caespitosa* and $F_{3,28}=1.36$, $p=0.27$ for *A. calycularis*, Figure 1.2.C and D, Table S3-Appendix I).

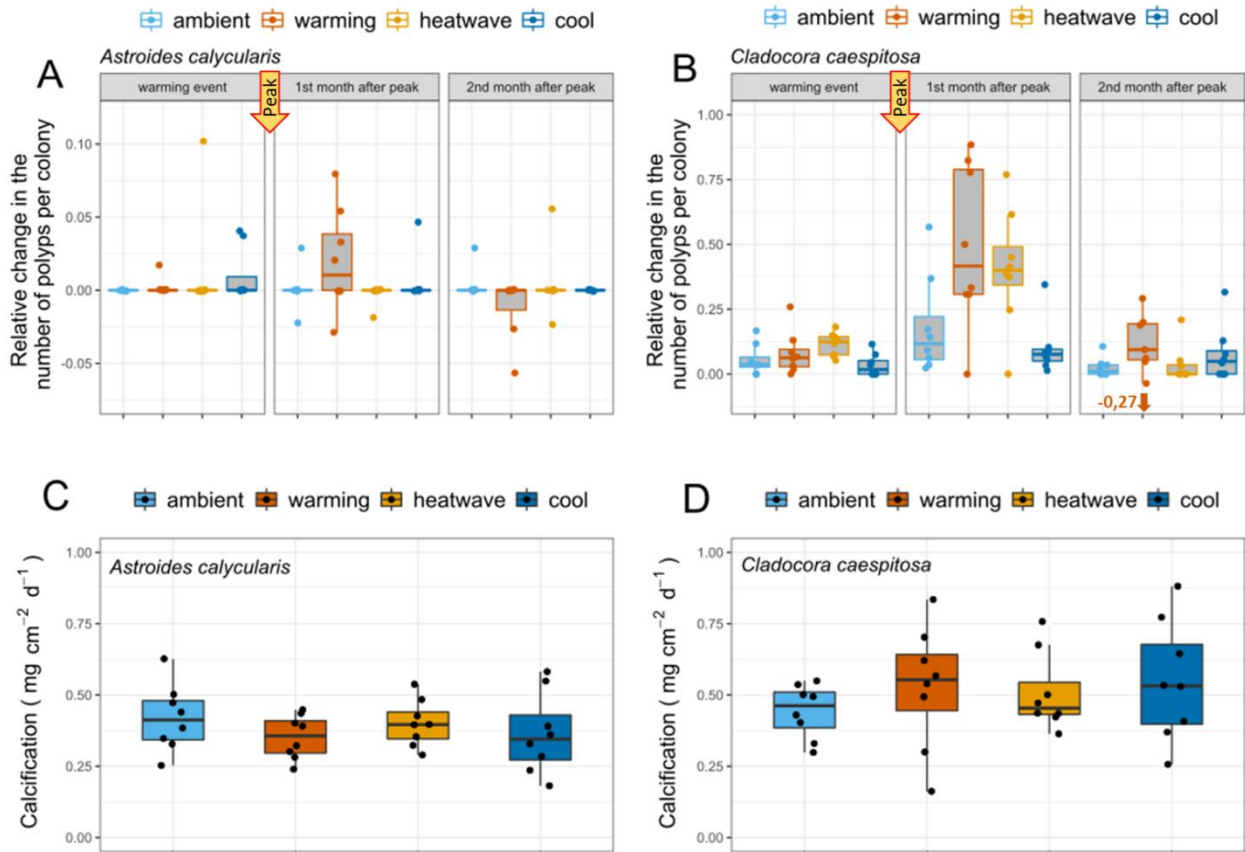


Figure 1.2 **Growth of *Astroides calycularis* and *Cladocora caespitosa* colonies under 3-month exposition to the temperature treatments.** Panels (A) and (B) show the monthly relative change in the number of polyps per colony before the peak of temperature (16 August 2020), the first month after the peak and the second month after the peak, panels (C) and (D) show the calcification rates by buoyant method for the first 3 months of experiment. Dots represent the relative change in number of polyps and calcification rates of each colony per experimental tank and boxes represent the median with the 25% and 75% quartiles. The color of the box indicates the temperature treatment. $n = 8$ per treatment. Note: -0.27 (outlier data point in Panel B) refers to a value we did not include in the graph to help reading.

3.2 Photosynthesis and respiration

The respiration rate of *C. caespitosa* was significantly affected by the interaction of temperature and time (HLM, $F_{9,110}=17.51$ $p<0.001$, Figure 1.3.A). It was 72% and 47% higher at the peak temperature than one month before and after the peak for all treatments, respectively. Corals in the Cool treatment presented lower respiration rates than in the other treatments (-0.013 $\text{mg O}_2 \text{ cm}^{-1} \text{ h}^{-1}$ for cool, -0.016 $\text{mg O}_2 \text{ cm}^{-1} \text{ h}^{-1}$ for Ambient and Warming, and -0.02 $\text{mg O}_2 \text{ cm}^{-1} \text{ h}^{-1}$ for Heatwave). Net photosynthesis was also significantly affected by the interaction of temperature and time (HLM, $F_{6,82}=15.02$ $p=0.02$, Figure 1.3.C), with

photosynthesis lower at the peak temperature than one month before or after the peak for all the treatments. However, gross photosynthesis was not impacted by temperature nor time, nor their interaction (Figure 1.3.D, Table. S2-Appendix I).

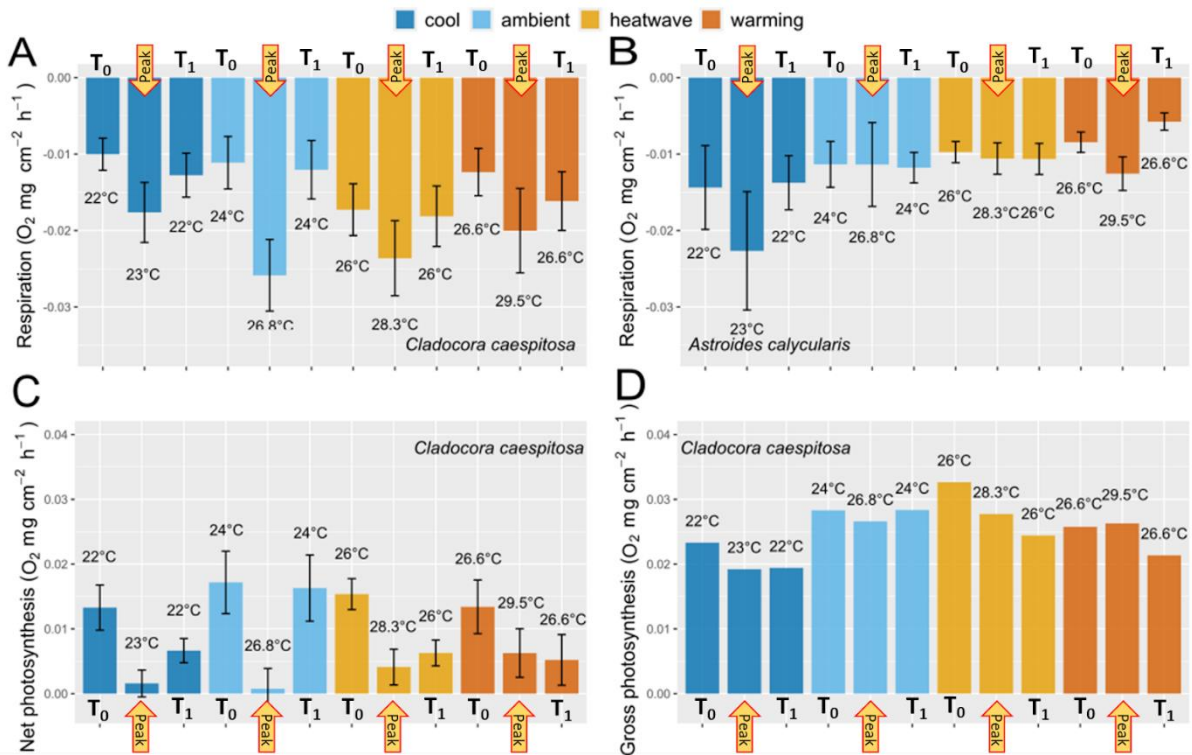


Figure 1.3 : Net and gross photosynthesis and dark respiration rates of *Cladocora caespitosa* and *Astroides calycularis* under the four temperature treatments at the beginning of the experiment (T₀), peak of temperature, and one month after the peak (T₁). Values are mean ± SE. Panel (A) shows dark respiration of *C. caespitosa*, panel (B) shows dark respiration of *A. calycularis*, panel (C) and (D) show net and gross photosynthesis of *C. caespitosa*. The color of the bars indicates the origin and the temperature treatment. n = 8 per treatment.

Concerning *A. calycularis*, respiration was affected by time (HLM, $F_{2,82}=7.24, p=0.03$). For the Cool and Warming treatments, respiration was 50% higher during the peak temperature, however for the Ambient and Heatwave treatments, no differences were found between the three dates of measurement (Figure 1.3.B, Table S2-Appendix I).

3.3 Response of zooxanthellae in symbiosis with *Cladocora caespitosa*

The effective quantum yield ($\Delta F/F_m$) of symbionts of *C. caespitosa* was significantly affected by the interaction between the treatments and time (PERMANOVA, $F_{3,1161}=3.01, p=0.03$, Figure 1.4.A, Table. S3-Appendix I). Colonies under the Warming treatment exhibited a decrease of $\Delta F/F_m$ a week after the peak temperature that reached a minimum of 0.615 ± 0.04 a month and a half after the peak. Under the Ambient and Heatwave treatments, the $\Delta F/F_m$

started to decrease a month after the peak temperature for a month down to 0.632 ± 0.028 and 0.642 ± 0.021 , respectively. No difference was observed on the $\Delta F/F_m$ under the Cool treatment over the all experiment. On 11 December 2022 (four months after the peak temperature), the $\Delta F/F_m$ values for all the colonies were back to values similar to the ones at the beginning of the experiment (before summer temperatures). A whitening of the colonies was observed in all the treatments except for the cool treatment. Percentage of colony's whiteness was significantly impacted by the temperature treatment and time ($F_{3,1325}=33.08$ $p<0.001$, Figure 1.4.B, Table. S2-Appendix I). The mean whiteness of the colonies at the beginning of the experiment was around 20%. It increased, three months after the peak temperature, to 40% in the Warming treatment, 37% under the Heatwave treatment and 35% in the Ambient treatment. The severely bleached colonies reached up to 60% of whiteness under the Warming treatment three months after the peak temperature. There was no significant difference in the percent whiteness in the Cool treatment.

The symbiont density also significantly changed in the combined treatments and over time (PERMANOVA, $F_{4,70}=3.11$ $p= 0.02$, Figure 1.4.C, Table S3-Appendix I). Zooxanthellae density was around 2×10^6 cells cm^{-2} at the beginning of the experiment. It decreased to 1×10^6 cells cm^{-2} under the four temperature treatments in the middle of the experiment (start of October) as well as for the *in situ* reference samples collected at the end of September. Interestingly, corals from all temperature treatments were able to regain their initial density (2×10^6 cells cm^{-2}) at the end of the experiment except in the Warming treatment, in which colonies remained about 1×10^6 cells cm^{-2} .

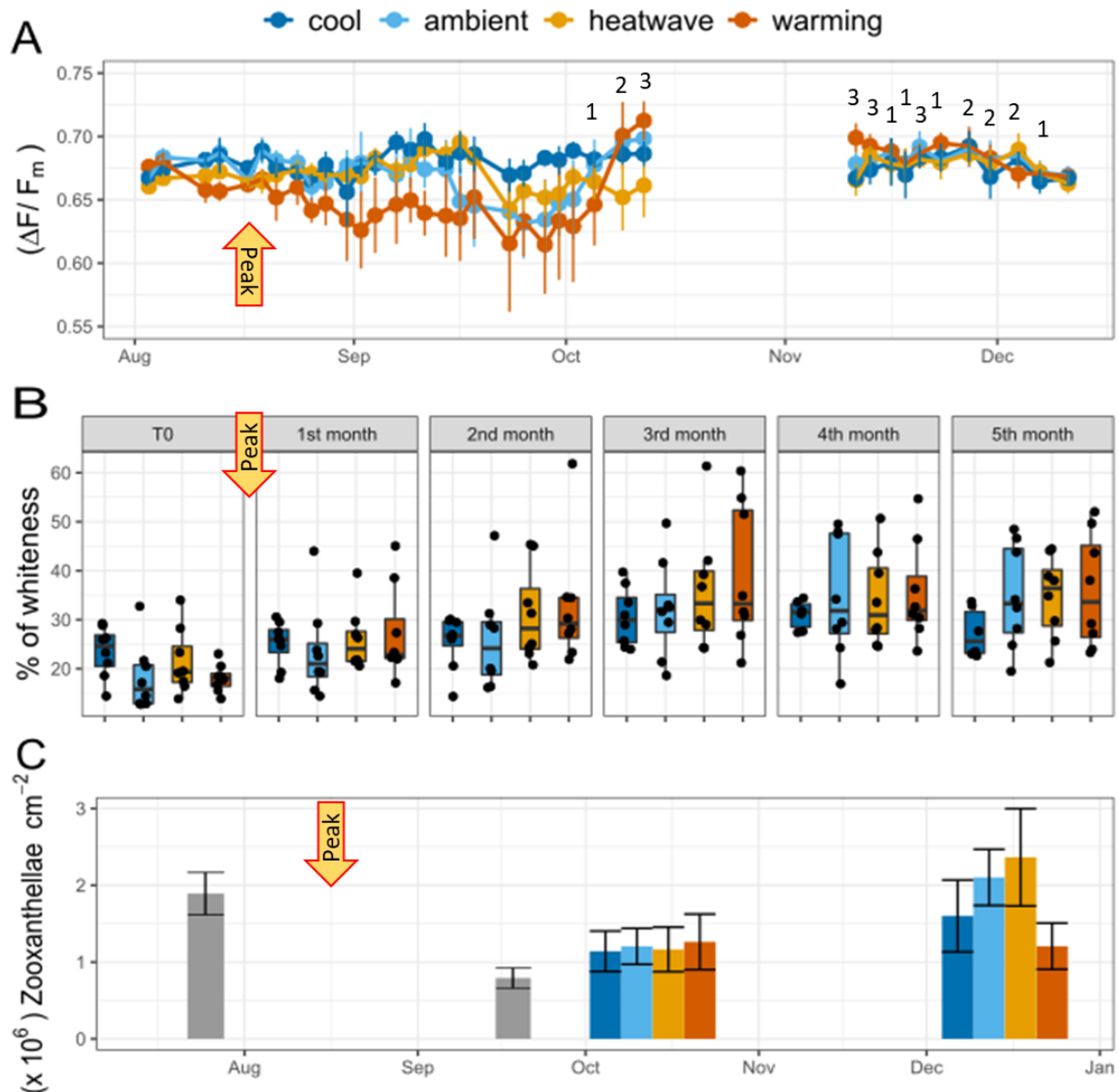


Figure 1.4 **Response of zooxanthellae of *Cladocora caespitosa* over time under the four temperature treatments.** Panel (A) shows the effective quantum yield profile over time. Values are mean \pm SE, numbers above the mean are the number of colonies in the warming treatment in which measurements with the diving PAM were not possible due to severe bleaching. Panel (B) shows the % of whiteness of the colonies over time. Dots represent the % of whiteness of each colony and boxes represent the median with the 25% and 75% quartiles. Panel (C) shows the density of zooxanthellae per photosynthetic surface. The gray bars indicate the “*in situ*” samples collected in the field. Values are mean \pm SE. The color of the box indicates the treatment. $n = 8$ per treatment. T0 = 27 July; Peak temperature = 16 August 2020, 1st month = 18 August; 2nd month = 17 September; 3rd month = 12 October; 4th month = 20 November and 5th month = 14 December 2022.

4. Discussion

For five months, colonies of the zooxanthellate coral *Cladocora caespitosa* and the azooxanthellate coral *Astroides calycularis* were exposed to four different laboratory

temperature treatments to assess the physiological response and the recovery after summer peak temperature. Our approach to apply the daily means of temperature based on nine years of field data with different warming scenarios and extreme events, such as MHWs, have never been applied to Mediterranean corals. It allowed to assess a realistic response of the two species to projected future temperature and their recovery during the natural decrease in temperature in the fall. This approach is all the more important, given that extreme events in the Mediterranean Sea are increasing, and summer 2022 might be presenting one of the worst MHWs, with sea surface temperature anomaly of + 5°C along the coast of France and Italy (<https://t-mednet.org/t-resources/2022-marine-heatwaves>). Our results showed an impact of high temperature on the number of polyps and respiration of both species and on the response of the symbionts of *C. caespitosa*. However, both species recovered with respect to respiration, regrowth of polyps of *A. calycularis*, and regain of pigmentation after bleaching for *C. caespitosa*. Combined, these results suggest a resilience of both species to the summer peak temperature, however, given the opposite responses on different parameters no clear generalizations can be made on the difference in tolerance between zooxanthellate or azooxanthellate species.

High temperature regimes are known to favour the increase of budding of *C. caespitosa* (Rodolfo-Metalpa *et al.* 2008, Kersting and Linares 2019), as observed in this study for both *C. caespitosa* and *A. calycularis*. However, *A. calycularis* presented a much lower increase in number of polyps than *C. caespitosa* possibly due to their difference in trophic strategy. *A. calycularis* being an azooxanthellate species, it relies solely on heterotrophy for nutrient provision. However, in summer, due to the establishment of a thermocline and overall low hydrodynamics, particulate organic matters are severely reduced in the Mediterranean Sea (Mena *et al.* 2019). Even if corals were fed three times a week during the experiment, summer might be a low growth period for *A. calycularis*. Moreover, *A. calycularis*' reproduction occurring from April to the end of June (Goffredo *et al.* 2011) and being an energy consuming process, no energy may have been possible to allocate to other functions such as growth by new polyps in summer (Coma *et al.* 2000). In contrast, summer could be a period of active growth for *C. caespitosa*, in which the maximum increase of calix height occurs between July and September (Peirano *et al.*, 2005). In the Warming treatment, the second month after the peak, one colony of *C. caespitosa* showed partial necrosis and lost six polyps while four colonies of *A. calycularis* lost up to 14 polyps. This necrosis was also observed *in situ* for both species under summer MHWs with temperatures which could reach 29°C (Kersting *et al.*

2013, Bisanti *et al.* 2022). *C. caespitosa* also showed an increase of respiration with the increase of temperature. Our results are in agreement with an increase in respiration of *C. caespitosa* from cold temperatures in winter to higher temperatures during summer (Rodolfo-Metalpa *et al.* 2010) or during a heat stress experiment at 26 and 28°C (Rodolfo-Metalpa *et al.* 2006). Temperature is commonly linked to an increase in oxygen consumption i.e. respiration by increasing the activity of enzymes (Clarke and Fraser, 2004). Even if temperature affected the number of polyps per colony of both species and the respiration rates for *C. caespitosa*, no impact was found on the one calcification rate for the two species. The three-month calcification rate was similar to the calcification recorded over a year, previously observed in these species (Rodolfo Metalpa *et al.* 2006, 2008, Movilla *et al.* 2016). However, in previous studies, both species showed a decrease in growth rate at temperatures between 24°C and 28°C (Rodolfo Metalpa *et al.* 2006, Movilla *et al.* 2016). In the present study, colonies of *C. caespitosa* were less impacted than reported by Rodolfo-Metalpa *et al.* (2006), which all died through necrosis at 26 and 28°C for 48 days.

As a proxy of warming, a latitudinal gradient of temperature along the coast of Italy where zooxanthellate and azooxanthellate corals can be found (8° range) have been studied and presented differences between the two trophic strategy (Goffredo *et al.* 2009).

Azooxanthellate corals were more resistant to increased temperature, with *Leptopsammia pruvoti* and *Caryophyllia inornata* having the same growth rate (Caroselli *et al.* 2012 and 2016) and the same reproductive efficiency among the different sites (Airi *et al.* 2017, Marchini *et al.* 2020). Zooxanthellate corals were affected with an inhibition of calcification and a decrease in reproduction efficiency along the gradient in *Balanophyllia europaea*, possibly due to inhibition of photosynthesis at elevated temperature (Goffredo *et al.* 2007, Airi *et al.* 2014). Since, in the present study calcification of both species was unaffected by temperature, one cannot conclude on a difference in tolerance. Furthermore *A. calycularis* exhibited a higher necrosis than *C. caespitosa* and a higher tolerance of respiration to temperature.

Data from our study confirmed bleaching of *C. caespitosa* colonies. Depending on the site in the Mediterranean Sea, *C. caespitosa* bleach (Kruzic *et al.* 2016 in Croatia, Mačić *et al.* 2019 in Montenegro) or presents direct necrosis with loss of colored tissue under heat stress (Rodolfo-Metalpa *et al.* 2005 in Italy, Kersting *et al.* 2013 in Spain) or both (Jimenez *et al.* 2016 in Cyprus). In our study, the loss of polyps by necrosis only happened after bleaching. The microbiome of the coral may play a role in these different responses to increasing

temperature. Necrosed colonies of *C. caespitosa* are associated with *Vibrio* pathogens while healthy colonies are not (Rubio-Portillo *et al.* 2018). *Vibrio* is a thermodependent bacteria, whose abundance increases with increasing temperature (Vezzulli *et al.* 2012). Furthermore, under warming seawater, the inoculation of *Vibrio* AK-1 in *Oculina patagonica* led to bleaching after 10 days (Kushmaro *et al.* 1997). It would thus be interesting to assess changes in the microbiome of *C. caespitosa* across locations. Exploratory options to limit bleaching or necrosis during MHWs in coral reefs could be to alter the microbiome of *in situ* populations (Peixoto *et al.* 2021). In the present study, the percent whiteness was also associated with the density of zooxanthellae. In all temperature treatments, we observed a decrease in symbiont density from the start of the experiment to one month after peak temperature. This was also observed *in situ*. This decrease in symbiont during summer to the beginning of fall and a recovery in winter is a natural seasonal variation in temperate corals, controlled by changes in irradiance and temperature (Rodolfo-Metalpa *et al.* 2008)

The first sign of the impact of temperature on the symbiosis of *C. caespitosa* was a decrease in the photosynthetic efficiency. Elevated temperatures are known to induce a saturation of photosynthetic electron transport leading to the degradation of the photosystem II and promoting the generation of reactive oxygen species that trigger bleaching by losing symbiont or by photopigment degradation (Oakley and Davy 2018). Effective quantum yield in the Warming, Heatwave and Ambient treatments, decreased after peaks at 30°C, 28.3°C and 26.8°C, respectively. Under cool treatment, it remained constant under temperatures never above 24°C. This is consistent with the fact that a temperature threshold of 24°C was reported in *C. caespitosa* (Rodolfo-Metalpa *et al.* 2006). Interestingly, gross photosynthesis did not change with time nor across temperature treatments for the three first months. Under summer temperature with a peak around 25°C, Rodolfo-Metalpa *et al.* (2010) observed an increase of net photosynthesis on *C. caespitosa* while a decrease was measured by Jurriaans *et al.* (2021). In this study, as most of the symbiotic response measured (decrease in zooxanthellae density and quantum yield and increase in the percent whiteness) occurred after this period of first three months, it might explain why gross photosynthesis was not impacted yet. However, as expected with the decreasing values of quantum yield, bleaching occurred three months after peak temperature in the Ambient and Heatwaves treatments, and severe bleaching occurred in the Warming treatment.

For both species a quick recovery of respiration after the peak temperature was observed. In all treatments, respiration rates were similar at same temperature, one month before and one

month after the peak, even if respiration increased during the peak. The same response was observed in *C. caespitosa* during a recovery phase of 14 days at 24°C after a heat stress at 26°C (Rodolfo-Metalpa *et al.* 2006). In the same experiment, but with a heat stress of 28°C, respiration of *C. caespitosa* was not back to initial rates after recovery. The fact that recovery was higher in our study compared to the experiment of Rodolfo-Metalpa *et al.* (2006) might be due to our recovery time being twice longer between the heat stress and the respiration measurements and because the heat stress applied in our study was a natural gradient profile and not a constant elevated thermal threshold. Furthermore, due to the increasing number and intensity of future warming events, Osborne *et al.* (2017) have projected that coral would need a longer recovery time between the events because of reduced capacity for coral to regain from repetitive stress.

C. caespitosa also recovered from heat stress. After three months post-peak, colonies from Heatwave, Ambient and Cool treatments had returned to the same effective quantum yield and density in zooxanthellae than at the beginning of the experiment and showed a decrease in the percentage of whiteness, thus a full recovery of symbiont population health after bleaching. The severely bleached colonies from the warming treatment recovered little pigmentation (Figure S4) and showed at the end a quantum yield back to the value of the beginning of the experiment but symbiont density was not back to the initial levels after four months. This is in agreement with the results of Rodolfo-Metalpa *et al.* (2006) who showed that after 14 days of recovery phase at 24°C corals exposed to heat stresses of 26 and 28°C, recovered their photosynthetic efficiency at the end of the experiment, while their density of symbiont remained almost five times lower than at the beginning of the experiment.

After losing polyps by necrosis (Figure S4-Appendix I), *A. calycularis* presented a regeneration of fully functioning polyps grown from remaining live tissue in the outline of dead polyps (Figure S3-Appendix I). This phenomenon was observed by Kramarsky-Winter and Loya (1996), if the mouth was necrosed, a new mouth was developed in the remaining tissue in the calyx of the necrosed polyp. A similar procedure was also observed in *C. caespitosa* when after a MHW, polyps retracted inside the corallites as a transitory resistance phase, which facilitated faster recovery of colonies in the field by reducing calyx diameter (Kersting and Linares 2019). Another regenerative mechanism has been observed on both studied species when elevated temperatures initiates necrosis: polyp bail-out or propagules (Kruzic *et al.* 2007, Serrano *et al.* 2018) and a full polyp can detach from its skeleton forming single skeletonless polyps (Serrano *et al.* 2018) to preserve tissue in order to regenerate a full

colony after a stress. These regenerative mechanisms are important for recovery and maintenance of the populations' genetic biodiversity, in order to extend the capacity for future adaptation of coral populations by natural selection (Bay and Palumbi 2014).

This study highlights a moderate impact of marine heatwaves on both *C. caespitosa* and *A. calycularis* with recovery of the impacted parameters during heat. However, the projected warming of +3.2°C for the end of the century was more aggressive, with severe bleaching of *C. caespitosa* and higher necrosis of *A. calycularis* leading to a partial recovery after four months. Future sea surface warming is expected to increase the number of MHWs (Smale *et al.* 2019). Thus, both species will be exposed to conditions similar to the ones of the present study in the future. Moreover, the direct impact of warming and MHWs on the physiological traits of the two studied species is not the only threat. Increase of temperature during MHWs also leads to the development of mucilaginous aggregate blooms on benthic communities (Schiaparelli *et al.* 2007). These macroalgae can smother *C. caespitosa* beds, causing a reduction of light and local hypoxia, and leading to necrosis (up to 55%) and 80% loss of coverage (de Biasi *et al.* 2021). Ocean warming also generates ocean deoxygenation because of warming-induced reduction in O₂ solubility, increase of biological O₂ demand and increased stratification (Oschlies *et al.* 2018), and is known to lead coral reefs into “dead zones” (Altieri *et al.* 2017). Furthermore, ocean warming is projected to increase along with ocean acidification (Kwiatkowski *et al.* 2020). Warming seems to be the main factor leading to the decrease of calcification and increase of respiration of adult colonies of *C. caespitosa* and *A. calycularis* (Rodolfo-Metalpa *et al.* 2010, Movilla *et al.* 2016). However, the combined effect of ocean warming and acidification decreases recruitment and post settlement growth of early life stages of *A. calycularis* (Carbonne *et al.* 2022), decreasing population connectivity and genetic diversity (Figueiredo *et al.* 2022). Understanding of how future temperature will impact these two Mediterranean habitat-forming species is essential in order to be able to preserve them as well as coastal benthic ecosystems

Authors contributions

C.C., N.T., S.C. and J.-P.G designed the study. C.C. and N.T. were involved with fieldwork. C.C., K.P and S.S. performed the experiments, S.S. designed the set of macros to measure the symbiont density, C.C. analyzed the data. C.C. wrote the first draft of the manuscript which was then finalized by all co-authors.

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Colony of *Astroides calycularis* at the CO₂ vent site Grotta del Mago

Chapter 2: Two temperate corals are tolerant to low pH regardless of previous exposure to natural CO₂ vents

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Abstract

Ocean acidification is perceived to be a major threat for many calcifying organisms, including scleractinian corals. Here we investigate (1) whether past exposure to low pH environments associated with CO₂ vents could increase corals tolerance to low pH and (2) whether zooxanthellate corals are more tolerant to low pH than azooxanthellate corals. To test these hypotheses, two Mediterranean colonial corals *Cladocora caespitosa* (zooxanthellate) and *Astroides calycularis* (azooxanthellate) were collected from CO₂ vents and reference sites and incubated in the laboratory under present-day (pH on the total scale, pH_T 8.07) and low pH conditions (pH_T 7.70). Rates of net calcification, dark respiration and photosynthesis were monitored during a six-month experiment. Monthly net calcification was assessed every 27 to 35 d using the buoyant weight technique, whereas light and dark net calcification was estimated using the alkalinity anomaly technique during 1 h incubations. Neither species showed any change in net calcification rates, respiration, and photosynthesis regardless of their environmental history, pH treatment and trophic strategy. Our results indicate that *C. caespitosa* and *A. calycularis* could tolerate future ocean acidification conditions for at least 6 months. These results will aid in predicting species' future responses to ocean acidification, and thus improve the management and conservation of Mediterranean corals.

1. Introduction

Ocean acidification describes the shift in carbonate chemistry caused by the uptake of anthropogenic CO₂ by the ocean (Caldeira and Wickett 2003, Gruber *et al.* 2019). It causes an increase in dissolved inorganic carbon and bicarbonate ion concentrations, along with a concurrent decrease in pH, carbonate ion concentration and its associated saturation state (Ω ; Orr *et al.* 2005). Calcifying species are perceived to be particularly vulnerable to ocean acidification (Kroeker *et al.* 2013a). For example, corals are particularly sensitive to changes in carbonate chemistry as their skeleton is made of aragonite, a metastable form of calcium carbonate that is less stable than calcite (Erez *et al.* 2011, Foster and Clode 2016). As a result, coral calcification is expected to decrease as seawater acidity increases (Gattuso *et al.* 1999). Coral calcification decreases by on average 22% under pH values expected by the end of the century under the high-CO₂ emissions scenario (RCP 8.5 from AR5 of IPCC; Chan and Connolly 2013). However, a range of contradictory species- and experience-specific responses have been described (*e.g.* Kornder *et al.* 2018), with some species such as massive *Porites* spp. being tolerant to ocean acidification (Fabricius *et al.* 2011). This resistance to ocean acidification was also described in several temperate and cold-water corals (*e.g.* Rodolfo-Metalpa *et al.* 2010). Such tolerance is seen in many studied parameters such as net calcification rate, respiration, photosynthetic physiology, calcifying fluid pH (McCulloch *et al.* 2012a, Wang *et al.* 2020). According to Varnerin *et al.* (2020) even juveniles from the temperate *Oculina arbuscula* were only slightly affected by ocean acidification.

A central question when assessing the future of corals in a high-CO₂ world is whether they have the capacity to acclimatize and further adapt to ocean acidification. This question remains unanswered as corals are generally long-lived organisms for which it is extremely challenging to study acclimatization and adaptation processes over several generations. Acclimatization refers to reversible phenotypic changes limited by genotype, occurring under naturally varying conditions by a physiological readjustment of the organism's tolerance levels (Edmunds and Gates 2008). In contrast, adaptation refers to changes in the genetic composition of a population by natural selection, in which traits will be passed on to the next generation (Savolainen *et al.* 2013). In a recent study, Cornwall *et al.* (2020) conducted a one-year experiment showing that coralline algae have the capacity to acclimatize to low pH after seven generations of exposure. Replicating such a study on corals is virtually impossible as they are long-lived organisms which only reach sexual maturity after several years. CO₂ vents

therefore offer a unique opportunity to investigate the response of corals to ocean acidification over long periods of time. In such systems, volcanic CO₂ bubbles from the seafloor which acidifies the surrounding seawater and creates an environment, with carbonate chemistry conditions that mimic those expected in the future (Hall-Spencer *et al.*, 2008, Camp *et al.* 2018, González-Delgado and Hernández 2018). By investigating individuals, species, and communities along transects radiating from the sources of CO₂, it is possible to substitute time for space. It must be pointed out, however, that CO₂ vents are imperfect windows into the future because they generally do not mimic other climate-related changes such as ocean warming. Furthermore, it is not possible to disentangle any changes in the mean versus variability of seawater pH (Hall-Spencer *et al.* 2008). Overall, these systems exhibit significant decreases in trophic complexity and biodiversity, as well as a major decrease in the abundance of calcifying organisms (Kroeker *et al.* 2013b, Linares *et al.* 2015, Teixidó *et al.* 2018). Corals living in the vicinity of CO₂ vents have been exposed to low and high variability pH conditions for an extended period of time (years to decades). They are therefore ideal models to investigate acclimatization and adaptation to ocean acidification (Fantazzini *et al.* 2015, Foo *et al.* 2018). Populations or individuals living in these variable and more extreme environments are hypothesized to be more tolerant to suboptimal environmental conditions such as acidification (*e.g.* Cornwall *et al.* 2018).

CO₂ vents have been used for transplant experiments between acidified and nearby reference sites with ambient pH and no vent activity. For example, a decline in calcification was described in the zooxanthellate corals *Cladocora caespitosa* and *Balanophyllia europaea* transplanted to a CO₂ vent site in Ischia (Rodolfo-Metalpa *et al.* 2011). The skeletal growth of *Porites astreoides* living in a natural pH gradient showed no acclimatization to ocean acidification despite their life-long exposure to low pH (Crook *et al.* 2013). Few laboratory studies have focused on temperate scleractinian corals from natural CO₂ vents despite the relevance of studying the tolerance of temperate coral populations to ocean acidification (Trotter *et al.* 2011, Teixidó *et al.* 2020). Such studies are important for understanding and elucidating how these species will respond to the suboptimal pH conditions projected for the end of this century.

The symbiosis between scleractinian corals and endosymbiotic algae, commonly referred to as zooxanthellae, has been studied thoroughly (Davy *et al.* 2012). Photosynthetic products from zooxanthellae contribute significantly to the energy budget of the scleractinian host (Allemand *et al.* 2011, Muller-Parker *et al.* 2015) and can play an important role in

calcification (Erez *et al.* 2011, Inoue *et al.* 2018). However, not all corals harbor zooxanthellae (azooxanthellate corals). Gibbin *et al.* (2014) demonstrated that zooxanthellae regulate intracellular pH under acidified conditions, whereas azooxanthellate corals can suffer from reduced intracellular pH which can lead to intracellular acidosis. It can thus be expected that photosynthesis may enhance calcification by (1) providing energy for this energetically costly process and (2) by increasing pH at the site of calcification (Allemand *et al.* 2011). In a transplant experiment in the CO₂ vents of Panarea, Prada *et al.* (2017) showed that the calcification of two azooxanthellate corals *Leptopsammia pruvoti* and *Astroides calycularis* decreased at low pH, whereas calcification of the zooxanthellate coral *B. europaea* was unaffected. A similar result was observed in pH-controlled aquaria by Ohki *et al.* (2013) as polyps of *Acropora digitifera* with symbionts showed higher calcification rates than polyps without symbionts under low pH, suggesting that symbionts confer tolerance to ocean acidification. However, in a study led by McCulloch *et al.* (2012b), azooxanthellate cold-water corals presented higher intracellular pH regulation than shallow-water zooxanthellate corals under acidification. As it is not truly known whether or not symbionts promote the maintenance of elevated pH at the site of calcification, it is interesting to compare zooxanthellate and azooxanthellate corals as they may display contrasting responses to ocean acidification.

The present study focuses on two Mediterranean long-lived corals of key relevance for conservation, the zooxanthellate species *C. caespitosa* (Linnaeus, 1767) and the azooxanthellate species *A. calycularis* (Pallas, 1766). *Cladocora caespitosa* is a unique zooxanthellate reef-building coral that provides a valuable ecosystem service in the Mediterranean Sea which is lacking large bioconstructions (Kersting and Linares 2012). *Astroides calycularis* is commonly found in the southwestern Mediterranean Sea and can be highly abundant in low light, shallow rocky habitats (Zibrowius 1995). These two scleractinian corals naturally occur at two newly discovered CO₂ vent systems along the coast of Ischia, Italy. *In situ* data obtained by Teixidó *et al.* (2020) showed variable responses in skeletal structure and growth patterns of *A. calycularis*, with colonies from the CO₂ vent site characterized by smaller size, fewer polyps, and less porous, denser skeletons. Thus, these two scleractinian coral species grown under elevated pCO₂ environments are great model systems for assessing tolerance to acidification. Here, two hypotheses are tested: (1) past exposure to low pH conditions confers tolerance to ocean acidification and (2) the coral-algal symbiosis modulates this tolerance. To test these hypotheses, corals from natural CO₂ vent

systems and reference sites were exposed to present-day (pH on the total scale, $\text{pH}_T \sim 8.07$) and low pH conditions ($\text{pH}_T \sim 7.70$) during a six-month long laboratory experiment, in which calcification, respiration and photosynthesis rates were assessed.

2. Material and Methods

2.1. Study sites

We studied populations of the corals *Cladocora caespitosa* and *Astroides calycularis* that naturally occur at two distinct CO_2 vent sites with low pH, and two reference sites with ambient pH and no vent activity at the island of Ischia, Italy (Figure S1-Appendix II).

Cladocora caespitosa occurs near a CO_2 vent site adjacent to rocky reefs at about 10 m depth (Chiane del Lume, hereafter Vent 1), whereas *A. calycularis* occurs in another CO_2 vent site between 1 - 2 m depth (Grotta del Mago, a semi-submerged cave hereafter Vent 2, where CO_2 vents occur at 5 m depth). Vent gas compositions at Vent 1 and Vent 2 were predominantly CO_2 (mean \pm SE: 93.9 ± 0.6 for Vent 1, 93.4 ± 1.0 for Vent 2), with undetectable levels of hydrogen sulfide ($< 0.0002\%$), and did not elevate the temperature (for more information on gas measurements, see Teixidó *et al.* 2020). The reference sites without any visible vent activity and exhibiting ambient pH were located: i) approximately 100 m west from Vent 1 for *C. caespitosa* (hereafter Ambient 1, 10 m depth) and ii) 1.5 km away from Vent 2 for *A. calycularis* (San Pancrazio, an overhang, hereafter Ambient 2, 1-2 m depth) (Figure S1-Appendix II).

2.2. Coral field surveys

The *C. caespitosa* abundance was quantified using 12 quadrats of 50 cm x 50 cm, which were randomly placed along the rocky reef at 10 m depth at the two sites ($n = 24$ quadrats in total). Abundance was assessed by counting the number of colonies found in each quadrat. In addition, size of colonies was determined by measuring the major colony diameter with a plastic ruler (with an error of ± 1 cm) (Peirano *et al.* 2001). Major colony diameter was selected as the best colony size descriptor (Kersting 2012). The *A. calycularis* abundance was quantified using 12 photo-quadrats (25 x 25 cm) between 1 and 2 m depth in the two other sites ($n = 24$ quadrats in total, Vent 2, Ambient 2). Size frequency-distribution was calculated by counting the number of polyps of each colony and each colony was then pooled into one of five size classes (I: 1-5 polyps; II: 6-10 polyps; III: 11-15 polyps; IV: 16-20 polyps; V: > 20 polyps). The survey's depth for *C. caespitosa* and *A. calycularis* corresponded to the depth

range at which colonies were sampled for the experiment (see below). Abundance and size-frequency data for *A. calycularis* have been described previously in Teixidó *et al.* (2020).

2.3. *In situ* pH_T and carbonate chemistry associated with CO₂ vents and ambient pH sites

SeaFET™ Ocean pH sensors (Satlantic) were deployed to quantify variation in pH at the two CO₂ vent and the two ambient pH sites. Measurements were taken every 15 minutes. One sensor was deployed at the Vent 1 site from May 7 to 21, 2019, and one sensor was deployed at the Ambient 1 site during the same period. At the semi-submerged cave (Vent 2), sensors were deployed in May-June 2019 (before summer) and in September 2018 (after summer). Dates of deployment for Vent 2 were from September 8 to September 24, 2018 and from May 30 to June 18, 2019 at Vent 2. One sensor was deployed in the reference area Ambient 2 during the same period. Before deployment, the SeaFETs were calibrated with ambient pH water in the aquarium facilities at the Center Villa Dohrn (Ischia, Italy). The mean offset between calibration samples and calibrated SeaFET pH was ± 0.002 units, indicating high quality pH dataset. Discrete water samples were collected using Niskin bottles to measure the carbonate system parameters during the pH sensor deployment. Samples for total alkalinity (A_T) were collected using standard operating protocols. The HCl (0.1 M) titrant solution was calibrated against certified reference materials distributed by A.G. Dickson (CRM, Batches #153, #171, and #177). Precision of the A_T measurements of CRMs was < 2 and $< 1 \mu\text{mol kg}^{-1}$ from nominal values. A_T and pH_T were used to determine the remaining carbonate system parameters at *in situ* temperature and depth of each sampling period in the R package seacarb v3.2.12. For full details of pH sensors deployment, calibration, and total alkalinity standard operating protocols, see Teixidó *et al.* (2020).

2.4. Coral collection

A total of 81 colonies of *C. caespitosa* and *A. calycularis* were randomly sampled for the aquarium experiment. Fourteen (Vent 1) and sixteen (Ambient 1) colonies of *C. caespitosa* were collected at 10 m depth; twenty-seven (Vent 2) and fourteen (Ambient 2) colonies of *A. calycularis* were collected from 1 to 2 m depth. A portion of approximately 3 x 3 cm was gently dislodged from each colony using a hammer and chisel. They were placed in coolers filled with seawater changed regularly and maintained in Ischia for two days prior to transportation to the aquarium facilities of the *Laboratoire d'Océanographie de Villefranche*, France. The duration of the transfer was less than 10 h; colonies were wrapped in seawater-

soaked paper tissues. The colonies were cleaned, tagged with a number stuck under it using water-resistant epoxy glue, and maintained at an ambient pH_T of ~ 8.05 during 2 weeks.

2.5. Experimental set-up and treatments

Colonies of *C. caespitosa* and *A. calycularis* were maintained in two constant pH treatments during six months (202 days), one “present-day” treatment with a $\text{pH}_T = 8.08 \pm 0.01$ (mean \pm SE, $n = 348$, total number of weekly pH_T measures in each 12 present-day experimental tank) and one “low pH” treatment with a $\text{pH}_T = 7.72 \pm 0.01$ (mean \pm SE, $n = 348$, total number of weekly pH_T measures in each 12 low pH experimental tank) corresponding to the pH values expected by the end of the century under the RCP 8.5 CO_2 emissions scenario. More specifically, 14 colonies from Vent 1 and 16 colonies from Ambient 1 of *C. caespitosa* and 27 from Vent 2 and 14 from Ambient 2 of *A. calycularis* were used for this experiment. The vent-low pH condition was used to test the acclimation or long-term tolerance of the colonies to low pH in the lab, after a life-time of exposure to *in situ* low pH. The ambient-low pH condition was used to test the short-term tolerance to low pH of colonies that have not been exposed to low pH before. The vent-present day condition tested the response to ambient pH of colonies exposed in the long-term to *in situ* low pH. The ambient-present day condition was the control. There were six independent 5 L experimental tanks for each of the four conditions (origin \times pH treatment), for a total of 24 experimental tanks. Each experimental tank contained one to three colonies of each species, depending on their size, to get approximately the same total amount of polyps per tank (Table S1-Appendix II). Seawater pumped from Villefranche Bay at 5 m depth was continuously flowing into six 25 L header tanks, used as water storage and to maintain gravity pressure for water flow into the experimental tanks. Three header tanks were maintained at $\text{pH}_T \sim 8.07$ (present-day treatment) and the other three at $\text{pH}_T \sim 7.7$ (low pH treatment). pH was controlled in the header tanks using pH controllers (APEX, Neptune Systems) which regulated the delivery of pure CO_2 . Each header tank gravity-fed four experimental four-liter tanks at a rate of 100 ml min^{-1} (Figure 2.1).

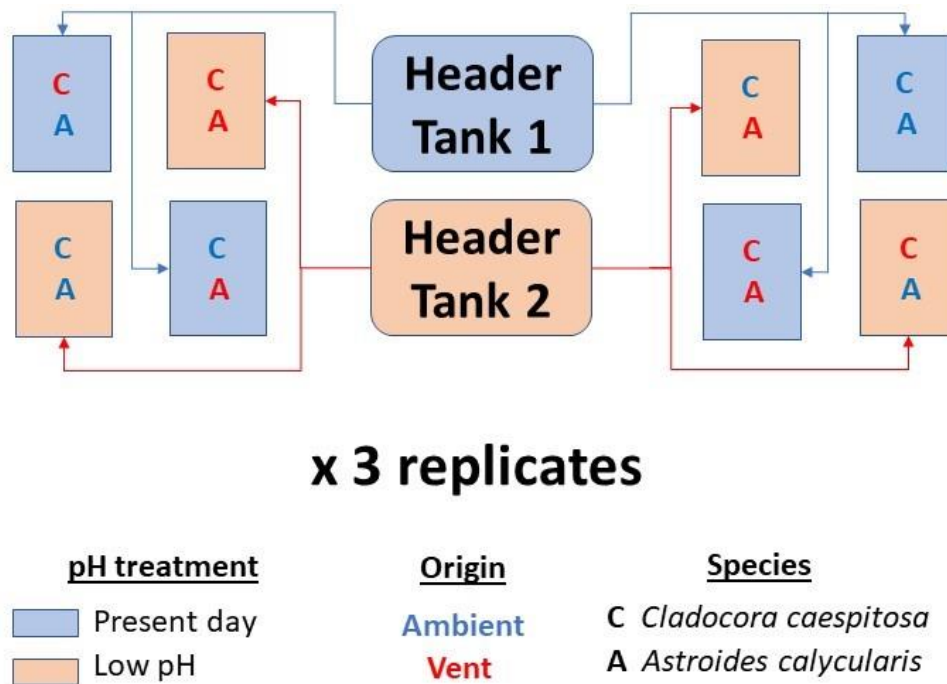


Figure 2.1 : Experimental set-up used to test the effects of origin (ambient vs vent site) and pH treatment (present day, $\text{pH}_T = 8.07 \pm 0.01$ and low pH, $\text{pH}_T = 7.72 \pm 0.01$) on the coral *C. caespitosa* and *A. calycularis*. The experimental set-up was repeated 3 times, resulting in 6 header tanks, and 24 experimental tanks in which corals were randomly assigned.

Light was provided by 89 W LED light bars (Aquadistik, Aqualumix). Irradiance gradually increased from 0 at 06:00 to a maximum irradiance of $180 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ between 11:00 and 14:00, and gradually decreased to 0 at 18:00. According to the attenuation coefficients of PAR in Villefranche Bay calculated in Martin and Gattuso (2009), the maximum irradiance levels used in the study ($180 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) is within the irradiance range observed at 10 m, where *Cladocora caespitosa* can be found in the Mediterranean Sea (154 to $449 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). As *A. calycularis* were collected in a semi-submerged cave and an overhang, dark plastic bags covered one side of the experimental tanks to shade colonies from direct light. The 24 experimental tanks were kept in 6 water baths under controlled temperature following Ischia's natural changes (Figure S2-Appendix II, APEX, Neptune Systems). Submersible pumps (NEWA) provided water motion in each experimental tank. Three times a week, each tank was provided with a 30 mL solution of freshly hatched brine shrimps (*Artemia* sp.) for feeding.

2.6. Health status of experimental colonies

With pictures at the start of the experiment, each colony has been classified into two categories: colonies with loss of coenosarc (tissue between polyps) and colonies with tissue

connecting their polyps. The number of dead polyps per colony (complete disappearance of tissue in the calice) was counted by comparing the pictures of the same colony at the start and the end of the experiment.

2.7. Carbonate chemistry in experimental tanks

pH in the header and experimental tanks was measured weekly using a handheld pH-meter (826 pH mobile, Metrohm) calibrated with a TRIS buffer (batch #T33 provided by A. Dickson, Scripps Institution of Oceanography, USA) before each set of measurements. Total alkalinity (A_T) of each experimental and header tank was measured weekly during the first four weeks in order to ascertain whether conditions were altered by metabolic activity. Subsequently, and for the remainder of the experiment, A_T was measured every month in four randomly selected tanks and one header. A_T was determined by potentiometric titration using a Metrohm 888 Titrande following the method of (Dickson *et al.* 2007). Titrations of certified reference material (Batch #186) provided by A. Dickson were used to assess the accuracy of the measurements and were within $6.5 \mu\text{mol kg}^{-1}$ of the reference value. Ischia's temperatures are between 0.87 to 2.67°C higher than in the Bay of Villefranche during the studied period. Water pumped on the Bay of Villefranche was heated to be as close as possible to Ischia's temperatures. Salinity at Ischia and in the Bay of Villefranche-sur-Mer are similar. Salinity data were gathered from the salinity recorded weekly in the Bay of Villefranche by the *Service d'Observation Rade de Villefranche, SO-Rade*, of the *Observatoire Océanologique and the Service d'Observation en Milieu Littoral, SOMLIT/CNRS-INSU*. pH_T , temperature, A_T and salinity were used to calculate the other carbonate chemistry parameters using the R package seacarb (Gattuso *et al.* 2020) (Table 1, Table S2-Appendix II).

2.8. Six-month and monthly calcification

Calcification rate was assessed using the buoyant weight technique (Davies 1989). Weighing was done every 27 to 35 days. Changes in wet weight were converted to dry weight using the following equation:

$$\text{dry weight} = \frac{\text{wet weight}}{\left(1 - \frac{\text{water density}}{\text{aragonite density}}\right)}$$

with an aragonite density of 2.93 g cm^3 . Six-month calcification rates corresponding to the entire experiment duration were determined as the change in dry weight between the first and the last weighing, normalized by the corals living tissue surface at the end of the experiment

and the number of days of the experiment (202 days). Regular rates of calcification over time were normalized to the surface of the corals and to the number of days between weighing (~30 d). Surfaces were determined by the aluminum foil technique for *C. caespitosa* (Marsh 1970) because these 3D colonies were impossible to analyze with 2D pictures. *A. calycularis* surfaces were measured by photographic analysis with the software ImageJ for (IMAGEJ, NIH US Department of Health and Human Services) as they had flat shape. Surface measurements were completed at the start and end of the experiment. Marginal changes in surface area were observed between the beginning and end of the experiment.

2.9. Dark respiration, gross photosynthesis and short-term calcification

Dark respiration, net photosynthesis and short-term calcification were determined during less than 1 h incubations in 500 ml transparent perspex chambers, placed in a temperature-controlled water bath to maintain a constant temperature. Incubations were carried out a month after the start of the experiment (hereafter “start”) and a month before the end of the experiment (hereafter “end”) at the same temperature of 20°C. Magnetic stirrers were used to provide water motion in the incubation chambers. Each colony was transferred to an individual chamber filled with water from the experimental tanks from which the corals were taken for the incubation. A blank incubation with no coral colony and filled with water from the same header tank was performed for each four experimental tanks. Incubations lasted 30 to 60 min depending on the magnitude of the metabolic activity. To measure oxygen uptake during respiration, colonies were maintained in darkness by covering the chamber with an opaque plastic bag and incubations started after a 10 min acclimation period. Production of oxygen by photosynthesis was only measured on colonies of *C. caespitosa* under an irradiance of 180 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Oxygen saturation within the chambers was measured every 5 s using a fiber optic oxygen sensor (PreSens, OXY-4 mini), which was calibrated at 0% saturation in an oxygen-free solution created by saturating seawater with sodium sulfite, and at 100% saturation in an oxygen-saturated seawater obtained by bubbling ambient air during 5 min. The difference in oxygen saturation between the beginning and end of the incubation was converted to $\text{O}_2 \text{ mg L}^{-1}$ using the `gas_O2sat` function of the `marelac R` package (Soetaert *et al.* 2020). Oxygen consumption was then normalized by coral surface area, incubation time and chamber volume to obtain the rates of respiration (negative in dark conditions) and net photosynthesis (oxygen production in excess of the respiratory demand under light conditions). The rate of gross photosynthesis was calculated by subtracting the

respiration rate in dark to the rate of net photosynthesis, with the assumption that respiration rate is the same under light and dark conditions.

Water samples were collected at the beginning and end of the incubation for each colony for calcification determination using the alkalinity anomaly technique (Smith and Key 1975). This method relies on the stoichiometric relationship between total alkalinity and calcification, where the precipitation of one mole of CaCO_3 lowers the total alkalinity by two moles. Alkalinity anomalies were normalized by the coral surface area, incubation time, and chamber volume to calculate light and dark calcification rates. The alkalinity anomaly method was only applied to the zooxanthellate *C. caespitosa*.

2.10. Data analysis

Biological surveys: A generalized linear mixed model was used to test for differences in abundance (poisson family) as a function of site (fixed factor, 2 levels) and quadrat as a random effect. Chi-square contingency tables were used to compare the size–frequency distributions between sites. *Laboratory experiment:* The assumptions of normality and equality of variance were evaluated through graphical analyses of residuals using QQ plot functions in R software. Mean values of the colonies in the experimental tank were used as a replicate for six-month calcification. Each incubation was treated as a statistical replica because they were done in individual chambers on single individuals. The effect of header tanks was first tested with an ANOVA and since its significance was negligible ($p > 0.05$), it was not considered in subsequent analyses. A two way-ANOVA was then used to test for differences in net calcification caused by the origin (vent or ambient pH sites) and the pH treatments (present-day or low pH) for both *C. caespitosa* and *A. calycularis*. For net calcification as a function of time, a three-way ANOVA was applied, with origin, pH treatment, and time as fixed factors. Tukey’s post hoc tests were conducted when significant differences were detected. Three-way repeated measure ANOVA was used for dark respiration, gross photosynthesis and short-term calcification since the same colonies of *C. caespitosa* and *A. calycularis* were used at the start and end of the incubations. Results have been reported as means \pm standard error of the mean (SE) hereafter, unless otherwise noted.

3. Results

3.1. *In situ* environmental conditions and populations of corals

Water carbonate chemistry and *in situ* monitoring of seawater pH_T (pH on the total scale) at the CO_2 vent and reference sites with ambient pH showed mean $\text{pH}_T = 7.91$ at Vent 1, $\text{pH}_T = 7.91$ at Vent 2, $\text{pH}_T = 8.05$ at Ambient 1, and $\text{pH}_T = 7.97$ at Ambient 2, respectively (Table 2.1). The field surveys showed significant lower abundance of *C. caespitosa* at Vent 1 compared to Ambient 1 (1.8 ± 0.16 colonies versus 3.3 ± 0.35 , $Z = 2.2$, $p < 0.02$, Figure S3-Appendix II). No large colonies of more than 21 cm were found at Vent 1 (Figure S1-Appendix II). This reduction of size was also observed for *A. calycularis* at Vent 2 (92 % had up to 10 polyps versus 72 % in Ambient 2), which differed significantly between sites ($\chi^2 = 76.7$, $p < 0.0001$). Abundance of *A. calycularis* was lower in Vent 2 (67.1 ± 10.3 at Vent 2 versus 80.3 ± 14.5 at Ambient 2, $Z = 2.65$, $p < 0.08$).

3.2. Experimental conditions

Weekly temperature in the tanks varied according to natural changes in the summer temperature profile at 10 m depth in Ischia, from 19.08 ± 0.06 °C at the end of May 2019 (mean \pm SE, $n = 24$) to 21.51 ± 0.07 °C at the beginning of December (mean \pm SE, $n = 24$) with a maximum value of 25.17 ± 0.15 °C in August (mean \pm SE, $n = 24$, Figure S2 Supplementary materials). The “present-day” treatment was maintained at a mean pH_T of 8.06 ± 0.06 ($n = 347$) whereas “low pH” treatment had a pH_T of 7.73 ± 0.09 ($n = 348$). Salinity was 37.8 ± 0.5 ($n = 25$).

Colonies of *Astroides calycularis* had physical differences depending on their site of origin. 85% (23/27 colonies) of vent and 14% (2/14 colonies) of ambient colonies did not present coenosarc (i.e. tissue between polyps). Furthermore, by comparing photos from the beginning and end of the experiment, it was observed that none of the *A. calycularis* colonies from ambient suffered polyp death, while three vent colonies showed 2/5, 1/7 and 4/9 dead polyps (number of dead polyps / total number of polyps in the colony). No polyp loss was observed in *Cladocora caespitosa*.

TABLE 2.1. Measured and estimated seawater physiochemical parameters at the CO₂ vent sites, reference areas with ambient pH and both pH treatment in the experimental tanks for salinity (S), temperature (T), total alkalinity (A_T), dissolved inorganic carbon (C_T), pH_T, pCO₂, calcite (Ω_c) and aragonite (Ω_a) saturation. Values are means ± SD with 25th and 75th percentiles. Calculated concentrations of C_T, pCO₂, Ω_c and Ω_a are shown. 1: Parameters measured from discrete water samples; 2: parameters measured with *in situ* sensors. pH conditions: vent system (low pH); ambient pH; ambient pH experimental treatment (present day); acidified pH experimental treatment (low pH).

Local name	pH conditions	S	T (°C)	A _T (μmol kg ⁻¹)	C _T (μmol kg ⁻¹)	pH _T	pCO ₂ (μatm)	Ω _c	Ω _a
Vent1	Low pH	37.9 ¹ ± 0	16.8 ² ± 0.4	2532 ¹ ± 16	2324 ± 47	7.91 ²	626 ± 311	3.69 ± 0.61	2.39 ± 0.39
		(37.9, 37.9), n=3	(16.6, 17.1), n=1326	(2522, 2545), n=25	(2292, 2341), n=1326	(7.89, 7.99), n=1326	(497, 643), n=1326	(3.43, 4.13), n=1326	(2.22, 2.67), n=1326
Ambient 1	Ambient pH	37.3 ¹ ± 0	17.3 ² ± 0.4	2618 ¹ ± 15	2338 ± 20	8.05 ²	448 ± 42	4.80 ± 0.30	3.11 ± 0.19
		(37.2, 37.3), n=3	(17.0, 17.6), n=1331	(2607, 2633), n=14	(2324, 2350), n=1331	(8.03, 8.07), n=1331	(417, 471), n=1331	(4.61, 5.01), n=1331	(2.98, 3.24), n=1331
Vent 2 (Sept 2018)	Low pH	37.3 ¹ ± 0.2	25.9 ² ± 0.2	2564 ¹ ± 7	2542 ± 79	7.65 ²	2905 ± 1664	1.68 ± 0.59	1.11 ± 0.39
		(37.2, 37.5), n=9	(25.8, 26.0), n=1530	(2561, 2566), n=9	(2477, 2585), n=1530	(7.58, 7.90), n=1530	(1724, 3438), n=1530	(1.21, 2.21), n=1530	(0.80, 1.47), n=1530
Vent 2 (June 2019)	Low pH	37.8 ¹ ± 0	21.8 ² ± 2.1	2541 ¹ ± 20	2352 ± 89	7.74 ²	983 ± 868	3.56 ± 1.05	2.33 ± 0.69
		(37.8, 37.8), n=7	(19.8, 23.8), n=1841	(2533, 2550), n=7	(2289, 2389), n=1841	(7.74, 7.93), n=1841	(590, 978), n=1841	(2.96, 4.39), n=1841	(1.94, 2.88), n=1841
Ambient 2	Ambient pH	37.9 ¹ ± 0	26.4 ² ± 1	2642 ¹ ± 17	2324 ± 21	7.97 ²	556 ± 57	5.53 ± 0.32	3.67 ± 0.22
		(37.9, 37.9), n=7	(25.9, 27.0), n=1691	(2629, 2659), n=17	(2310, 2338), n=1691	(7.94, 8.00), n=1691	(513, 597), n=1691	(5.31, 5.75), n=1691	(3.53, 3.82), n=1691
Experimental tanks	Present day	37.8 ± 0.5	22.6 ± 1.9	2542 ± 54	2206 ± 63	8.06 ± 0.06	415 ± 70	5.65 ± 0.69	3.71 ± 0.46
		(37.3, 38.3), n=25	(20.7, 24.5), n=347	(2488, 2596), n=347	(2143, 2269), n=347	(8.00, 8.12), n=347	(345, 485), n=347	(4.96, 6.34), n=347	(3.25, 4.17), n=347
Experimental tanks	Low pH	37.8 ± 0.5	22.7 ± 1.9	2542 ± 54	2389 ± 65	7.73 ± 0.09	1048 ± 237	2.99 ± 0.56	1.97 ± 0.37
		(37.3, 38.3), n=25	(20.8, 24.6), n=348	(2488, 2596), n=348	(2324, 2454), n=348	(7.64, 7.82), n=348	(811, 1285), n=348	(2.43, 3.55), n=348	(1.60, 2.34), n=348

3.3. Six-month, monthly and short-term calcification rates

The rates of six-month calcification of *C. caespitosa* and *A. calycularis* were not affected by the origin and the pH treatment (Figure 2.2; Table S3-Appendix II; Two-way ANOVA, $F_{1,20}=1.778$, $P = 0.2$ for *C. caespitosa*, $F_{1,20}= 0.13$, $P = 0.72$ for *A. calycularis*).

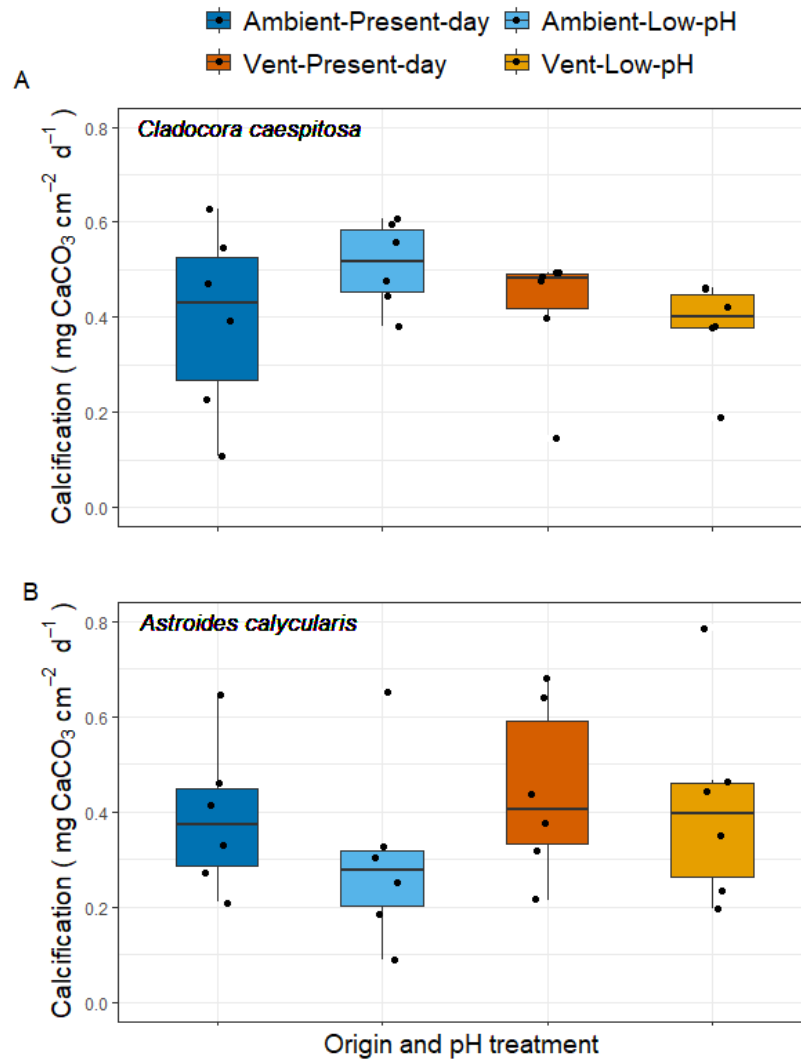


Figure 2.2 Six-month calcification rates of *C. caespitosa* and *A. calycularis* collected from vent and ambient sites exposed to the two pH treatments (pHT = 8.08 ± 0.01 , present day; pHT = 7.72 ± 0.01 , low pH) during the entire duration of the experiment (202 days). Panel A) shows *Cladocora caespitosa* and panel B) shows *Astroides calycularis*. Dots represent the mean calcification rates of colonies per experimental tank and boxes represent the median and the 25 and 75% quartiles. The color of the box indicates the origin and the treatment. n = 6 per pH treatment and origin.

No statistically significant effect of origin and pH treatment on calcification rate was found in *C. caespitosa* over time (Figure 2.3.A; $F_{1,110} = 1.919$, $P = 0.17$). However, different calcification rates with time were found for *A. calycularis* (Figure 2.3.B, $F_{1,112} = 26.2$, $P < 0.001$), with significantly higher calcification measured during the first month of the experiment (1.0 ± 0.2 mg CaCO₃ cm⁻² d⁻¹). The mean monthly calcification rate was 0.2 ± 0.1 mg CaCO₃ cm⁻² d⁻¹ during the following months of experiment.

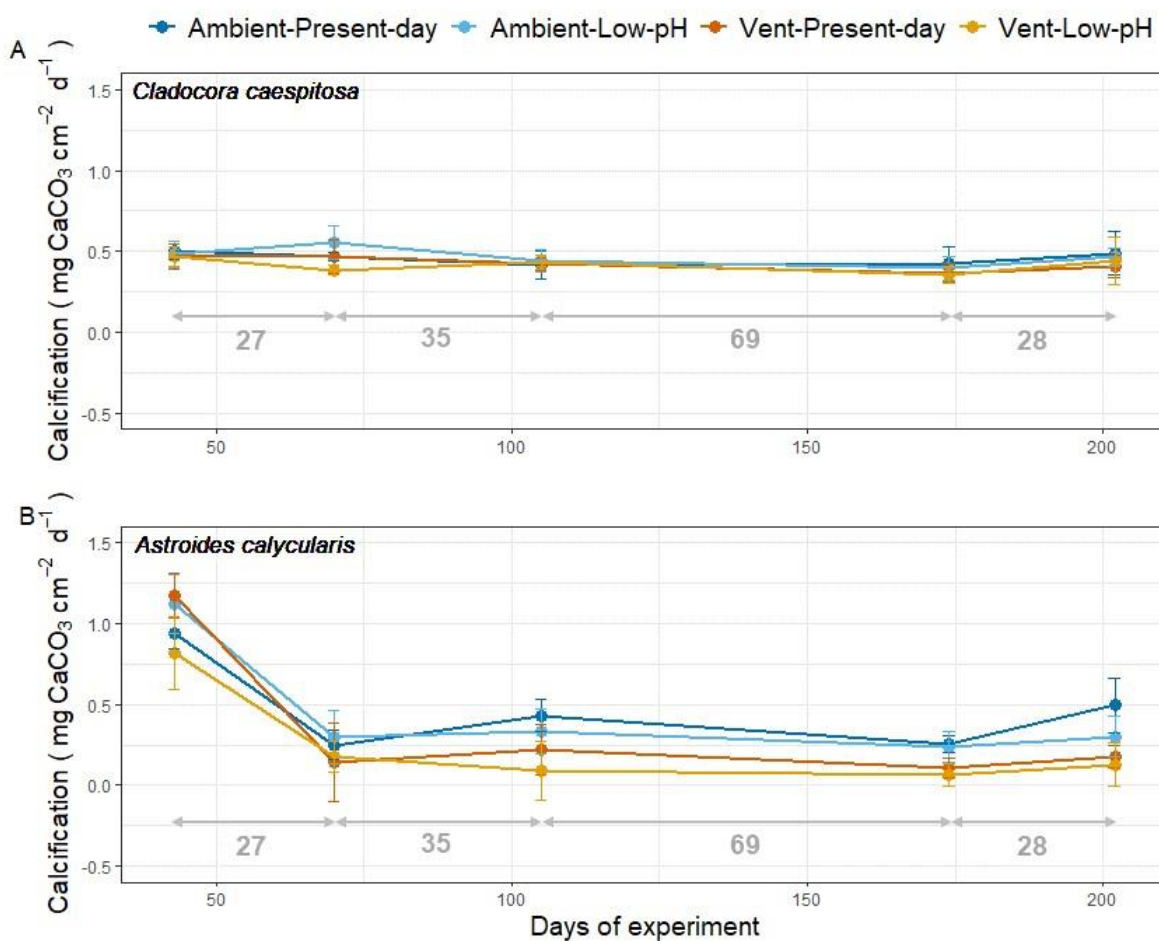


Figure 2.3 Calcification rates of *C. caespitosa* and *A. calycularis* from vent and ambient sites under the two pH treatments ($\text{pH}_T = 8.08 \pm 0.01$, present day; $\text{pH}_T = 7.72 \pm 0.01$, low pH) over time (every 27 to 35 days). Values are means \pm SE, Panel A) *Cladocora caespitosa* and B) *Astroides calycularis*. The color of the dots and lines indicates the origin and the treatment. We had a technical problem with day 139 weighing which is therefore not represented here. Numbers below arrows between dots represent the number of days between measurements. $n = 6$ per pH treatment and origin.

Short-term calcification of *C. caespitosa* in the dark and in the light was not significantly different at the start (Figure 2.4.A and 2.4.B; 10 ± 1.1 and $15.65 \pm 1.85 \mu\text{g CaCO}_3 \text{ cm}^{-2} \text{ h}^{-1}$, respectively) and the end of the experiment (Figure 2.4.C and 2.4.D; 11.42 ± 1.40 and $25.21 \pm 3.03 \mu\text{g CaCO}_3 \text{ cm}^{-2} \text{ h}^{-1}$, respectively). Colonies light and dark calcification were also not affected by origin and pH treatment (Figure 2.4; $F_{1,40} = 0.07$, $P = 0.795$).

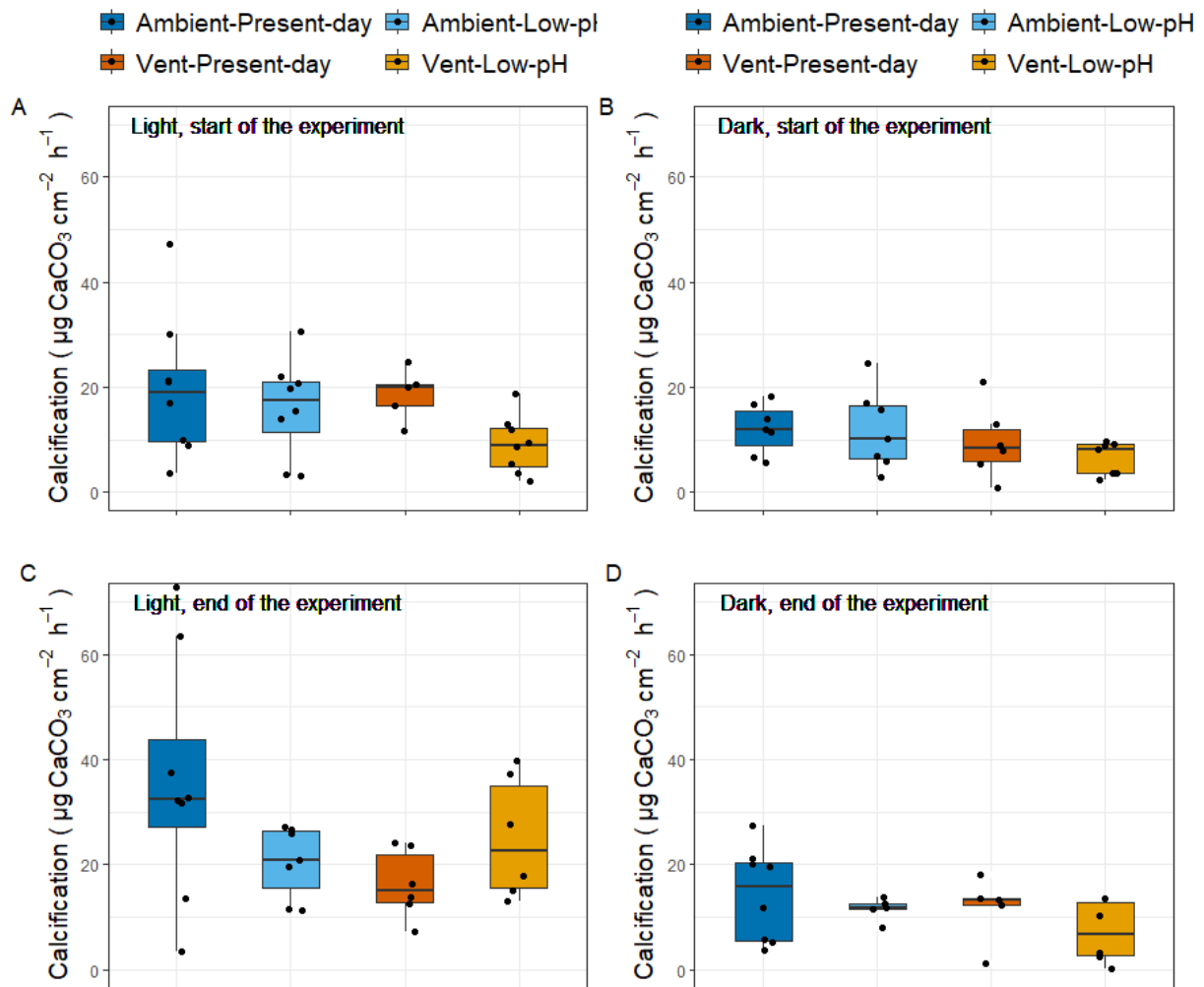


Figure 2.4 Short-term calcification rates for *Cladocora caespitosa* from vent and ambient sites under the two pH treatments ($\text{pH}_T = 8.08 \pm 0.01$, present day; $\text{pH}_T = 7.72 \pm 0.01$, low pH) in the light and dark at the start (27th of June 2019) and end of the experiment (24th of October 2019). Values are means \pm SE. Rates were calculated using alkalinity anomaly method following short term incubations (< 1 h) at 20°C . Panel A) and C) under light conditions, B) and D) under dark conditions. Dots represent the calcification rates of the coral colonies and boxes represent median and 25 and 75% quartiles. The color of the box indicates the origin and the treatment. $n = 5$ to 8 per pH treatment and origin.

3.4. Dark respiration and gross and net photosynthesis

Net photosynthesis of *C. caespitosa* was not affected by origin and pH treatment (Figure 5A; $F_{1,40} = 2.36$, $P = 0.134$ and $F_{1,40} = 1.24$, $P = 0.272$, respectively), but was different between the beginning and the end of the experiment (Figure 2.5.A; $F_{1,40} = 13.14$, $P < 0.001$). Gross photosynthesis of *C. caespitosa* did not exhibit any statistically significant difference between origin, pH treatment and time (Figure 2.5.B; $F_{1,24} = 0.17$, $P = 0.683$). Mean gross photosynthesis for all pH treatments was similar at the start and end of the experiment (0.024 ± 0.001 vs 0.020 ± 0.002 mg O₂ cm⁻² h⁻¹, n = 30). As light and dark respiration may differ, gross photosynthesis measurements are uncertain, and most likely underestimated.

Dark respiration rate of *C. caespitosa* was not significantly different between origin and pH treatment (Figure 2.5.C, $F_{1,24} = 0.008$, $P = 0.93$ and $F_{1,24} = 3.09$, $P = 0.091$, respectively), but was significantly different between the beginning and end of the experiment ($F_{1,24} = 9.45$, $P = 0.005$). In June, mean dark respiration rates for all conditions (origin and pH treatment) was -0.0079 ± 0.0003 mg O₂ cm⁻² h⁻¹ (n = 30) while in November it was -0.0097 ± 0.0006 mg O₂ cm⁻² h⁻¹ (n = 28), which represents a 23% of increase in dark respiration.

At the start of the experiment, the dark respiration of *A. calycularis* did not differ between origin or pH treatment (Figure 2.5.D, $F_{1,37} = 0.32$, $P = 0.58$). At the end of the experiment (Figure 2.5.D), a significant difference between site of origin was observed ($F_{1,36} = 18.5$, $P < 0.0001$), with corals from the ambient site having a mean dark respiration of -0.038 ± 0.003 mg O₂ cm⁻² h⁻¹ (n = 13) whilst colonies from the CO₂ vent site had a rate of -0.087 ± 0.008 mg O₂ cm⁻² h⁻¹ (n = 27). The overall (all treatments) mean dark respiration rate were also different at the start and at the end of the experiment, with a lower mean dark respiration at the start (-0.026 ± 0.002 vs -0.071 ± 0.007 mg O₂ cm⁻² h⁻¹; $F_{1,36} = 52.3$, $P < 0.0001$).

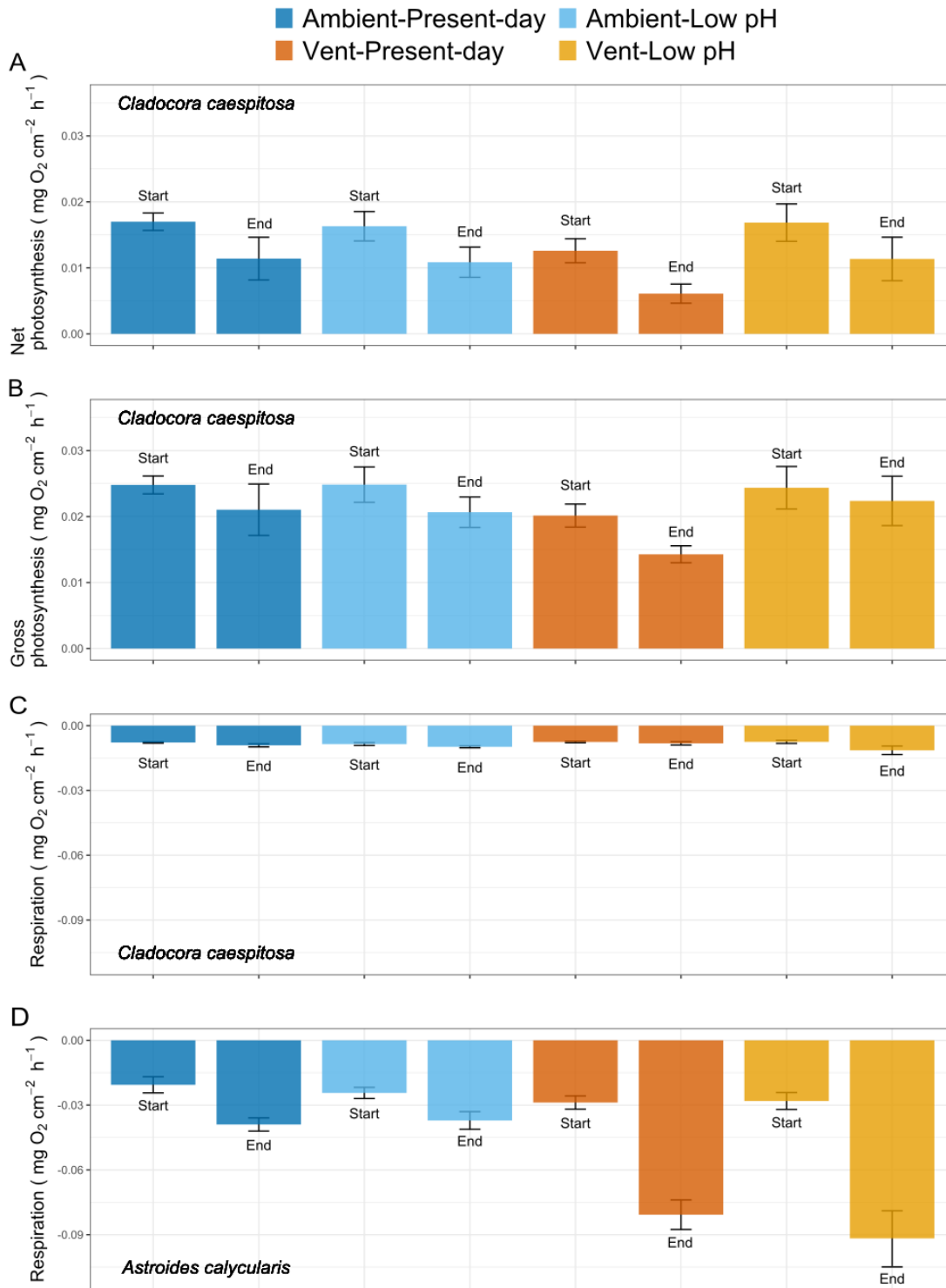


Figure 2.5 Net and gross photosynthesis and dark respiration rates of *C. caespitosa* and *A. calycularis* from the vent and ambient sites under the two pH treatments (pH_T = 8.08 ± 0.01, present day; pH_T = 7.72 ± 0.01, low pH) at the start (27th of June 2019) and end of the experiment (24th of October 2019). Values are means ± SE, Panel A) *Cladocora caespitosa*'s net photosynthesis, B) *Cladocora caespitosa*'s gross photosynthesis, C) *Cladocora caespitosa*'s dark respiration. D) *Astroides calycularis*' dark respiration. The color of the bars indicates the origin and the treatment. n = 6 to 15 per pH treatment and origin.

4. Discussion

During this six-month laboratory experiment, we tested whether colonies of the zooxanthellate coral *Cladocora caespitosa* and the azooxanthellate coral *Astroides calycularis* growing in naturally acidified waters were more tolerant to low pH than colonies from ambient pH sites or acclimated. In contrast to our initial hypothesis, calcification rates and metabolic responses did not change for any coral species no matter the site of origin and pH treatment. Therefore, our results suggest that both species, independently of environmental history and trophic strategy, can tolerate low pH conditions for six months, under controlled conditions in aquaria. In contrast, *in situ* surveys revealed lower abundance and smaller sizes for both corals at CO₂ vent sites compared with ambient sites.

The hypothesis that corals from vent sites would be less impacted by low pH stems from reports that organisms experiencing highly variable or low pH in their natural habitat may be more tolerant to ocean acidification (Cornwall *et al.* 2018). Corals living in suboptimal environmental conditions may possess physiological and potentially genetic mechanisms that confer tolerance to such environmental conditions (Camp *et al.* 2018, Teixidó *et al.* 2020). For example, microhabitats experiencing periodic temperature extremes are potential habitats for heat tolerant corals (Schoepf *et al.* 2015), yet these habitats can exhibit a mosaic of populations, harboring species previously reported with high to low thermal tolerance (Ainsworth *et al.* 2016). Such physiological mechanisms and intraspecific variability may exist in *C. caespitosa* and *A. calycularis* which highlights the importance of studying the influence of suboptimal environmental conditions on tolerance to stressors.

Previous research has shown a decrease in calcification when corals were transplanted from ambient pH sites to CO₂ vents (Rodolfo-Metalpa *et al.* 2011; Prada *et al.* 2017). However, previous studies on temperate/cold-water coral including *C. caespitosa* and *A. calycularis* have also shown a greater tolerance to ocean acidification than most tropical corals (see Table S5 Supplementary materials). Calcification rates reported in the present study agree with earlier studies. For *C. caespitosa*, Rodolfo-Metalpa *et al.* (2010) observed a yearly calcification rate of 0.51 mg CaCO₃ cm⁻² d⁻¹, which is similar to what we measured during this six-month experiment (0.43 ± 0.03 mg CaCO₃ cm⁻² d⁻¹). The calcification rates of *A. calycularis* measured here are also of the same order of magnitude to those reported by Movilla *et al.* (2016) (0.39 ± 0.04 and 0.47 ± 0.06 mg CaCO₃ cm⁻² d⁻¹, respectively). In contrast to temperate and cold-water corals, tropical corals tend to be more affected by ocean

acidification, as they exhibit a mean decrease in calcification of 10-25% when exposed to the pCO₂ expected by the end of the century under the high-CO₂ emissions scenario (Chan and Connolly 2013; Cornwall *et al.* 2021). Most tropical corals investigated so far are fast calcifiers, growing in warm waters, therefore as seawater pH declines, they may be unable to maintain these energetically costly fast rates of calcification (Comeau *et al.* 2014). In contrast, temperate and cold-water corals are slower calcifiers with lower energy requirements for mineralization, thus making them more likely to maintain constant calcification rates at lower pH (Rodolfo-Metalpa *et al.* 2010, Comeau *et al.* 2014). Ries *et al.* (2010), also suggested that the effect of acidification on the calcification of temperate corals, such as *Oculina arbuscula*, may manifest at a lower pH threshold compared to tropical corals. The capacity of *C. caespitosa* and *A. calycularis* to control the carbonate chemistry at their site of calcification could explain this tolerance to low pH. For example, using the geochemical proxy $\delta^{11}\text{B}$ it was shown that the temperate coral *Balanophyllia europaea* at the CO₂ vents of Panarea Island (Italy) can maintain constant calcifying fluid pH when exposed to pH ranging from 8.07 to 7.74 (Wall *et al.* 2019). This confirms the capacity of some corals to maintain internal pH homeostasis and utilize this as a way of calcifying under reduced seawater pH (Comeau *et al.* 2019).

From this study no conclusions can be drawn as to whether colonies from the vent sites are acclimated to low pH, as colonies from both vent sites and the ambient sites were not affected by low pH in the laboratory. However, controlling the transport of calcium and inorganic carbon to the calcifying medium and producing the skeletal organic matrix are all energy-demanding processes (Allemand *et al.* 2011, Wall *et al.* 2019), therefore the maintenance of calcification rates under these treatments may have come at a cost. For example, Wall *et al.* (2019) suggest that the inactivation of some calciblastic cells could compensate for the energy required to regulate internal pH and maintain calcification rates under low seawater pH, but at the cost of increased skeletal porosity. This is supported by a study that showed a constant growth rate of the temperate coral *B. europaea* in acidified conditions at the expense of skeletal density (Fantazzini *et al.* 2015), which in return increases the sensitivity to bioerosion and susceptibility to damage (Crook *et al.* 2013). Skeletal porosity was not measured in the present study. However, increased sensitivity to bioerosion can also be linked to the tissue coverage of the skeleton. Rodolfo-Metalpa *et al.* (2010) observed that following transplantation to low pH conditions, skeleton dissolution was observed in *Cladocora caespitosa* which had exposed skeleton, and not in *Balanophyllia europaea* which had less

exposed skeleton. In our study, we observed that most of the *A. calycularis* colonies collected at the CO₂ vent sites did not possess coenosarc (i.e. coral tissue between polyps) and exhibited polyp death during our experiment. These phenomena were also observed in natural colonies of *A. calycularis* (Teixidó *et al.* 2020) and the corals *Pocillopora damicornis* and *Oculina patagonica* when incubated at low pH (Kvitt *et al.* 2015). The increased sensitivity to bioerosion of *A. calycularis* and *C. caespitosa* with exposed skeleton could explain in part the field observation.

Field observations confirmed the difference between the population of the CO₂ vent and ambient sites, as both corals were less abundant and had smaller colonies at the vent sites (Figure S3 Supplementary materials, Teixidó *et al.* 2020). These contrasting results between field and laboratory observations, could be explained by differences in pH variability, which is much greater in the field with short periods of very low pH. Alternatively, other environmental parameters such as food availability or biological interactions (*e.g.*, competition with algae) may contribute to these differences. pH variability at the CO₂ vent sites can be very high, as pH_T can range from 6.70 to 8.13 in a day (Foo *et al.* 2018, Teixidó *et al.* 2020), whereas pH_T was very stable during our pH-controlled laboratory experiment (pH_T = 7.72 ± 0.01 for low pH treatment). It may be that no differences in calcification and photosynthesis were observed in the laboratory, due to the fact that both corals are slow-growing and long-lived species that have had a lifetime to acclimatize to their environmental pH. Therefore, the six-month time scale of the experiment may not have been enough to trigger any visible variation. However, even if our results did not show any statistical difference between treatments during the laboratory experiment, different trends in calcification between conditions can be observed for *A. calycularis*. The colonies from ambient-low pH condition seem to have lower calcification rate than the control colonies of ambient-present day condition (Figure 2), suggesting a possible impact of low pH on calcification in the short term. The same trend is observed with the colonies from the vent site, as the vent-low pH presents reduced calcification rate compared to the vent-present day condition. Moreover, calcification was similar in the vent-low pH and the control ambient-present day conditions. This trend suggests a possible acclimation to low pH in corals growing at the CO₂ vent site

Zooxanthellate and azooxanthellate corals were both used in the present study to assess the role that photosynthesis could play in mitigating the effects of ocean acidification on calcification. In zooxanthellate corals, light-enhanced calcification describes the relationship

between calcification, photosynthesis and irradiance (Gattuso *et al.* 1999; Allemand *et al.* 2011). Four main mechanisms explaining light-enhanced calcification were proposed: photosynthesis could (1) supply energy to the animal through the translocation of photosynthates, (2) increase pH at the site of calcification by using CO₂, (3) remove phosphate, which is poison to carbonate crystallization, and (4) facilitate the production of the organic matrix which serves as a scaffold in which CaCO₃ is precipitated (Allemand *et al.* 2011). Here, short-term calcification of *C. caespitosa* was higher in light than in the dark, with a mean light:dark calcification ratio of 2.38 (See Table S4-Appendix II), which is close to the mean ratio of 3.0 obtained by Gattuso *et al.* (1999). However, neither light nor dark calcification rates differed with experimental pH conditions, thus we cannot conclude that photosynthesis did provide better tolerance to ocean acidification. Furthermore, no difference in the response of calcification to ocean acidification was observed between the zooxanthellate (*C. caespitosa*) and azooxanthellate species (*A. calycularis*). The data from the *in situ* survey at Ischia did not highlight a difference between the zooxanthellate and azooxanthellate corals either, as the size and abundance of colonies from CO₂ vent sites were lower for both species. Contrastingly, Prada *et al.* (2017) reported that the zooxanthellate coral *B. europaea* was unaffected by low pH conditions, whereas the azooxanthellate corals *Leptopsammia pruvoti* and *A. calycularis* showed a negative effect of pH on calcification. However, the study of Prada *et al.* (2017) was conducted *in situ*, on corals transplanted next to CO₂ vents in Panarea Island, whereas our study is based on a laboratory experiment and field observations. Feeding could also explain this difference as it can mitigate the negative effects of environmental stressors on coral physiology (Fox *et al.* 2018). In our experiment, corals were fed freshly hatched *Artemia nauplii* three-times a week therefore the demand of energy required to maintain calcification under low pH was likely sustained by heterotrophy (Edmunds 2011). Kornder *et al.* (2018) showed that in studies where corals were fed, the negative impacts of ocean acidification on corals were mitigated. Therefore, the fact that Movilla *et al.* (2012) observed a decrease in calcification at low pH in *C. caespitosa* may be explained by the fact that the colonies were only fed once per week.

No difference in photosynthesis was observed between site of origin or pH treatment. This aligns with the observation that there is a limited impact of environmentally relevant decreases in pH on photosynthesis (Comeau *et al.* 2017), and that low pH rarely causes bleaching (Anthony *et al.* 2008). Moreover, Mediterranean corals harbor Clade B *Symbiodinium*, that have growth rates, photosynthetic capacities and respiration rates that are

unaffected by acidification (Brading *et al.* 2011). Respiration was also not affected by the pH treatment in *C. caespitosa*, confirming previous observations for this species (Rodolfo-Metalpa *et al.* 2010), and other coral species (Comeau *et al.* 2017). However, colonies of *A. calycularis* from the vent site exhibited a higher rate of respiration at the end of the experiment (November) for both low and present-day pH treatments. This result contrasts with studies that show a decrease in respiration at lower pH (*e.g.* Comeau *et al.* 2014), or no impact on respiration (Rodolfo-Metalpa *et al.* 2010; Comeau *et al.* 2017). However, an increase in respiration could be due to the increased energetic demand associated with maintaining calcification rates at lower pH (Comeau *et al.* 2017). For example, a decrease in seawater pH_T of 0.7 units requires an additional $1\text{--}2 \text{ kJ of energy mol}^{-1}$ of CaCO_3 precipitated (McCulloch *et al.* 2012a). For zooxanthellate corals, this energy is less than 1% of the energy provided by photosynthesis, however, for azooxanthellate corals such as *A. calycularis*, respiration is the only way to up-regulate this energy demand. Moreover, the increase of respiration from colonies of vent sites of *A. calycularis* could be partly due to the smaller size of colonies at the vent site than at the ambient site (Edmunds and Burgess 2016).

Although the corals studied here showed tolerance in the laboratory to pH conditions expected by 2100, it is a fragile equilibrium that can be disrupted by the inclusion of changes in other environmental variables such as temperature that could lead to a greater impact on physiological processes and survival, as observed in the *in situ* survey and predicted by the negative trend on the calcification of *A. calycularis*. For example, marine heatwaves can lead to mass mortality of *C. caespitosa* and *A. calycularis* (Rodolfo-Metalpa *et al.* 2005) and are projected to be more frequent and severe in the future (Galli *et al.* 2017, Smale *et al.* 2019). Moreover, the impact of ocean acidification on reproduction as well as larval and juvenile survival, which can limit the persistence and dispersion of a species remains to be investigated. The present study contributes to our knowledge of *Cladocora caespitosa* and *Astroides calycularis*' ability to survive under future ocean acidification conditions. Such information will improve our ability to manage and conserve the biodiversity of Mediterranean calcifying species.

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Author contributions

S.C., N.T., B.M., and J.-P.G designed the study. N.T., S.C., A.M. and B.M. were involved with fieldwork. C.C, B.M., T.G., S.C., and N.T performed the experiments. C.C., N.T. and A.M. analyzed the data. C.C. wrote the first draft of the manuscript which was then finalized by all co-authors.

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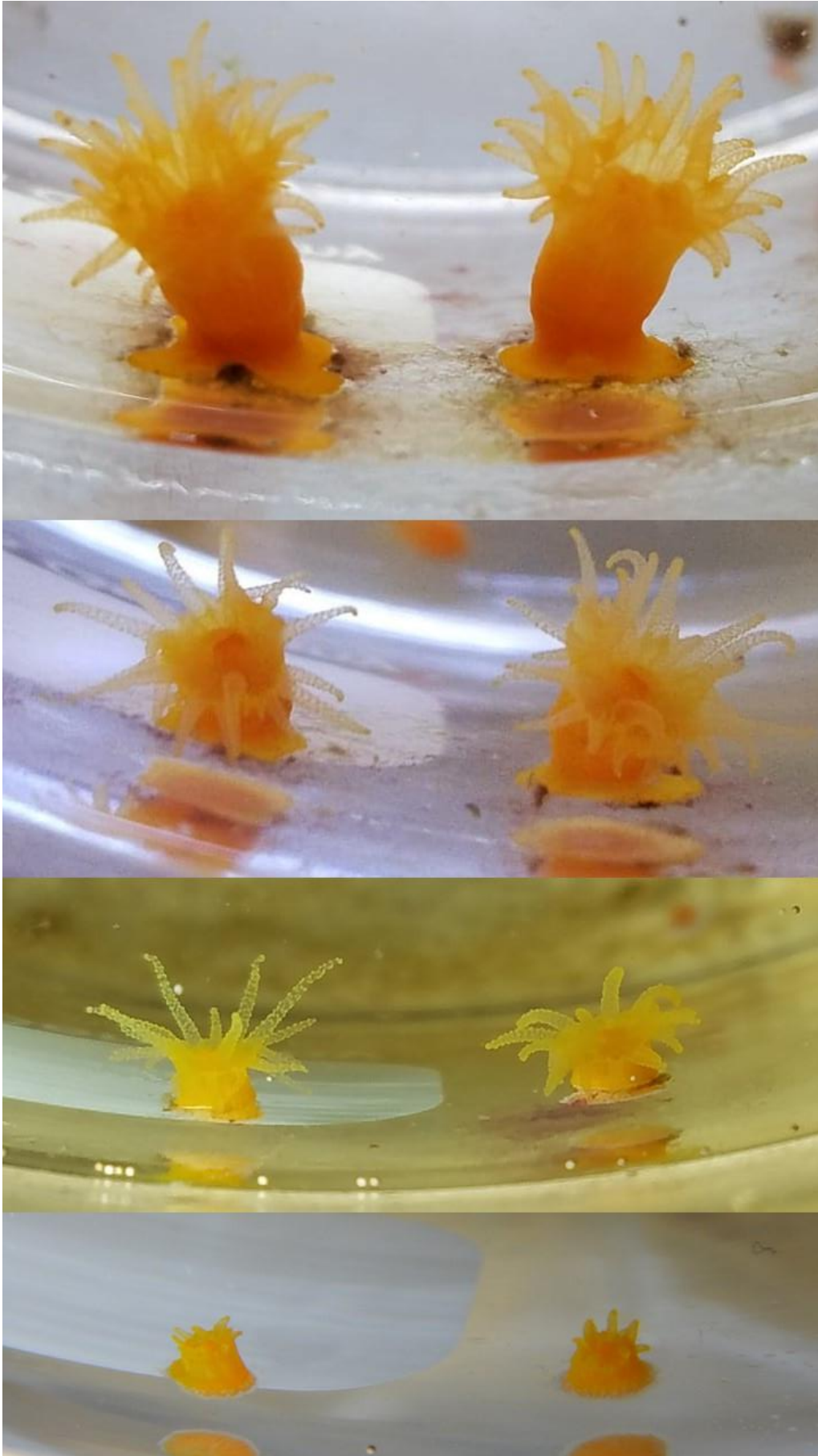
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Growth of recruits of *Astroides calycularis* over 9 months

Chapter 3: Early life stages of a Mediterranean coral are vulnerable to ocean warming and acidification

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Abstract

The ability of coral populations to recover from disturbance depends on larval dispersion and recruitment. While ocean warming and acidification effects on adult corals are well documented, information on early life stages is comparatively scarce. Here, we investigate whether ocean warming and acidification can affect the larval and recruit development of the Mediterranean azooxanthellate coral *Astroides calycularis*. Larvae and recruits were raised for 9 months at ambient (23°C) and warm (26°C) temperatures and ambient (8.0) and low pH (7.7, on the total scale). The timing of the larvae metamorphosis, growth of the recruit polyp by linear extension and budding, and skeletal characteristics of the 9-month-old polyps were monitored. Settlement and metamorphosis were more successful and hastened under a warm temperature. In contrast, low pH delayed the metamorphosis and affected growth of the recruits by reducing the calcified area of attachment to the substrate, as well as by diminishing the skeleton volume and the number of septa. However, skeleton density was higher under low pH and ambient temperature. The warm temperature and low pH treatment had a negative impact on the survival, settlement, and growth of recruits. This study provides evidence of the threat that represents ocean warming and acidification for the larval recruitment and the growth of recruits of *A. calycularis*.

1 Introduction

Anthropogenic atmospheric CO₂ emissions are driving major global threats for corals such as ocean warming and acidification (Kleypas *et al.* 2001). Under the high-CO₂ emission scenario SSP5 - RCP 8.5, sea surface temperature is expected to increase globally by +3.2°C and ocean pH to decrease by 0.3 units by the end of the century (Kwiatkowski *et al.* 2020). Ocean warming has well-documented negative impacts on tropical reefs where it can induce coral bleaching, which leads to a reduction in growth, reproduction, recruitment, and high mortality over large spatial scales (McClanahan *et al.* 2009; Sully *et al.* 2019). Ocean acidification, the change in carbonate chemistry resulting from the uptake of atmospheric CO₂ by the ocean (Gattuso and Hansson 2011), is responsible for decreasing seawater pH and calcium carbonate saturation state (Orr *et al.* 2005). Calcifying species such as corals are perceived to be especially threatened by ocean acidification as many studies have shown a decline in adult tropical coral calcification and potential accretion with a pH reduction (Cornwall *et al.* 2021). Mediterranean anthozoans, including scleractinian corals (6 species) and gorgonians (7 species), have been suffering regular mass mortalities due to the increased intensity, duration and frequency of marine heatwaves (Garrabou *et al.*, 2022). However, temperate corals seem to be more tolerant to ocean acidification than tropical corals as their calcification is rarely affected by low pH (Rodolfo-Metalpa *et al.*, 2011, Carbonne *et al.*, 2021). The recovery of coral populations depends not only on adult resilience but also on successful sexual reproduction, larval development and recruitment (Bahr *et al.*, 2020).

Sexual reproduction is essential for the dynamics of populations since it maintains genetic diversity and favors adaptation to changing environmental conditions (Bay and Palumbi, 2014). It also facilitates recovery by replenishing populations after disturbances and maintaining resilience of marine communities (Hughes *et al.*, 2019). Effective sexual reproduction is a complex process defined by different life-history factors: (1) gamete production and fertilization leading to high pelagic larval dispersal, (2) habitat selection by recognition of suitable substrate and high recruitment by settlement, (3) and post-settlement growth and survival (Ritson-Williams *et al.*, 2009, Albright *et al.*, 2011). Although the production of larvae and early life stages are critical processes for rebuilding adult populations, corals in these early stages can be particularly sensitive to ongoing and projected environmental changes (Adjeroud *et al.*, 2016). However, little is known on the response of coral larvae and recruits to ocean warming and acidification.

Ocean warming can be deleterious to coral larvae. For example, in early development, high temperatures can lead to embryonic abnormality (Woolsey *et al.*, 2013). Furthermore, the survival of larvae and recruits is frequently reduced under ocean warming (Baria *et al.*, 2015, Bahr *et al.*, 2020). Elevated sea surface temperature is commonly related to higher metabolic rates, resulting in faster metamorphosis (Chua *et al.*, 2013) and settlement (Nozawa and Harrison, 2007), increasing larval retention to the native population.

Unlike warming, the response of coral early life stages to ocean acidification is equivocal. In some cases, lower pH leads to decreasing larval metabolism, metamorphosis, and settlement (Albright 2011, Nakamura *et al.*, 2011). However, Chua and colleagues (2013) have shown that embryonic development and metamorphosis of two coral species is differently affected by elevated pCO₂ (low pH). On the other hand, most of the literature reports little impact of acidification on the survival of larvae and recruits (Suwa *et al.*, 2010; Chua *et al.*, 2013). As for adult corals, low pH has a negative impact on post-settlement calcification (Suwa *et al.*, 2010, Varnerin *et al.*, 2020) and induces skeletal deformities in recruits (Foster *et al.*, 2016).

Although studies of the effects of warming and acidification in isolation are few, even less have assessed the combined effects of both stressors in early-life stages. Regarding adult corals, the combined effects of both high temperature and low pH are generally dependent on their intensities. For example, a moderate warming can counteract the impact of acidification ending up with a neutral effect on calcification of adult corals (Kornder *et al.*, 2018). Warming and acidification will have an additive effect (higher than the individual effects) on calcification rates under the RCP 4.5 and a synergistic effect (more than the sum of the individual effects) under RCP 8.5 for the end of the century (Cornwall *et al.*, 2021). In the early life stages, warming and acidification induce divergent responses with decreased or enhanced calcification (Anlauf *et al.* 2011, Foster *et al.*, 2015). In contrast, studies have reported no effects of combined warming and acidification on larval survival, metamorphosis and settlement (Anlauf *et al.*, 2011, Chua *et al.*, 2013, Foster *et al.*, 2015). However, most of the studies of the impact of both ocean warming and acidification on early life stages have focused on tropical zooxanthellate coral species (*e.g.*, Albright *et al.*, 2011, Baria *et al.*, 2015), while studies on temperate corals are scarce (Caroselli *et al.*, 2019, Varnerin *et al.*, 2020).

Here, we assess the response of early life stages of the temperate azooxanthellate coral, *Astroides calycularis*, to the combined effects of elevated temperature and low pH. This colonial scleractinian coral is endemic to the Mediterranean Sea and found between 0 and 50

m depth, more commonly in shallow rocky habitats (Zibrowius, 1995). Colonies are gonochoric (Goffredo *et al.*, 2011) and fertilization occurs from April to May, with sperm release coinciding with increasing photoperiod and water temperature (Goffredo *et al.*, 2011). *A. calycularis* is an internal brooder as egg fertilization takes place in the coelenteron and female broods the embryos until they are fully developed into mature swimming larvae. Larvae are released at the beginning of summer when temperature increases (~23°C, Goffredo *et al.*, 2011). Previous studies on *A. calycularis* adult colonies have shown that this coral is tolerant to ocean warming and acidification (Movilla *et al.*, 2016, Teixido *et al.*, 2020; Carbonne *et al.*, 2021).

In the present study, we hypothesize that elevated temperature and low pH have an additive or synergistic effect on the development of the larvae and growth by linear extension and polyp budding of recruits of *A. calycularis*. To test this hypothesis, larvae of *A. calycularis* were exposed in a fully factorial design to ambient and warm temperatures (23 and 26°C) and ambient and low pH (pH_T 8.05 and 7.7). They were maintained in the laboratory for 9 months to study the impact of these conditions on the development of larval stages, growth of recruit polyps, and skeleton density of the 9-month-old polyps.

2 Materials and Methods

2.1 Sampling site and larval release

Fifty-six colonies of *Astroides calycularis* (~5 cm of diameter) were collected on the 1st of July 2020 by scuba diving in Ischia, Italy, at the site Sant'Angelo (40°41'31.1"N 13°53'35.0"E, Figure S1-Appendix III). They were maintained in a 30-liter tank with water motion provided by a NEWA mini 606 pump and maintained in the dark. Larvae were observed by transparency in the gastrovascular cavity and tentacles of the female colonies (Figure S2-Appendix III). Larvae were released from the mouth of female polyps when the body contracted or when the colonies were touched (Figure 3.1.A and Video 1, DOI after the discussion). The release took place on the night of 1st-2nd of July 2020; *in situ* seawater temperature was around 23°C. Released larvae were fully mature (Video 2, DOI after the discussion). They were collected with a pipette, pooled, stored into two 300 mL airtight plastic boxes filled with seawater, and transported to the aquarium facilities of the Laboratory of Villefranche-sur-Mer, France, in less than 12h.

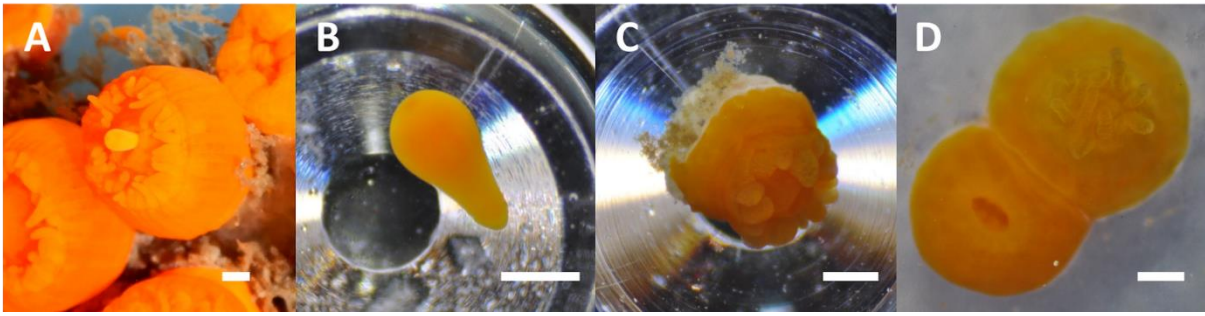


Figure 3.1 **Early life stages of *Astroides calycularis*** A) spawning of a larva from the mouth of a female polyp, B) a larva after 15 days, C) not-settled metamorphosed polyp after 1 week, and D) settled metamorphosed polyp with 2 polyps after 1 week. Scale: 1 mm. See also videos S1, S2, and S3

2.2 Experimental setup and treatments

After 72 h of acclimation to the laboratory at ambient pH ($\text{pH}_T = 8.05$) and temperature (23°C), the 180 larvae were maintained in the laboratory under 2 constant pH and 2 constant temperature treatments for 9 months. pH treatments were “ambient pH” ($\text{pH}_T \sim 8.05$), and a “low pH” ($\text{pH}_T \sim 7.70$), corresponding to a decline of pH projected by the end of the century under the RCP 8.5 CO_2 emissions scenario (Kwiatkowski *et al.*, 2020). The two temperature treatments were “ambient temperature” which was the *in situ* temperature at the time of sampling (23°C) and “warm temperature” $\sim 26^\circ\text{C}$, which was shown to induce thermal stress of benthic species in the Mediterranean Sea (Gómez-Gras *et al.*, 2019). The larvae were divided into 12 x 300 mL crystallizers (Duran®), as triplicates with 15 larvae for each of the four treatment conditions (2 pH x 2 temperatures). Each crystallizer was submerged in an independent 5 L experimental tank. While larvae were still swimming, water was changed every day and crystallizers were covered with parafilm® to prevent changes in pH and the escape of larvae. When all larvae metamorphosed and settled, the crystallizers were covered with a $45\ \mu\text{m}$ plankton net to allow water exchange with the experimental tank. The experimental tanks were gravity-fed ($50\ \text{ml}\ \text{min}^{-1}$) from six 25 L header tanks (3 at each pH treatment) continuously supplied by seawater pumped from Villefranche Bay at 5 m depth. pH was controlled using a pH controller (APEX, Neptune Systems) which regulated the delivery of pure CO_2 . Temperature was regulated in the experimental tanks by temperature controllers (APEX, Neptune Systems) and 300 W ThermoControl-e heater (Eheim), and both pH and temperature were measured every week (see below).

Light was provided by 24W Aquablue Special T5 (ATI). Irradiance was constant from 7 to 19h at $16\ \mu\text{mol}\ \text{photons}\ \text{m}^{-2}\ \text{s}^{-1}$. Submersible pumps (NEWA) provided water motion in each

experimental tank. When recruits polyps had tentacles (approximately after a month, from the 5th of August 2020), they were fed twice a week with a 10 mL solution of freshly hatched brine shrimps (*Artemia* sp.). 24 h after feeding, the crystallizers were cleaned with a painting brush and the water changed to remove any detritus.

2.3 Carbonate chemistry

pH in the header and experimental tanks was measured weekly using a handheld pH-meter (826 pH mobile, Metrohm) calibrated with a seawater pH TRIS buffer (batch #T33 provided by A. Dickson, Scripps Institution of Oceanography, USA) before each set of measurements. Temperature was also measured weekly in each tank with a Traceable™ digital thermometer (FisherBrand). Salinity and total alkalinity (A_T) data during the experiment were obtained from the weekly measurements performed in the in the Bay of Villefranche by the *Service d'Observation Rade de Villefranche, SO-Rade*, of the *Institut de la mer de Villefranche and the Service d'Observation en Milieu Littoral, SOMLIT/CNRS-INSU*. This was possible because we worked in an open system where seawater from the Bay of Villefranche was continuously delivered. To confirm that A_T was not altered by metabolic activity, it was measured in each experimental and header tank at the beginning and end of the experiment. It was determined by potentiometric titration using a Metrohm 888 Titrando following the method of Dickson *et al.* (2007). Titrations of certified reference material (Batch #186) provided by A. Dickson were used to assess the accuracy of the measurements and were within $6.5 \mu\text{mol kg}^{-1}$ of the reference value. pH_T , temperature, A_T and salinity were used to calculate the other carbonate chemistry parameters using the R package seacarb (Gattuso *et al.*, 2021).

2.4 Early life-stages monitoring

The life stages of each 15 individuals (larvae) per crystallizer were recorded every 2 days when swimming larvae were present, and every week after settlement. The different stages were: “planula”, when the larvae were still swimming (Figure 3.1.B, Video 3 (DOI after the discussion)), “settled polyp” when larvae had metamorphosed and settled (Figure 3.1.D), “non-settled polyp” when larvae had metamorphosed but not settled or detached from the substrate after settling (Figure 3.1.C), and “dead”. Missing larvae were assumed dead as coral larvae lyse within 24 h after death (Baird *et al.*, 2006).

Pictures of each larva from each crystallizer were taken every 2 to 3 days with a stereomicroscope (SteREO, Discovery V.12, Zeiss) coupled with a camera (D5100, Nikon)

until no swimming planula was present. For each polyp attached to the glass of the crystallizer, a picture was taken every 2 weeks from below with a camera (Coolpix W300, Nikon) and a ruler used as scale (Figure 3.3.A). All pictures were analyzed with ImageJ to measure the maximum length and maximum width of the larvae and the surface of the polyp base (Figure 3.3.A). Polyp budding, the appearance of new polyps from one initial polyp was recorded and reported as the number of polyps in one colony (Figure 3.3.C).

2.5 Skeleton analysis

After 9 months, the polyps were collected and placed in 5% sodium hypochlorite for 2 h and then rinsed with MilliQ water to remove organic tissue from the skeleton. The skeletons were dried at ambient temperature for two days. The perpendicular diameters (D and d) and the height (h) were measured using a digital caliper. The volume of the skeleton was calculated using the following equation: $V = \pi * D * d * h$. To count the number of septa, a picture of each skeleton was taken from the top with a stereomicroscope (SteREO, Discovery V.12, Zeiss) mounted with a camera (D5100, Nikon).

Six skeletons of each condition (total of 24) were scanned in a micro-CT scanner (GE Healthcare, eXplore Locus RS, see Micro-CT scanner setup in Appendix III, Figure S3-Appendix III). Micro-CT imaging produced a 3D distribution of linear attenuation coefficients that was stored as an x-ray volume image for each coral specimen, defined by the coral polyp base along the X-Y in-plane axis, and the growth axis following the Z-axis (Figure 3.5.A). Sample visualization and analysis was performed using MicroView Standard 2.5.0–2702 (Parallax Innovations Inc., 2015) to reconstruct each specimen in 3D, and to digitize a region of interest (ROI delineating the region to be analyzed). Six coral specimens were imaged in every micro-CT scan, each representing one of the four laboratory treatments (temperature x pH). The ROI was plotted as a square based prism spanning the entire specimen to crop each coral sample out for individual analysis. A threshold greyscale value of 2500 HU was carefully selected to remove pore spaces from coral skeleton. This approach assured consistent sampling focused on skeletal aragonite only. Voxels with a greyscale values below the threshold of 2500 HU were deemed to be empty space and excluded from the skeletal density calculation. However, small pore spaces (< 20 μm) were included in the skeletal density calculation since they were unresolvable at 20 μm resolution. The skeletal density for each coral polyp (mg/cm^3) was determined using the fractional mineral content of each voxel (3D pixel) above the greyscale threshold (to exclude pore spaces), averaged over

all the voxels contained within each region of interest, and linearly rescaled to units of pure crystal aragonite (density = 2.95 g/cm³).

2.6 Data analysis

Linear mixed-models with a hierarchical structure were used to evaluate the treatment effects through time on the number of larvae in each life stage (planula, settled polyp, non-settled polyp, dead), and the length and width of larvae. Hierarchical linear models were used since data were compiled from repeated measures of the same pool of larvae over time. The models were fitted using the function `lmer` of the R package `lme4` (Bates *et al.*, 2015). The fixed factors of the models were temperature, pH and time, and crystallizers and time were assigned as random effects. The structure of the random term was selected by comparing models with different error structures using the Akaike information criterion (Table S1). For parameters that are not time-dependent, generalized linear mixed models (GLMM) were used with a Gaussian distribution to test for the effects of the treatments on the surface of the polyps' base, the skeletal density and volume, and a Poisson distribution for the number of polyps per colony and the number of septa. Temperature and pH were fixed factors and crystallizers of each condition a random factor.

3 Results

3.1 Experimental conditions

The ambient pH treatment was maintained at a mean pH_T of 8.05 ± 0.09 (mean \pm SD, $n=324$) and the low pH treatment at a mean pH_T of 7.78 ± 0.10 (mean \pm SD, $n=324$, total number of weekly pH_T discrete measures in each ambient pH experimental tank, Table 3.1, Figure S4.B-Appendix III). The *ambient temperature* treatment was maintained at 23.5 ± 1.6 °C (mean \pm SD, $n=342$) and the *warm temperature* treatment at 26.6 ± 0.8 °C (mean \pm SD, $n=342$, Table 3.1, Figure S4.A-Appendix III). Salinity and total alkalinity did not vary much during the duration of the experiment with a salinity of 37.84 ± 0.17 (mean \pm SD, $n=71$, Table 3.1) and a total alkalinity of 2548 ± 13 $\mu\text{mol kg}^{-1}$ (mean \pm SD, $n=71$, Table 3.1).

Table 3.1 Measured and estimated seawater physicochemical parameters of the two pH treatments in the experimental tanks for salinity (S), temperature (T), total alkalinity (A_T), dissolved inorganic carbon (C_T), pH_T , pCO_2 , calcite (Ω_c) and aragonite (Ω_a) saturation. Values are means \pm SD with 25th and 75th percentiles. Calculated concentrations of C_T , pCO_2 , Ω_c and Ω_a are shown. 1: Parameters measured from discrete water samples. pH and temperature conditions are the experimental pH and temperature treatments.

pH condition	Temperature condition	pH_T	T (°C)	A_T ($\mu\text{mol kg}^{-1}$)	C_T ($\mu\text{mol kg}^{-1}$)	Salinity	pCO_2 (μatm)	Ω_c	Ω_a
low	warm	7.77 \pm 0.11 n=102	26.32 \pm 0.87 n=111	2548 ¹ \pm 13 n=71	2350 \pm 62 n=102	37.84 ¹ \pm 0.17 n=71	964.42 \pm 295.57 n=102	3.69 \pm 0.83 n=102	2.45 \pm 0.56 n=102
ambient	ambient	8.07 \pm 0.05 n=102	23.23 \pm 1.65 n=111	2548 ¹ \pm 13 n=71	2202 \pm 42 n=102	37.84 ¹ \pm 0.17 n=71	404.43 \pm 59.18 n=102	5.83 \pm 0.60 n=102	3.84 \pm 0.41 n=102
low	ambient	7.76 \pm 0.10 n=102	23.13 \pm 1.70 n=111	2548 ¹ \pm 13 n=71	2375 \pm 57 n=102	37.84 ¹ \pm 0.17 n=71	962.62 \pm 269.51 n=102	3.30 \pm 0.73 n=102	2.17 \pm 0.49 n=102
ambient	warm	8.04 \pm 0.07 n=102	26.51 \pm 0.76 n=111	2548 ¹ \pm 13 n=71	2188 \pm 54 n=102	37.84 ¹ \pm 0.17 n=71	442.88 \pm 100.72 n=102	6.10 \pm 0.78 n=102	4.05 \pm 0.52 n=102

3.2 Development of early life stages

Larvae started metamorphosing into settled and non-settled polyps after 5 days under *warm temperature - ambient pH* and the last larva settled after 41 days under *ambient temperature - low pH* condition. The proportion of larvae was significantly affected by the interaction of pH, temperature and time (Figure 3.2.A, $F_{1,145}=4.437$, $p=0.035$, Table S2-Appendix III), as larvae under *warm temperature* conditions metamorphosed faster: 50% in 15 days, while *ambient temperature - ambient pH* and *ambient temperature - low pH* conditions reached 50% of metamorphosis after 19 and 25 days, respectively (Figure 3.2.A). Settlement of larvae differed between conditions, only 25% of settlement occurred in the *warm temperature - low pH* condition and more than 40% in the two *ambient pH* treatment conditions (Figure 3.2.C) due to a significant interaction among the three factors pH, temperature and time ($F_{1,397}=29.155$, $p<0.001$, Figure 3.2.C, Table S2-Appendix III). There was a significant effect of pH, temperature and time on the proportion of metamorphosed but non-settled larvae ($F_{1,397}=36.369$, $p<0.001$, Table S2-Appendix III). The two *low pH* treatment conditions presented an opposite outcome. The *warm temperature - low pH* treatment had 36% of non-settled polyps at the beginning of the experiment and almost 0% after 3 months (Figure 3.2.D). On the other hand, the *ambient temperature - low pH* condition presented a progressive detachment of polyps as the proportion of non-settled polyps increased from 9% after a month until reaching 25% after 6 months (Figure 3.2.D). Mortality of larvae and polyps increased for every condition during the first two months of the experiment, and then reached a plateau around 50% (Figure 3.2.B). The *warm temperature - low pH* condition presented higher mortality compared to the other conditions, reaching up to 71% with a significant effect of the

combined pH, temperature and time ($F_{1,397}=110.9$, $p<0.001$, Figure 3.2.B, Table S2). The treatments did not affect larval size (Figure S7-Appendix III).

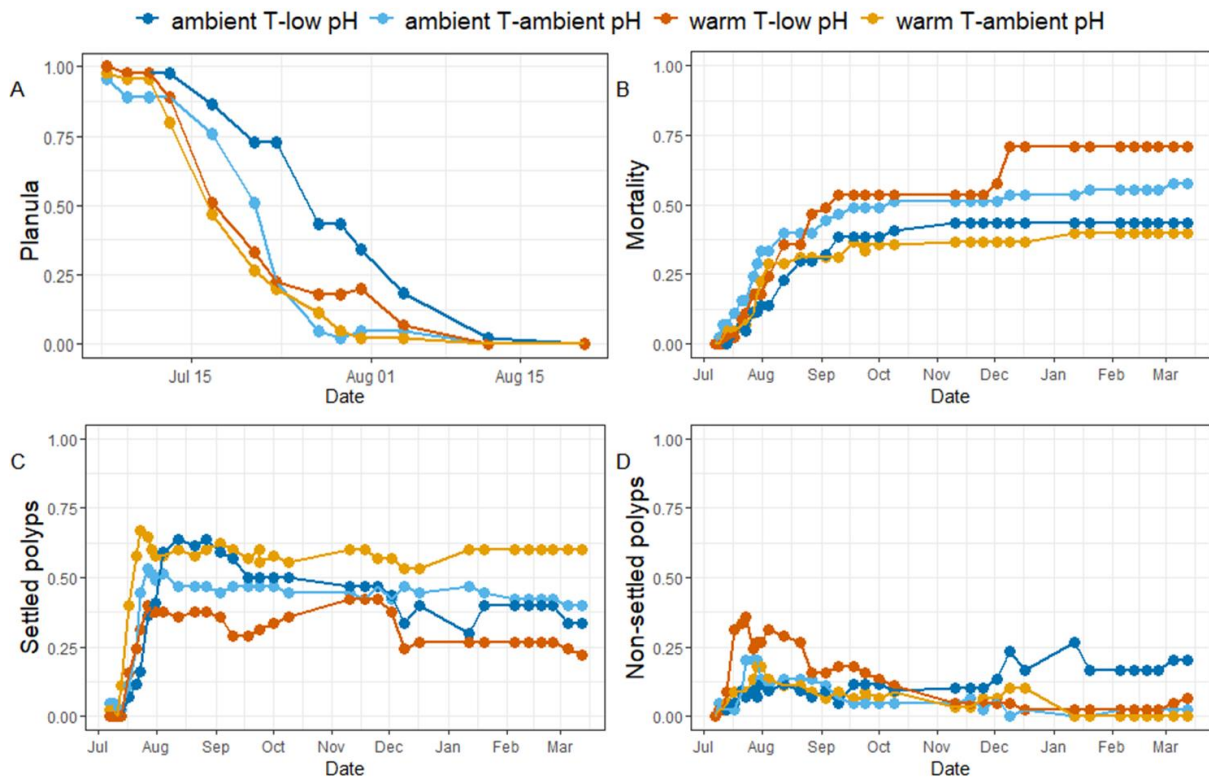


Figure 3.2 Proportions of four different stages of *A. calycularis* under the two temperature treatments (23°C for ambient Temperature and 26°C for warm Temperature) and the two pH treatments ($pH_T = 8.05 \pm 0.09$ for ambient pH and $pH_T = 7.78 \pm 0.10$ for low pH) during 9 months. Panel A) The proportion of planulas (swimming larvae), B) the proportion of mortality (dead larvae and polyps), C) the proportion of settled polyp (metamorphosed and fixed), and D) the proportion of non-settled polyp (metamorphosed but not fixed). The proportion of non-settled polyp includes when larvae metamorphosed into a free polyp and detachment of a settled polyp. $n = 180$ original larvae, 3 replicates per temperature and pH treatments.

3.3 Growth of the recruits by linear extension

The surface of the polyp bases was significantly affected by pH ($F_{1,35} = 23.6$, Table S3-Appendix III). The base surface in the *ambient pH* treatment were significantly larger than under *low pH*, respectively reaching $32.7 \pm 3.6 \text{ mm}^2$ (mean \pm SE, $n=18$) and $13 \pm 2 \text{ mm}^2$ ($n=21$) after 9 months of experiment (Figure 3.3.B, Figure S5-Appendix III).

3.4 Development of new polyps by budding

New polyps started to bud on the periphery of the recruits at the end of December 2020 (Figure S6-Appendix III). The number of polyps per colony after 9 months of experiment was not

significantly different across treatments ($F_{1,55}= 2.3$, $p=0.135$, Table S3-Appendix III). However, in contrast to all other treatments, recruits in the *warm temperature – low pH* treatment did not develop new polyp during the 9-month of experiment (Figure 3.3.D). The recruits in the *warm temperature – ambient pH* treatment exhibited new polyps first as well as the highest number of polyps per colony at the end of the experiment with a mean of 1.33 polyps per recruit.

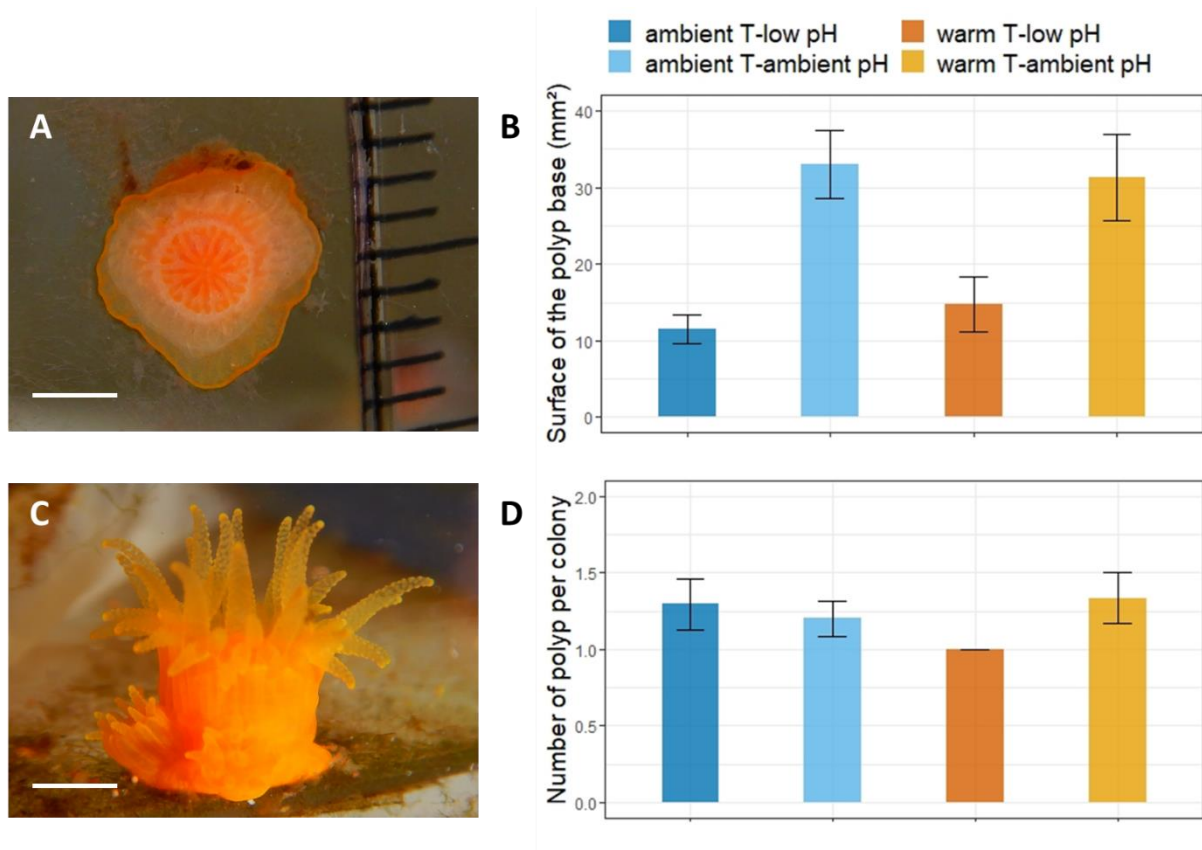


Figure 3.3 Mean surface of the recruits' base and mean number of polyps per colony of *A. calycularis* under the two temperature treatments (23°C for ambient Temperature and 26°C for warm Temperature) and the two pH treatments ($pH_T = 8.05 \pm 0.09$ for ambient pH and $pH_T = 7.78 \pm 0.10$ for low pH) after 9 months. Values are means \pm SE. A) Picture of the base of a recruit through the glass of the crystallizer, B) Mean surface of the recruits' base at 9-month old, C) Picture of a recruit with two new polyps on the outskirts, D) Mean number of polyps per colony. The color of the bars indicates the temperature and pH treatment. $n= 9-20$ per condition. Scale : 2 mm.

3.5 Skeleton analysis

The size of the skeleton was significantly impacted by the pH treatment ($F_{1,46}=4.05$, $p=0.044$, Table S3-Appendix III), with a mean volume of 0.12 cm³ in the *ambient pH* treatment and 0.1 cm³ under *low pH* treatment (Figure 3.4.B). The number of septa per skeleton was significantly lower under low pH ($F_{1,48}=10.5$, $p=0.002$, Table S3-Appendix III). The skeleton had less

developed septa (mean \pm SE, n=29) under low pH treatment 8.2 ± 0.6 septa than under ambient pH (10.2 ± 0.6 septa, n=23, Figure 3.4.C).

The combined effect of pH and temperature had a significant impact on skeletal density ($F_{1,19}=7.6$, $p=0.012$, Table S3-Appendix III). The skeleton of polyps exposed to *ambient temperature – low pH* condition presented a higher density with 1739 ± 19 mg cm⁻³ (mean \pm SE, n=6) than in the other conditions in which it was lower than 1690 mg cm⁻³ (n=6, Figure 3.4.A and 3.4.D).

4 Discussion

This experiment shows that settlement and metamorphosis of the larvae are faster under warm temperatures. In contrast, low pH delayed the metamorphosis and affected the skeletal properties of the recruits. The surface of the base of the skeleton and the number of septa per polyp were lower under low pH conditions, whereas the skeleton density was higher. Both drivers combined had a negative synergistic impact on survival and settlement, and an additive impact on growth by linear extension and polyp budding. Therefore, our results suggest that both warming and acidification, individually or combined, affect the early life stages of *Astroides calycularis*.

Larvae were released by the female colonies at the beginning of summer (end of June), when the sea surface temperatures reached 23°C. Metamorphosis and settlement were accelerated in the warm treatment. Similar results were observed in the majority of similar studies in which tropical coral larval development was noticeably increased at warmer temperatures (Edmunds *et al.*, 2001, Nozawa and Harrison, 2007, Chua *et al.*, 2013). In contrast, few studies reported a decline in settlement and development of tropical coral larvae under warming (*e.g.* Randall and Szmant, 2009), possibly due to the high temperature (31°C and 33°C) that reduced larval mobility and increased mortality. Higher temperatures accelerate biochemical processes by increasing the activity of enzymes until a threshold is reached (Clarke and Fraser, 2004). As a result, metabolic rates increase, shortening the duration of the larval phase and reducing the distance of dispersal by causing metamorphosis and settlement before larvae are exported from their population of origin (O'Connor *et al.*, 2007, Figueiredo *et al.*, 2014). Local retention of the larvae has a direct impact on connectivity and genetic diversity, which potentially reduces the resilience to disturbances and future global change (Bay and Palumbi, 2014). Warmer temperatures are also commonly associated with an increased mortality of early life stages (Baria *et al.*, 2015). Here, the mortality of larvae and recruits was not affected by temperature.

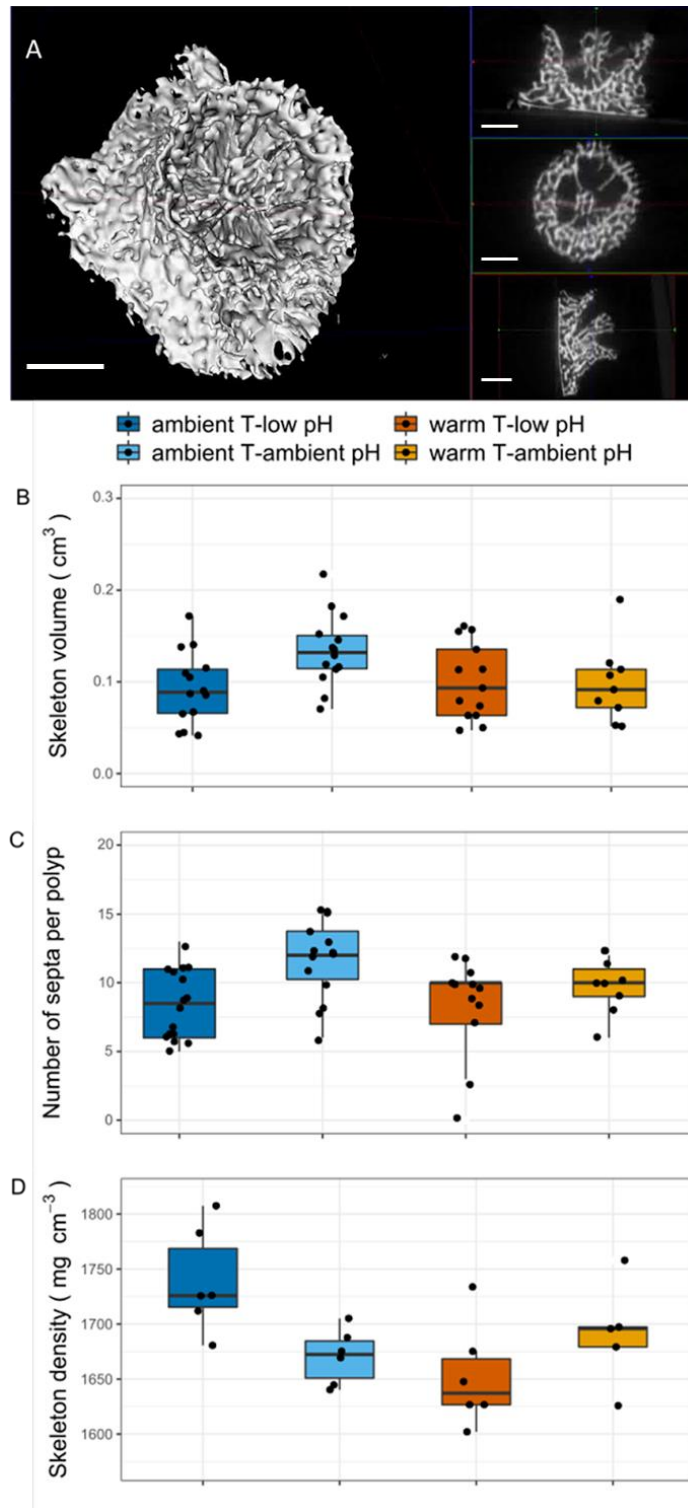


Figure 3.4 9 month-recruit skeletons of *A. calycularis* under the two temperature treatments (23°C for ambient Temperature and 26°C for warm Temperature) and the two pH treatments ($\text{pH}_T = 8.05 \pm 0.09$ for ambient pH and $\text{pH}_T = 7.78 \pm 0.10$ for low pH). A) 3D picture of a recruit skeleton by micro CT scanning, scale: 1 mm See also video S4, B) Volume of the recruit. n=9-16 per temperature and pH treatment. C) Number of septa per skeleton. n=9-16 per temperature and pH treatment. D) Skeleton density. Values have been obtained by micro CT scanning. n=6 per temperature and pH treatment. Dots represent the number of septa per recruit coral skeleton and the density of the recruit's coral skeleton and boxes represent median and 25 and 75% quartiles. The color of the boxes indicates the temperature and pH treatment.

This might be because *A. calycularis* is a known thermophilic species, with a South-Western distribution in the Mediterranean Sea. The warm temperature used for the experiment, 26°C, is the mean temperature during the summer months in Ischia (July and August, Teixido *et al.*, 2020). The colonies of *A. calycularis* have been suffering mass mortalities in Ischia and North of Sicily in summer when temperatures reach 28°C (Gambi *et al.*, 2018, Bisanti *et al.*, 2022).

The surface area of the base of the skeleton was highly impacted by acidification, decreasing by 60% under *low pH* compared to the *ambient pH* treatment. The skeletal volume was also reduced by 19% under acidified conditions. Albright and Langdon (2011) also observed a decrease of post-settlement linear growth of 50% in recruits of *Porites astreoides* with a similar pH_T treatment of 7.7. This difference in growth might be due to a decrease in metabolism under low pH (Albright and Langdon 2011). The formation of the skeleton was also impacted by acidification as the number of septa was less abundant and not presenting a radial symmetry under low pH. This is in agreement with other studies which have reported porous corallite walls, thinner basal plate, and asymmetric skeleton under similar low pH conditions (Foster *et al.*, 2016). This impact on the linear extension and skeleton structure can likely explain the increased detachment of polyps five months after the beginning of the experiment. A large number of recruits under low pH had the skeleton base exposed, retracting their tissue towards the upper section of the corallite (Figure S8-Appendix III). While 17 out of 36 polyps exposed to *low pH* had an exposed skeleton at the base, none of the 33 polyps under *ambient pH* exhibited tissue retraction. The polyps were thus unable to extend their skeleton on the substrate, and the exposed skeleton was likely experiencing dissolution, as described before with *Cladocora caespitosa* by Rodolfo-Metalpa *et al.* (2011). This phenomenon of being more predisposed to dissolution may be compared to the loss of coenosarc (the living tissue connecting the polyps) in adult colonies of *A. calycularis* that naturally occur in a CO₂ vent site in Ischia, where seawater is naturally acidified. (Teixido *et al.*, 2020). As a response to low pH, colonies in the CO₂ vent showed a reduction of coenosarc compared to ambient pH sites. Interestingly, while the linear extension and structure of the skeleton were affected by low pH, the density of the skeleton was higher. These results are in accordance with the higher density observed on adult colonies living in the CO₂ vent site in Ischia (Teixido *et al.*, 2020). It suggests that *A. calycularis* responds to low pH by increasing the density of their skeleton, perhaps for greater resistance to mechanical stress, at the expense of other physiological parameters such as growth and skeleton structure. These results suggest that acidification mainly had

repercussions on calcification of the recruits, whereas larval development and survival were not impacted, as observed by Foster *et al.* (2015).

The proportion of settlement of *A. calycularis* larvae was significantly lower in the warm temperature and low pH treatment. In contrast, other studies reported no effect of such treatment (*e.g.* Anlauf *et al.*, 2011, Foster *et al.*, 2015). This difference can be explained by the higher magnitude of the warming and acidification treatments used in the present study compared with other studies (+ 3 vs + 1-2°C and -0.3 vs -0.2 pH unit) (Anlauf *et al.*, 2011, Chua *et al.*, 2013, Bahr *et al.*, 2020). Furthermore, all previous studies focused on tropical zooxanthellate corals which obtain additional energy by photosynthetic products translocated from their symbionts (Davy *et al.* 2012) and are not subject to high seasonal variability of temperature as Mediterranean species (around 14°C in winter to 26°C in summer in Ischia). The low settlement rate of *A. calycularis* under the warm and acidified treatment is related to the high number of larvae experiencing metamorphosis with no settlement during the first days of the experiment. In the first 15 days, 36% of the larvae metamorphosed without settling. After metamorphosing, the individuals floated for a week before sinking, then calcification started and the tentacles appeared. Some of them eventually attached to the bottom after three months. This phenomenon could also explain the high mortality rate observed in the *warm temperature – low pH* condition as non-settled polyps could not feed properly. Recruits were therefore less abundant, and their size was smaller than in any other condition. Warming and acidification presented an additive effect on the surface base of the recruits as the combined effects of warm and acidified waters lead to a 63% reduction in linear growth, while the low pH treatment caused a 55% decrease and the warm treatment a 6% decrease, compared to the control condition. Anlauf *et al.*, (2011) found a synergistic effect of warming and acidification on the calcification of *Porites panamensis* recruits. The calcification of the recruits was 30% smaller in a combined warm and low pH treatment, while only a 3% decrease was observed under acidification alone and 0% under warming. For *Acropora spicifera* recruits, warming mitigated the impact of acidification as calcification was lower (-60%) under sole acidification than under both warming and acidification (-48%, Foster *et al.*, 2015). The impact of stressors on calcification is considered species-specific (Comeau *et al.*, 2013). However, methodological differences could also explain the range of responses observed. The duration of our experiment (9 months) was longer than previous ones (6 weeks, Anlauf *et al.*, 2011 and 5 weeks, Foster *et al.*, 2015). For example, in our study, the different responses to the treatments on *A. calycularis* recruits' surface base were only observed 2 months post settlement. Finally, under *warm temperature – low pH*

condition, 63% of the recruits presented an exposed skeleton at the base, more than under *ambient temperature - low pH* condition. However, new polyps of *A. calycularis* bud from the tissue at the base of the recruit periphery. This particular characteristic explains why the recruits under the double stress condition were the only one not presenting new polyps. Thus, warm temperature and low pH together harmed the development of recruits into colonies.

Our findings highlight that warm temperatures and acidification have distinct impacts on the early life stages of *Astroides calycularis*. Temperature acts on larval development while pH acts on the linear growth and calcification of the recruits as observed in previous studies on tropical species (*e.g.* Albright and Langdon 2011, Chua *et al.*, 2013). The combined effect of warming and acidification early life stages will negatively impact the resilience and resistance of the Mediterranean populations by decreasing dispersion, recruitment and post-settlement growth. In order to better predict the future of *A. calycularis* populations at the end of the century under global change, further research needs to be done to evaluate if acclimatization or adaptation to warming or acidification can occur in early life stages.

Video supplement

Video 1 Spawning of a larva from the mouth of *Astroides calycularis* female polyp

<https://doi.org/10.5446/58541>

Video 2 One-day old swimming larvae of *Astroides calycularis* <https://doi.org/10.5446/58542>

Video3 Sequence of early life stages development of *Astroides calycularis*

<https://doi.org/10.5446/58543>

Author contributions

C.C., S.C., N.T., and J.-P.G designed the study. C.C. and N.T. were involved with fieldwork. C.C., P.C. and K.P. performed the experiments. C.C., N.T. and K.P. analyzed the data. C.C. wrote the first draft of the manuscript which was then finalized by all co-authors.

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General Discussion

As the results of the chapters 1, 2 and 3 have been discussed in their respective sections, the goals of this general discussion is, first, to resume the results of the five studies on the impact of ocean warming and acidification on *Cladocora caespitosa* and *Astroides calycularis*, second to compare the response to global change of the different life stages, third to discuss the mechanisms of acclimatization to ocean acidification at CO₂ vents, and finally to compare the response of zooxanthellate and azooxanthellate corals to ocean warming and acidification.

Summary of the results

With the studies presented in this thesis, I contributed to increase knowledge on the responses to global environmental change of the two habitat-forming Mediterranean corals, the zooxanthellate *Cladocora caespitosa* and the azooxanthellate *Astroides calycularis*, which are of key relevance for marine biodiversity in the Mediterranean Sea.

In chapter 1, we assessed the physiological responses and potential recovery of adult colonies of *C. caespitosa* and *A. calycularis* to different warming scenarios and marine heatwave events, based on nine years of temperature data at the collection site. Across the two species studied, we observed a similar physiological response to heat stress. During the three first months of warming and MHW experiment, the calcification rates did not differ between the treatments for both species, nor the gross photosynthesis of *C. caespitosa*. Respiration rates and the relative number of polyps per colony increased during the summer peak for every treatment of temperature for *C. caespitosa*. In the case of *A. calycularis*, necrosis started on some polyps after the peak temperature under Warming treatment. We were able to observe that *C. caespitosa* from Ischia bleached at high temperatures like it has been reported in Croatia, Montenegro and Cyprus (Jiménez *et al.* 2016; Kružić *et al.* 2016; Mačić *et al.* 2019), but colonies did not show direct necrosis as observed in Columbretes Island and Ligurian Sea (Rodolfo-Metalpa *et al.* 2005; Rubio-Portillo *et al.* 2018).

In addition to ocean warming, ocean acidification can severely affect marine organisms. In Chapter 2, I aimed to study whether previous exposition to low pH at CO₂ vent sites provided tolerance to adult colonies of both coral species. For this purpose, colonies from the CO₂ vents

and ambient pH sites were exposed to ambient and low pH treatments in the laboratory. In contrast to our initial hypothesis, we did not observe any change in net calcification rates, respiration, and photosynthesis regardless of their environmental history, pH treatment and trophic strategy, making it difficult to conclude in any acclimation to acidification. In contrast, *in situ* surveys revealed lower abundance and smaller sizes for both corals at CO₂ vent sites compared with ambient pH sites.

Adult stage of the two corals presented a moderate response to warming and acidification, however early life stages are generally more sensitive to global environmental change and are generally less studied than adult colonies. In chapter 3, we tested whether larval development and survival, and growth of recruits of *A. calycularis* were affected by warm temperature and low pH in the laboratory. Our results showed that warm temperature hastened settlement and metamorphosis of the larvae. In contrast, low pH delayed the metamorphosis and affected the post-settlement growth by reducing linear extension, budding, volume of the skeleton, and number of septa. Interestingly, skeletal density of recruits under low pH and ambient temperature was higher. Finally, combined drivers had a negative synergistic impact on survival, settlement and linear extension.

As acidification had an impact on early life stages of *A. calycularis* and reproduction was happening at the CO₂ vent site, we wondered whether acclimation to low pH was acquired by early life stages whose parents originated from CO₂ vents. In Appendix IV, larvae from CO₂ vent and ambient pH populations were exposed to different pH in the laboratory. The main differences were observed between the site of origin of the larvae, with smaller size and lower survival for the larvae from the CO₂ vents regardless of pH treatment. Larvae from the CO₂ vent site had also a lower settlement success at low and extreme low pH than the larvae from the ambient pH site. The analysis of the transcriptome of five-month-old recruits confirms a difference in gene expression between origin sites and an upregulation of differentially expressed genes such as cytoskeleton regulation genes for the recruits from the CO₂ vents.

Sexual reproduction is one of the mechanisms that can be impaired under acidification. Moreover, reproduction of *C. caespitosa* still has a lot of unknowns. Thus, in Appendix V, we studied the impact of acidification on gametogenesis and we investigated the timing of the spawning of *C. caespitosa* of Ischia. For this purpose, samples from CO₂ vent and ambient sites were obtained in July and September 2020 to analyze the progression of gametogenesis on selected colonies. The sex ratio and the size of the gametes did not differ from a site to

another. However, in September, the colonies of both sex in the ambient site had released their gametes while an asynchrony of spawning was exposed the CO₂ vent site, as the male had not spawned. This asynchrony could lead to an impairment of the fertilization and thus, the number of larvae which are critical factors to maintain or replenish population after disturbance.

Difference of impact of ocean warming and acidification between life stages

In order to have a healthy population, not only the survival of the adult colonies is important, but also an effective sexual reproduction with a great number of eggs and larvae, a successful metamorphosis and a high post-settlement survival and growth of recruits (Albright 2011). Along this thesis, I was able to study the impact of warming, acidification separately or in combination on different life stages of *C. caespitosa* and *A. calycularis*. Here, I will discuss the difference or similarity of responses observed between life stages.

Ocean Warming

As found in the majority of studies of warming on larvae (*e.g.* Edmunds *et al.* 2001; Chua *et al.* 2013), temperature had an impact on the development of *A. calycularis*' larvae by speeding the settlement and metamorphosis (Chapter 3), possibly due to an increase of metabolism and therefore respiration (Edmunds *et al.* 2001). Increase of metabolism could shorten the duration of the larval phase and reduce the distance of dispersal by causing metamorphosis and settlement before larvae travel out of their population of origin (O'Connor *et al.* 2007; Figueiredo *et al.* 2014). Local retention of the larvae has a direct impact on connectivity and genetic diversity, which potentially reduces the resistance to disturbances (Bay and Palumbi 2014). Increase of metabolism with temperature is known to be one of the first answers to warming in adults, due to the increased activity of enzymes (Clarke and Fraser 2004). However, respiration of adult colonies of *A. calycularis* from Chapter 1, did not increase with temperature as it would have been expected. Adult colonies of *C. caespitosa* showed in contrast an increase of respiration at the summer peak temperature in all temperature treatments (Chapter 1), or after 20 days at 26 and 28°C (Rodolfo-Metalpa *et al.* 2006). This increase of respiration was also linked with the increase in budding in *C. caespitosa* just after the peak temperature. *A. calycularis* also showed an increase of polyps by budding, but only for the Warming treatment, which reached a peak of summer temperature of 30°C. The same was observed in early life stages of *A. calycularis* in Chapter

3, as recruits under warm treatment of 26°C and ambient pH were the first to present budding and had a higher mean number of polyps per new colony.

As larvae have little reserves of energy, the increase of metabolism usually increases mortality (Bassim and Sammarco 2003; Randall and Szmant 2009; Woolsey *et al.* 2013). In Chapter 3, larvae under warm temperature and ambient pH did not present a higher mortality, though. As *A. calycularis* is a thermophilic species, larvae might be tolerant to warm temperatures, as adult colonies do. The warm temperature was 26°C for the larvae (Chapter 3) on the larvae and adult colonies did not present necrosis before the peak temperature of 30°C (Chapter 1). Our results were less severe than the one obtained in a previous laboratory experiment, where *A. calycularis* presented a 25% decrease in calcification after a summer peak of 24°C (Movilla *et al.* 2016). A peak of 30°C did not change the calcification rate of the colonies in our study (Chapter 1) and linear extension of the recruits was not impacted by temperature (Chapter 3).

Under higher temperatures, adult colonies and early life stages showed similar responses as they were both tolerant to warm temperatures. However, the acceleration of larval development might have repercussions on the connectivity and genetic diversity of populations and thus their capacities to react to changes in the environment.

Ocean acidification

Contrary to warming, acidification either has no effect on the development of larvae (Anlauf *et al.* 2011; Chua *et al.* 2013) or decreases the metabolism of larvae (Albright 2011). A decrease in metabolism can decrease settlement and metamorphosis (Albright *et al.* 2010; Albright 2011; Chua *et al.* 2013). In chapter 3, larvae of *A. calycularis* were no exception and settled less at low pH. However, adult colonies of *A. calycularis* did not present any decrease in metabolism, as respiration was not affected, neither for the colonies of *C. caespitosa* (Chapter 2, Carbonne *et al.* 2021) or in the study of Rodolfo-Metalpa *et al.* (2010). Generally, respiration of adult corals is not affected by acidification contrary to calcification that is known to be impacted by low pH in adults and recruits (Foster *et al.* 2015; Cornwall *et al.* 2021; Leung *et al.* 2022). The *A. calycularis* recruits did present an impact on calcification, with a decrease in linear extension, volume of the skeleton, and number of septa under low pH (Chapter 3). However, calcification of adult *A. calycularis* was not affected by low pH (Chapter 2, Carbonne *et al.* 2021) as obtained by Movilla *et al.* (2016). The same tolerance of calcification to acidification was observed on *C. caespitosa* adults that were not impacted by

low pH (Chapter 2). These results are not surprising as calcification of temperate corals is rarely affected by low pH, making them more tolerant to ocean acidification than tropical corals (Ries *et al.* 2010; Rodolfo-Metalpa *et al.* 2011, 2015).

Tolerance to ocean acidification after previous exposition to CO₂ vents: difference between adult colonies and early life stages

Aside from assessing the impact of warming and acidification on early life stages and adult colonies of *C. caespitosa* and *A. calycularis*, one of the main questions of this thesis was to know if the two studied corals found at the CO₂ vents were acclimatized to ocean acidification. For this purpose, study of *in situ* colonies and acidification experiments in the laboratory with adult colonies or larvae from CO₂ vent and ambient sites have been conducted.

In chapter 2, adult colonies of *C. caespitosa* and *A. calycularis* presented no difference in calcification, respiration, and photosynthesis (this last only for *C. caespitosa*), regardless of the pH treatment in the lab and the site of origin (CO₂ vents or ambient pH sites). Thus, for the parameters tested, we cannot conclude any acclimation to acidification. However, some acclimation might have been acquired by recruits from the CO₂ vents (Appendix IV), as they presented a higher growth rate under ambient pH treatment than recruits from ambient pH site. Besides this result, all the other treatments presented a similar growth rate. This greater linear extension might be due to a higher metabolism, as observed by Putnam and Gates (2015), where larvae from parents living in a high pCO₂ and temperature environment produced larvae with a higher metabolism to cope with unfavorable environments.

During the experiment of Chapter 2, corals did not show acclimation, however, *in situ*, adult colonies of *A. calycularis* presented signs of acclimatization with a change in skeletal structure. Teixido *et al.* (2020) observed an increase in bulk density, micro-density and a decrease in porosity, producing encrusting skeletons to face low pH at the CO₂ vents compared to the ambient pH sites. Interestingly, recruits from Chapter 3 under ambient temperature-low pH also showed a higher density. Usually, the opposite is found on corals exposed to low pH, with a decrease in bulk density and an increase in porosity to maintain linear extension. For example, four species of corals living in CO₂ vents of Papua New Guinea (Prada *et al.* 2021) or the Mediterranean solitary coral *Balanophyllia europaea* in the CO₂ vents of Panarea, Italy (Fantazzini *et al.* 2015) presented a lower bulk density and higher porosity. Lower skeletal density under low pH can be explained by the inactivation of some

calycoblastic cells to save energy and limit the decrease of pH in the calcifying fluid (Wall *et al.* 2019).

Even if some acclimatization of the skeletal structure can be observed in adult colonies, a very important difference in structure of the colonies and population have also been observed *in situ*. Colonies of *A. calycularis* at Grotta del Mago are smaller, they have fewer polyps with few coenosarc (tissue between polyps, Teixido *et al.* 2020). In chapter 3, under low pH recruits also presented loss of tissue, with the polyp retracting in the calix and exposing the base of the skeleton. The base of the exposed skeleton could likely experience dissolution, as described before with *C. caespitosa* by Rodolfo-Metalpa *et al.* (2011) and polyps could detach from the substrate (Chapter 3). This might explain why colonies of *A. calycularis* found near CO₂ vents presented a decrease in coverage with decreasing pH at this site with depth (Teixido *et al.* 2020). The fact that larvae from CO₂ vent parents were smaller (-30%), can be related to the amount of energy reserve and their survivorship (Isomura and Nishihira 2001). This difference in larval energy reserves might be explained by a possible tradeoff in energy allocation of the parents. Calcification and metabolism were maintained on adult colonies from the CO₂ vents under low pH, possibly at a cost (Chapter 2, Carbonne *et al.* 2021). Thus, only low energy might have been able to be allocated to reproduction. In addition to larval mortality, a wide range of benthic organisms such as gastropods and bivalves have a decrease of settlement in the CO₂ vents (Cigliano *et al.* 2010). Corals are no exception, in particular because acidification alters the chemical cues from microbial communities or from crustose coralline algae, reducing induction of settlement and metamorphosis in larvae (Harrison 2011; Webster *et al.* 2011; Doropoulos and Diaz-Pulido 2013). The higher mortality and lower settlement rates might result in a lower recruitment at the CO₂ vent site in the Grotta del Mago and explain the lower coverage with lower pH. This is particularly true for brooding species such as *A. calycularis* that usually have a short dispersal and low input of larvae from other populations.

Besides a possible reduced recruitment capacity of *A. calycularis* larvae from the CO₂ vent site of Ischia, Marchini *et al.* (2021) have found that reproduction of *A. calycularis* was impacted by low pH with a delay in development of spermary by using colonies from ambient pH and transplanted to CO₂ vents in Panarea. Gametogenesis was not impacted by low pH conditions (Marchini *et al.* 2021) as gametes had the same sizes and numbers under ambient and low pH, such as in other studies (Albright 2011; Gizzi *et al.* 2017; Caroselli *et al.* 2019). Because larvae of *A. calycularis* from the CO₂ vents were smaller, a difference of gamete size

would be expected. Future analysis of colonies of *A. calycularis* from CO₂ vents and ambient pH sites in Ischia might bring some answers on the difference in the larval size. Overall, very little is known about sexual reproduction of *C. caespitosa* and about the impact of acidification on it. In this Thesis, I confirmed that *C. caespitosa* in Ischia was gonochoric, with colonies having separate sex and polyps presenting only one type of gamete as observed in Columbretes Island (Kersting *et al.* 2013c) and differed from colonies from the Adriatic Sea where are reported to be hermaphroditic (Kružić *et al.* 2008). In appendix V, the gametogenesis analysis of *C. caespitosa* of Ischia shows the same size of gametes for colonies from the CO₂ vent and ambient pH sites. The analysis of gametes in September allowed us to conclude that spawning occurs after summer, as observed by Kersting *et al.* (2013c) in Columbretes Islands, and that an asynchrony of spawning occurs in the CO₂ vent site. Indeed, in September, colonies of the ambient pH site had released all their gametes besides one male (5/5 females, 4/5 males), while colonies from the CO₂ vent site only females had released (5/5 females, 1/5 males). This result of asynchrony also occurred to *A. calycularis* in the CO₂ vents of Panarea (Marchini *et al.* 2021). This asynchrony prevents fertilization and could lead to very low recruitment and replenishment of the population.

The populations of *A. calycularis* from the CO₂ vent site and the ambient pH sites did not differ only in coverage and morphology of the colonies, but they also showed a strong genetic structure using transcriptomic data (Teixido *et al.* 2020). Indeed, the population from the CO₂ vent differed with the ambient pH populations by presenting high levels of divergence linked to calcification (Teixido *et al.* 2020). Similar results regarding gene expression and calcification were observed in the temperate coral *Balanophyllia elegans* in high upwelling sites where pH is lower (Griffiths *et al.* 2019). The gene expression analyses of five-month old recruits of *A. calycularis* (Appendix IV) showed a difference between the two populations by using 20 transcripts. The transcriptomes analyses indicated that gene expression differed significantly between the CO₂ vent and the ambient pH site regardless of the pH treatments. A comparison between sites explains 98.8% of the differentially expressed genes, contrary to the comparison between pH treatment that only explains 1.2%. Interestingly, *Acropora millepora* of Papua New Guinea did not present any consistent change in gene expression between CO₂ vents and ambient pH sites (Kenkel *et al.* 2018). The difference might be in the fact that *A. millepora* studied in Papua New Guinea is a broadcast spawner with long larval dispersion in an open environment where recruitment from other sites can happen, while *A. calycularis* is

an internal brooder with short dispersal that occurs in a semi-closed cave in Ischia where lower genetic connectivity can be expected.

Synergistic impact of warming and acidification

Both warming and acidification combined had a negative synergistic impact on growth of the recruits (Chapter 3). However, on adult colonies of *A. calycularis*, no combined effect has been observed, only temperature decreased the calcification of corals (Movilla *et al.* 2016). A 33% decrease in coral net calcification is expected for the end of the century under RCP8.5 with both warming and acidification (Cornwall *et al.* 2021). The combined stress also worsens the low settlement rate, and induces unusual behavior, where larvae metamorphosed before settlement (Chapter 3), leading to free polyps with very little survival chances. Furthermore, the polyps that were able to settle under warm temperatures and low pH, were the only ones that did not present budding. Which present a far more negative picture of future recruitment of *A. calycularis* than with acidification alone.

Difference of impact between zooxanthellate and azooxanthellate corals

The zooxanthellate *C. caespitosa* suffered an increase of respiration and bleaching under Ambient, Heatwave and Warming treatment. Whereas the azooxanthellate *A. calycularis* only presented a response under the Warming treatment with an increase of number of polyps during the peak temperature and some polyp necrosis in four colonies after the peak (Chapter 1). This difference in tolerance between species might be due to the fact that photosynthetic efficiency and density of *C. caespitosa*'s symbionts is reduced above 24°C (Rodolfo-Metalpa *et al.* 2006, Chapter 1). Thus, *A. calycularis* not being dependent on symbionts for nutrient input, have a higher temperature threshold than *C. caespitosa*. Among Mediterranean corals, azooxanthellate corals presented a better tolerance to increasing temperatures than zooxanthellate corals (Goffredo *et al.* 2007; Caroselli *et al.* 2012, 2016; Airi *et al.* 2014, 2017; Marchini *et al.* 2020), possibly due to photosynthesis inhibition of symbiotic zooxanthellae at high temperature.

Warming has not only an impact on adult coral symbiosis, but also in larvae. High temperature can decrease the first establishment of the symbiosis on larvae (Schnitzler *et al.* 2012), but also induce bleaching (Bahr *et al.* 2020) and reduce photosynthesis (Edmunds *et al.* 2001). No difference of survival was found between symbiotic and aposymbiotic larvae of

Acropora muricata at different high temperatures (Baird *et al.* 2006). However, when symbiotic larvae bleach under high temperature, they are disadvantaged compared to aposymbiotic, as it results in an oxidative stress with cellular damage that induce mortality (Yakovleva *et al.* 2009). On the contrary, Rivest *et al.* (2018), through a transcriptomic analysis, have found that symbiosis may enhance the physiological capacity of larvae of *Pocillopora damicornis* to cope with warming and acidification without expressing additional protective mechanisms.

Opposite results can be found on adult colonies, with some species having a decrease in symbionts with acidification (Hii *et al.* 2009; Kaniewska *et al.* 2012; Mason 2018) and other no impact (Wall *et al.* 2016). Here, *C. caespitosa* and *A. calycularis*, did not present any difference in respiration and calcification, and photosynthesis in *C. caespitosa*, under low pH. The presence of symbionts might give an advantage to zooxanthellate corals as the photosynthetic activity might help decrease the intracellular CO₂, and recover from cellular acidosis induced by environmental acidification (Gibbin *et al.* 2014).

Warming, by inducing bleaching, might have a greater impact on zooxanthellate corals, however, no clear answer can be given on the impact of acidification, as very few comparisons have been done between them under low pH and the very variable responses between species.

Conclusion and perspectives

Conclusion

The principal aim of this Ph.D. Thesis was to determine the effects of present and future coastal environmental conditions and variability on Mediterranean corals. I was able to answer the principal questions through the analysis of environmental data and field surveys at the CO₂ vents of Ischia combined with laboratory experiments under controlled temperature and pH on the two Mediterranean corals *Cladocora caespitosa* and *Astroides calycularis*.

How do present-day and future temperature and/or pH affect the physiology of *Cladocora caespitosa* and *Astroides calycularis* at every life stage (adult, gametogenesis, larvae, and recruits)?

=> Adult colonies of *C. caespitosa* and *A. calycularis* showed a tolerance to acidification for respiration, photosynthesis and calcification. However, it is a fragile equilibrium, because this

tolerance is out a cost. The addition of another stressor might be fatal or induce a trade-off between survival, growth and reproduction. Warming is already a threat for *C. caespitosa* and *A. calycularis*, as most of the MHWs lead to mass mortalities. In our laboratory conditions, the colonies of both species were able to recover after summer heat peak. However, warming and MHWs can go along with a strong thermocline, decrease in nutrients, a deoxygenation or development of harmful *Vibrios* which were not tested in our experiment, but will be suffered *in situ*. Larvae suffered from warming and low pH, which impaired, separately and additively, the settlement, the metamorphosis and the post-settlement growth and increased mortality. Which are pessimistic results, as reproduction is the only natural way to recolonize a decreasing population.

Are the different life stages equally impacted by future environmental conditions?

=> The impacts of warming and acidification on adult colonies *C. caespitosa* and *A. calycularis* were moderated, showing tolerance and resilience (defined here as the ability to recover after a disturbance). However, the impact of global change on the early life stages of *A. calycularis* is worrying as it mostly causes a decrease in settlement and survival. These negative responses could lead to a crash of the populations if mass mortality events of adults increase with MHWs.

Does exposure to low pH at CO₂ vents enhance the tolerance of corals to future acidification?

=> Given our results, with the studied parameters, the CO₂ vents sites of Ischia do not seem to favor acclimatization for *C. caespitosa* and *A. calycularis*. Contrariwise, they seem to have a negative impact on the individual physiology (reproduction, early life stages development and recruitment, morphology of the colony) and a shift on species' ecological traits and population structure (massive versus encrusting forms and smaller sizes).

Perspectives

Concerns on the effects of global environmental change on corals have been raised due to the increasing mass mortalities and bleaching events across geographical regions and the effect of ocean acidification on their calcification. During this thesis, the main objective was to deepen the knowledge on the response of two important Mediterranean habitat-forming species. However, as it often occurs in research, answering some questions leads to new ones that would need to be assessed in future research.

What to pursue at the individual level?

More research is still needed to understand why the adult colonies were tolerant to warming and ocean acidification at the individual level. One of the paths to follow could be to look at the reserves of energy and the contribution of heterotrophy to it. Most of the maintenance of the physiological parameter under warming and acidification has generally a metabolic cost (Wood *et al.* 2008). One of the hypotheses is that corals increase their heterotrophy to increase the input of energy and increase the energy reserves (Hughes and Grottoli 2013; Towle *et al.* 2015). In order to test this hypothesis, the colonies from the experiment of Chapter 2 have been sent to Ann Marie Hulver, Ph.D. student under the supervision of Prof. Andrea Grottoli from the Ohio State University (USA). Energy reserves such as carbohydrates and lipids are being analyzed by Ann Marie Hulver along with stable isotopes ($\delta^{13}\text{C}$) in order to determine if the origin of the corals or the pH treatment have an impact on the reserves or on the heterotrophy of *Cladocora caespitosa*. The same type of measurements could be done with the colonies from Chapter 1, to see if there is a link between energy reserves, heterotrophy and recovery of colonies after the exposition to different summer temperatures and MHWs. Furthermore, as colonies of *C. caespitosa* from the same temperature treatment did not bleach equally (Chapter 1), it would be interesting to analyze the zooxanthellae and the microbiome communities to understand this individual variability in responses. This analysis could be also applied to other sites where *C. caespitosa*'s populations are found in the Mediterranean Sea to understand why some colonies bleach or not and why some of them suffer direct necrosis.

The fact that calcification was maintained under low pH also needs more investigation. Assessing the pH and carbonate chemistry of the calcifying fluid of *C. caespitosa* and *Astroides calycularis* from the CO₂ vent and ambient pH sites under different pH in the laboratory might help us to understand if pH and DIC upregulation at the calcifying site are mechanisms that they used to maintain calcification under low pH. To complete the analysis, some single cell transcriptomics could be done on calycoloblastic cells in order to study the expression of genes that could help maintain the calcification under low pH.

Large scale studies: from a population to the Mediterranean basin level

We observed that the reproduction of *C. caespitosa* was impaired by low pH at the CO₂ vents. In order to confirm that only colonies found in the ambient pH site are able to reproduce, a genetic analysis to assess the genetic fluxes of the two populations could be

done. Reproduction of *C. caespitosa* still has a lot of grey areas. Some efforts of *in situ* monitoring could be done around the theoretical period of spawning in both sites, in order to identify the exact moment of spawning and collect gametes. To confirm our hypothesis concerning the potential decrease of recruitment and growth of early life stages of *A. calycularis* under acidification, *in situ* surveys over time with permanent transects could be performed at the vent site and in an ambient site, to quantify the number of new recruits, their survivorship, and their growth.

This thesis focused on the study of the acclimatization to acidification at CO₂ vents. However, the future of populations depends on their ability to adapt to their environments. To assess the adaptation of the CO₂ vent population, *in situ* reciprocal transplant experiments should be done with an ambient pH site. A reciprocal transplant experiment has been conducted by Dr. Núria Teixidó with *A. calycularis* in Ischia and results are in progress, however, nothing has been done with *C. caespitosa* so far. A similar transplant experiment with colonies from CO₂ vent and Ambient sites could also be performed with *Cladocora* in order to reveal whether the mechanisms of local adaptation are species specific or more generalizable.

Overall, comparison of populations of both corals could be very interesting, for example in a latitudinal and longitudinal gradients of temperature (*e.g.* north and south, west and east). Calcification, respiration, photosynthesis, reserves of energy, reproduction and genomics could be analyzed on sites with different temperature regimes.

Conservation

C. caespitosa and *A. calycularis* have been suffering repetitive mass mortality events due to MHWs. Monitoring environmental data by deploying long-term sensors at a large scale is important to help projecting realistic future warming scenarios. In parallel, monitoring the mass mortality events is also essential to identify key sites where the corals are more or less impacted. These natural sites less impacted by MHWs could be perfect candidates for Marine Protected Areas. During this thesis, 100 colonies of *C. caespitosa* have been tagged in CO₂ vent and ambient pH sites of Ischia. The yearly monitoring of these colonies could bring precious information on the impact of MHWs on *C. caespitosa*. Furthermore, Ischia is the current northern distribution of the thermophilic *A. calycularis* in the Mediterranean Sea.

Monitoring the progression of this species toward the North could be a way to track the warming of the Mediterranean Sea.

Résumé en français

Introduction

Les émissions de gaz à effet de serre d'origine anthropique, tels que le CO₂, ont augmentées depuis le début de la Révolution Industrielle, passant de 277 parts par million (ppm) en 1750 (Joos and Spahni, 2008) à 416 ppm de nos jours (actualisé à partir de Keeling and Keeling, 2017, Archive 2022-06-01). Les futures projections du Groupe d'experts intergouvernemental sur l'évolution du climat (GIEC) sont modélisées à partir de scénarios basés sur les émissions possibles de CO₂ futures. Les prévisions de concentrations de CO₂ atmosphérique montent à ~ 600 ppm sous les scénarios SSP2-4.5 et RCP4.5 jusqu'à ~ 1000 ppm sous RCP8.5 et ~ 1150 ppm sous SSP5-8.5 pour la fin du siècle (Kwiatkowski *et al.* 2020). La pression partielle de CO₂ (pCO₂) des océans tend à s'équilibrer avec la pCO₂ atmosphérique, faisant de l'océan un puit à CO₂. L'augmentation de CO₂ atmosphérique contribue donc à des changements physico-chimiques de l'océan, avec d'importantes conséquences sur ses écosystèmes et services fournis aux humains (Gattuso et al 2015).

Le réchauffement de l'océan et les vagues de chaleur marines.

L'augmentation de la concentration des gaz à effet de serre dans l'atmosphère réchauffe la surface de la Terre (Hegerl 2005), cependant, 90% de cette chaleur est accumulée dans l'océan, menant à son réchauffement (Cheng *et al.* 2022). Les projections sous le scénario RCP8.5, prévoit un possible réchauffement de 3.04°C à la surface de l'océan pour 2100 (Kwiatkowski *et al.* 2020, Figure 1).

En plus du réchauffement progressif et à long-terme, une augmentation de fréquence et d'intensité d'événement climatiques extrêmes est attendue, tels que les vagues de chaleur marine (Smale et al 2019, Oliver et al 2021). Ces événements exceptionnels de masses d'eau chaude sont définis par une température de l'eau de mer dépassant pendant au moins 5 jours, un seuil défini comme étant le 90^{ème} centile de la moyenne climatologique (Hobday *et al.* 2016, figure 2). Les vagues de chaleur marines ont des effets dévastateurs sur les espèces et les écosystèmes, menant à des changements écologiques et physiologiques majeurs, comme des événements de mortalité de masse et d'extinctions locales (Smale et al 2019, Smith et al 2021, Garrabou *et al.* 2022).

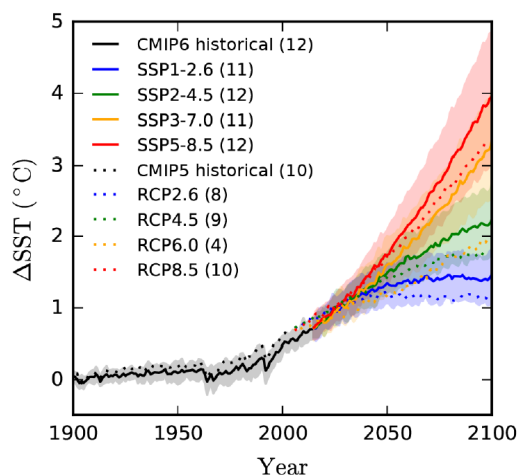


Figure 1: Moyenne globale de la température de surface de l'océan entre 1900–2100 relatif à la période 1870-1899 en fonction des différents scénarios. Figure adaptée de la Figure 1 de Kwiatkowski *et al.* 2020).

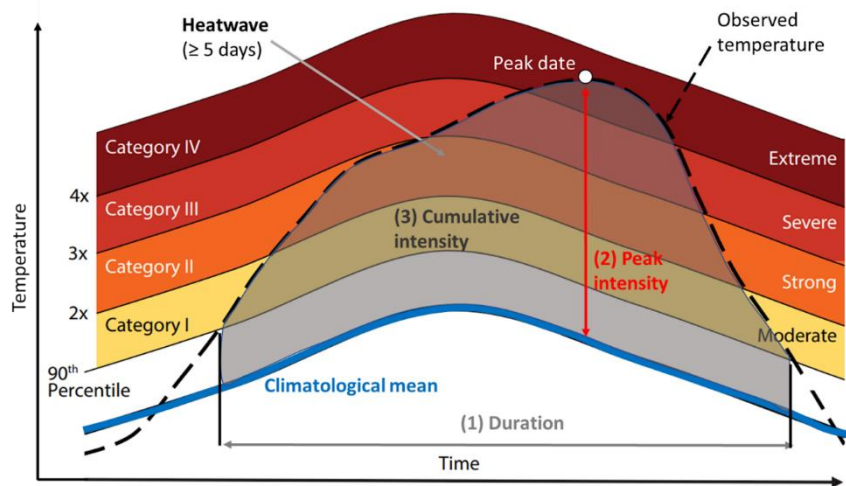


Figure 2: Schéma des caractéristiques utilisées pour définir les vagues de chaleur marines. Ici sont présentés la température observée (ligne pointillée), la moyenne climatologique (ligne bleue) et le 90ème centile de la moyenne (ligne fine). Les trois propriétés d'une vague de chaleur marine sont: (1) sa durée, le nombre consécutif de jours de l'évènement, (2) l'intensité maximum, l'anomalie la plus élevée en °C, et (3) l'intensité cumulée, l'addition de toutes les intensités journalières de l'évènement (Hobday *et al.* 2016). Quatre categories sont définies à partir du 90ème centile afin de mieux évaluer les caractéristiques de la vague de chaleur et pour définir son évolution. La limite de chaque catégorie est un multiple du 90ème centile.

Acidification de l'océan

L'acidification de l'océan est causée par l'absorption de CO₂ atmosphérique par l'océan. Lorsque le CO₂ se dissout dans l'eau de mer, il modifie la chimie des carbonates et cause une diminution du pH, de la concentration en ions carbonates (CO₃²⁻) et de l'état de saturation du carbonate de calcium. Ces paramètres jouent un rôle clé dans le processus de formation des squelettes et coquilles d'organismes calcifiants marins (Gattuso and Hansson 2011). Le pH de surface a déjà diminué de 0.1 unités depuis le début de l'ère industrielle (Caldeira and Wickett, 2003, Orr *et al.* 2005). Etant donné que les émissions de CO₂ augmentent, les projections sous RCP8.5 suggèrent une décroissance supplémentaire de 0.38 unités de pH pour la fin du siècle (AR5 du GIEC, Kwiatkowski *et al.* 2020, Figure 3). Les études analysant l'impact de l'acidification sur les espèces calcifiantes, telles que les coraux, les algues calcaires ou les bivalves suggèrent qu'un pH bas induit une décroissance de leur calcification (Andersson *et al.* 2011, Leung *et al.* 2022). De nos jours, la majorité des études sur l'impact de l'acidification sur les espèces benthiques ont été menées lors d'expériences en laboratoire dans des conditions contrôlées (Boyd *et al.* 2019). Les systèmes naturellement acidifiés, tels que les événements de CO₂, reflètent les effets de l'acidification sur l'ensemble de l'écosystème sur de longues échelles de temps (10 à 18 000 ans), fournissant ainsi des informations cruciales sur les effets de l'acidification sur la biodiversité et le potentiel d'acclimatation ou d'adaptation des espèces en combinant les travaux de terrain avec des expériences en laboratoire ou des transplantations croisées *in situ* (Rodolfo-Metalpa *et al.* 2010, Calosi *et al.* 2013, Olivé *et al.* 2017, Gonzalez-Delgado *et al.* 2018). Les systèmes d'événement de CO₂ volcaniques provoquent une acidification locale de l'eau de mer en

faisant buller du gaz contenant 90 % à 99 % de CO₂ depuis le fond de la mer et affectent l'écosystème environnant (Hall-Spencer *et al.* 2008, Gonzalez-Delgado et Hernandez 2018).

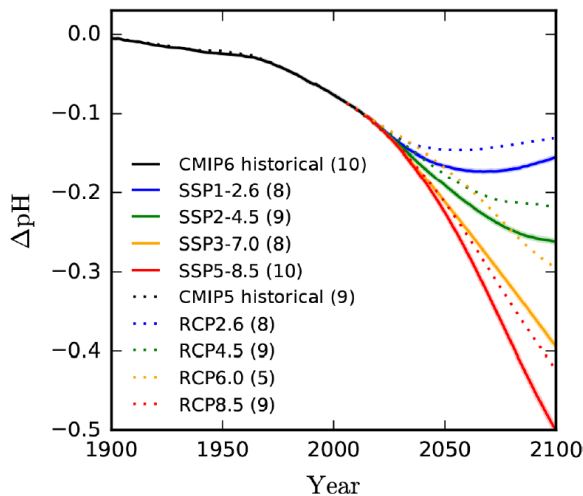


Figure 3: Moyenne globale du pH de surface entre 1900 et 2100 relatif à la période 1870-1899 en fonction des différents scénarios. Adapté de la Figure 1 Kwiatkowski *et al.* 2020).

La mer Méditerranée : un point chaud du changement global

En raison de sa condition de mer semi-fermée, le réchauffement futur de la mer Méditerranée devrait être supérieur de 20 % (annuel) et de 50 % (été) aux projections de la moyenne mondiale (Lionello et Scarascia 2018). La température de surface de la mer a déjà augmenté jusqu'à 0,44°C et une augmentation de 0,8 à 3,8°C est prévue pour la fin du siècle selon les régions (Darmaraki *et al.*, 2019). Les vagues de chaleur marines en mer Méditerranée dans le cadre du RCP8.5 devraient avoir, à la fin du siècle, une durée trois mois plus longue et l'intensité quatre fois plus élevée d'actuellement (chapitre 9 du WGI AR6). Ces événements extrêmes entraînent la mortalité massive d'espèces à travers les habitats et les profondeurs, où le phylum le plus touché est celui des cnidaires (55%), dont les coraux scléactiniaires (Garrabou et al 2022).

Les coraux

Cette thèse est principalement axée sur les coraux de l'ordre des Scleractinia. La plupart de ces coraux des récifs tropicaux peu profonds sont zooxanthellés, c'est-à-dire qu'ils réalisent une symbiose intracellulaire avec des algues dinoflagellées du genre *Symbiodinium*, également appelées zooxanthelles (Davy *et al.* 2012). Ce symbionte unicellulaire est hébergé dans les cellules du corail et échange de façon intercellulaire des nutriments avec son hôte (Oakley et Davy 2018 (Weis 2008, Davy *et al.* 2012). Certains coraux sont azooxanthellés (non symbiotiques), l'hétérotrophie est alors la seule stratégie trophique, en mangeant du zooplancton et des débris organiques qu'ils capturent avec leurs tentacules.

Reproduction des coraux

Les coraux ont un cycle de vie composé d'une phase benthique dominante sous forme de polype, prédominée par la croissance des tissus et du squelette, de cycles réguliers de reproduction sexuelle et parfois asexuée, et d'une courte phase larvaire planctonique (Harrison 2010, Figure 7). La reproduction sexuelle des coraux est très importante pour la connectivité

des populations et la diversité génétique car les larves coralliennes constituent la seule phase pélagique du cycle de vie des coraux (Bay et Palumbi 2014).

Deux modes de sexualité peuvent être observés chez les coraux scléactiniaires, gonochorique - où les colonies sont soit femelles soit mâles - et hermaphrodite - où les colonies ou les polypes produisent les deux types de gamètes, sperme et ovocytes. Au fur et à mesure de la gamétogenèse, les gonades, ovaires (femelles) et testicules (mâles), s'étendent avec le développement des ovocytes et des spermatozoïdes, respectivement (Glynn et al 2016).

Lorsque les gamètes arrivent à maturité, deux modes de développement peuvent être observés chez les coraux, la couvaison (brooding) et le frai diffus (broadcast spawning). Chez les coraux couveurs, la fécondation des ovocytes par les spermatozoïdes et le développement embryonnaire ont lieu à l'intérieur des polypes parentaux jusqu'à maturité des larves (Szmant 1986). En revanche, le frai diffus (broadcast spawning) est une libération synchrone des gamètes femelles et mâles, qui par flottabilité vont se retrouver à la surface où la fécondation se produit (Harrison 2010).

Après la dispersion de la larve, la fixation se produit lorsque les larves ont sélectionné leur substrat (Ritson-Williams *et al.* 2016). Une fois installée, la larve se métamorphose et devient une recrue sous forme de polype (Hadfield *et al.* 2001). La croissance des recrues est basée sur le bourgeonnement des polypes et crée une colonie génétiquement identique (Harrison 2010).

Impact du réchauffement et de l'acidification sur les coraux

Au cours des 30 dernières années, le réchauffement de la température de la surface de la mer a déclenché un nombre croissant de blanchiments massifs de coraux, notamment des événements pan-tropicaux en 1998, 2010 et 2015/2016 (Heron *et al.* 2016, Hughes 2018). Le blanchiment est la rupture de la relation symbiotique entre le corail (hôte) et ses zooxanthelles endosymbiotiques ou une perte des pigments photosynthétiques de ces symbiotes microalgues (Davy *et al.* 2012). Les coraux zooxanthellés blanchis ont une capacité réduite à satisfaire les besoins métaboliques car la plupart des besoins en carbone sont obtenus à partir des produits photosynthétiques transférés par les symbiotes (Goldberg 2018). Les coraux sont contraints de puiser dans leurs réserves énergétiques (lipides et glucides) et de faire un trade-off entre survie, croissance et reproduction (Shore-Maggio et al 2018).

Le blanchiment perturbe l'homéostasie du calcium et diminue la calcification (DeSalvo *et al.*, 2008). Outre cette possible diminution du taux de croissance, le réchauffement et le blanchiment des adultes ont également un impact sur la vulnérabilité des populations coralliennes en perturbant la reproduction (Hagedorn *et al.* 2016) et en diminuant la connectivité entre les populations en réduisant la dispersion des larves, diminuant ainsi la diversité génétique (Figuereido *et al.* 2022). En effet, les larves soumises à des températures élevées présentent un développement accéléré, entraînant des anomalies (Woolsey *et al.* 2013), un taux de survie plus faible (Baria *et al.*, 2015, Bahr *et al.*, 2020), une métamorphose et une fixation plus rapides (Nozawa et Harrison, 2007, Chua *et al.* 2013), ce qui augmente la rétention des larves dans la population indigène.

La réponse des coraux zooxanthellés et azooxanthellés tempérés de la Méditerranée face aux événements répétitifs de mortalité massive dus aux MHW (par exemple, Kersting *et al.* 2013, Kruzic *et al.* 2016, Bisanti *et al.* 2022) est beaucoup moins étudiée que les coraux tropicaux. Il a été constaté que les coraux azooxanthellés méditerranéens sont plus résistants à l'augmentation de la température que les coraux zooxanthellés. Les espèces azooxanthellées *Leptopsammia pruvoti* et *Caryophyllia inornata* n'ont présenté aucune différence de taux de croissance et de reproduction le long d'un gradient de températures croissantes (de 16,74 à 19,15°C en moyenne annuelle selon les sites) le long des côtes italiennes (Caroselli *et al.* 2012 et 2016, Marchini *et al.* 2020), tandis que l'espèce zooxanthellée *Balanophyllia europaea* a montré une diminution de la reproduction et une inhibition de la calcification probablement due à l'inhibition de la photosynthèse à température élevée (Goffredo *et al.* 2007, Airi *et al.* 2014). Les coraux zooxanthellés méditerranéens présentent également un blanchiment induit par la chaleur comme cela a été observé chez *Oculina patagonica*, *Madracis pharensis* et *Cladocora caespitosa* (Shenkar *et al.* 2005, Kružić et Popijač 2015, Jimenez *et al.* 2014). Le réchauffement a également un rôle dans l'augmentation de l'abondance de certaines bactéries pathogènes appartenant aux genres *Vibrio* et qui pourraient être responsables de la nécrose sur *Cladocora caespitosa* ou du blanchiment sur *Oculina patagonica* (Kushmaro *et al.* 1997, Vezzulli *et al.* 2012, Rubio-Portillo *et al.* 2018). Cependant, le réchauffement et son lien intime avec l'augmentation du nombre de MHW (Smale *et al.* 2019) n'est pas la seule menace pour les coraux.

L'acidification des océans est responsable d'une diminution de 15 % en moyenne de la calcification des coraux sous les valeurs de pH attendues d'ici la fin du siècle selon le scénario RCP 8.5 (Cornwall *et al.* 2021). La calcification des coraux scléactiniaires a lieu dans le fluide de calcification, un environnement semi-fermé entre le squelette et les couches cellulaires calicoblastiques (Wall *et al.* 2019). La chimie du carbonate du fluide calcifiant est physiologiquement régulée par des mécanismes cellulaires tels que des protéines transmembranaires. Cela permet au pH du fluide calcifiant de se maintenir à un niveau beaucoup plus élevé que celui de l'eau de mer environnante, ce qui facilite la précipitation du carbonate de calcium (Comeau *et al.* 2017). Cependant, l'acidification des océans peut diminuer l'efficacité de ces mécanismes, ce qui explique la diminution de la calcification avec l'acidification, observée chez de nombreux coraux. Comme décrit par Wall *et al.* (2019), pour maintenir la calcification, une dépense d'énergie plus importante est déployée pour assurer l'homéostasie du fluide de calcification. Il en résulte d'un squelette moins dense et plus poreux.

Dans des conditions d'acidification, la gamétogenèse n'est généralement pas affectée, mais elle peut induire une asynchronisation du frai (spawning) (Olischläger et Wild 2020, Liberman *et al.* 2021). De plus, la fécondation peut être diminuée sous acidification (Albright *et al.* 2010), en raison d'une réduction de la motilité flagellaire (Morita *et al.* 2010). Un pH bas peut également diminuer le métabolisme des larves (Albright *et al.* 2011), et une diminution de leur fixation en perturbant le film microbien dans les algues coralliennes encroûtantes (Webster *et al.* 2011, Doropoulos et Diaz-Pulido 2013). Les recrues et les juvéniles sont impactés par l'acidification, avec une diminution de 50% de la croissance sous un pH bas (Albright et Langdon 2011) ou un squelette déformé et poreux (Foster *et al.*, 2016).

Cependant, les coraux tempérés semblent être plus tolérants à l'acidification de l'océan que les coraux tropicaux car leur calcification est rarement affectée par un pH bas (Rodolfo-Metalpa *et al.*, 2011, Carbonne *et al.*, 2021).

Le réchauffement des océans devrait s'accroître en même temps que l'acidification des océans (Kwiatkowski *et al.* 2020) et pourrait, en synergie, réduire la précipitation de carbonate de 33 % d'ici la fin du siècle et augmenter la mortalité des coraux (Hoegh-Guldberg *et al.* 2007, Prada *et al.* 2017, Cornwall *et al.* 2021).

Plasticité phénotypique et adaptation locale aux changements environnementaux

La façon dont les populations et les espèces répondent aux conditions environnementales est essentielle à leur persistance dans les conditions climatiques actuelles et futures. Les espèces peuvent s'adapter à ces nouvelles conditions environnementales grâce à la plasticité phénotypique, s'adapter par sélection naturelle ou migrer vers des conditions plus favorables. La plasticité phénotypique est la capacité d'un organisme individuel à modifier son phénotype physiologique, comportemental ou morphologique avec son génotype existant, en réponse à un environnement variable (Putnam *et al.* 2016). La plasticité phénotypique est importante car elle peut constituer une réponse rapide à un changement environnemental soudain et est habituellement réversible (Chevin *et al.* 2010); cependant, elle est maintenue à un coût, elle est fortement énergivore (Jones et Berkelmans, 2011). La sélection naturelle réduit ces coûts au cours de l'évolution (DeWitt, 1998). L'adaptation est un changement génétique dans une population dû à la sélection naturelle pour améliorer les fonctions dans un environnement donné, et où ces changements sont héréditaires et transmis à la génération suivante (Edmunds et Gates 2008).

Coraux scléactiniaires de Méditerranée

La mer Méditerranée a une longue histoire avec les coraux scléactiniaires révélée dans les archives fossiles que plus de 100 espèces de coraux scléactiniaires (>80 genres) formaient des récifs coralliens (Vertino *et al.* 2013, Zibrovius 1995). Cependant, à la fin du Miocène, la crise de salinité messinienne (dessiccation presque complète de la mer Méditerranée entre 5,96 et 5,33 millions d'années) a entraîné la disparition des récifs coralliens et de presque tous les coraux zooxanthellés (Perrin et Bosellini 2012). Aujourd'hui, il ne reste plus que 33 espèces (25 genres) dans la mer Méditerranée (Zibrovius 1980). Cette thèse se concentre sur deux coraux méditerranéens d'une importance capitale pour la conservation : l'espèce zooxanthellée *C. caespitosa* (Linnaeus, 1767, Box *Cladocora caespitosa*) et l'espèce azooxanthellée *A. calycularis* (Pallas, 1766, Box *Astroides calycularis*).

Cladocora caespitosa

Cladocora caespitosa (Linnaeus 1767) est l'unique corail zooxanthellé endémique de la mer Méditerranée capable de former des structures proches des récifs coralliens (Zibrovius 1980). Il construit des structures ressemblant à des récifs, des bancs, qui recouvrent plusieurs mètres carrés de surface, comme par exemple à l'île Columbretes (Espagne, Kersting and Linares 2012) et au parc National Mljet (Croatie, Kružić and Benković 2008). Par contre, il forme généralement des colonies semi-sphériques de 10 à 30 cm de diamètre, disperses et séparées,

appelés des lits (Peirano *et al.* 1998). *C. caespitosa* a été fortement touché par des vagues de chaleur marine de plus en plus fréquentes. Les colonies présentent soit des nécroses directes (Rodolfo-Metalpa *et al.* 2005, Kersting *et al.* 2013) ou du blanchiment (Kruzic *et al.* 2014, Jimenez *et al.* 2016, Mačić *et al.* 2019).



Photographie d'une colonie de *Cladocora caespitosa in situ* (10m, Ischia, Italie) présentant une perte de pigmentation et des polypes sortis (colonie d'environ 20 cm de diamètre).

La reproduction de *C. caespitosa* est peu connue. Quelques fraies (broadcast spawning) ont été observés en mer Adriatique au début de l'été et identifiés comme étant hermaphrodiques (Schiller 1993, Kružić *et al.* 2008). Mais des analyses histologiques de colonies de Columbretes suggèrent que c'est une espèce gonochorique présentant un frai à la fin de l'été (Kersting *et al.* 2013).

Astroides calycularis

A. calycularis (Pallas, 1766) est un corail azooxanthellé endémique de la mer Méditerranée et caractérisé par sa couleur orange fluo (Zibrowius, 1995). Il est communément retrouvé dans des habitats rocheux peu profonds à faible lumière (des parois verticales, l'entrée de grottes ou surplombs, Zibrowius, 1995). Sa distribution géographique est limitée au sud-ouest de la mer Méditerranée (Zibrowius, 1995). Ce corail est considéré comme étant une espèce thermophile avec comme limite de température minimale 14°C pendant l'hiver (Bianchi, 2007). Des événements de mortalité d'*A. calycularis* ont été observés à la fin de l'été au niveau de la côte d'Ischia et des îles Pélages (Bisanti *et al.* 2022, Garrabou *et al.* 2022).



Photographie d'une colonie d'*Astroides calycularis in situ* (2 m, Ischia, Italie), caractérisée par sa couleur orange fluo (colonie d'environ 15 cm de diamètre).

A. calycularis est une espèce gonochorique qui couve ses larves (internal brooder) (Goffredo *et al.* 2010). Le frai du mâle se produit entre avril et mai, et suite à une fécondation et à un développement embryonnaire dans la femelle, les larves sont relâchées fin juin, lorsque l'eau de mer atteint environ 23°C (Goffredo *et al.* 2011, Casado-Amezua *et al.* 2013). Les larves relâchées font environ 2mm, matures et prêtes à se fixer (Goffredo *et al.* 2010).

Ischia

Les sites d'étude de cette thèse sont situés le long de la côte de l'île d'Ischia, en Italie. A Ischia du CO₂ provenant du magma sort du plancher océanique (Vezzoli 1988) créant des événements de CO₂ qui acidifient localement l'eau de mer.

Deux sites de CO₂ nouvellement découverts ont été les principaux sites d'étude de cette thèse : la Grotta del Mago, une grotte semi-submergée peu profonde (5 m de profondeur) avec un pHT entre 7,88 et 7,60 (de 2 à 4 m de profondeur, Teixido *et al.* 2020) et Chiane del Lume, un récif rocheux à 10 m de profondeur, où le pHT est de 7,99 à 7,89 (Carbonne *et al.* 2021). Ces deux sites de CO₂ ont été mis sous surveillance avec des capteurs haute résolution de pH, de température et de P.A.R. Il est intéressant de noter que des populations de coraux scléactiniaires sont naturellement présentes dans ces sites d'événement de CO₂ : le corail zooxanthellé *Cladocora caespitosa* à Chiane del Lume (10 m de profondeur) et le corail azooxanthellé *Astroides calycularis* dans la Grotta del Mago (5 m).

Objectifs et approches scientifiques de la thèse

Le but principal de cette thèse est de déterminer les effets de conditions environnementales présentes et futures sur les coraux méditerranéens. Cette thèse utilise des coraux provenant de deux sites d'événements de CO₂ à Ischia (Italie), qui causent une acidification locale de l'eau de mer. Le pH y est proche de celui attendu pour la fin du siècle. Mes recherches se sont focalisées sur des populations naturelles de coraux méditerranéens de ces événements de CO₂, qui sont des espèces clés pour la conservation, étant des espèces créatrices d'habitat : *Cladocora caespitosa* et *Astroides calycularis*. Plus en particulier, les objectifs étaient d'intégrer les données environnementales et des études de terrain au niveau des événements de CO₂ et de sites de référence avec de expériences de laboratoire avec une température et un pH contrôlé.

Les principales questions qui ont guidé la thèse sont :

- Comment les température et pH présents et futurs affectent la physiologie de *Cladocora caespitosa* et *Astroides calycularis* à chaque stade de vie (adultes, gamétogenèse, larves et recrues) ?
- Est-ce que les différents stades de vie sont impactés de manière égale par les futures conditions environnementales ?
- Est-ce que l'exposition à un pH bas au niveau des événements de CO₂ augmente la tolérance des coraux à une future acidification ?

Afin de répondre à ces questions, la thèse s'articule autour de 5 études dont 3 sont présentés comme chapitre de cette thèse et 2 sont en annexes car ils nécessitent des analyses complémentaires de leurs résultats.

Chapitre 1 Réponse de deux coraux scleractiniaires tempérés à un réchauffement et à des vagues de chaleur marine.

La mer Méditerranée est considérée comme un point chaud du changement climatique où des effets écologiques importants sur la biodiversité marine sont prévus (Giorgi *et al.* 2006, Cramer *et al.* 2018). En plus d'un réchauffement graduel des océans, la fréquence et la gravité des événements climatiques extrêmes tels que les vagues de chaleur marines ont considérablement augmenté (Smale *et al.* 2019). Les coraux zooxanthellés sont menacés par l'augmentation de la température de l'eau de mer entraînant un stress thermique qui provoque le blanchiment (Hughes *et al.* 2017). Le blanchiment entraîne une réduction de la capacité à satisfaire les besoins métaboliques (McLachlan *et al.* 2020). La plupart des connaissances accumulées sur l'impact du réchauffement sur les coraux ont été obtenues à partir de coraux tropicaux. La réponse des coraux méditerranéens tempérés aux événements répétitifs de mortalité massive dus aux vagues de chaleur marines (par exemple, Kersting *et al.* 2013, Kruzic *et al.* 2016, Bisanti *et al.* 2022) est en comparaison bien moins comprise.

Nous avons étudié la performance physiologique et la récupération de colonies du corail zooxanthellé *Cladocora caespitosa* et du corail azooxanthellé *Astroides calycularis*, aux températures estivales actuelles et futures. Plus précisément, l'objectif était (1) d'étudier les impacts physiologiques sur ces coraux des températures actuelles et futures sur la base de neuf années de données de terrain, (2) de déterminer si la symbiose amplifie la sensibilité à la température élevée chez *C. caespitosa*, et (3) de tester le potentiel de récupération des deux espèces à une gamme de températures estivales. Des colonies des deux espèces ont été maintenues dans quatre traitements de températures actuelles et futures, basés sur neuf ans de données de température sur le terrain : 1) la moyenne quotidienne (2012-2019) à -10m, 2) les températures de l'année la plus chaude à -10m (2018), 3) la moyenne quotidienne à -10m +3,2°C prévu à la fin du siècle (RCP8.5) et 4) la moyenne quotidienne à -15m.

Pour les deux espèces étudiées, nous avons observé une réponse physiologique similaire au stress thermique. Pendant les trois premiers mois de l'expérience de réchauffement et de MHW, les taux de calcification n'ont pas différencié entre les traitements pour les deux espèces, ni la photosynthèse brute de *C. caespitosa*. Les taux de respiration et le nombre relatif de polypes par colonie ont augmenté pendant le pic estival pour chaque traitement de température pour *C. caespitosa*. Dans le cas de *A. calycularis*, la nécrose a commencé sur certains polypes après le pic de température sous le traitement le plus chaud (3). La symbiose de *C. caespitosa* a été altérée par la température avec une diminution du rendement quantique effectif, de la densité des zooxanthelles et une augmentation du pourcentage de blancheur dans tous les traitements sauf le traitement le plus froid. Nous avons pu observer que *C. caespitosa* d'Ischia blanchissait à des températures élevées comme cela a été rapporté en Croatie, au Monténégro et à Chypre (Kruzic *et al.* 2014, Jimenez *et al.* 2016, Mačić *et al.*

2019), mais les colonies ne présentaient pas de nécrose directe comme cela a été observé sur l'île de Columbretes et en mer Ligure (Rodolfo-Metalpa *et al.* 2005, Kersting *et al.* 2013). Cependant, les deux espèces se sont rétablies en ce qui concerne la respiration, la repousse des polypes d'*A. calycularis*, et le rétablissement de la pigmentation et des zooxanthelles après le blanchiment pour *C. caespitosa*. L'ensemble de ces résultats suggère que, dans le cadre de la tendance actuelle et prévue au réchauffement, ces espèces de coraux méditerranéens seront capables de se rétablir après la chaleur de l'été.

Chapitre 2 Deux coraux tempérés sont tolérants à un pH bas indépendamment d'une exposition à des événements naturels de CO₂

L'acidification est perçue comme étant l'une des plus grandes menaces pour beaucoup d'espèces calcifiantes marines, dont les coraux (Gattuso *et al.* 1999). En effet, la calcification des coraux décroît en moyenne de 22% avec un pH attendu pour la fin du siècle sous le scénario RCP8.5 (Kwiatkowski *et al.* 2020). Néanmoins certaines espèces sont capables de résister à l'acidification, comme par exemple les *Porites sp.* massifs (Fabricius *et al.* 2011), ainsi que chez plusieurs coraux tempérés (*e.g.*, Rodolfo-Metalpa *et al.* 2010). Certains coraux forment une symbiose avec des micro-algues, les zooxanthelles, qui contribuent significativement au budget énergétique du corail (Allemand *et al.* 2011; Muller-Parker *et al.* 2015). Lors de la photosynthèse des zooxanthelles seraient en mesure de réguler le pH intracellulaire (Gibbin *et al.* 2014). Dans le contexte d'acidification, la symbiose avec des zooxanthelles pourrait nuancer l'impact sur la calcification. L'une des questions centrales concernant le futur des coraux dans un monde avec de hautes concentrations de CO₂ est de savoir si une acclimatation voire même une adaptation à l'acidification est possible. Etudier ces procédés sur des animaux longévives tels que les coraux est compliqué en laboratoire car il faut pouvoir maintenir un système aquarium sur plusieurs générations. Les événements de CO₂ offrent une opportunité unique d'étudier la réponse de coraux à l'acidification dans la durée. Dans ces systèmes, les bulles de CO₂ d'origine volcanique acidifient localement l'eau de mer et reproduisent des conditions prévues dans le futur (Hall-Spencer *et al.* 2008; Camp *et al.* 2018; Gonzalez-Delgado and Hernandez 2018).

Cette étude se focalise sur deux espèces des coraux Méditerranéens *Cladocora caespitosa* et *Astroides calycularis* qui ont des populations naturelles au niveau d'événements de CO₂ à Ischia. Les hypothèses testées sont : (1) une exposition passée à des conditions de pH bas confère une tolérance à l'acidification et (2) une symbiose algue-corail régule cette tolérance. Dans le but de vérifier ces hypothèses, des colonies des coraux *C. caespitosa* et *A. calycularis* ont été prélevés de sites d'événements de CO₂ et de sites ambiants et disposés en laboratoire dans un pH_T bas (7.7) ou dans un pH_T ambiant (8.0). Les taux de calcification, de respiration et de photosynthèse ont été contrôlés au cours des 6 mois de l'expérience.

Aucune des deux espèces n'a présenté de changement de calcification, respiration ou photosynthèse indépendamment du site d'origine, du traitement de pH subit en laboratoire ou de la stratégie trophique (présentant une symbiose avec micro-algue ou pas).

Chapitre 3 Les premiers stades de vie d'un corail méditerranéen sont vulnérable au réchauffement et à l'acidification de l'océan

La reproduction sexuelle est essentielle pour la dynamique des populations et afin de renouveler les populations après des perturbations (Hugues *et al.* 2019). Néanmoins, peu d'études ont été menées sur les réponses des larves et recrues de coraux face au réchauffement et à l'acidification. Le réchauffement peut avoir un impact délétère sur les larves de coraux en menant à des déformations des embryons (Woolsey *et al.*, 2013), à une diminution de la survie des larves et des recrues (Baria *et al.*, 2015, Bahr *et al.*, 2020) ainsi qu'à l'augmentation du métabolisme des larves, menant à une métamorphose et une fixation plus rapide (Nozawa and Harrison, 2007, Chua *et al.*, 2013). Par contre, l'acidification provoque des réponses mitigées. Dans certains cas, un pH bas décroît le métabolisme et la fixation (Albright 2011, Nakamura *et al.*, 2011). D'autre part, il n'a aucun impact sur la survie des larves et des recrues (Suwa *et al.*, 2010; Chua *et al.*, 2013). L'acidification a néanmoins un impact négatif sur la calcification des recrues et induit des difformités du squelette, comme chez les coraux adultes (Suwa *et al.*, 2010, Foster *et al.*, 2016, Varnerin *et al.*, 2020). Les études sur l'impact combiné du réchauffement et de l'acidification sur les premiers stades de vie sont rares. La combinaison des deux facteurs semble avoir des effets opposés, avec par exemple une diminution ou une augmentation de la calcification (Anlauf *et al.* 2011, Foster *et al.*, 2015), alors que certaines études n'ont reporté aucun impact sur la survie, la métamorphose et la fixation (Anlauf *et al.*, 2011, Chua *et al.*, 2013, Foster *et al.*, 2015).

Dans cette étude nous évaluons la réponse des premiers stades de vie d'un corail azooxanthellé, *Astroides calycularis* aux effets combinés de température élevée et de pH bas. Nous posons l'hypothèse qu'une température élevée et un pH bas ont un effet additif ou synergétique sur le développement et la croissance des larves et recrues d'*A. calycularis*. Afin de tester cette hypothèse des larves et recrues ont été élevées pendant 9 mois sous une température ambiante (23°C) ou chaude (26°C) et sous un pH_T ambiant (8.0) ou pH_T bas (7.7). Le moment de la métamorphose des larves, la croissance des recrues et les caractéristiques de leur squelette ont été contrôlés.

Les résultats montrent une métamorphose et une fixation plus rapide face à une température élevée. Au contraire, un pH bas, ralenti la métamorphose et a un impact négatif sur la croissance des recrues en réduisant l'aire d'attachement au substrat, ainsi que le volume et le nombre de septa. Par contre, la densité du squelette est plus élevée sous un pH bas et une température ambiante. La température chaude et le pH bas combinés ont un effet négatif sur la survie, la fixation et la croissance des recrues. Cette étude met en évidence le fait que la température chaude et le pH bas ont des impacts différents sur les premiers stades de vie d'*A. calycularis*. La température agit sur le développement larvaire tandis que le pH agit négativement sur la croissance des recrues. La combinaison des deux facteurs pourrait avoir un impact négatif sur la tolérance et la résilience des populations méditerranéennes en diminuant la dispersion larvaire, le recrutement et la croissance post-fixation.

Annexe IV L'exposition parental à des événements naturels de CO₂ modifie la réponse à un pH bas de premiers stades de vie d'un corail tempéré.

L'étude des premiers stades de vie tels que le développement larvaire, la colonisation, la survie et la croissance des recrues est essentielle pour mieux comprendre la résilience et la persistance des populations coralliennes. Si les effets délétères de l'acidification des océans sur la calcification et la croissance des coraux adultes sont bien connus, les impacts sur les premiers stades de vie sont encore peu documentés. Ici, nous cherchons à savoir si l'exposition passée des colonies parentales à des environnements naturellement acidifiés dans des événements de CO₂ peut augmenter la tolérance de la progéniture à un faible pH.

Des larves du corail azooxanthellé méditerranéen *Astroïdes calycularis* ont été obtenues à partir de colonies parentales collectées sur des sites à pH faible et ambiant à Ischia, en Italie. Les larves ont été exposées en laboratoire à trois traitements de pH : ambiant (pHT ~8,05), bas (pHT ~7,7, pH prévu pour la fin du siècle sous le scénario RCP 8,5) et extrêmement bas (pHT ~7,5, pH dans des conditions extrêmes étant le pH du site des événements de CO₂ - 0.3 unité de pH, uniquement pour les larves provenant du site de l'événement de CO₂). Plusieurs caractéristiques des premiers stades de vie ont été suivies pendant cinq mois : taille des larves, succès de la fixation, survie et croissance des recrues. Des différences importantes ont été observées entre le site d'origine des larves et le site des événements de CO₂. Par exemple, les larves provenant du site des événements de CO₂ étaient plus courtes et présentaient un taux de mortalité plus élevé que les larves provenant du site à pH ambiant, quel que soit le traitement du pH auquel elles étaient exposées. De plus, les larves provenant du site d'événements de CO₂ avaient un succès de colonisation plus faible à un pH bas et extrêmement bas. Nous avons également analysé la transcriptomique des recrues de cinq mois. Les différences d'expression génétique ont été principalement observées entre le site d'origine et non entre les traitements de pH en laboratoire. Nos résultats suggèrent que les futures conditions d'acidification auront un impact sur la survie et la fixation des premiers stades de vie. Ces découvertes fournissent de nouvelles informations sur le recrutement des coraux et leur capacité à répondre aux conditions actuelles et futures d'acidification de l'océan.

Annexe V Les événements naturels de CO₂ retardent le frai d'un corail tempéré.

La reproduction sexuée est l'un des mécanismes qui peuvent être altérés par l'acidification. De plus, la reproduction de *C. caespitosa* comporte encore beaucoup d'inconnues. Ainsi, nous avons étudié l'impact de l'acidification sur la gamétogenèse et nous avons essayé d'identifier le timing du frai de *C. caespitosa* d'Ischia. À cette fin, des échantillons provenant de l'événement à CO₂ et du site ambiant ont été obtenus en juillet et septembre 2020 pour analyser la progression de la gamétogenèse sur des colonies sélectionnées. Le sex-ratio et la taille des gamètes ne différaient pas d'un site à l'autre. En septembre, les colonies des deux sexes du site ambiant ont libéré leurs gamètes, alors qu'au niveau du site des événements de CO₂, le mâle n'a pas frayé, entraînant donc un asynchronisme de frai entre mâles et femelles. Cette asynchronie pourrait conduire à un affaiblissement de la fécondation et donc de la quantité de larves ce qui

représente un facteur critique pour maintenir ou reconstituer la population après une perturbation.

Discussion

Différence d'impact du réchauffement et de l'acidification de l'océan entre les différents stades de vie

Afin d'avoir une population saine, non seulement la survie des colonies adultes est importante, mais aussi une reproduction sexuelle efficace avec un grand nombre d'œufs et de larves, une métamorphose réussie ainsi que la survie et la croissance élevée des recrues après la fixation (Albright, 2011). Tout au long de cette thèse, j'ai pu étudier l'impact du réchauffement, de l'acidification séparément ou combinés sur les différents stades de vie de *C. caespitosa* et *A. calycularis*. Ici, je discuterai de la différence ou de la similarité des réponses observées entre les stades de vie.

Réchauffement de l'océan

Comme cela a été constaté dans la majorité des études du réchauffement sur les larves (par exemple Edmunds *et al.* 2001, Chua *et al.* 2013), la température a eu un impact sur le développement des larves d'*A. calycularis* en accélérant la fixation et la métamorphose (Chapitre 3), probablement en raison d'une augmentation du métabolisme et donc de la respiration (Edmunds *et al.* 2001, 2011). L'augmentation du métabolisme pourrait raccourcir la durée de la phase larvaire et réduire la distance de dispersion en provoquant la métamorphose et la fixation avant que les larves ne quittent leur population d'origine et donc la connectivité et diversité génétique diminue aussi (O'Connor *et al.*, 2007, Figueiredo *et al.*, 2014). L'augmentation du métabolisme avec la température est connue pour être l'une des premières réponses au réchauffement chez les adultes, en raison de l'augmentation de l'activité des enzymes (Clarke et Fraser, 2004). Cependant, la respiration des colonies adultes d'*A. calycularis* du chapitre 1, n'a pas augmenté avec la température comme cela aurait été attendu. Les colonies adultes de *C. caespitosa* ont en revanche montré une augmentation de la respiration au pic de température estival dans tous les traitements de température (chapitre 1), ou après 20 jours à 26 et 28°C (Rodolfo-Metalpa *et al.* 2006). Cette augmentation de la respiration était également liée à l'augmentation du bourgeonnement chez *C. caespitosa* juste après le pic de température. *A. calycularis* a également montré une augmentation des polypes par bourgeonnement, mais seulement pour le traitement qui a atteint un pic de température estival de 30°C. La même chose a été observée dans les premiers stades de vie d'*A. calycularis* au chapitre 3, car les recrues sous traitement chaud de 26°C et pH ambiant étaient les premières à présenter un bourgeonnement et avaient un nombre moyen de polypes plus élevé par colonie.

Comme les larves ont peu de réserves d'énergie, l'augmentation du métabolisme augmente généralement la mortalité (Bassim et Sanmarco 2003, Randall et Szmant 2009, Woolsey et al 2013). Dans le chapitre 3, les larves sous température chaude et pH ambiant n'ont, cependant pas présenté une mortalité plus élevée. Comme *A. calycularis* est une espèce thermophile, les

larves pourraient être tolérantes aux températures chaudes, comme les colonies adultes. Le traitement température chaude était de 26°C pour les larves (Chapitre 3) et les colonies adultes n'ont pas présenté de nécrose avant une température de 30°C (Chapitre 1). Un pic de 30°C n'a pas modifié le taux de calcification des colonies dans notre étude (chapitre 1) et l'extension linéaire des recrues n'a pas été impactée par la température (chapitre 3).

Acidification de l'océan

Contrairement au réchauffement, l'acidification n'a soit aucun effet sur le développement des larves (Anlauf *et al.* 2010, Chua *et al.* 2013), soit diminue le métabolisme des larves (Albright *et al.* 2011). Une diminution du métabolisme peut réduire la fixation et la métamorphose (Albright *et al.* 2010, 2011, Nakamura *et al.* 2011). Dans le chapitre 3, les larves d'*A. calycularis* n'ont pas fait exception et se sont moins fixées à un pH bas. Cependant, les colonies adultes d'*A. calycularis* ne présentaient pas de diminution du métabolisme, la respiration n'étant pas affectée, ni pour les colonies de *C. caespitosa* (chapitre 2, Carbonne *et al.* 2021). En général, la respiration des coraux adultes n'est pas affectée par l'acidification contrairement à la calcification qui est connue pour être impactée par un faible pH chez les adultes et les recrues (Foster *et al.* 2015, Cornwall *et al.* 2021, Leung *et al.* 2022). Les recrues d'*A. calycularis* présentaient bien un impact sur la calcification, avec une diminution de l'extension linéaire, du volume du squelette et du nombre de septa sous un faible pH (chapitre 3). Cependant, la calcification de l'*A. calycularis* adulte n'a pas été affectée par un pH bas (chapitre 2, Carbonne *et al.* 2021) comme obtenu par Movilla *et al.* (2016). La même tolérance de la calcification à l'acidification a été observée sur les adultes de *C. caespitosa* qui n'ont pas été impactés par un faible pH (Chapitre 2).

Tolérance à l'acidification de l'océan après une exposition antérieure à des événements de CO₂ : différence entre les colonies adultes et les premiers stades de vie

L'une des principales questions de cette thèse était de savoir si les deux coraux étudiés trouvés dans les événements de CO₂ étaient acclimatés à l'acidification des océans. Dans le chapitre 2, les colonies adultes de *C. caespitosa* et *A. calycularis* n'ont présenté aucune différence dans la calcification, la respiration et la photosynthèse (cette dernière uniquement pour *C. caespitosa*), indépendamment du traitement du pH en laboratoire et du site d'origine (événements de CO₂ ou sites à pH ambiant). Ainsi, pour les paramètres testés, nous ne pouvons conclure à une acclimatation à l'acidification. Cependant, une certaine acclimatation pourrait avoir été acquise par les recrues provenant des événements de CO₂ (Annexe IV), puisqu'elles ont présenté un taux de croissance plus élevé sous traitement au pH ambiant que les recrues provenant du site à pH ambiant. Cette plus grande extension linéaire pourrait être due à un métabolisme plus élevé, comme l'ont observé Putnam et Gates (2015), où les larves provenant de parents vivant dans un environnement à température et pCO₂ élevées ont produit des larves avec un métabolisme plus élevé pour faire face aux environnements défavorables.

Au cours de l'expérience du chapitre 2, les coraux n'ont pas affiché une réelle acclimatation, cependant, *in situ*, les colonies adultes d'*A. calycularis* ont présenté des signes d'acclimatation avec un changement de la structure du squelette. Teixido et al (2020) ont observé une augmentation de la densité apparente, de la microdensité et une diminution de la porosité,

produisant des squelettes encroûtants pour faire face au faible pH des événements de CO₂ par rapport aux sites à pH ambiant. Il est intéressant de noter que les recrues du chapitre 3 soumises à une température ambiante et à un faible pH présentaient également une densité plus élevée. Habituellement, on observe le contraire sur les coraux exposés à un faible pH, avec une diminution de la densité apparente et une augmentation de la porosité pour maintenir l'extension linéaire (Fantazzini *et al.* 2015, Prada *et al.* 2021).

Même si une certaine acclimatation de la structure du squelette peut être observée dans les colonies adultes, une différence très importante dans la structure des colonies et de la population a été observée *in situ*. Les colonies d'*A. calycularis* à Grotta del Mago sont plus petites, elles ont moins de polypes avec peu de coenosarc (tissu entre les polypes, Teixido *et al.* 2020). Dans le chapitre 3, sous un pH faible, les recrues présentaient également une perte de tissu, le polype se rétractant dans le calix et exposant la base du squelette. La base du squelette exposé pourrait probablement subir une dissolution, comme décrit précédemment avec *C. caespitosa* par Rodolfo-Metalpa *et al.* (2011) et les polypes pourraient se détacher du substrat (chapitre 3). Cela pourrait expliquer pourquoi les colonies d'*A. calycularis* trouvées près des événements de CO₂ présentaient une diminution de la couverture avec la diminution du pH sur ce site avec la profondeur (Teixido *et al.* 2020). Le fait que les larves provenant des parents des événements de CO₂ étaient plus petites (-30%), peut être lié à la quantité de réserve d'énergie et à leur survie (Isomura et Nishihira 2001). Cette différence dans les réserves d'énergie des larves pourrait être expliquée par un trade-off possible dans l'allocation d'énergie des parents. La calcification et le métabolisme ont été maintenus sur les colonies adultes provenant des événements de CO₂ sous un faible pH, peut-être à un coût (chapitre 2, Carbonne *et al.* 2021). Ainsi, seule une faible énergie a pu être allouée à la reproduction. La diminution de la fixation pourrait s'expliquer par le fait que l'acidification modifie les signaux chimiques des communautés microbiennes ou des algues coralligènes encroûtantes, réduisant l'induction de la fixation et de la métamorphose chez les larves (Harrison 2010, Webster *et al.* 2011, Doropoulos et Diaz-Pulido 2013). La mortalité plus élevée et les taux de fixation plus faibles pourraient entraîner un recrutement plus faible sur le site de l'événement de CO₂ dans la Grotta del Mago.

Globalement, on sait très peu de choses sur la reproduction sexuelle de *C. caespitosa* et sur l'impact de l'acidification sur celle-ci. Dans cette thèse, j'ai confirmé que *C. caespitosa* à Ischia était gonochorique, avec des colonies ayant un sexe séparé et des polypes ne présentant qu'un seul type de gamète comme observé dans l'île de Columbretes (Kersting *et al.* 2013). Dans l'annexe V, l'analyse de la gamétogenèse de septembre nous a permis de conclure que le frai a lieu après l'été, comme l'ont observé Kersting *et al.* (2013) dans les îles Columbretes, et qu'une asynchronie de la ponte se produit dans le site de l'événement de CO₂. En effet, en septembre, seules les colonies femelles du site d'événements de CO₂ n'avaient frayé. Ce résultat d'asynchronie s'est également produit chez *A. calycularis* dans les événements de CO₂ de Panarea (Marchini *et al.* 2021). Cette asynchronie empêche la fécondation et pourrait conduire à un recrutement et un renouvellement de la population très faibles.

Les populations d'*A. calycularis* du site de l'événement de CO₂ et des sites à pH ambiant ne différaient pas seulement par la morphologie des colonies, mais elles ont également montré une forte différence de transcriptomique (Teixido *et al.* 2020). En effet, la population de l'événement à CO₂ différait des

populations à pH ambiant en présentant des niveaux élevés de divergence liés à la calcification (Teixido *et al.* 2020). Des résultats similaires concernant l'expression génique et la calcification ont été observés chez le corail tempéré *Balanophyllia elegans* dans des sites à forte remontée d'eau où le pH est plus faible (Griffiths *et al.* 2019). Les analyses de l'expression génique des recrues de cinq mois d'*A. calycularis* (annexe IV) ont également montré que l'expression des gènes différait significativement entre l'événement de CO₂ et le site à pH ambiant, quels que soient les traitements du pH. Il est intéressant de noter que *Acropora millepora* de Papouasie-Nouvelle-Guinée n'a pas présenté de changement cohérent dans l'expression des gènes entre les événements de CO₂ et les sites à pH ambiant (Kenkel *et al.* 2018).

Impact synergique du réchauffement et de l'acidification

Le réchauffement et l'acidification combinés ont eu un impact synergique négatif sur la croissance des recrues (chapitre 3). Cependant, sur les colonies adultes d'*A. calycularis*, aucun effet combiné n'a été observé, seule la température a diminué la calcification des coraux (Movilla *et al.* 2016). Une diminution de 33 % de la calcification nette des coraux est attendue pour la fin du siècle sous RCP8.5 avec à la fois le réchauffement et l'acidification (Cornwall *et al.* 2021). Le stress combiné aggrave également le faible taux de fixation, et induit un comportement inhabituel, où les larves se métamorphosent avant la fixation (chapitre 3), conduisant à des polypes libres avec de très faibles chances de survie. De plus, les polypes qui ont pu s'installer sous des températures chaudes et un pH bas, étaient les seuls à ne pas présenter de bourgeonnement. Ce qui présente une image beaucoup plus négative du recrutement futur d'*A. calycularis* que l'acidification seule.

Conclusion

L'objectif principal de cette thèse de doctorat était de déterminer les effets des conditions environnementales côtières actuelles et futures et de leur variabilité sur les coraux méditerranéens. J'ai pu répondre aux principales questions grâce à l'analyse de données environnementales et d'enquêtes de terrain dans les événements de CO₂ d'Ischia, combinées à des expériences de laboratoire sous température et pH contrôlés sur les deux coraux méditerranéens *Cladocora caespitosa* et *Astroides calycularis*.

Comment la température et/ou le pH actuels et futurs affectent-ils la physiologie de *Cladocora caespitosa* et *Astroides calycularis* à tous les stades de leur vie (adulte, gamétogénèse, larves et recrues) ?

=> Les colonies adultes de *C. caespitosa* et *A. calycularis* ont montré une tolérance à l'acidification pour la respiration, la photosynthèse et la calcification. Cependant c'est un équilibre fragile, car cette tolérance a un coût. L'ajout d'un autre facteur de stress pourrait être fatal ou induire un trade-off entre la survie, la croissance et la reproduction. Le réchauffement est déjà une menace pour *C. caespitosa* et *A. calycularis*, car la plupart des vagues de chaleur marines entraînent des mortalités massives. Dans nos conditions de laboratoire, les colonies des deux espèces ont pu se rétablir après le pic de chaleur estival. Cependant, le réchauffement et les vagues de chaleur marine peuvent s'accompagner d'une forte thermocline, d'une diminution des nutriments, d'une désoxygénation ou du développement de *Vibrios* nuisibles qui n'ont pas été testés dans notre expérience, mais qui seront subis *in situ*. Et le plus inquiétant est le fait que les larves ont souffert d'un réchauffement et d'un pH bas, ce qui a entravé, séparément et de manière additive, la fixation, la métamorphose et la croissance post-fixation et a augmenté la mortalité. Ces résultats sont pessimistes, car la reproduction est le seul moyen naturel de recoloniser une population en déclin.

Les différents stades de vie sont-ils également touchés par les conditions environnementales futures ?

=> Les impacts du réchauffement et de l'acidification sur les colonies adultes *C. caespitosa* et *A. calycularis* ont été modérés, montrant une tolérance et une résilience (définie ici comme la capacité à se rétablir après une perturbation). Cependant, l'impact du changement global sur les premiers stades de vie d'*A. calycularis* est inquiétant car il entraîne surtout une diminution de la fixation et de la survie. Ces réponses négatives pourraient conduire à un effondrement des populations si les événements de mortalité massive des adultes augmentent avec les vagues de chaleur marines.

L'exposition à un pH faible dans les événements de CO₂ améliore-t-elle la tolérance des coraux à l'acidification future ?

=> D'après nos résultats, avec les paramètres étudiés, les sites d'événements de CO₂ d'Ischia ne semblent pas favoriser l'acclimatation de *C. caespitosa* et *A. calycularis*. Au contraire, ils semblent avoir un impact négatif sur la physiologie individuelle (reproduction, développement et recrutement des premiers stades de vie, morphologie de la colonie) et une modification des traits écologiques et de la structure de la population des espèces (formes massives versus encroûtantes et tailles plus petites).

Perspectives

Les effets du changement environnemental global sur les coraux ont suscité des inquiétudes en raison de l'augmentation des mortalités massives et des événements de blanchiment dans les régions géographiques et de l'effet de l'acidification des océans sur leur calcification. Au cours de cette thèse, l'objectif principal était d'approfondir les connaissances sur la réponse de deux espèces importantes formant l'habitat méditerranéen. Cependant, comme cela se produit souvent dans la recherche, répondre à certaines questions en amène de nouvelles qui devront être évaluées dans des recherches futures.

Que poursuivre au niveau individuel ?

Des recherches supplémentaires sont encore nécessaires pour comprendre pourquoi les colonies adultes étaient tolérantes au réchauffement et à l'acidification des océans au niveau individuel. L'une des pistes à suivre pourrait être d'examiner les réserves d'énergie et la contribution de l'hétérotrophie à celles-ci. La plupart du maintien des paramètres physiologiques sous réchauffement et acidification a généralement un coût métabolique (Wood et al. 2008). Une des hypothèses est que les coraux augmentent leur hétérotrophie pour augmenter l'apport d'énergie et accroître les réserves énergétiques (Hughes et Grottoli 2013 ; Towle et al. 2015). Afin de tester cette hypothèse, les colonies de l'expérience du chapitre 2 ont été envoyées à Ann Marie Hulver, doctorante sous la supervision du professeur Andrea Grottoli de l'Ohio State University (États-Unis). Les réserves énergétiques telles que les glucides et les lipides sont analysées par Ann Marie Hulver avec des isotopes stables ($\delta^{13}C$) afin de déterminer si l'origine des coraux ou le traitement du pH ont un impact sur les réserves ou sur l'hétérotrophie de *Cladocora caespitosa*. Le même type de mesures pourrait être effectué avec les colonies du chapitre 1, afin de voir s'il existe un lien entre les réserves énergétiques, l'hétérotrophie et la récupération des colonies après l'exposition à différentes températures estivales et à des vagues de chaleur marines. De plus, comme les colonies de *C. caespitosa* soumises au même traitement de

température n'ont pas blanchi de la même manière (chapitre 1), il serait intéressant d'analyser les zooxanthelles et les communautés microbiennes pour comprendre cette variabilité individuelle des réponses. Cette analyse pourrait également être appliquée à d'autres sites où l'on trouve des populations de *C. caespitosa* en Méditerranée afin de comprendre pourquoi certaines colonies blanchissent ou non et pourquoi certaines d'entre elles souffrent de nécrose directe.

Le fait que la calcification ait été maintenue sous un pH bas doit également être étudié plus en détail. L'évaluation du pH et de la chimie des carbonates du fluide de calcification de *C. caespitosa* et *Astroïdes calycularis* provenant de sites d'événements de CO₂ et de sites à pH ambiant sous différents pH en laboratoire pourrait nous aider à comprendre si la régulation du pH et de la DIC au niveau du site de calcification sont des mécanismes qu'ils ont utilisés pour maintenir la calcification sous un pH bas. Pour compléter l'analyse, une analyse transcriptomique unicellulaire (single cell transcriptome) pourrait être réalisée sur des cellules calycoblastiques afin d'étudier l'expression des gènes qui pourraient contribuer au maintien de la calcification à faible pH.

Études à grande échelle : d'une population au niveau du bassin méditerranéen

Nous avons observé que la reproduction de *C. caespitosa* était altérée par un faible pH dans les événements de CO₂. Afin de confirmer que seules les colonies trouvées dans le site à pH ambiant sont capables de se reproduire, une analyse génétique visant à évaluer les flux génétiques des deux populations pourrait être effectuée. La reproduction de *C. caespitosa* présente encore de nombreuses zones d'ombre. Des efforts de suivi *in situ* pourraient être réalisés autour de la période théorique de ponte dans les deux sites, afin d'identifier le moment exact de la ponte et de collecter les gamètes. Pour confirmer notre hypothèse concernant la diminution potentielle du recrutement et de la croissance des premiers stades de vie d'*A. calycularis* sous l'effet de l'acidification, des études *in situ* dans le temps avec des transects permanents pourraient être réalisées sur le site de l'événement et sur un site ambiant, afin de quantifier le nombre de nouvelles recrues, leur survie et leur croissance.

Cette thèse s'est concentrée sur l'étude de l'acclimatation à l'acidification au niveau des événements de CO₂. Cependant, l'avenir des populations dépend de leur capacité à s'adapter à leur environnement. Afin d'évaluer l'adaptation de la population des événements de CO₂, des expériences de transplantation réciproque *in situ* doivent être réalisées avec un site à pH ambiant. Une expérience de transplantation réciproque a été menée par le Dr. Núria Teixidó avec *A. calycularis* à Ischia et les résultats sont en cours d'analyse, cependant, rien n'a été fait avec *C. caespitosa* jusqu'à présent. Une expérience de transplantation similaire avec des colonies provenant de sites d'événements de CO₂ et de sites ambiants pourrait également être réalisée avec *C. caespitosa* afin de révéler si les mécanismes d'adaptation locale sont spécifiques à l'espèce ou plus généralisables.

Globalement, la comparaison des populations des deux coraux pourrait être très intéressante, par exemple dans un gradient latitudinal et longitudinal de température (par exemple nord et sud, ouest et est). La calcification, la respiration, la photosynthèse, les réserves d'énergie, la reproduction et la génomique pourraient être analysées sur des sites présentant des régimes de température différents.

Conservation

C. caespitosa et *A. calycularis* ont souffert d'événements répétés de mortalité massive dus aux vagues de chaleurs marines. La surveillance des données environnementales par le déploiement de capteurs à long terme à grande échelle est importante pour aider à projeter des scénarios réalistes de réchauffement futur. Parallèlement, le suivi des épisodes de mortalité massive est également essentiel pour identifier les sites clés où les coraux sont plus ou moins touchés. Ces sites naturels moins impactés par les MHWs pourraient être des candidats parfaits pour des Aires Marines Protégées. Au

cours de cette thèse, 100 colonies de *C. caespitosa* ont été marquées dans le site d'Ischia où se trouvent des événements de CO₂ et à un site de pH ambiant. Le suivi annuel de ces colonies pourrait apporter des informations précieuses sur l'impact des vagues de chaleur marine sur *C. caespitosa*. De plus, Ischia est l'actuelle distribution nord de l'espèce thermophile *A. calycularis* en Méditerranée. Le suivi de la progression de cette espèce vers le Nord pourrait être un moyen de suivre le réchauffement de la mer Méditerranée.

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Appendix I

Physiological response of two temperate scleractinian corals to ocean warming and heatwaves

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Supplementary materials

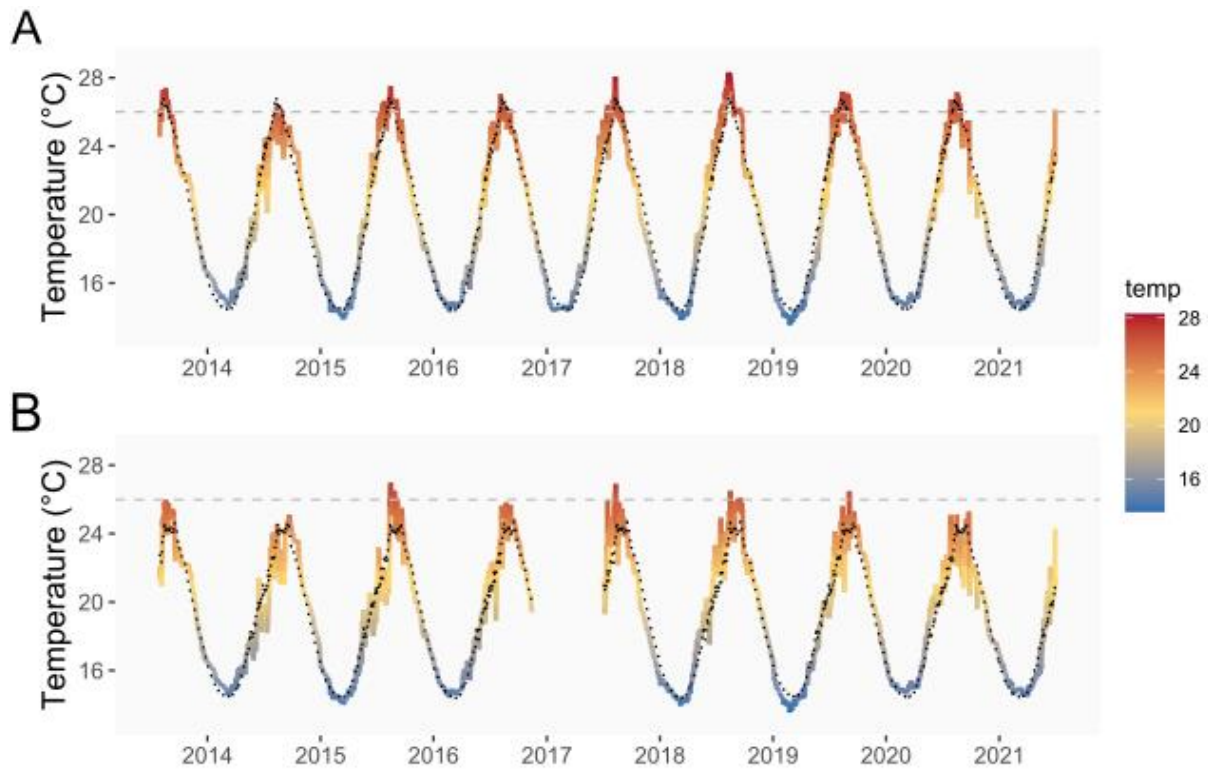


Figure S1-Appendix I: Temperatures of Ischia at (A) 10 and (B) 15 m depth between 2013 and 2021. Temperature was recorded every hour with yearly deployed HOBO Water Temperature Pro v2 Data Loggers (Onset). The black dotted line shows the daily mean temperature of 2013 to 2021 at 10 and 15 m depth. The gray dotted line shows the 26°C limit over the one mass mortalities of benthic communities are observed.

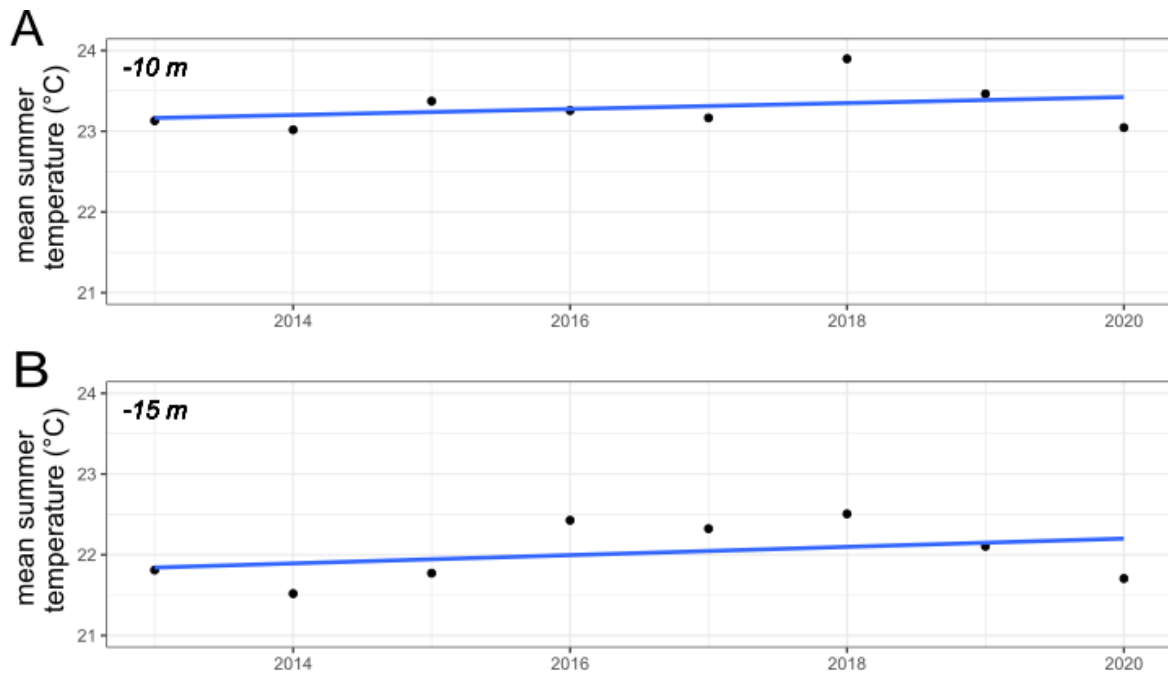


Figure S2-Appendix I Mean summer temperatures of Ischia from 2013 to 2020 (JJASON) (A) at 10 m and (B) at 15 m depth.

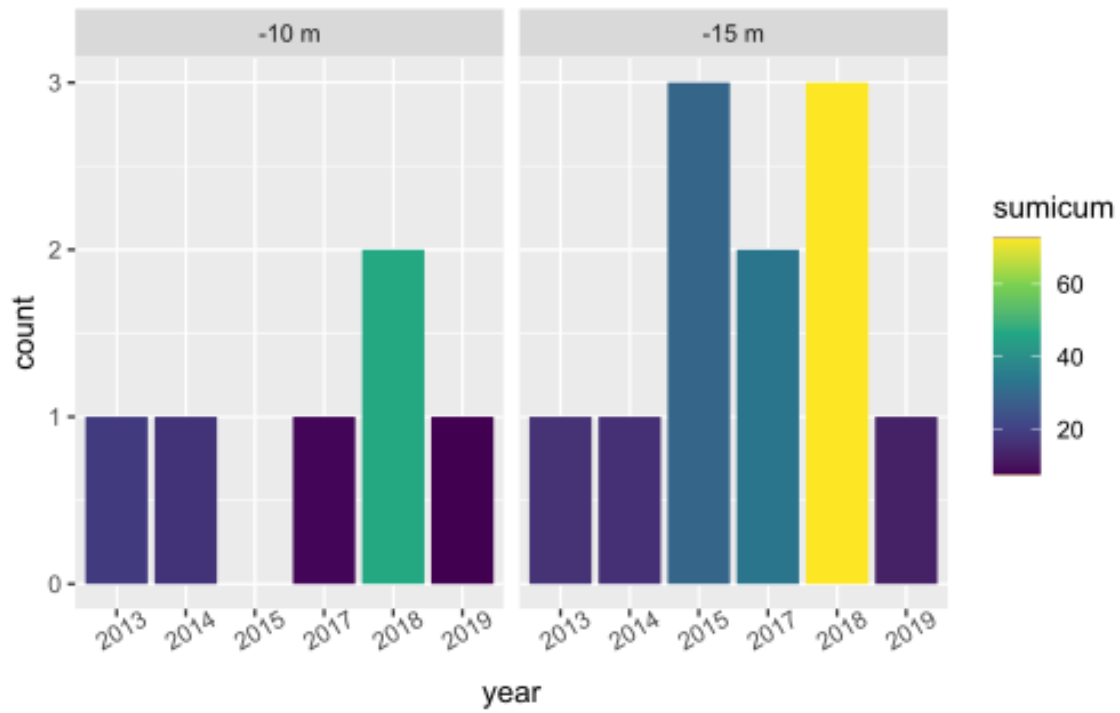


Figure S3-Appendix I. Marine heatwave events in Ischia at 10 and 15 m deep between 2013 to 2019 for the summer period (JJASON). “Count” shows the number of events and “sumicum” in °C shows the sum of the cumulative intensities of the events, with the cumulative intensity being the integral of temperature anomalies above the climatology, over an event. The analysis of the temperature data was done with the HeatwaveR package (Schlegel and Smit, 2018).

Method S1-Appendix I: Supplementary information about the macros of the automatic counting method on ImageJ software

After extraction of the tissue of one polyp per colony with an air-brush, tissues were homogenized with an ultra-turrax (IKA) in 0.5 ml of MilliQ water. Samples were then centrifuged at 800 g for 2 min (Centrifuge 5417C, Eppendorf®). The supernatant was removed, the pellet being symbiont material which was stored at -80°C in 1 mL of MilliQ water. Four 200 µL replicates of each sample were disposed in an haemocytometer (Malassez cell, Marienfeld) and images were collected on an inverted ZEISS Observer microscope (Zeiss, Jena, Germany) equipped with a 10X/0.3 EC Plan-Neofluar objective lens using a monochrome Flash 4 (Hamamatsu photonics, Japan). Chlorophyll fluorescence was acquired with 405 nm excitation and 659-701 nm emission. Mosaics of 9 mm² were typically acquired for brightfield and chlorophyll fluorescence using motorized stage driven under Zen software. We developed under ImageJ a set of macros to easily settle the parameters and analyze automatically in batch all the images providing a datasheet for statistics. Fluorescence images were thresholded with automatic but tunable parameters. Due to their high roundness, we applied a watershed filter to separate algae in contact. Finally, objects were filtered based on a range of diameter (here 4 to 14 µm). As Mallassez cells are 200 µm thick, we then can measure, among others, the absolute volumetric concentration. The macros are available on Github with tutorial and example : <https://github.com/SebastienSchaub/CounZoox/>.

Table S1-Appendix I Comparison among hierarchical linear mixed-effects models assessing the different measured parameters. The structure of the random term was selected by comparing models with different error structures using the Akaike information criterion (AIC). The selected model is highlighted in yellow.

Model	df	AIC
<i>Relative change in the number of polyps Astroides calycularis</i>		
Growth*temperature_treatment*time+(1 colony)	0	-504.78
Growth*temperature_treatment*time+(1 header_tank)	0	-504.43
Growth*temperature_treatment*time+(1 colony)+(1 header_tank)	1	-502.78
<i>Relative change in the number of polyps Cladocora caespitosa</i>		
Growth*temperature_treatment*time+(1 colony)	7	-151.69
Growth*temperature_treatment*time+(1 header_tank)	0	-72.19
Growth*temperature_treatment*time+(1 colony)+(1 header_tank)	0	-149.88
<i>Respiration rate Cladocora caespitosa</i>		
Respiration*temperature_treatment*time+(1 colony)	0	-671.15
Respiration *temperature_treatment*time+(1 header_tank)	0	-578.95
Respiration *temperature_treatment*time+(1 colony)+(1 header_tank)	1	-669.15
<i>Net photosynthesis rate Cladocora caespitosa</i>		
NetPhoto*temperature_treatment*time+(1 colony)	0	-628.94
NetPhoto *temperature_treatment*time+(1 header_tank)	0	-604.49
NetPhoto *temperature_treatment*time+(1 colony)+(1 header_tank)	1	-626.94
<i>Gross photosynthesis Cladocora caespitosa</i>		
GrossPhoto*temperature_treatment*time+(1 colony)	0	-575.00
GrossPhoto *temperature_treatment*time+(1 header_tank)	0	-489.56
GrossPhoto *temperature_treatment*time+(1 colony)+(1 header_tank)	1	-573.00
<i>Respiration rate Astroides calycularis</i>		
Respiration*temperature_treatment*time+(1 colony)	0	-629.02
Respiration*temperature_treatment*time+(1 header_tank)	0	-591.83
Respiration*temperature_treatment*time+(1 colony)+(1 header_tank)	1	-627.02
<i>% of whiteness Cladocora caespitosa</i>		
Whiteness*temperature_treatment*time+(1 colony)	0	10008.4
Whiteness*temperature_treatment*time+(1 header_tank)	0	10356.3
Whiteness*temperature_treatment*time+(1 colony)+(1 header_tank)	1	9911.8

Table S2-Appendix I Statistical results of the linear mixed-models with a hierarchical structure of the impact of temperature on *C. caespitosa* and *A. calycularis*. . = p<0.1, * = p< 0.05, ** = p<0.01, *** =p<0.001.

Species	Variable	Factors	df	F	p-value
<i>Cladocora caespitosa</i>	<i>Relative change in number of polyps</i>	temperature	3,110	14.02	2.87 10 ^{-3**}
		time	3,110	115.76	< 10 ^{-16***}
		temperature*time	9,110	41.22	4.56 10 ^{-6***}
<i>Astroides calycularis</i>	<i>Relative change in number of polyps</i>	temperature	3,108	2.75	0.43
		time	3,108	7.70	0.05 .
		temperature*time	9,108	14.10	0.12
<i>Cladocora caespitosa</i>	<i>Respiration rate</i>	temperature	3,110	1.41	0.70
		time	3,110	84.46	< 10 ^{-16***}
		temperature*time	9,110	17.51	7.57 10 ^{-3**}
<i>Cladocora caespitosa</i>	<i>Net photosynthesis rate</i>	temperature	3,82	1.17	0.76
		time	2,82	50.93	8.74 10 ^{-12***}
		temperature*time	6,82	15.01	0.02*
<i>Cladocora caespitosa</i>	<i>Gross photosynthesis rate</i>	temperature	3,82	1.05	0.79
		time	2,82	5.76	0.06 .
		temperature*time	6,82	4.49	0.61
<i>Astroides calycularis</i>	<i>Respiration rate</i>	temperature	3,82	3.51	0.32
		time	2,82	7.24	0.03*
		temperature*time	6,82	8.27	0.22
<i>Cladocora caespitosa</i>	<i>% whiteness</i>	temperature	3,1325	2.13	0.79
		time	1,1325	274.53	< 10 ^{-16***}
		temperature*time	3,1325	33.08	2.36 10 ^{-9***}

Table S3-Appendix I Statistical results of the PERMANOVA of the impact of temperature on *C. caespitosa* and *A. calycularis* calcification rates, and *C. caespitosa* effective quantum yield and density of zooxanthellae. . = $p < 0.1$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Species	Variable	Factors	df	F	p-value
<i>Cladocora caespitosa</i>	<i>Calcification rate</i>	temperature	3,28	0.42	0.74
<i>Astroides calycularis</i>	<i>Calcification rate</i>	temperature	3,28	1.36	0.27
<i>Cladocora caespitosa</i>	<i>Effective quantum yield</i>	temperature	3,1161	10.92	$1.00 \cdot 10^{-3}***$
		time	1,1131	8.44	$4.00 \cdot 10^{-3}**$
		temperature*time	3,1161	3.01	0.03*
<i>Cladocora caespitosa</i>	<i>Density of zooxanthellae</i>	temperature	4,70	0.79	0.55
		time	1,70	1.95	0.17
		temperature*time	4,70	3.11	0.02*

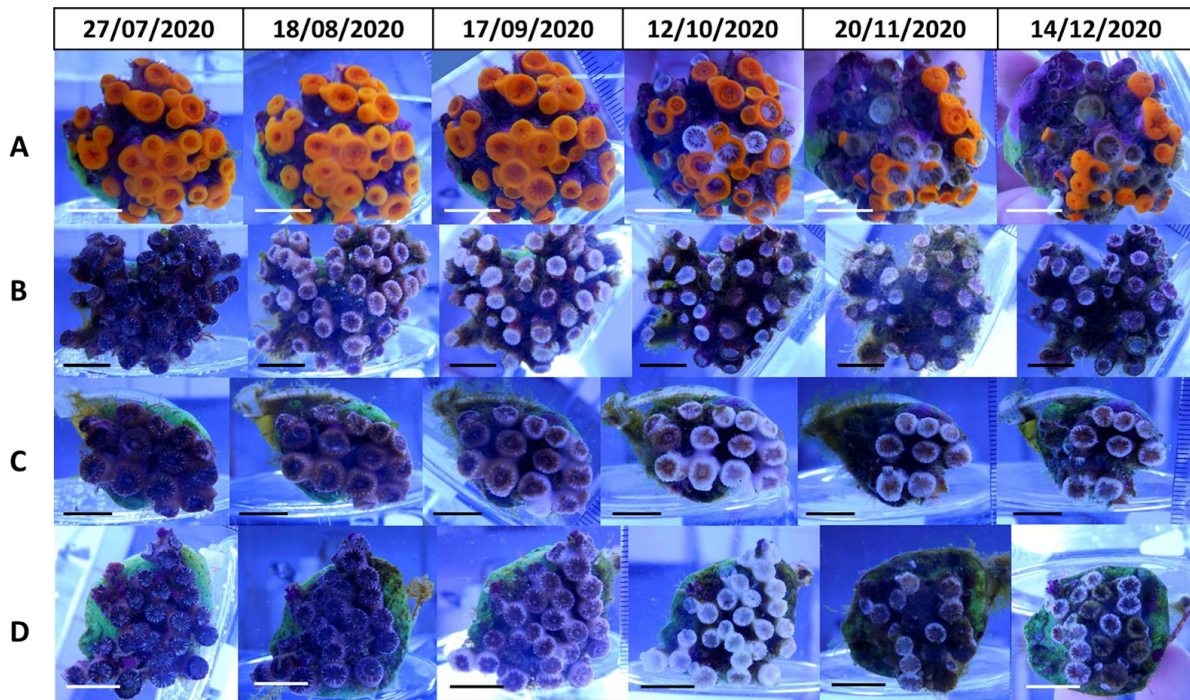


Figure S4-Appendix I Pictures of the impact of temperature on the colonies of *Cladocora caespitosa* (B, C, D) and *Astroides calycularis* (A) every month under the Warming treatment. Colonies presenting bleaching (B, C, D) and necrosis (A, D). Note: Polyps were sampled for analyses between 12 October and 20 November 2020 . Scale: 1 cm.

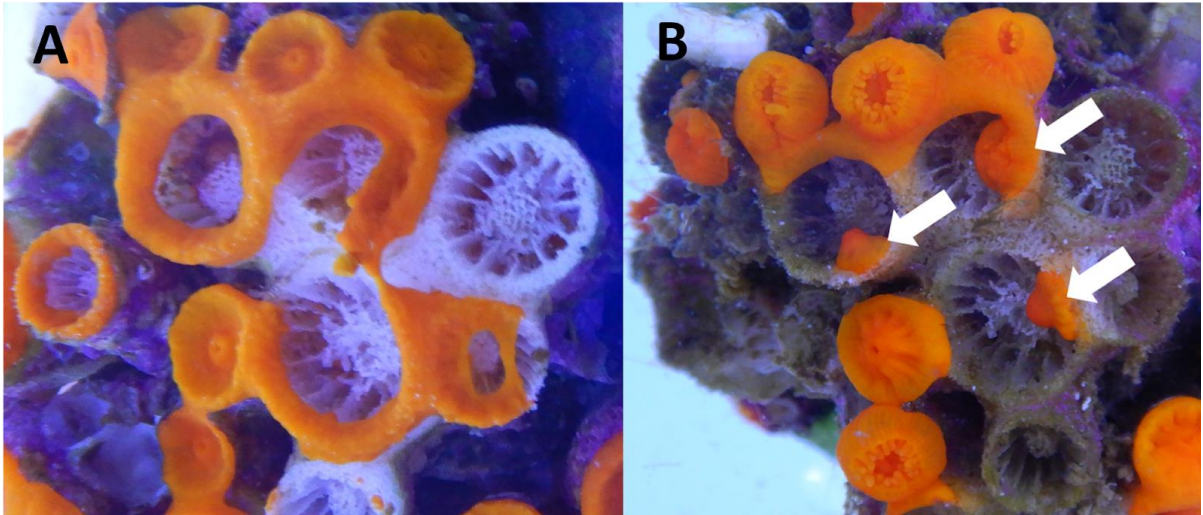


Figure S5-Appendix I Picture of a colony of *Astroides calycularis* (A) showing necrosis two months after the peak of temperature (12 October 2022), (B) and at the end of the experiment (14 December 2022) showing new polyps grown from residual tissue of necrosed polyps.

Appendix II

Two temperate corals are tolerant to ocean acidification regardless of previous exposure to natural CO₂ vents

Chloe Carbonne, Nuria Teixido, Billy Moore, Alice Mirasole, Thomas Gutierrez, Jean-Pierre Gattuso, Steeve Comeau

Supplementary materials

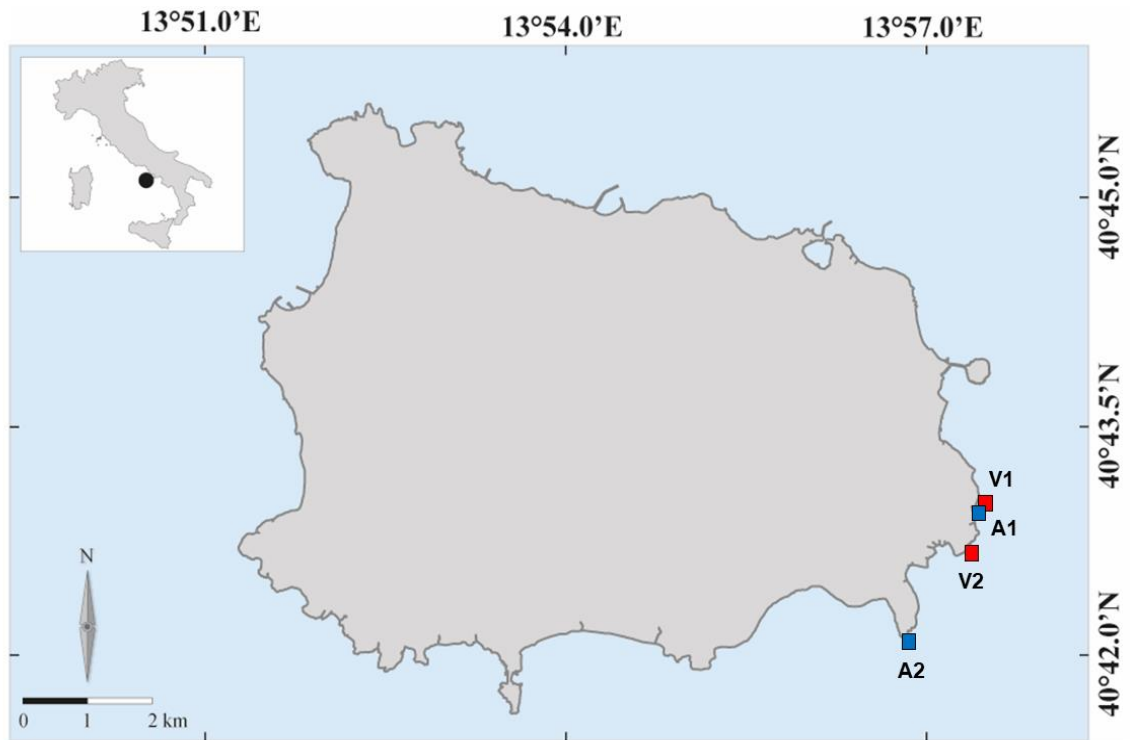


Figure S1-Appendix II. Map of the study sites along the coast of Ischia Island (Italy). *Cladocora caespitosa* and *Astroides calycularis* were sampled at the CO₂ vent systems named Chiane del Lume (V1, Vent1) and Grotta Mago (V2, Vent2) and the reference sites with ambient pH: Ambient1 (A1) and Ambient2 (A2), respectively.

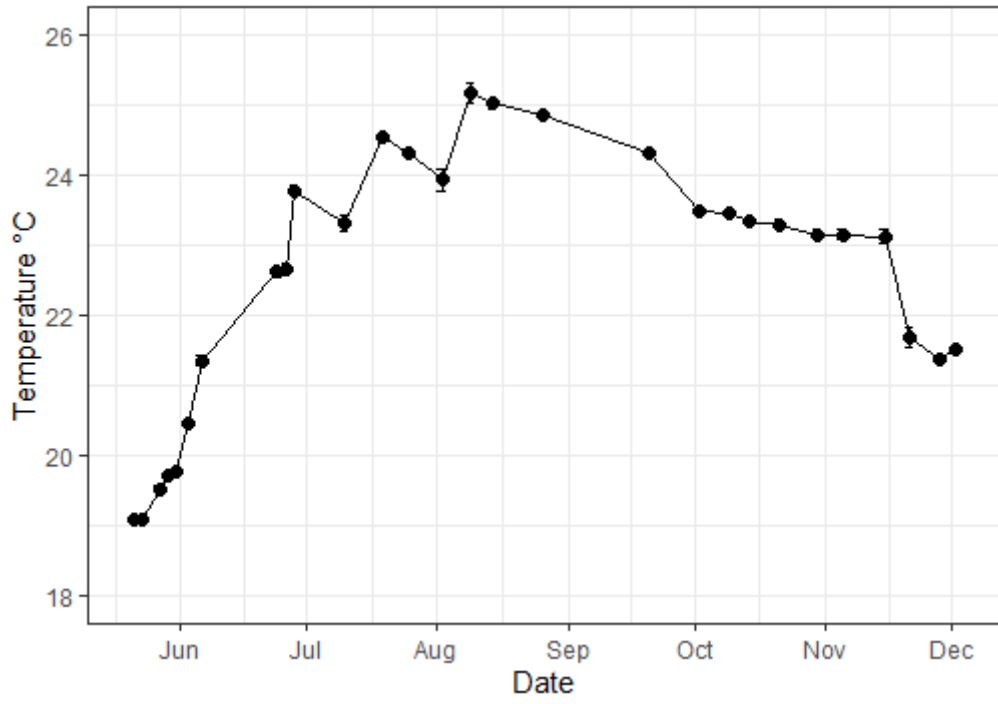


Figure S2-Appendix II. Evolution of the mean temperature in the experimental tanks (N=24). Mean \pm SE

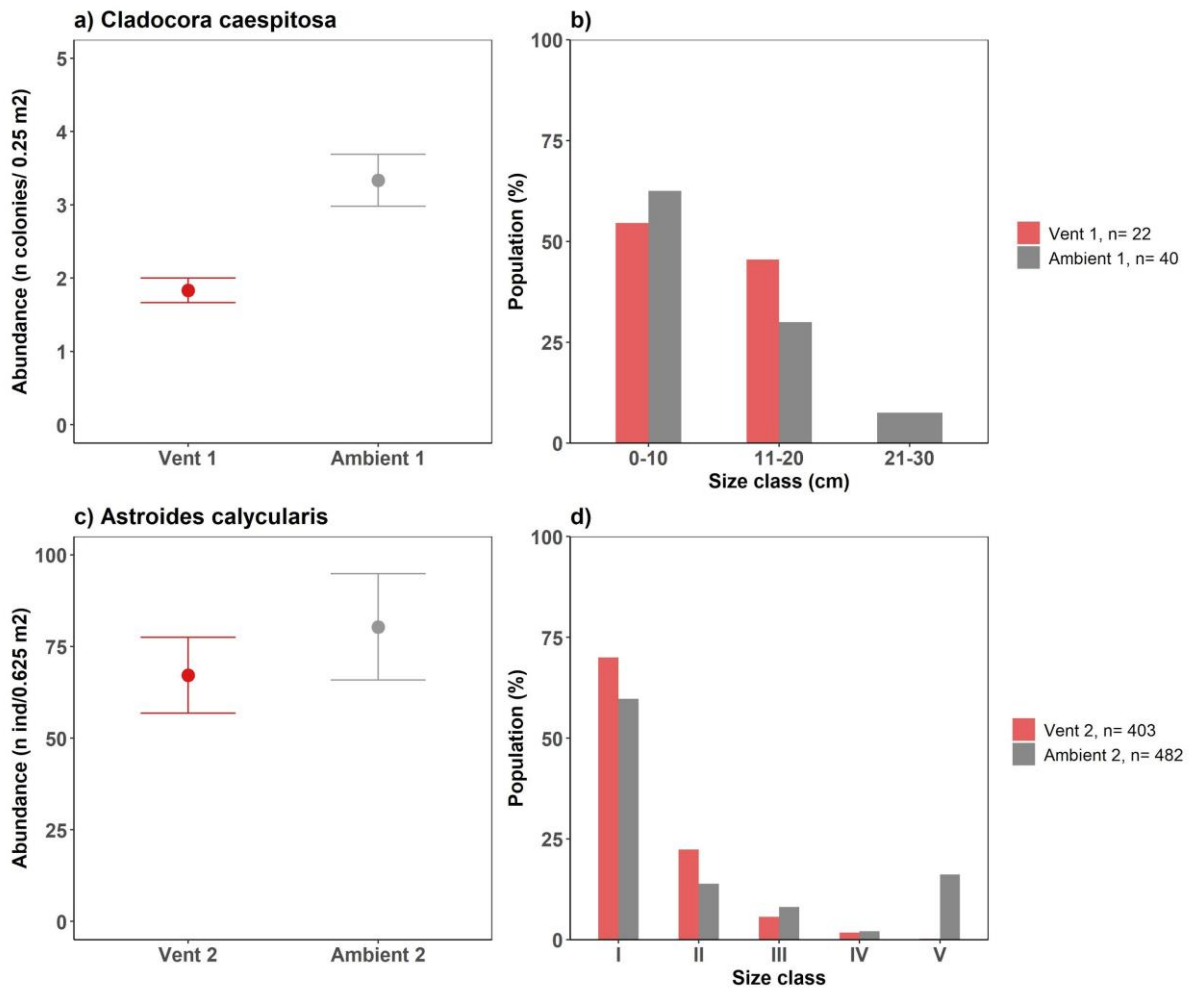


Figure S3-Appendix II. Abundance (mean \pm SE) and size-frequency of *Cladocora caespitosa* (a, b) and *Astroides calycularis* (c, d). *Cladocora caespitosa* was quantified using 12 quadrats (50 x 50 cm) per site, each colony was pooled into one of the three size classes: 0-10, 11-20, 21-30 cm; depth= 10 m. *Astroides calycularis* was quantified using 6 quadrats (25 cm x 25 cm) per site, each colony was pooled into one of five size classes (I: 1-5 polyps; II: 6-10 polyps; III: 11-15 polyps; IV: 16-20 polyps; V: > 20 polyps), depth= 1-2 m. n= number of total colonies analyzed per each site. Data of *Astroides calycularis* was originally reported in Teixido *et al.* 2020.

Table S1-Appendix II. Number of colonies of *Cladocora caespitosa* and *Astroides calycularis* per experimental condition (origin and pH treatment).

Condition	<i>Cladocora caespitosa</i>	<i>Astroides calycularis</i>
Ambient – Present day	N=8	N=6
Vent – Present day	N=6	N=12
Ambient – Low pH	N=8	N=7
Vent – Low pH	N=8	N=15

Table S2-Appendix II. Measured and estimated sea water physicochemical parameters per pH treatment and per month of experiment: temperature (T), salinity (S), total scale pH (pH_T), partial pressure of CO_2 ($p\text{CO}_2$), aragonite saturation (Ω_a) and total alkalinity (A_T). Values of T, A_T , S, CO_2 and Ω_a are monthly means \pm SE. pH_T values are monthly means with 25th and 75th percentile. T and pH_T were measured in each experimental tank, A_T was measured every month in randomly selected tanks, S is the sea surface salinity measured weekly in the Bay of Villefranche, and $p\text{CO}_2$ and Ω_a were calculated with the R package seacarb (Gattuso *et al.* 2020).

TableS2_carbonate_chemistry.csv (<https://doi.org/10.1002/lno.11942>)

Table S3-Appendix II. Mean long-term calcification, short-term calcification, respiration and gross photosynthesis per coral species, origin and pH treatment. Values are means \pm SE. Long-term calcification ($\text{mg CaCO}_3 \text{ cm}^{-2} \text{ d}^{-1}$) was assessed for 202 days of experiment, using the buoyant weight technique. Respiration, gross photosynthesis and short-term calcification were determined during short-term incubations ($< 1 \text{ h}$), a month after the start of the experiment (start) and a month before the end of the experiment (end). Respiration ($\text{mg O}_2 \text{ cm}^{-2} \text{ h}^{-1}$) and gross photosynthesis ($\text{mg O}_2 \text{ cm}^{-2} \text{ h}^{-1}$) were assessed using a fiber optic oxygen sensor under dark and light conditions, respectively. Short-term calcification ($\mu\text{g CaCO}_3 \text{ cm}^{-2} \text{ h}^{-1}$) was assessed on light and dark conditions using the alkalinity anomaly technique.

TableS3_mean_data.csv (<https://doi.org/10.1002/lno.11942>)

Table S4-Appendix II. Light to dark ratio of short-term calcification of *Cladocora caespitosa* from Vent and Ambient sites under the two pH treatments ($pH_T = 8.08 \pm 0.01$, Present day; $pH_T = 7.72 \pm 0.01$, Low pH), at the start (June 2019) and end of the experiment (end of October 2019). The ratio means were obtained with the model II regression major axis method, using lmodel2 function in R software. CI: 97.5% confidence interval. NA are confidence intervals too wide to be calculated.

Origin	Treatment	N	Light:Dark ratio (mean \pm CI 97.5%)
<i>Start of the experiment</i>			
Ambient	Present-day	7	2.7 ± 5.7
Ambient	Low pH	7	1.2 ± 6.3
Vent	Present-day	5	0.8 ± 2.4
Vent	Low pH	7	4.5 ± 1.4
<i>End of the experiment</i>			
Ambient	Present-day	7	2.0 ± 5.7
Ambient	Low pH	4	2.6 ± 7.8
Vent	Present-day	4	$1.0 \pm NA$
Vent	Low pH	5	$4.2 \pm NA$

Table S5-Appendix II. Summary of most of the available experimental and field data corals calcification response to ocean acidification

TableS5_calcification_response_corals_OA.csv (<https://doi.org/10.1002/lno.11942>)

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Appendix III

Early life stages of a Mediterranean coral are vulnerable to ocean warming and acidification

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Núria Teixidó^{1,4}

Supplementary materials

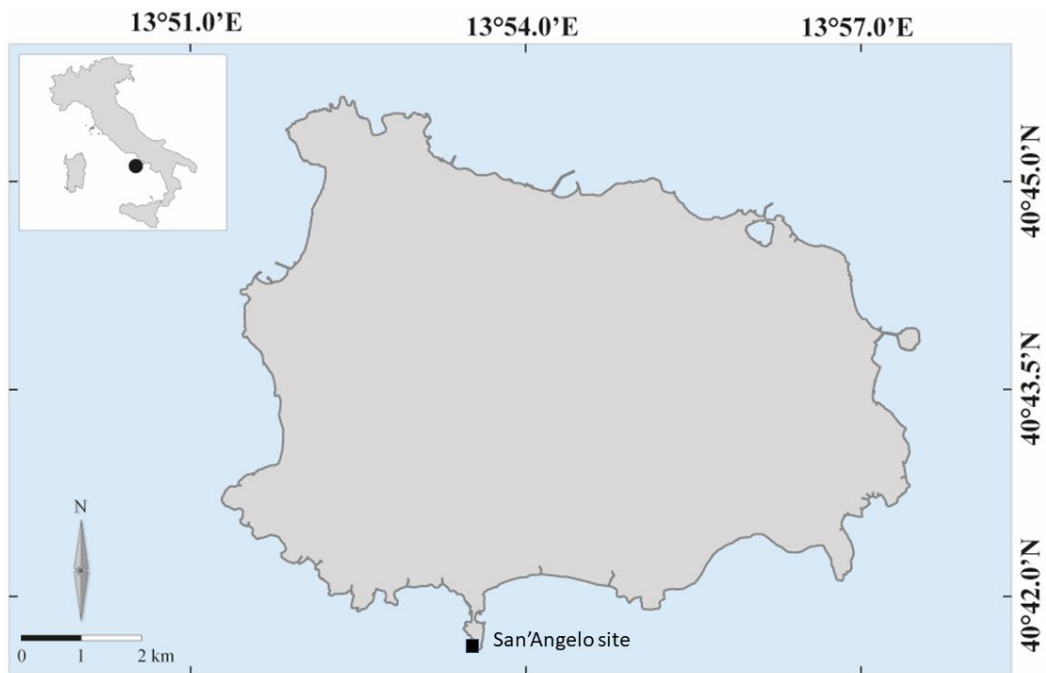


Figure S1-Appendix III Map of the study site along the Southern coast of Ischia Island (Italy). *Astroides calycularis* adult colonies were sampled at 10m depth at the site of Sant'Angelo (40°41'31.1"N 13°53'35.0"E) on the 1st of July 2020.

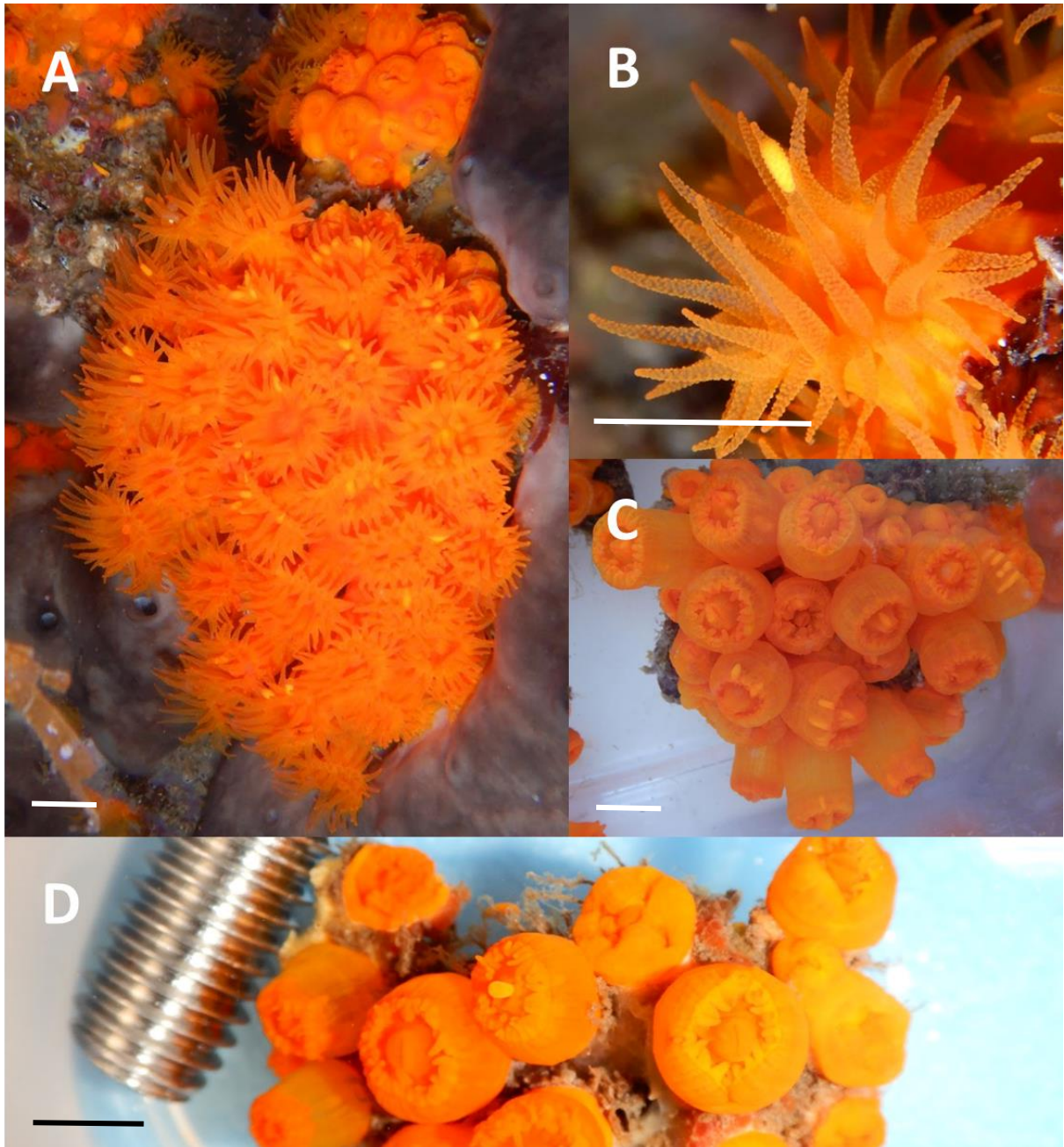


Figure S2-Appendix III Picture of the internal brooding reproduction last processes of *Astroides calycularis* A) *In situ* Female colony brooding larvae, where the larvae are the yellow oval structures in the tentacles of the polyps B) Close up on a larva inside the tentacle of a female polyp C) Close up of larvae inside the gastrovascular cavity of a female polyp and, D) Spawning of a larva from the mouth of a female polyp. Scale: 1 cm

Micro-CT scanner setup

Six skeletons of each condition (total of 24) were scanned in a micro-CT scanner (GE Healthcare, eXplore Locus RS) at a peak x-ray energy of 80 kVp and tube current of 450 μ A at a resolution of 20 μ m. 900 x-ray projections or views (image matrix size 2010 x 1920 pixels) were collected at 0.4-degree angular increments over a single 360-degree rotation. Each projection was 4500 ms in duration and 2 image frames were acquired at each view angle and averaged to reduce image noise resulting in a 2.75 hourlong scan. Each averaged x-ray projection was corrected for brightfield, darkfield, and geometric distortions and then a 3D volumetric image was re-constructed from the x-ray projection data using a filtered-back projection algorithm with isotropic 20.171 μ m voxels. Sample vials containing air, water, Hydroxyapatite SB3 cortical bone mimic $\text{Ca}_5(\text{PO}_4)_3(\text{OH})$, (Gammex RMI, density = 1.83 g/cm^3), calcite crystal (density = 2.71 g/cm^3), and aragonite crystal (density = 2.95 g/cm^3) were included in the scanner field of view (FOV) during acquisition and used for calibration purposes. The 3D data set was linearly rescaled into Hounsfield units (HU) so that air was represented by voxel greyscale values near -1000 and water by values near 0.

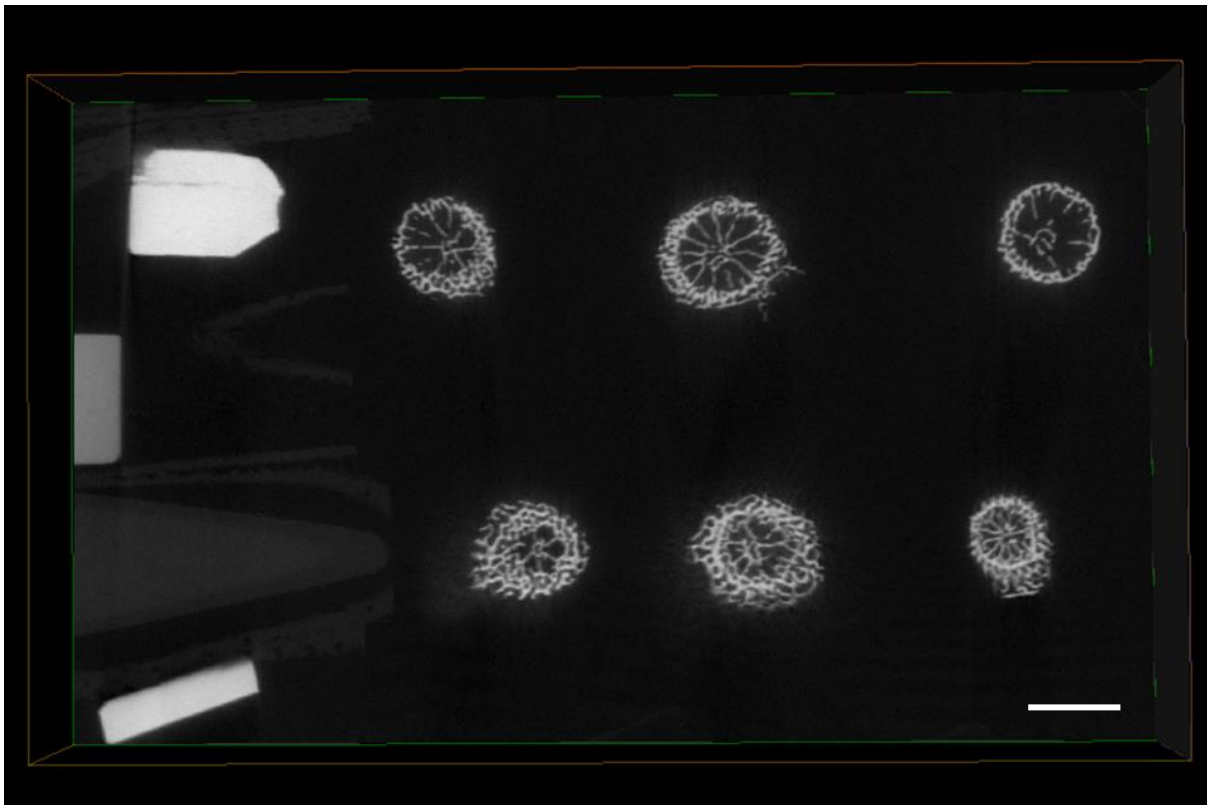


Figure S3-**Appendix III** **Picture of the micro-CT scan set up** with six nine-month old recruits skeletons (on the right), and calcite, aragonite crystals and water for calibration (on the left). Scale: 3 mm (on the bottom left)

Table S1-**Appendix III Comparison among hierarchical linear mixed-effects models assessing the different measured parameters.** The structure of the random term was selected by comparing models with different error structures using the Akaike information criterion (AIC).

Model	df	AIC
<i>Proportions of planula</i>		
Planula~pH_condition*temperature_condition*date + (1 date)	10	1707.0
Planula~pH_condition*temperature_condition*date + (1 cristallyser)	10	2325.8
Planula~pH_condition*temperature_condition*date + (1 date) + (1 cristallyser)	11	1693.4
<i>Proportion of settled polyps</i>		
Settled~pH_condition*temperature_condition*date + (1 date)	10	1996.0
Settled~pH_condition*temperature_condition*date + (1 cristallyser)	10	2126.4
Settled~pH_condition*temperature_condition*date + (1 date) + (1 cristallyser)	11	1950.5
<i>Proportion of non-settled polyps</i>		
Non-settled~pH_condition*temperature_condition*date + (1 date)	10	1530.2
Non-settled ~pH_condition*temperature_condition*date + (1 cristallyser)	10	1433.6
Non-settled ~pH_condition*temperature_condition*date + (1 date) + (1 cristallyser)	11	1395.3
<i>Mortality</i>		
Mortality~pH_condition*temperature_condition*date + (1 date)	10	1496.8
Mortality ~pH_condition*temperature_condition*date + (1 cristallyser)	10	1682.8
Mortality ~pH_condition*temperature_condition*date + (1 date) + (1 cristallyser)	11	1398.3

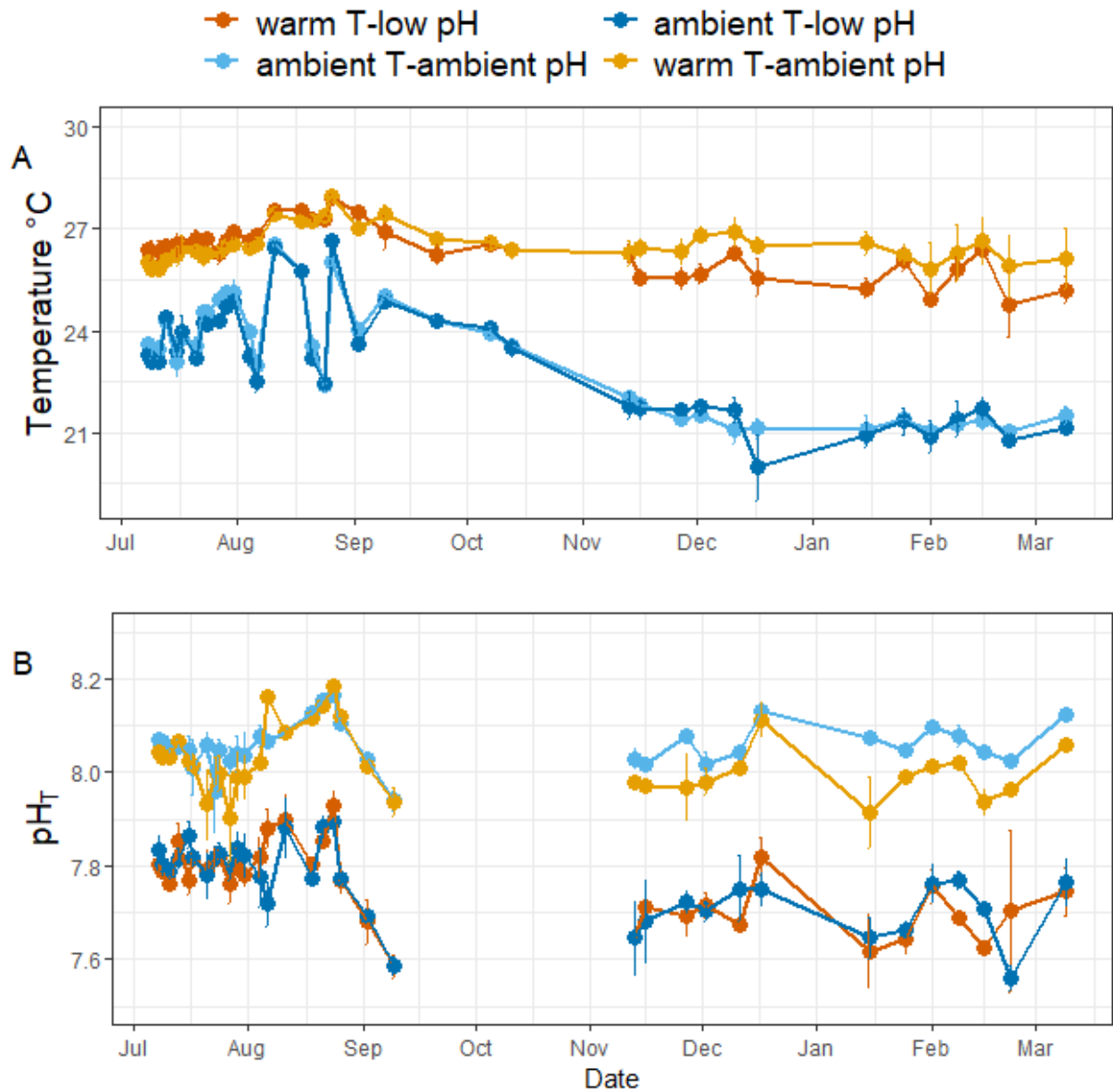


Figure S4-**Appendix III** Evolution of the mean temperature and pH_T in the experimental tanks under the two temperature treatments (23°C for ambient Temperature and 26°C for warm Temperature) and the two pH treatments (pH_T = 8.05 ± 0.09 for ambient pH and pH_T = 7.78 ± 0.10 for low pH) n=36. Mean ± SE

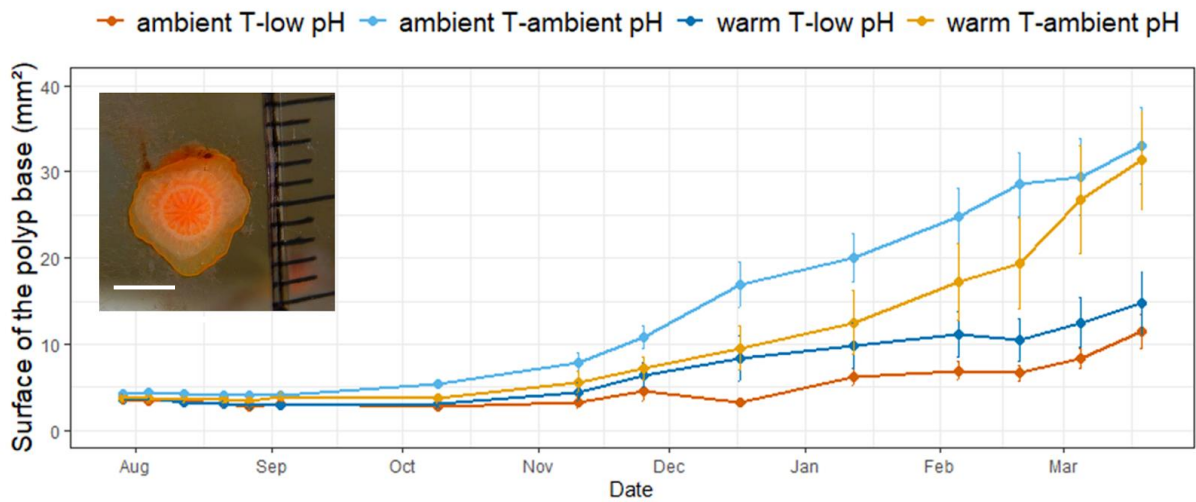


Figure S5-**Appendix III** Mean surface of the base of the juvenile polyps of *A. calycularis* under the two temperature treatments (23°C for ambient Temperature and 26°C for warm Temperature) and the two pH treatments ($\text{pH}_r = 8.05 \pm 0.09$ for ambient pH and $\text{pH}_r = 7.78 \pm 0.10$ for low pH) during 9 months. Values are means \pm SE. The surfaces were obtained by using ImageJ software on bi-monthly pictures of the base of the polyps through the glass of the crystallizers. The color of the dots and lines indicates the origin and the treatment. n= 33-65 per date.

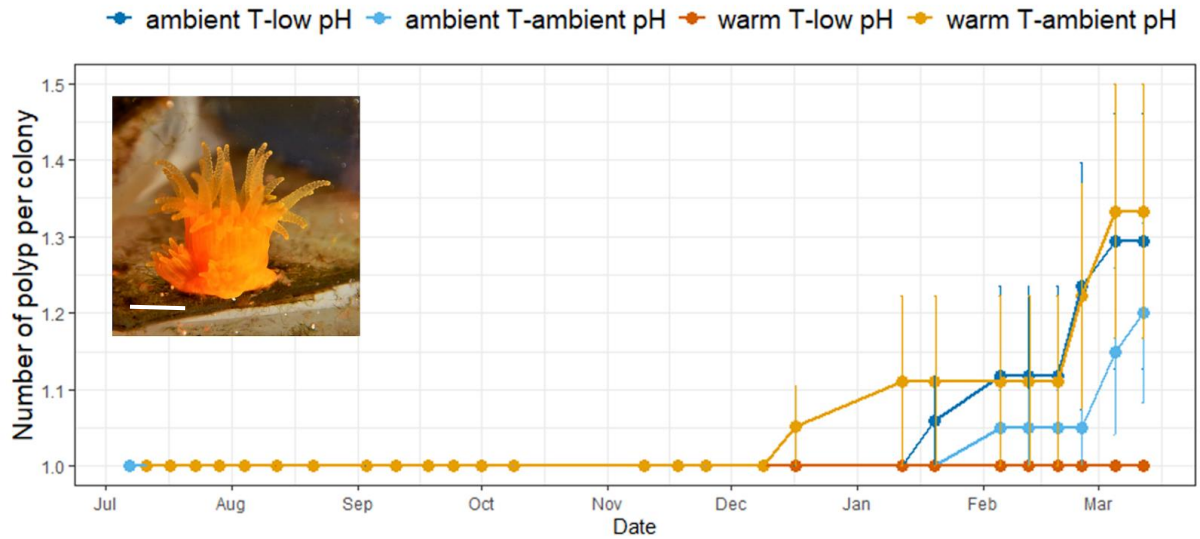


Figure S6-**Appendix III** Mean number of polyp per juvenile colony of *A. calycularis* under the two temperature treatments (23°C for ambient Temperature and 26°C for warm Temperature) and the two pH treatments ($pH_r = 8.05 \pm 0.09$ for ambient pH and $pH_r = 7.78 \pm 0.10$ for low pH) during 9 months. The number of polyp was recorded by counting the apparition of new polyps from the initial polyp. The color of the dots and lines indicates the origin and the treatment. n= 7-120 per date.

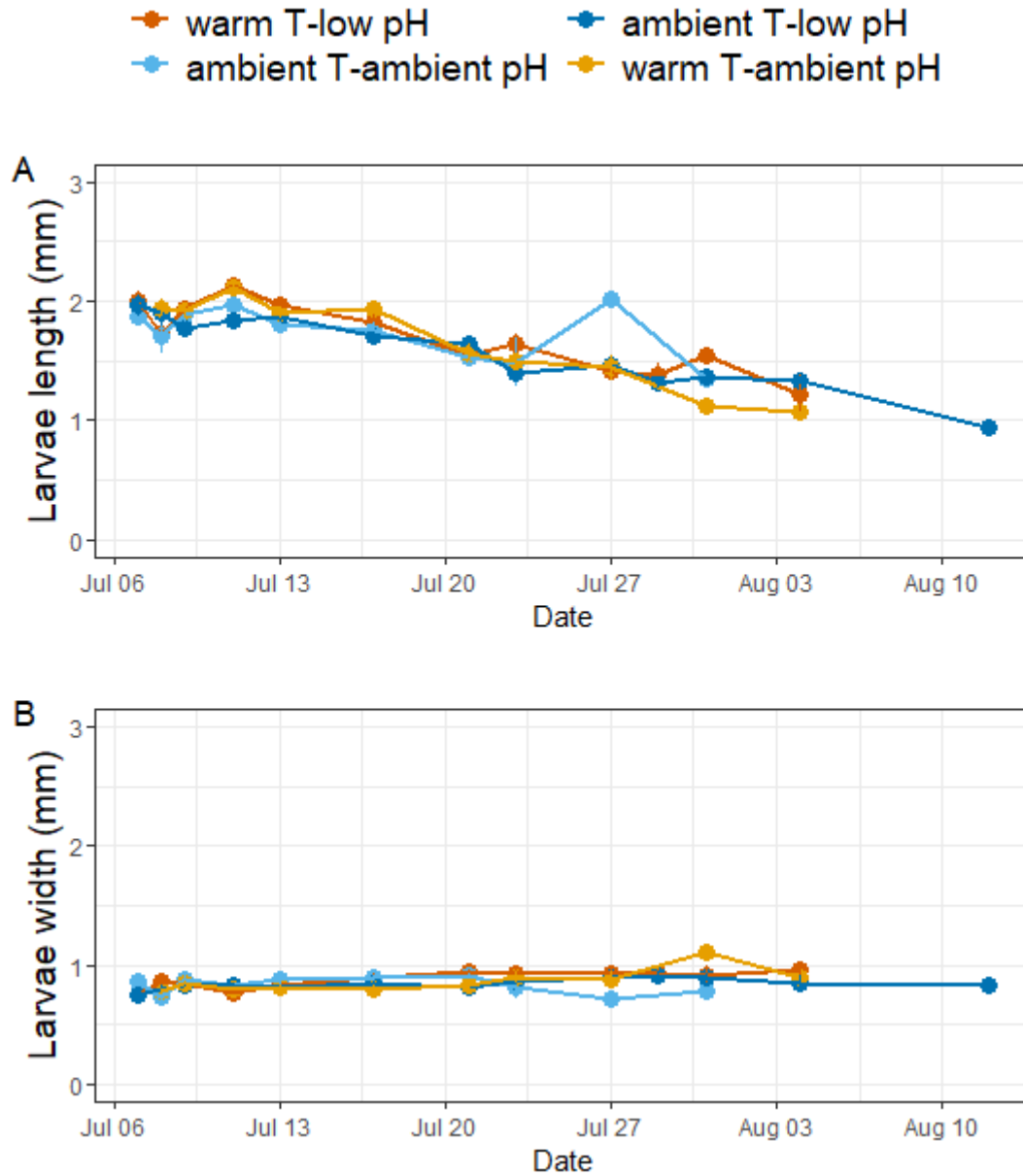


Figure S7-Appendix III Size of *Astroides calycularis* larvae monitored under the two temperature treatments (23°C for ambient Temperature and 26°C for warm Temperature) and the two pH treatments ($pH_r = 8.05 \pm 0.09$ for ambient pH and $pH_r = 7.78 \pm 0.10$ for low pH). A) Length of the larvae B) Width of the larvae. Mean \pm SE

Table S2-**Appendix III** Statistical results of the linear mixed-models with a hierarchical structure of the impact of temperature, pH and time on the larval development of *A. calycularis*. . = $p < 0.1$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Variable	Factor	df	F	p-value
Planula	pH	1,145	3.308	0.069 .
	temperature	1,145	2.215	0.137
	time	1,145	16.189	5.73 10 ⁻⁵ ***
	pH x temperature	1,145	0.878	0.349
	pH x time	1,145	16.999	3.75 10 ⁻⁵ ***
	temperature x time	1,145	11.695	6.30 10 ⁻⁴ ***
	pH x temperature x time	1,145	4.437	0.035 *
Settlement	pH	1,397	3.523	0.061 .
	temperature	1,397	0.091	0.763
	time	1,397	0.002	0.962
	pH x temperature	1,397	0.145	0.703
	pH x time	1,397	1.803	0.179
	temperature x time	1,397	12.833	3.41 10 ⁻⁴ ***
	pH x temperature x time	1,397	29.155	6.68 10 ⁻⁸ ***
Non-settled polyp	pH	1,397	1.236	0.266
	temperature	1,397	0.179	0.672
	time	1,397	11.802	5.91 10 ⁻⁴ ***
	pH x temperature	1,397	0.267	0.605
	pH x time	1,397	1.423	0.233
	temperature x time	1,397	39.293	3.65 10 ⁻¹⁰ ***
	pH x temperature x time	1,397	36.369	1.64 10 ⁻⁹ ***
Mortality	pH	1,397	0.409	0.523
	temperature	1,397	0.166	0.684
	time	1,397	71.417	<10 ⁻¹⁶ ***
	pH x temperature	1,397	18.498	1.70 10 ⁻⁵ ***
	pH x time	1,397	59.607	1.16 10 ⁻¹⁴ ***
	temperature x time	1,397	11.157	8.3 10 ⁻⁴ ***
	pH x temperature x time	1,397	110.880	< 10 ⁻¹⁶ ***
Larvae length	pH	1,933	0.108	0.947
	temperature	1,933	3.502	0.1736
	time	1,933	100.224	< 10 ⁻¹⁶ ***
	pH x temperature	1,933	0.021	0.885
	pH x time	1,933	0.038	0.845
	temperature x time	1,933	1.040	0.3079
	pH x temperature x time	1,933	0.375	0.540
Larvae width	pH	1,933	19.594	5.56 10 ⁻⁵ ***
	temperature	1,933	17.885	1.31 10 ⁻⁴ ***
	time	1,933	37.073	1.14 10 ⁻⁹ ***
	pH x temperature	1,933	13.756	2.08 10 ⁻⁴ ***
	pH x time	1,933	1.868	0.172
	temperature x time	1,933	4.870	0.027 *
	pH x temperature x time	1,933	0.734	0.392

Table S3-**Appendix III** Statistical results of the generalised mixed-models of the impact of temperature and pH skeleton parameter of *A. calycularis* 9-month recruits. . = p<0.1, * = p< 0.05, ** = p<0.01, *** =p<0.001.

Variable	Factor	df	F	p-value
Growth of the base	pH	1,35	23.631	2.4 10 ⁻⁵ ***
	temperature	1,35	0.381	0.541
	time	1,35	0.026	0.873
Number of polyps per colony	pH	1,55	0.307	0.582
	temperature	1,55	0.493	0.485
	time	1,55	2.298	0.135
Volume of skeleton	pH	1,46	4.051	0.044 *
	temperature	1,46	1.144	0.285
	pH x temperature	1,46	3.519	0.061
Number of septa	pH	1,48	10.549	0.002 **
	temperature	1,48	1.809	0.185
	pH x temperature	1,48	0.552	0.461
Density of skeleton	pH	1,19	0.785	0.387
	temperature	1,19	4.064	0.058
	pH x temperature	1,19	9.251	0.007 **

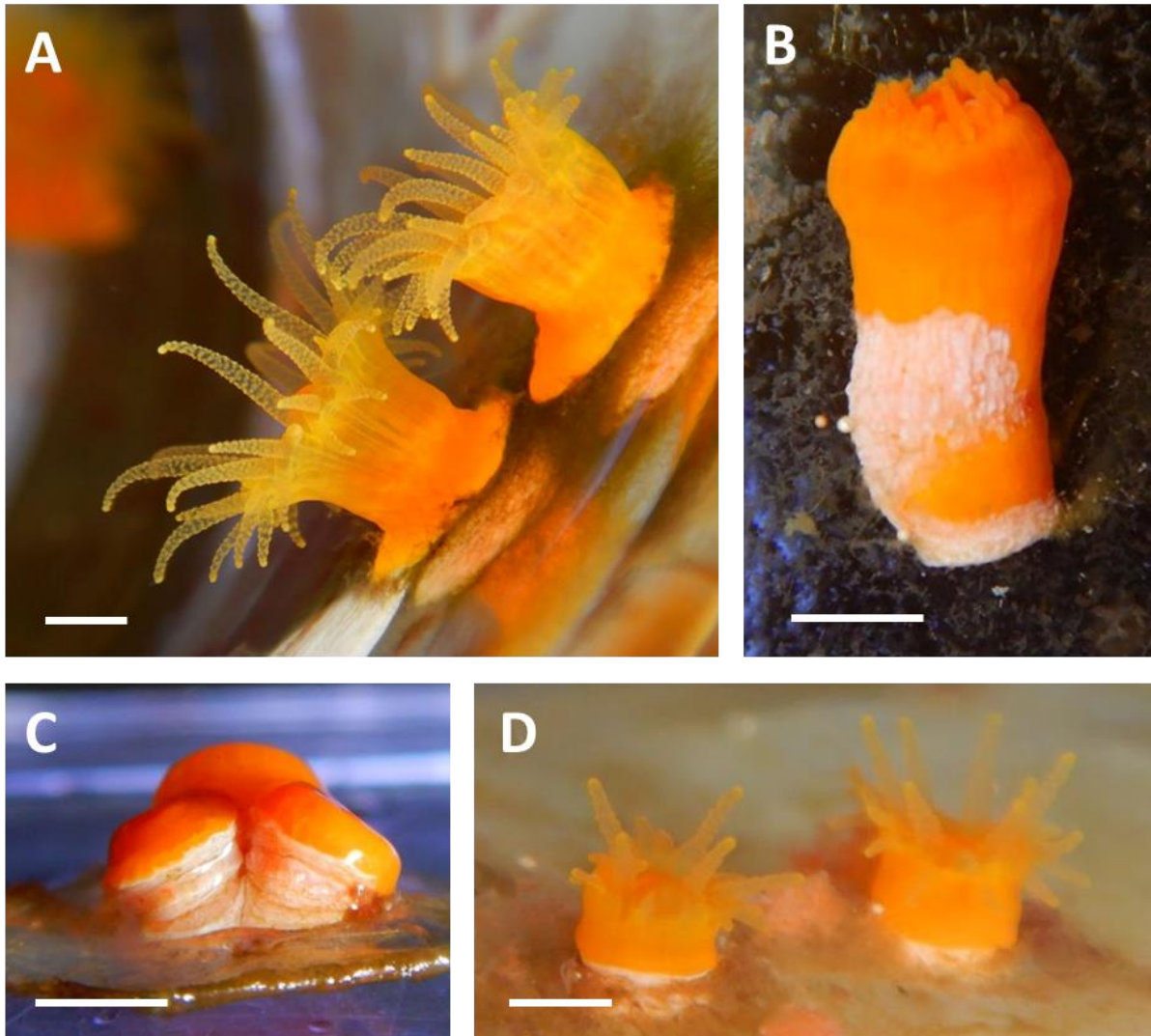


Figure S8-**Appendix III Exposed skeleton of nine-month old recruits in the low pH treatment** A) Picture of two healthy recruits under ambient pH treatment, with outskirts tissue covering the skeleton and invading the substrate, B) Picture of a non-settled recruit presenting an exposed skeleton under low pH treatment, C and D) Pictures of recruits presenting an exposed skeleton and tissue retracted to the top section of the corallite under low pH treatment. Scale : 3 mm

Appendix IV

Parental exposure to natural CO₂ vents alters the response to low pH of a temperate coral early life stages

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Keywords: ocean acidification, CO₂ vents, corals, early life stages, transcriptomics

Abstract:

The study of early life stages such as larval development, settlement, survivorship, and recruit growth is critical to better understand the resilience and persistence of coral populations. While the deleterious effects of ocean acidification on calcification and growth on adult corals are well known, the impacts on early life stages are still poorly documented. Here, we investigate whether past-exposure of parental colonies to naturally acidified environments at CO₂ vents can increase the offspring's tolerance to low pH. Larvae of the Mediterranean azooxanthellate coral *Astroides calycularis* were obtained from parent colonies collected from low and ambient pH sites in Ischia, Italy. Larvae were exposed in the laboratory to three pH treatments: ambient (pHT ~8.05), low (pHT ~7.7, pH projected for the end of the century under RCP 8.5) and extreme low (pHT ~7.5, pH in extreme condition representing vents pH - 0.3 unit, only for the larvae from the CO₂ vent site). Several traits of the early life stages were

monitored for five months: larval size, settlement success, survival and growth of the recruits. Key differences were observed between the site of origin of the larvae. For instance, larvae from the CO₂ vent site were shorter and exhibited a higher rate of mortality than the larvae from the ambient pH site regardless of the pH treatment they were exposed to. Also, larvae from the CO₂ vent site had a lower settlement success at low and extreme low pH. We also analyzed the transcriptomic of the five-month old recruits. Differences in gene expression were mostly observed between the site of origin and not between pH treatment in the laboratory. Our results suggest that future acidification conditions will have an impact on survival and settlement of early life stages. These findings provide new insights into coral recruitment and their ability to respond to present and future ocean acidification conditions.

Material and methods

Sampling site

Female adult colonies of *Astroides calycularis* (~5 cm of diameter) were collected by scuba diving the 20th of June 2021 in Ischia, Italy, when larvae were observed by transparency in their tentacles (Figure 1-Appendix IV). Thirty colonies were sampled in a CO₂ vent site, Grotta del Mago (GM, pH_T ~7.8, 40.711667, 13.964194) and 1.2 km away, at an ambient pH site, Punta San Pancrazio (SP, pH_T ~8.05, 40.701250, 13.954444). *In situ* seawater temperature was around 23°C at collection sites. The two sampled pools of colonies were maintained in separated 30-liter tanks with water motion provided by a NEWA mini 606 pump and maintained in dimly lit conditions. Fully mature larvae were released on the following night, from the mouth of female polyps. They were collected with a pipette, stored by groups of 100 into 250 mL plastic bottles filled with seawater, and transported to the aquarium facilities of the *Laboratoire d'Océanographie de Villefranche-sur-Mer*, France, in less than 12h.

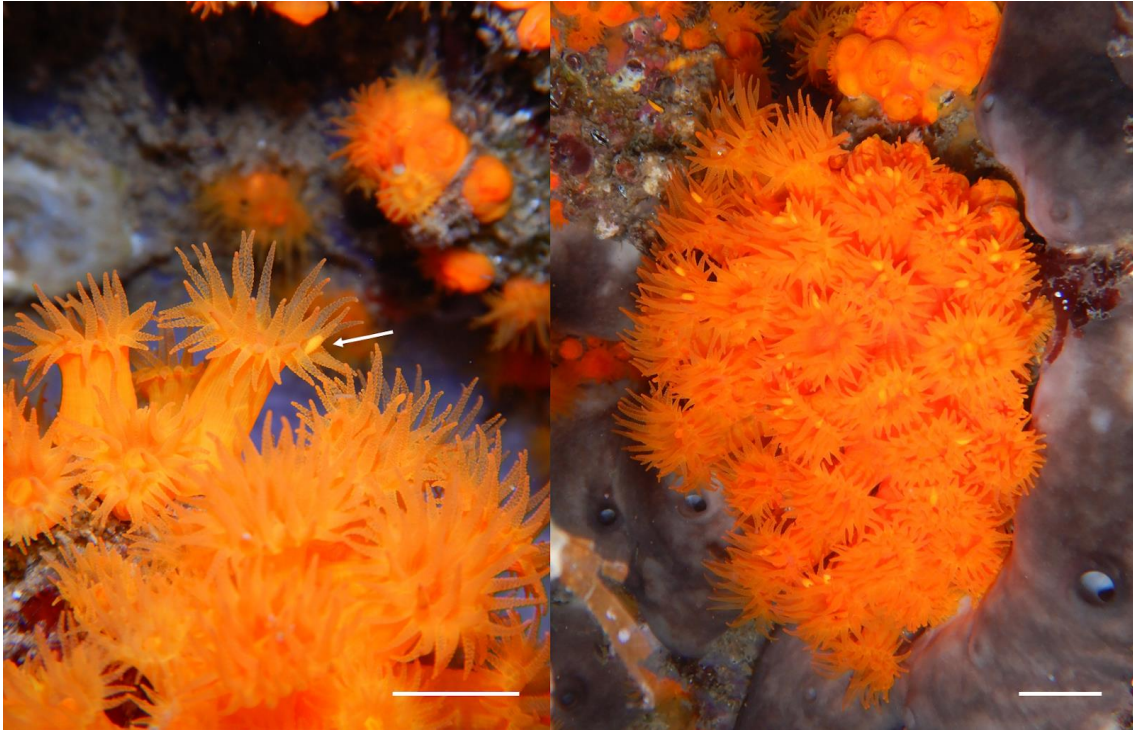


Figure 1-Appendix IV. Picture of female colonies of *Astroides calycularis* with larvae stored in the tentacles (yellow ovals) *in situ* (~2 m, Ischia). The white arrow shows a larva in the parent tentacle.

Experimental set-up and treatment

After 48h of acclimation in the laboratory at ambient pH ($pH_T = 8.05$) and temperature ($T = 23^\circ\text{C}$), the larvae were maintained for five months in the laboratory under three constant pH treatments. pH treatments were “ambient” pH ($pH_T \sim 8.05$), “low” pH ($pH_T \sim 7.70$), corresponding to a decline of 0.3 units of pH projected by the end of the century under the RCP 8.5 CO₂ emissions scenario (Kwiatkowski *et al.*, 2020), and “extreme low” pH ($pH_T \sim 7.5$), as the projected pH at the end of the century at the GM site ($7.8 - 0.3$ units of pH). The extreme low treatment was and only applied to the GM larvae. The larvae were divided into 20 x 300 mL crystallizers (Duran®), 12 with 20 larvae of GM (240 larvae) and 8 with 20 larvae from SP (160 larvae), as four replicates for each of the 5 treatment conditions (GM x 3 pH + SP x 2 pH). Temperature was the same for each tank and followed the daily mean temperature of Ischia at 5 m depth between 2012 and 2020. Each crystallizer was submerged in an independent 5 L experimental tank, gravity-fed (50 ml min^{-1}) from six 25 L header tanks (two for each pH treatment) continuously supplied by seawater pumped from Villefranche Bay at 5 m depth. While larvae were still swimming, water was changed every day and crystallizers were covered with parafilm® to prevent changes in pH and the escape of larvae. When all larvae metamorphosed and settled, the crystallizers were covered with a 45 μm

plankton net to allow water exchange with the experimental tank. pH was controlled in the header tanks using a pH controller 110 (APEX, Neptune Systems) which regulated the delivery of pure CO₂. Temperature was regulated in the experimental tanks by temperature controllers (APEX, Neptune Systems) and 300 W ThermoControl-e heater (Eheim), and both pH and temperature were measured three times a week (see below).

Light was provided by 89 W LED light bars (Aqualistik, Aqualumix). Irradiance gradually increased from 0 at 06:00 to a maximum of 7 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ at 12:30 and gradually decreased to 0 at 21:30. When juvenile polyps had tentacles (approximately after a month), they were fed three times a week with a 10 mL solution of freshly hatched brine shrimps (*Artemia* sp.). 24 h after feeding, the crystallizers were cleaned with a painting brush and the water changed to remove any detritus.

Carbonate chemistry

Header and experimental tanks pH were measured 3 times a week using a handheld pH-meter (913 pH mobile, Metrohm) calibrated with seawater pH TRIS buffer (batch #T33 provided by A. Dickson, Scripps Institution of Oceanography, USA) before each set of measurements. Temperature was measured at the same time in each tank with a Traceable™ digital thermometer (FisherBrand). Total alkalinity (AT) of 3 random experimental tanks and one header tank was measured weekly to confirm that AT was not altered by metabolic activity. AT was determined by potentiometric titration using a Metrohm 888 Titrando following the method of Dickson *et al.* (2007). Titrations of certified reference material (Batch #186) provided by A. Dickson were used to assess the accuracy of the measurements and were within 6.5 $\mu\text{mol kg}^{-1}$ of the reference value. Salinity data during the experiment were obtained from the weekly measurements performed in the in the Bay of Villefranche by the *Service d'Observation Rade de Villefranche, SO-Rade*, of the *Institut de la mer de Villefranche and the Service d'Observation en Milieu Littoral, SOMLIT/CNRS-INSU*. This was possible because we worked in an open system where seawater from the Bay of Villefranche was continuously delivered. pHT, temperature, AT and salinity were used to calculate the other carbonate chemistry parameters using the R package seacarb (Gattuso *et al.*, 2021).

Early life-stages monitoring

The life stages of each 20 individuals (larvae) per crystallizer were recorded every 2 days when swimming larvae were present, and every week after settlement. The different stages

were: “planula”, when the larvae were still swimming, “settled polyp” when larvae had metamorphosed and settled, “non-settled polyp” when larvae had metamorphosed but not settled or detached from the substrate after settling, and “dead”. Missing larvae were assumed dead as coral larvae lyse within 24 h after death.

Pictures of larvae from each origin site were taken before the beginning of the experiment (28 June 2021) with a stereomicroscope (SteREO, Discovery V.12, Zeiss) coupled with a camera (D5100, Nikon). For each polyp attached to the glass of the crystallizer, a picture was taken the 1 October 2021 (T1) and at the end of the experiment the 2 November 2021 (Tf), from below with a camera (Coolpix W300, Nikon) and a ruler used as scale. All pictures were analyzed with ImageJ to measure the maximum length and maximum width of the larvae and the surface of the polyp base. Growth rate of the extension of the base of the recruits were measured following this equation: $\text{base size (Tf)} - \text{base size (T1)} / \text{base size (T1)}$.

Transcriptomics

RNA Isolation and mRNA Sequencing

The 29th of November 2021, one polyp per flask was collected for RNA extraction (four replicate from each of the five conditions, GM x 3 pH + SP x 2 pH). The RNeasy kit (Invitrogen) was used for RNA extraction and then samples were purified with the RNeasy kit (QIAGEN). The quality and quantity of RNA from each extract were assessed with a Agilent D2100 Bioanalyzer. The samples were then stored in -80°C before being sent to BGI Genomica sequencing platform in Hong Kong in less than 72 h on dry-ice. The sequences in FASTQ format and an MD5 file were then retrieved via a cloud.

First, the integrity and quality of the raw reads was checked using the md5deep and FastQC tools. In order to proceed to the quantification of the transcripts, the reads were mapped on a transcriptome of *Astroides calycularis*. The reference transcriptome was made with 98,838 contigs; National Center for Biotechnology Information (NCBI) NCBI Transcriptome Shotgun Assembly (TSA) database at DDBJ/ENA/GenBank under accession number GIRZ01000000 (<https://www.ncbi.nlm.nih.gov/nucleotide/GIRZ01000000>).

Gene Expression Analysis

Libraries representing 20 individual Illumina lanes were aligned to the reference transcriptome. After quality check, a total of 12 lanes (4 Ambient pH, 4 Low pH, and 4 Extreme Low pH; $n = X$ individuals) from the Grotta del Mago population (CO₂ vent site) and 8 lanes (4 Ambient pH and 4 Low pH; $n =$ individuals) from the San Pancrazio population (Ambient pH site) were used for gene expression analyses. The quantification of the transcripts was performed with the Kallisto^[4] tool, then the differential expression of the quantified transcripts (counts) was performed with the R package DESeq2. Counts were normalized using Variance-Stabilizing Transformation (VST) for data visualization and inferential statistical analysis with PERMANOVAs. Low-expression (\log_2 fold change <10) contiguous sequences (i.e., “contigs”) were excluded from analyses to avoid potential artifacts. Several sample designs were constructed to analyze differences in gene expression regarding different factors. The first corresponded to all individuals with two factors, site and pH treatment, and was used for exploratory purposes. The second was similar to the first, but individuals from Grotta del Mago that experienced a pH of 7.5 were removed in order to get a symmetrical design for statistical purposes. The third was dedicated to a simulation of natural conditions, and only individuals from Grotta del Mago with a pH treatment of 7.7 and San Pancrazio with a pH treatment of 8.0 were kept. The functional enrichment analysis of genes was performed with several tools: 1) TransDecoder and hmmsearch to obtain the first functional annotation of the genes named Pfam; 2) the Ontologizer to obtain the second functional annotation based on Gene Ontology (GO) terms, which was obtained from the pfam2go database; and 3) the Functional Genes Analysis (FGA), a tool to filter the statistically significant results of DESeq2 and Ontologizer and combine them to obtain the genes whose expression and function are verified. All the scripts used are available on the GitHub repository https://github.com/MarcMeynadier/Astroides_transcriptome.

Data analysis

Linear mixed-models with a hierarchical structure were used to evaluate the treatment effects through time on the number of larvae in each life stage (planula, settled polyp, non-settled polyp, dead). Hierarchical linear models were used since data were compiled from repeated measures of the same pool of larvae over time. The models were fitted using the function lmer of the R package lme4 (Bates *et al.*, 2015). The fixed factors of the models were origin, pH and time, and header, flask and time were assigned as random effects. The structure of the

random term was selected by comparing models with different error structures using the Akaike information criterion (Table S1). For parameters that are not time-dependent, generalized linear mixed models (GLMM) were used with a Gaussian distribution to test for the effects of the treatments on the growth of the base of the recruits, the length and width of the larvae. Origin and pH were fixed factors and crystallizers of each condition a random factor.

Results

Carbonate chemistry

Table 1-Appendix IV. Measured and estimated seawater physicochemical parameters of the five conditions (origin and pH treatments) in the experimental tanks for salinity (S), temperature (T), total alkalinity (A_T), dissolved inorganic carbon (C_T), pH_T , pCO_2 , calcite (Ω_c) and aragonite (Ω_a) saturation. Values are means \pm SD. Calculated concentrations of C_T , pCO_2 , Ω_c and Ω_a are shown. 1: Parameters measured from discrete water samples. pH conditions are the experimental pH treatments. GM and SP are the origin sites, respectively Grotta del Mago and San Pancrazio in Ischia, Italy.

Origin	pH condition	pH_T	T (°C)	A_T ($\mu\text{mol kg}^{-1}$)	C_T ($\mu\text{mol kg}^{-1}$)	Salinity	pCO_2 (μatm)	Ω_a	Ω_c
GM	ambient	8.07 \pm 0.01 n=180	25.26 \pm 0.1 n=180	2569 ¹ \pm 10 n=22	2199 \pm 2 n=180		404.17 \pm 6.20 n=180	4.16 \pm 0.03 n=180	6.28 \pm 0.04 n=180
GM	low	7.70 \pm 0.01 n=180	25.29 \pm 0.1 n=180	2579 ¹ \pm 12 n=20	2413 \pm 3 n=180		1140.06 \pm 15.48 n=180	2.06 \pm 0.03 n=180	3.11 \pm 0.04 n=180
GM	extreme low	7.51 \pm 0.01 n=180	25.30 \pm 0.1 n=180	2577 ¹ \pm 26 n=20	2491 \pm 3 n=180		1815.16 \pm 25.62 n=180	1.42 \pm 0.02 n=180	2.14 \pm 0.03 n=180
SP	ambient	8.07 \pm 0.00 n=180	25.26 \pm 0.1 n=180	2571 ¹ \pm 12 n=24	2199 \pm 2 n=180		403.98 \pm 6.03 n=180	4.16 \pm 0.03 n=180	6.28 \pm 0.04 n=180
SP	low	7.69 \pm 0.01 n=180	25.31 \pm 0.1 n=180	2569 ¹ \pm 13 n=18	2418 \pm 3 n=180		1165.07 \pm 13.83 n=180	2.01 \pm 0.02 n=180	3.04 \pm 0.04 n=180

The ambient pH treatment was maintained at a mean pH_T of 8.07 \pm 0.01 (mean \pm SD, n=360), the low pH treatment at a mean pH_T of 7.70 \pm 0.01 (n=360), and the extreme low pH treatment at a mean pH_T of 7.51 \pm 0.01 (mean \pm SD, n=180, total number of weekly pH_T discrete measures in each ambient pH experimental tank, Table 1-Appendix IV, Figure 2-Appendix IV). All the treatments were at the same temperature, which was following the daily mean temperature of the period 2012-2020 of Ischia at 5m (Figure 2-Appendix IV). The mean temperature in the tanks was 25.29 \pm 0.1 °C (n=900, Table 1-Appendix IV). The total alkalinity was not different between treatments and did not vary much during the duration of the experiment, with a mean of 2573 $\mu\text{mol kg}^{-1}$ (n=104, Table 1-Appendix IV).

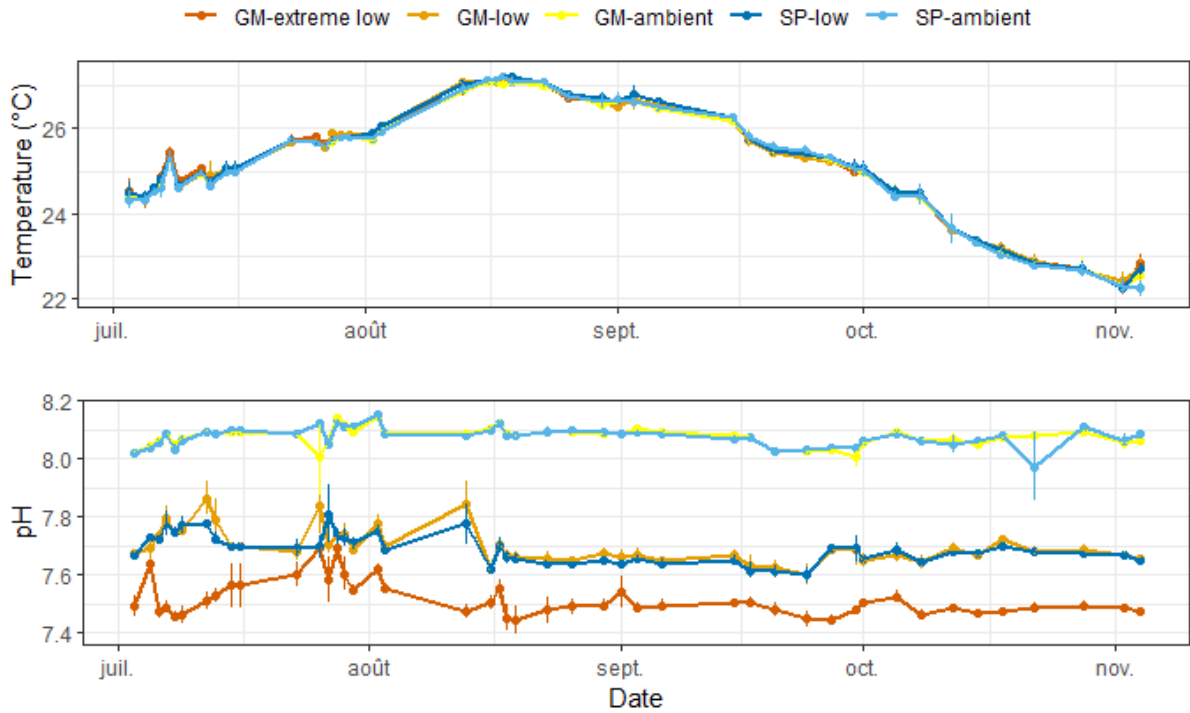


Figure 2-Appendix IV. Evolution of the mean temperature and pH_T in the experimental tanks under the three pH treatments (Ambient pH ~8.05, low pH ~7.7 and extreme low pH ~7.5).

Early life stages monitoring

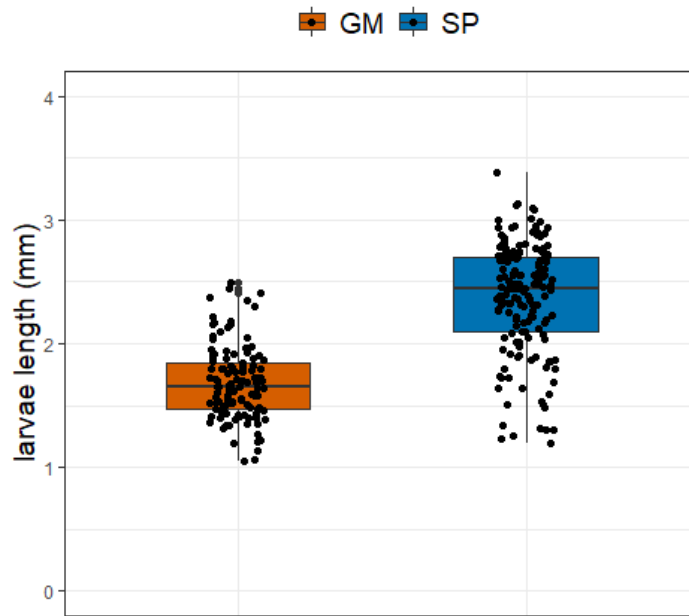


Figure 3-Appendix IV. Length of *A. calycularis* larvae according to site of origin, GM (Grotta Mago, a CO₂ vent site) and SP (San Pancrazio, ambient pH site). Dots represent the length of the larvae per origin site and boxes represent the median and the 25% and 75% quartiles. n=281 larvae

The length of the larvae from GM, 1.68 ± 0.03 mm, was significantly smaller than the larvae from SP, 2.37 ± 0.04 ($p < 0.001$, Figure 3-Appendix IV).

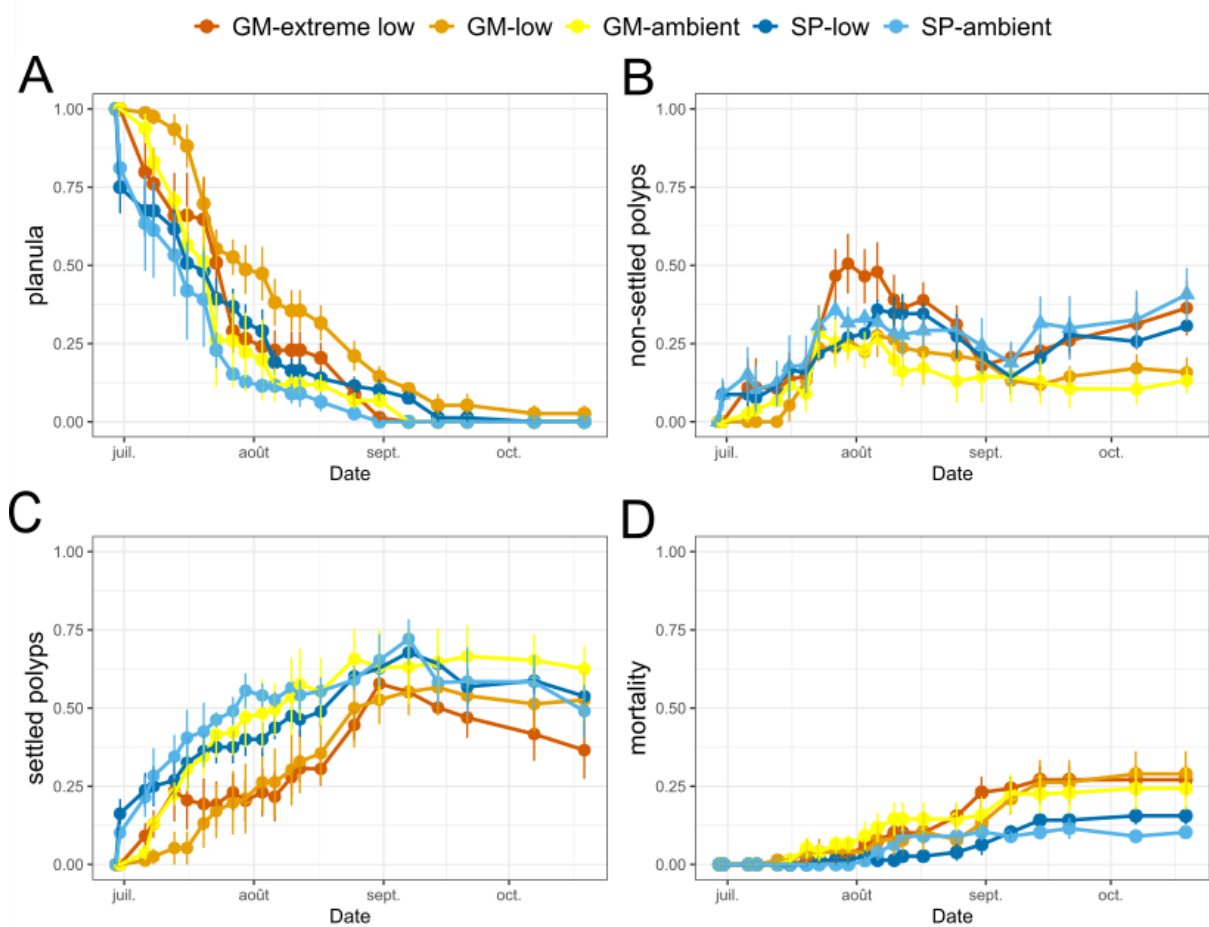


Figure 4-Appendix IV. Proportions of planula, non-settled, settled, and mortality of *A. calycularis* collected from a CO₂ vent site (GM) and an ambient pH site (SP) exposed to three pH treatments ($pH_T = 8.07 \pm 0.05$ for ambient pH, $pH_T = 7.69 \pm 0.07$ for low pH and $pH_T = 7.51 \pm 0.08$ for extreme low pH) during 4 months. Panel (A) shows the proportion of planulas (swimming larvae). Panel (B) shows the proportion of non-settled polyp, when larvae metamorphosed into a free polyp (not attached to the substrat) and settled polyp that subsequently detached. Panel C) show the proportion of settled polyps (metamorphosed and fixed), and D) the proportion of mortality (dead larvae and polyps). Points are mean \pm SE. $n = 400$ original larvae, 4 replicates per condition (origin and pH treatment).

Larvae of SP started settlement and metamorphosis only after 1 day in the flask, while the larvae from GM did not start before a week (Figure 4.A-Appendix IV). The settlement of the larvae from GM was significantly lower under low and extreme low treatments from the beginning of the experiment, to September 2021, while under ambient pH they show no difference with larvae from SP (Figure 4.C-Appendix IV). From September to the end of the experiment, the early life stages of GM presented a higher mortality, regardless of the pH treatment in the laboratory (Figure 4.D-Appendix IV).

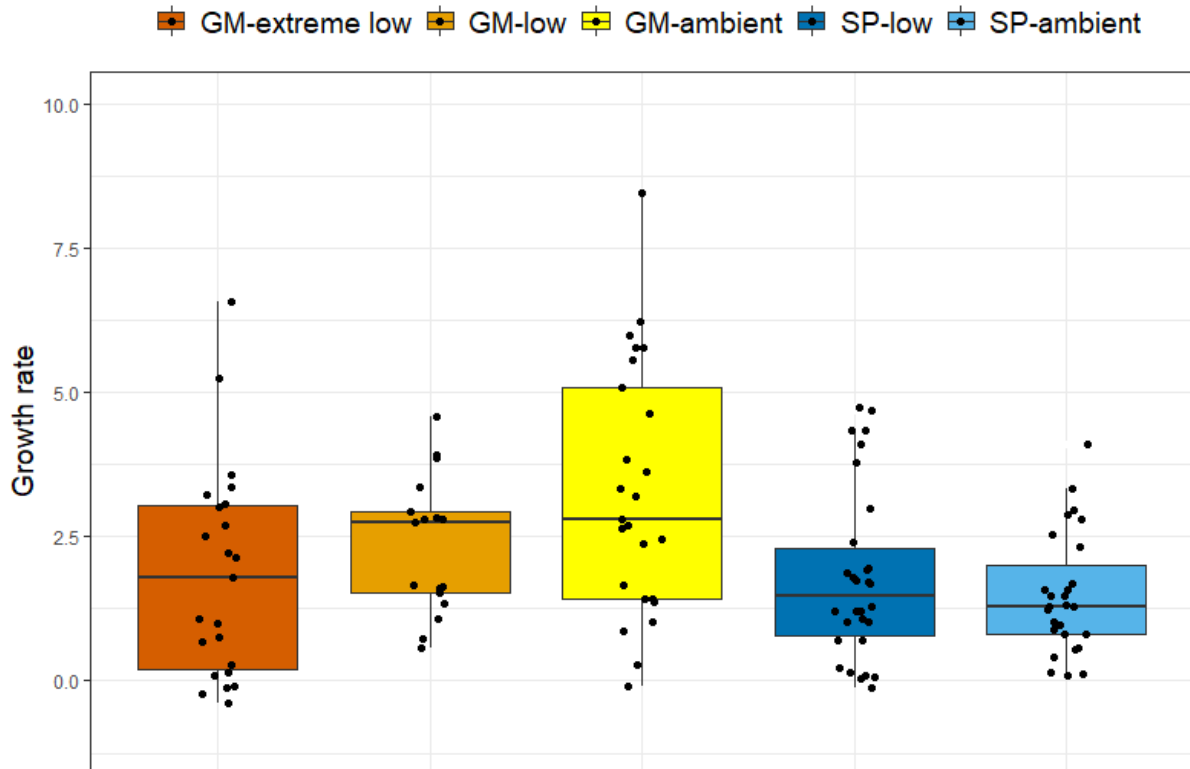


Figure 5-Appendix IV. Three-month growth rate of *A. calycularis* recruit collected from a CO₂ vent site (GM) and an ambient pH site (SP) exposed to three pH treatments ($pH_T = 8.07 \pm 0.05$ for ambient pH, $pH_T = 7.69 \pm 0.07$ for low pH and $pH_T = 7.51 \pm 0.08$ for extreme low pH). The growth rate was measured as the extension of the base of the recruit polyps on the crystallizer. Dots represent the individual growth rates and boxes represent the median, the 25%, and 75% quartiles. The color of the box indicates the origin and the treatment. n = 125 recruits

The growth rate of the base of the polyps was significantly different between site ($p < 0.001$) and pH treatment ($p = 0.01$). Regardless of the pH treatment, the recruits of GM had a mean growth rate of 2.90 ± 0.3 , while the recruits of SP were at 1.65 ± 0.2 . At ambient pH treatment, the recruits of GM had a growth rate 2.4 times higher than recruits of SP in the same treatment, and at low pH, they had a growth rate 1.8 times higher than SP (Figure 5-Appendix IV).

Transcriptomic analysis

Concerning the exploratory results of juvenile corals, gene expression across 93,585 reference contigs showed differences in both the CO₂ vent and ambient site populations (Figure 6.A-Appendix IV, PCA). Those differences were statistically confirmed by PERMANOVA, where both site and pH were taken as factors. Difference in gene expression was more dependent on

the site (p-value < 0.001) rather than pH treatment (p-value = 0.660). Concerning the statistically verified differentially expressed genes, most of them depend on the comparison of site (888, 98.8%) rather than pH treatment (11, 1.2%), where none of those genes were shared among those two comparisons (Figure 6.B-Appendix IV, Venn diagram). The result of the expression of functionally enriched genes in juveniles was performed on a single contrast, which corresponded to the simulation of the original natural conditions (Figure 6.C-Appendix IV, barplot). The totality of the biological functions found were downregulated for corals from San Pancrazio, compared to those of Grotta del Mago. These functions corresponded to the activity of catabolic and binding enzymes, as well as genetic and cytoskeletal regulation. It is important to note that for each function, the expression values were low and few genes were found.

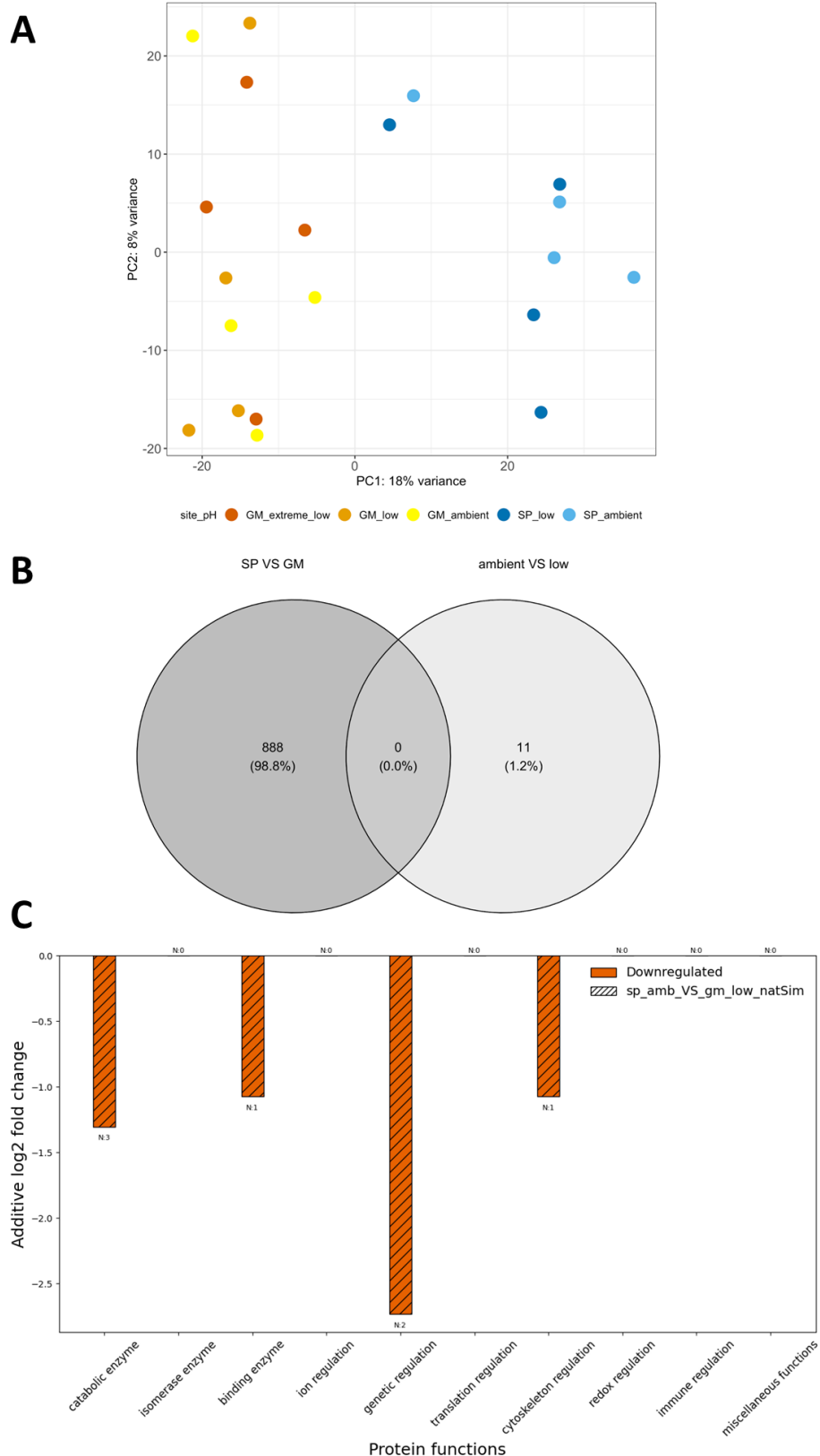


Figure 6-Appendix IV. Transcriptomic analysis of 5-month old recruits of *Astroides calycularis* collected from a CO₂ vent site (GM) and an ambient pH site (SP) exposed to three pH treatments (pHT = 8.07 ± 0.05 for ambient pH, pHT = 7.69 ± 0.07 for low pH and pHT = 7.51 ± 0.08 for extreme low pH). Panels (A) Principal component analysis performed on the results of the differential expression analysis for all the 93 585 transcripts. Panel (B) shows the Venn diagram of differentially expressed

genes through the different contrasts performed by DESeq2 (origin: SP vs GM, and pH treatment: ambient vs low pH), panel (C) shows a Bar chart of genes expression values quantified with additive log2 fold change according to the functions of the proteins associated with the genes. n=20 recruits, 4 replicates per condition (origin and pH treatment).

Appendix V

Exposure to natural CO₂ vents delays the spawning of a temperate coral

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Keywords: ocean acidification, CO₂ vents, corals, reproduction, gametogenesis, histological analysis

Abstract:

Sexual reproduction is one of the mechanisms that can be impaired under acidification. Moreover, reproduction of *C. caespitosa* still has a lot of unknowns. Thus, we studied the impact of acidification on gametogenesis and we investigated the timing of the spawning of *C. caespitosa* of Ischia. For this purpose, samples from CO₂ vent and ambient sites were obtained in July and September 2020 to analyze the progression of gametogenesis on selected colonies. The sex ratio and the size of the gametes did not differ from a site to another. However, in September, the colonies of both sex in the ambient site had released their gametes while an asynchrony of spawning was exposed the CO₂ vent site, as the male had not spawned. This asynchrony could lead to an impairment of the fertilization and thus, the number of larvae which are critical factors to maintain or replenish population after disturbance.

Material & Methods

Description of the site and sampling

The studied populations of *Cladocora* are found along the Island of Ischia, near Naples, Italy, where natural CO₂ vents locally acidify the surrounding environment, approaching the conditions predicted by the IPCC under the RCP 8.5 scenario. *C. caespitosa* naturally occurs near a CO₂ vent system adjacent to rocky reefs, at Chiane del Lume (40.717444, 13.967278), at around 10 m depth where low pH can be found (pHT ~7.90, Teixido *et al.*, 2018). The reference site with ambient pHT (~ 8.05) and no venting activity is located at 100 m from the Vent site.

One hundred colonies were individually marked in both sites with numbered tags, attached with marine epoxy by scuba diving. In the 1st July 2020, 4 to 6 polyps were randomly selected from 20 colonies from the CO₂ vent and 28 colonies from the ambient pH site to determine the sex. The difference in the number of colonies sampled between the two sites was to minimize the impact of sampling on the CO₂ vent colonies which have a smaller population. After the identification of the sex for the initial 48 colonies sampled, 5 female and 5 male colonies were chosen per site. On 18th September 2020, 4 to 6 polyps were sampled on the chosen colonies in both sites. July and September samples were used to study gametogenesis. The dates were selected to correspond to before and after the spawning for *C. caespitosa*, which is known to spawn at the beginning of September (Kersting *et al.*, 2013). All samples were transported from Ischia to the Laboratoire d'Océanographie de Villefranche-sur-Mer on dry ice and stored at -80° C.

Sex and gametes analysis

In Villefranche-sur-Mer, polyps' tissues were fixed with 10% formaldehyde in seawater for 48h before decalcification with a 5% HCl solution for a 4-6h hours until complete dissolution of the skeleton. The tissues were then stored in 70% Ethanol for 48h. Before inclusion in Surgipath Paraplast® (Leica Biosystems, Richmond, IL, USA), the tissues were dehydrated in increasing concentrations of Ethanol solutions (80%, 90%, and 100%) and then stored in 100% Butanol at 4°C for 24h. Transverse sections of 8 µm were obtained with a manual microtome (Leitz 1212) and placed on glass slides (Figure 1-Appendix V) for staining with the Hematoxylin (Mayer's Hematoxylin, Sigma-Aldrich) and Eosin (Eosin Y, Sigma-Aldrich) method. After staining, each slide was mounted with Permount Mounting Media (Fisher Chemical) and a cover slide.

720 slides and 21 600 histological cuts were analyzed for the samples of July 2020. For September 2020, 300 slides and 9000 cuts were performed. In total, 30 600 cuts were analyzed.

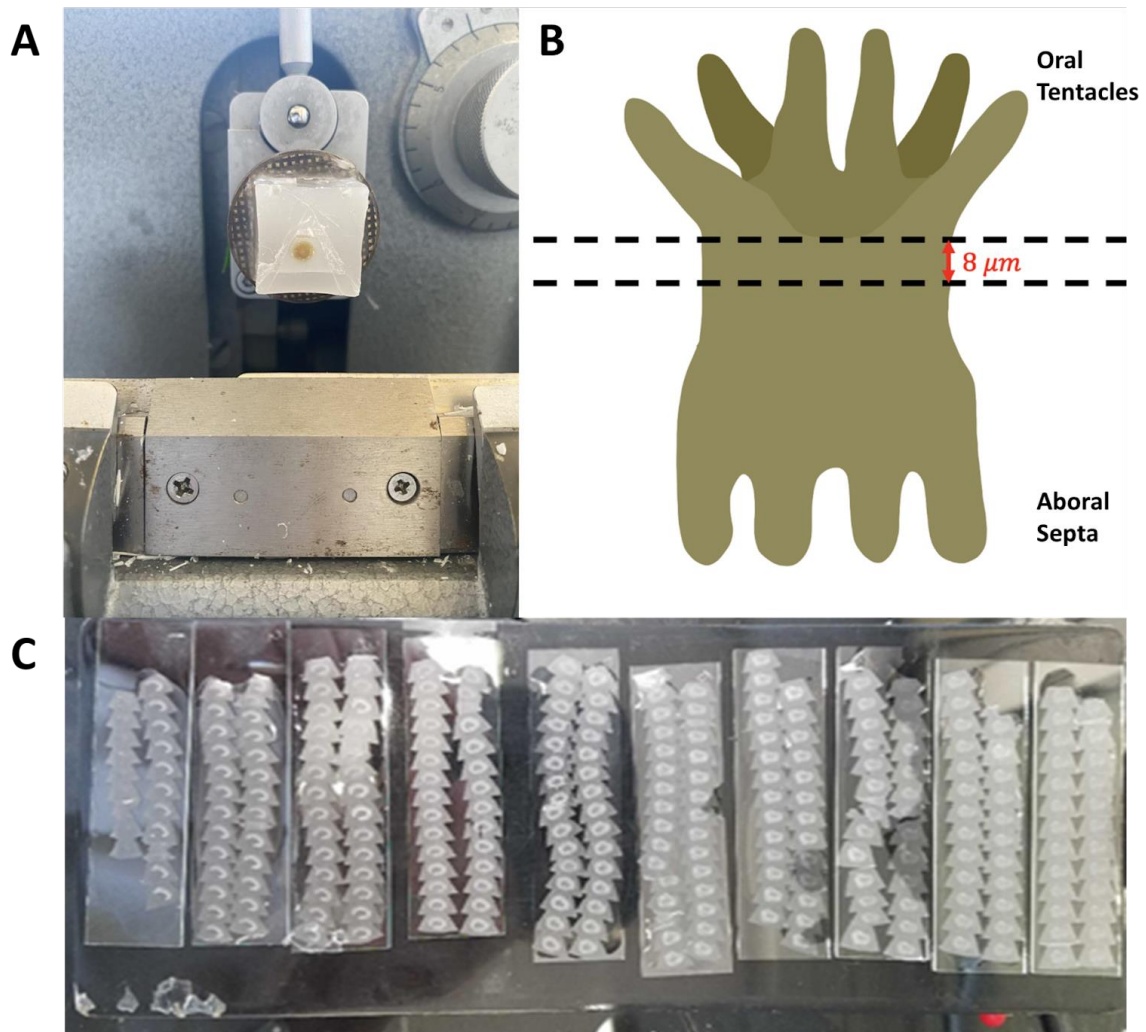


Figure 1-Appendix V. Polyp in Paraplast and placed in a manual microtome (A). The polyps were sliced with sections of 8 μm (B). Polyp sections disposed in slides for posterior staining (C).

First, 48 colonies (20 from the Vents site and 28 from the Ambient site) were analyzed using a microscope (Alphaphot-2 YS2, Nikon) to determine their sex in both sites (Figure 2-Appendix V). From the identified colonies, five females and five male colonies, in each site, were selected to count oocytes and spermaries, respectively. The counting was performed by randomly choosing six mature mesenteries and counting the oocytes and spermaries along the entire length of the mesentery (Figure 3-Appendix V). To browse an entire mesentery to measure the gametes all the cuts of one colony have to be analyzed one by one, from oral to aboral (~360 cuts). The obtained mean per mesentery was multiplied by the number of mature mesenteries

present to obtain the mean per polyp. For the size, the oocytes were measured when the visible nucleolus was at its largest size (Figure 2-Appendix V). Likewise, spermaries' measures were performed when they presented their most considerable size. Then, the mean of both measures was computed and considered as the final size.

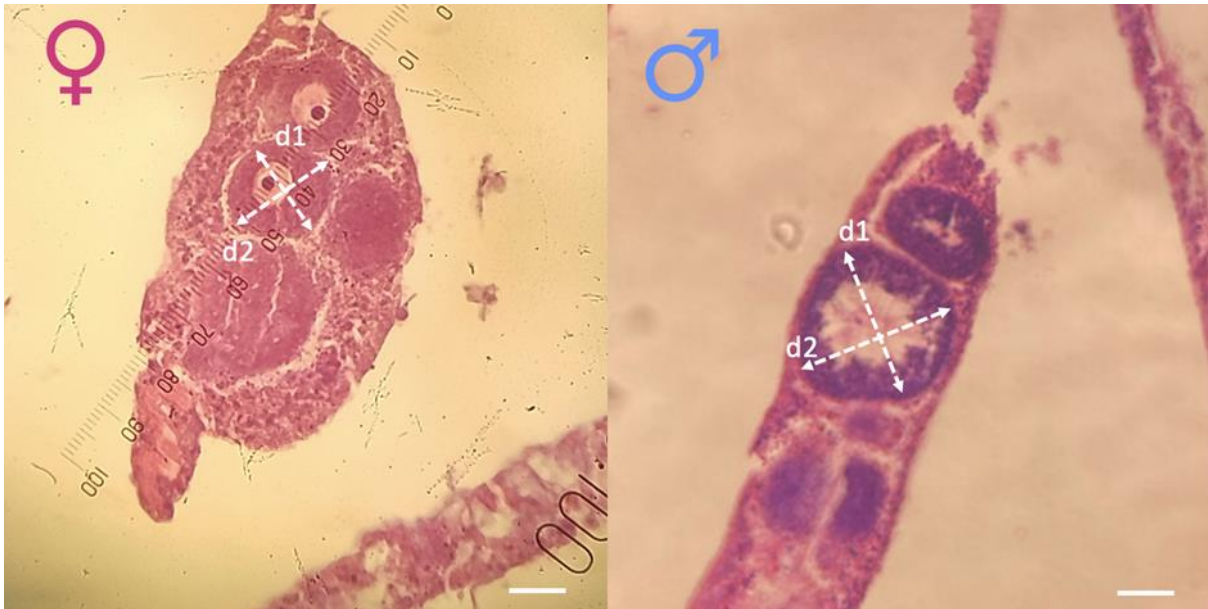


Figure 2-Appendix V. Scheme of the measurement of the gametes of *Cladocora caespitosa*. The picture on the left is an oocyte and the picture on the right is a spermary containing the spermatocytes. The schematic was done based on the microscope observation (x400, scale = 25 μm for both photos). The size was determined by measuring the largest length of the gamete (d1) and the perpendicular length (d2). Both diameters were used to compute the diameter mean.

Statistical analyses

All the statistical analyses were performed using R software. A Chi-square test was used to compare the sex proportions between the two sites. A Generalized Linear Mixed Model (gaussian family) followed by an ANOVA (test = “Chisq”) was used to test the possible differences in the size and number of gametes as a function of the site. The results are reported as mean \pm standard error.

Results

Sex identification, proportions, and gametogenesis steps

The gametes were observed in the mesenteries located in the septa of the polyps (Figure 3-Appendix V). The histological analysis showed that the coral colonies of *C. caespitosa* living in Ischia are monoecious with gonochoric polyps: only one sex of gametes was found in each polyp and all the polyps of the same colony have the same sex. The female gametes, (oocytes) are spherical, composed of a nucleus surrounded by a nucleolus and a vitellogenic layer that grows during the maturation of the gamete (Figure 4-Appendix V). The different stages of the oocytes are identified by the size of the nucleolus, however, no clear stage was differentiated with only a one-month histological analysis. The male gametes (spermatozoids) are contained in spherical spermaries when they are maturing (Figure 5-Appendix V). When the male gametes are close to maturation, a lumen begins to form in the center of the spermatocyte, and the spermatozoids' flagella can be observed.

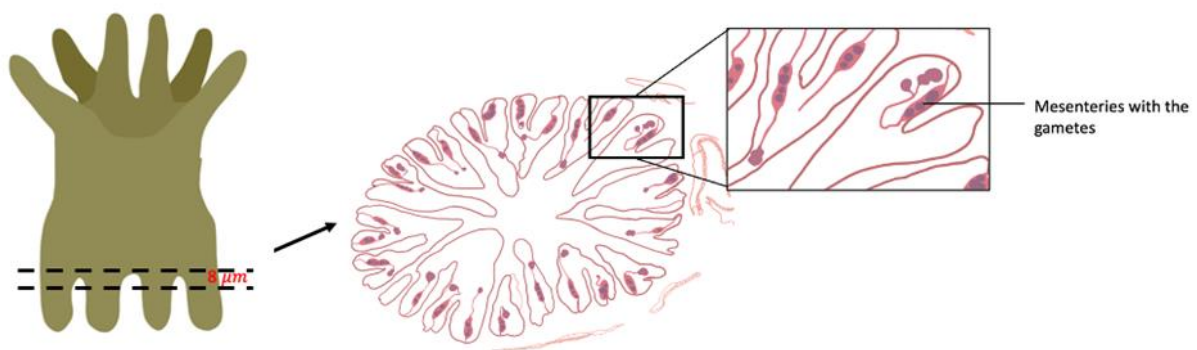


Figure 3-Appendix V. Schematic representing where the gametes are located in the polyp. gametes can be observed inside the mesenteries (schematic) that start from the beginning of the septa.

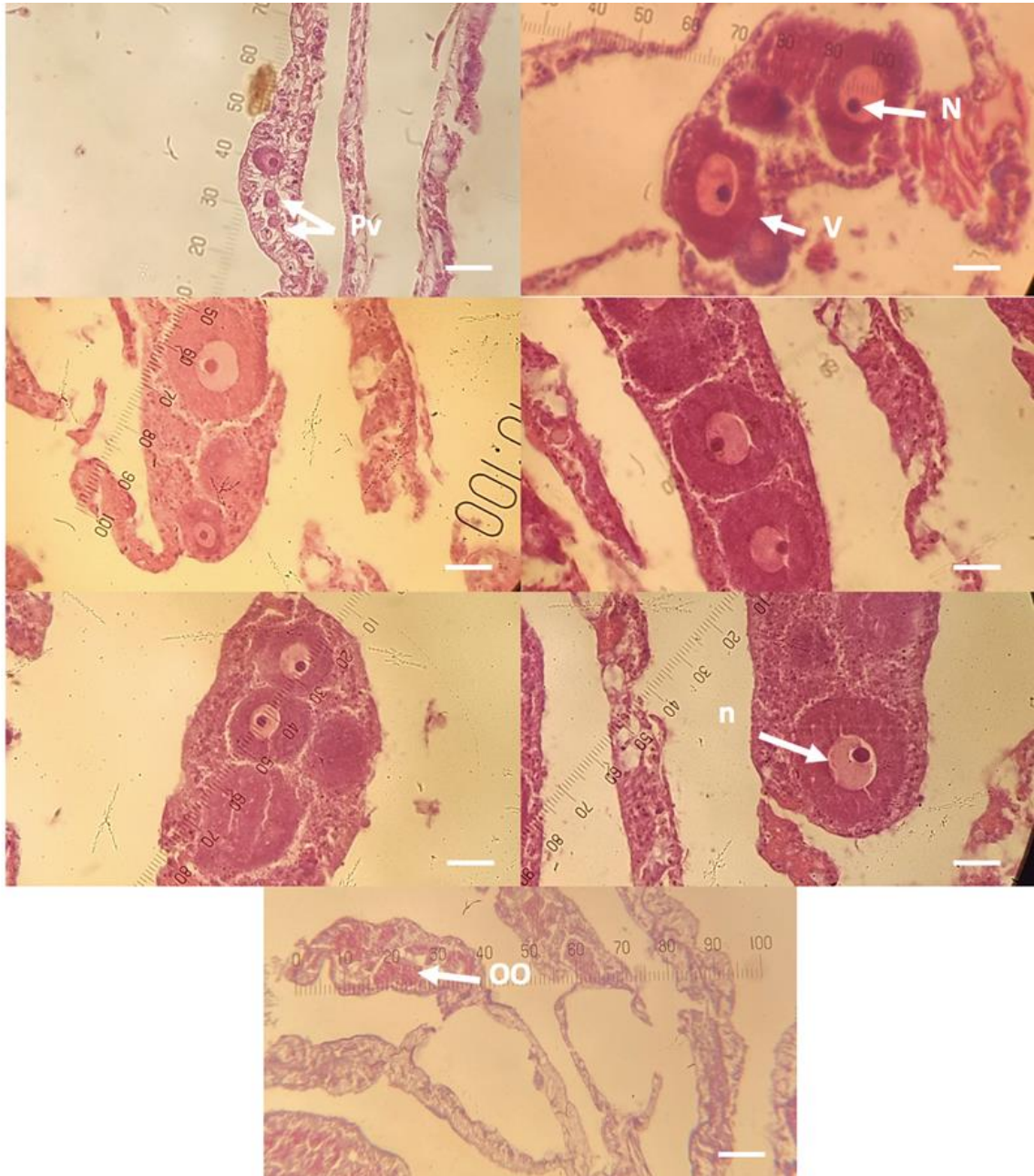


Figure 4-Appendix V. Female polyps' histological cuts of 8 μm after hematoxylin and eosin staining method. Gametes were observed in a microscope (x 400, scale = 25 μm). Labels: Pv = previtellogenic oocyte; V = vitellogenic oocyte; OO = oocyte in oosorption; N = nucleolus; n = nucleolus

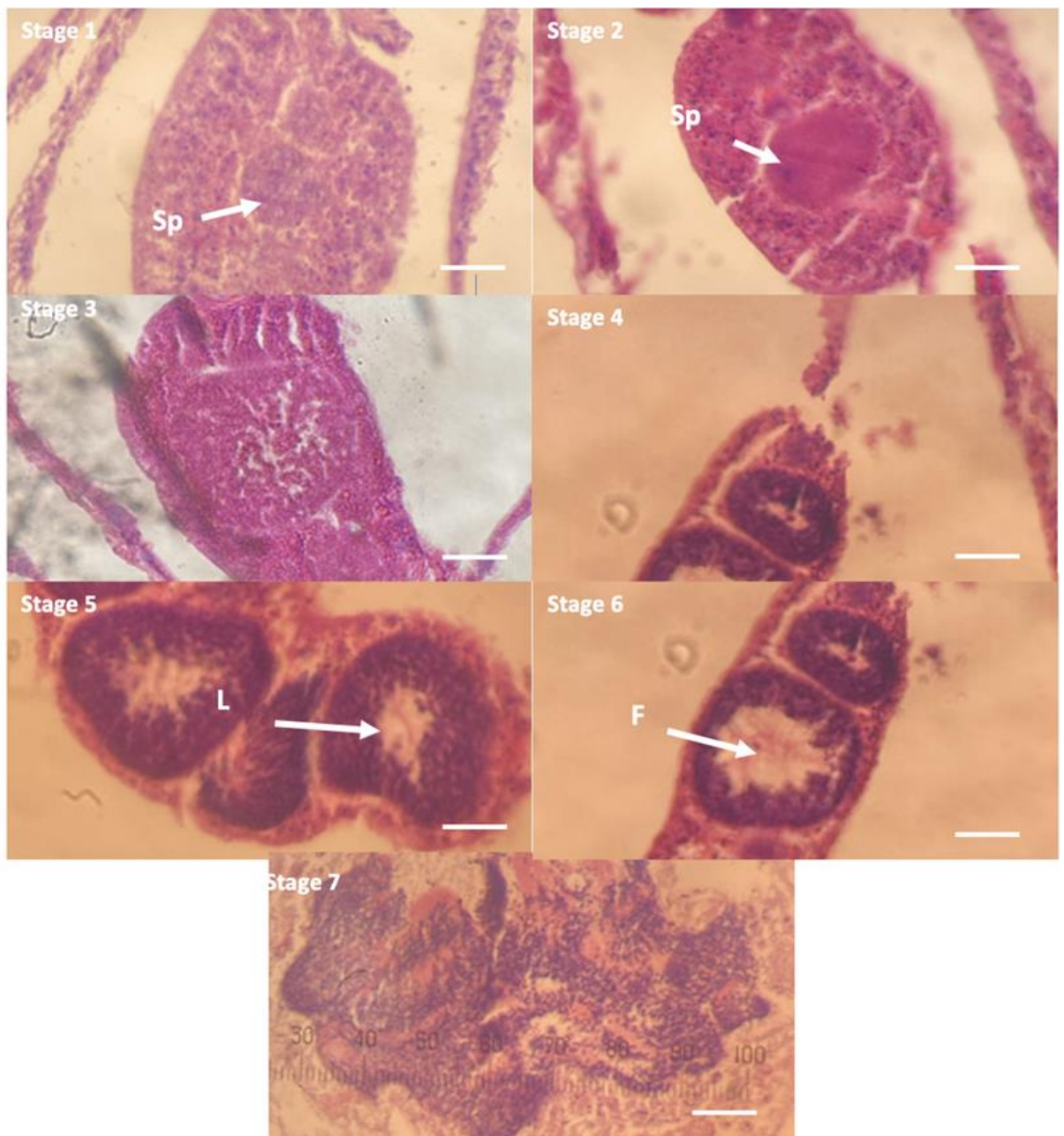


Figure 5-Appendix V. Male polyps' histological cuts of 8 μm after hematoxylin and eosin staining method. Gametes were observed in a microscope (x 400, scale = 25 μm). Labels: Sp = spermary; L = lumen; Fl = flagella.

The histological analysis of July 2020 (48 colonies) allowed to compare the sex proportions in each of the sites (Table 1-Appendix V). No significant difference in the proportions between the sites was found (Chi-squared test, $p=0.992$). The sex ratio in the Vent site was 8:10 (males:females) while in the Ambient pH site the ratio was 15:11 (males:females).

Table 1-Appendix V. Female and Male ratio of the Vent colonies (n= 20) and Ambient colonies (n= 28). The undetermined colonies correspond to the colonies that did not present visible and differentiated gametes.

	Number of polyps	Proportions
Vent samples	20	
Females	10	0.5
Males	8	0.4
Undetermined	2	0.1
Ambient pH samples	28	
Females	15	0.54
Males	11	0.39
Undetermined	2	0.07

The visual analysis of July 2020 allowed to establish the timing of spawning in both sites. In September 2020, in the Vent site, four male over five in total had not released their gametes while all the females had already spawned. For the Ambient site, all the observed polyps had spawn except for one male. Moreover, even if the histological slides of September 2020 were not used for counting and measuring of the gametes, differences were noticed. While in July all the visible oocytes were previtellogenic or vitellogenic oocytes, in September, the oocytes were undergoing oosorption (Figure 6-Appendix V). The oosorption was identified when the oocyte shape-like mass did not present a nucleus. This phenomenon of oosorption was observed in both Ambient and Vent samples.

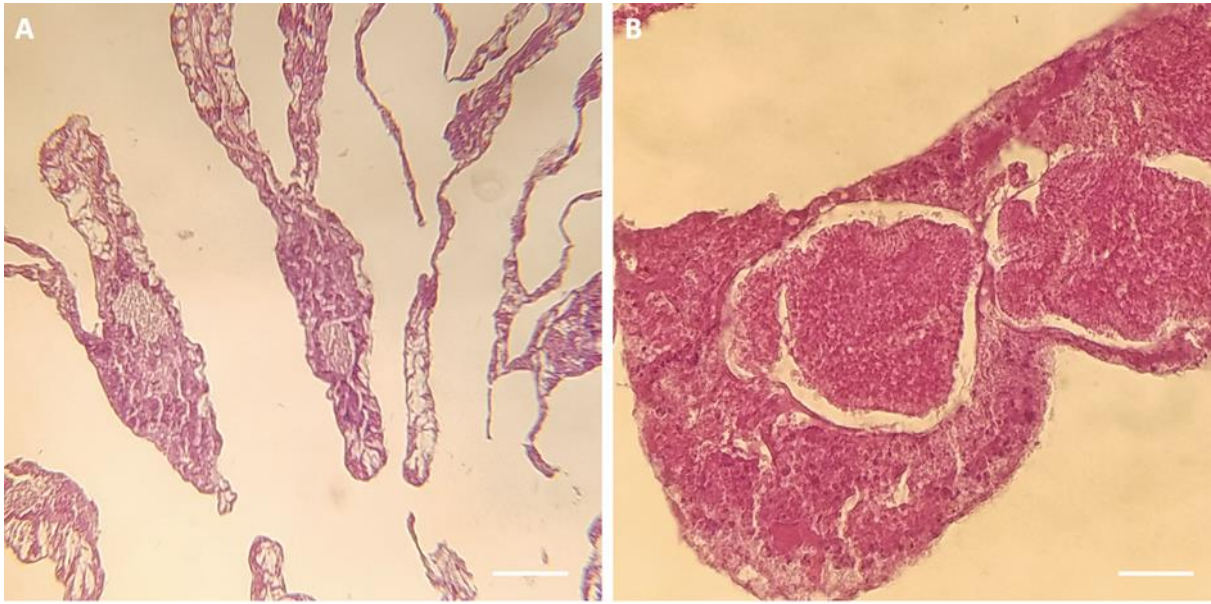


Figure 6-Appendix V. Microscopy photographs of female mesenteries with non-nucleated oocytes. Scale: 100 μm (A) and 25 μm (B). Samples of September 2020.

Number and size of the gametes

The female colonies presented a mean of 1456 ± 1014 gametes per polyps at the Vent site, and 1960 ± 603 at the Ambient pH site. The number of gametes per polyps were statistically similar from a site to another in female colonies ($p = 1$).

The histological analysis of the July 2020 samples showed no significant difference in size between the sites for neither female nor male gametes (Figure 7-Appendix V, $p = 0.497$). For the female polyps, the oocytes' mean size for the Ambient site was $0.316 \pm 0.090 \mu\text{m}$ while in the Vent site the mean size was $0.265 \pm 0.097 \mu\text{m}$. For the spermaries, the mean size in the Ambient site was $0.388 \pm 0.093 \mu\text{m}$ versus $0.358 \pm 0.067 \mu\text{m}$ in the Vent site.

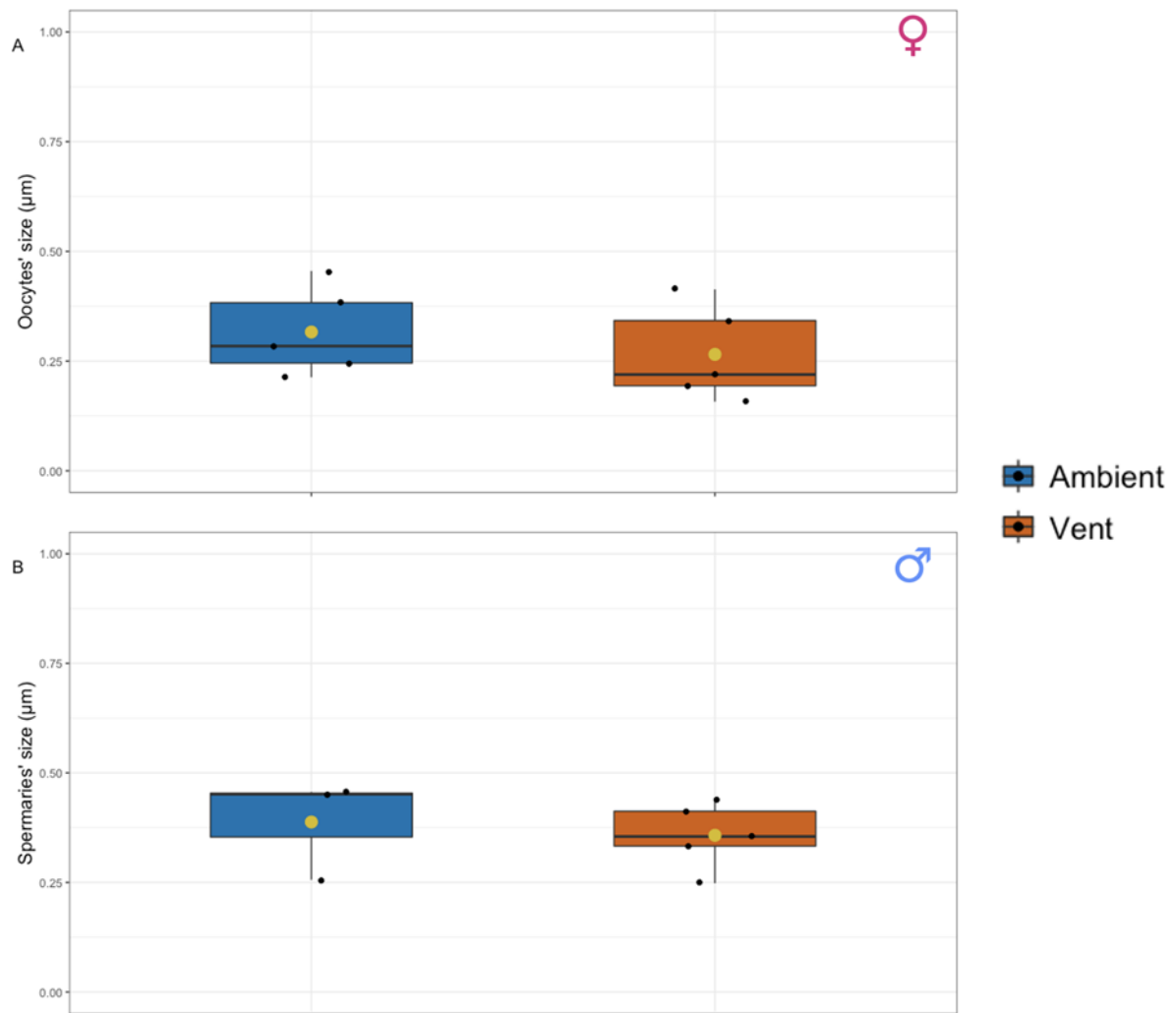


Figure 7-Appendix V. Gametes' size, females and males of *C. caespitosa* collected from the Vent and Ambient sites in July 2020. Panel A shows the sizes of female polyps, and panel B shows the sizes of male polyps. Dots represent the mean size of the female (n = 1577) and male (n = 1360) gametes of the polyp of the colonies per site and sex; boxes represent the median and the 25% and 75% quartiles. n = 5 per sex and study site.

Role of environmental variability on the response of Mediterranean corals to global change

Abstract :

The increase of atmospheric CO₂ is driving changes in the ocean's physical and chemical properties. The projections by the IPCC under the high CO₂ emission scenario RCP 8.5 suggest global sea surface temperature warming of 3.2°C and a decrease in pH of 0.3 units by the end of the century. Such changes have important consequences for ecosystems and the services they provide to humans. The Mediterranean Sea is a hot-spot of global change with warming sea surface temperature projected to be 20% higher than global projections and an increase in extreme events such as marine heatwaves.

The main goal of this thesis is to determine the effects of present and future coastal environmental conditions on Mediterranean corals. Natural populations of corals from two volcanic CO₂ vents in Ischia (Italy) are used. Local acidification of seawater occurs around the vents with pH values close to those expected by the end of the century. This research is focused on two Mediterranean long-lived corals that have key relevance for conservation as habitat-forming species: the zooxanthellate *Cladocora caespitosa* and the azooxanthellate *Astroides calycularis*.

The main research questions are: (1) how present-day and future warming and/or acidification affect the physiology of *Cladocora caespitosa* and *Astroides calycularis* at every life stage (adult, gametogenesis, larvae, and recruits) and (2) does previous exposure to low pH at CO₂ vents enhance their tolerance to future ocean acidification. To answer these questions, we combined environmental data, ecological field surveys at CO₂ vent sites and reference sites with ambient pH, and laboratory experiments with controlled temperature and pH.

This thesis provides new insights into the responses of adult colonies to warming on. I assess the physiological impact and potential recovery to different warming scenarios and heatwaves events, based on nine years of temperature data collected at the study site. I then study whether past exposure to low pH conditions confers tolerance to ocean acidification on the two species, with colonies sampled at the CO₂ vent and ambient pH sites and maintained in the laboratory under present day and low pH conditions.

I also focus on sexual reproduction and early life stages of the two Mediterranean corals to better understand how they respond to ocean warming and acidification. First, I characterize the development and post-settlement growth of larvae of *A. calycularis* under high and ambient temperatures and ambient and low pH. I then take a closer look at tolerance to acidification by exposing larvae from the CO₂ vents and ambient pH site to different pH conditions. I also explore whether differences in gene expression between the populations and pH treatments exist. Finally, I compare gametogenesis of *C. caespitosa*'s colonies from the CO₂ vent and ambient sites through histological analysis to test the impact of low pH on gametogenesis and spawning.

The results suggest that the impacts of warming and acidification on adult colonies of *C. caespitosa* and *A. calycularis* are moderate, with a tolerance to acidification and a recovery after summer heat. However, the impact of global change on the early life stages of *A. calycularis* and on the reproduction of *C. caespitosa* is worrying and could compromise the sustainability of the populations. My results suggest that *C. caespitosa* and *A. calycularis* living at the CO₂ vents sites of Ischia are not acclimatized to ocean acidification. Furthermore, exposure to low pH at the vent sites impairs individual's physiology (reproduction, early life stages development, morphology of the colony) and population growth. Given the susceptibility of both species to global change, the results highlight the risk of changes in the composition of Mediterranean communities in the near future and the urge to establish general conservation measures and long-term monitoring.

Key-words: Global change, ocean acidification, ocean warming, coral, Mediterranean Sea, CO₂ vents

Rôle de la variabilité environnementale sur la réponse des coraux méditerranéens aux changements globaux

Résumé :

L'augmentation du CO₂ atmosphérique conduit à des changements physiques et chimiques de l'océan. Les projections du GIEC selon le scénario à fortes émissions RCP8.5, suggèrent un réchauffement de la surface des océans de 3.2°C et une diminution de 0.3 unités de pH à la fin du siècle. Ces changements environnementaux s'accompagnent d'importantes conséquences sur les écosystèmes et les services rendus aux humains. La mer Méditerranée est un « hotspot » du changement global avec une prévision d'augmentation de la température de surface de la mer de +20% comparé aux projections globales, accompagné d'une augmentation d'événements extrêmes comme les vagues de chaleur marines.

L'objectif principal de cette thèse est de déterminer les effets de la variabilité environnementale présente et future sur des coraux Méditerranéens. Des populations naturelles de coraux vivant au niveau d'événements de CO₂ d'origine volcanique à Ischia (Italie) sont utilisées. Une acidification locale de l'eau de mer survient autour de ces événements, avec des valeurs de pH proches de celles qui sont attendues pour la fin du siècle. Les deux espèces étudiées : *Cladocora caespitosa* (zooxanthellé) et *Astroïdes calycularis* (azooxanthellé), ont une grande importance pour la conservation de par leur statut d'espèces fondatrices.

Les principales questions de l'étude sont : (1) comment les conditions présentes et futures de températures et/ou de pH affectent la physiologie des deux espèces à différents stades de vie (adultes, gamétogenèse, larves et recrues) et (2) est-ce que l'exposition à un pH bas peut augmenter la tolérance des coraux face à une future acidification. Afin de répondre à ces questions, nous avons combiné l'analyse de données environnementales, l'étude sur le terrain et des expériences de laboratoire avec contrôle de température et de pH.

Ma thèse se focalise en premier lieu sur la réponse des colonies adultes face au réchauffement de l'océan, avec l'acquisition des réponses physiologiques et le rétablissement des deux coraux à un réchauffement et à des vagues de chaleur marines simulées en laboratoire à partir de données réelles. Nous avons ensuite étudié, si l'exposition au pH bas des événements de CO₂ procurait une tolérance à l'acidification chez les deux espèces.

Cette thèse s'applique également à identifier l'impact du changement global sur la reproduction sexuelle et les premiers stades de vie des deux espèces de coraux. Dans un premier temps, j'ai étudié le développement et la croissance post fixation de larves d'*A. calycularis* face au réchauffement et à l'acidification. Puis, j'ai testé l'hypothèse selon laquelle des larves provenant des événements de CO₂ présentaient une tolérance à l'acidification. L'expression des gènes entre les populations et les traitements de pH en laboratoire a également été étudiée. Enfin, une comparaison des gamètes de *C. caespitosa* provenant des événements de CO₂ et d'un site ambiant a été effectuée par analyse histologique afin de savoir si le pH a un impact sur la gamétogenèse et le ponté.

Nos résultats suggèrent que les impacts du réchauffement et de l'acidification sur les colonies adultes de *C. caespitosa* et *A. calycularis* sont modérées, avec une tolérance à l'acidification et un rétablissement après les températures estivales. Par contre, l'impact sur les premiers stades de vie d'*A. calycularis* et la reproduction de *C. caespitosa* est inquiétant et pourrait compromettre le maintien des populations. Mes résultats suggèrent que *C. caespitosa* and *A. calycularis* vivants au niveau des événements de CO₂ de Ischia, ne sont pas acclimatés à l'acidification de l'eau de mer. De plus, cette exposition à des pH bas affecterait la physiologie des individus et la croissance de la population. Étant donné la sensibilité des deux espèces de coraux au changement global, ma thèse souligne un risque de changement de composition des communautés méditerranéennes dans un futur proche et une urgence à établir des mesures de conservation et un suivi à long terme.

Mots clés : changement global, acidification de l'océan, réchauffement de l'océan, coraux, mer Méditerranée, événements de CO₂