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Habitat choice and settlement of coral larvae of high latitude coral species under ocean acidification



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## Habitat choice and settlement of coral larvae of high latitude coral species under ocean acidification.

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### Introduction

Tropical coral reef communities are threatened by both biophysical stressors and anthropogenic activities. Overfishing (Jackson *et al.*, 2001), outbreaks of coral-eating predators (De'ath *et al.*, 2012), disease (Hughes 1994), coastal water pollution (McCulloch *et al.* 2003), hurricanes (Hughes, 1994), extreme temperatures and large-scale coral bleaching (Hoegh-Guldberg, 1999) are all causing a decline in the cover of scleractinians at a global scale (Jackson *et al.*, 2014); resulting in shifts in species composition towards macroalgae and turf algae (Bruno *et al.*, 2009; Ruzicka *et al.*, 2013). At the same time, species range shifts are being observed, with corals moving to marginal subtropical/temperate areas in an attempt to follow their thermal envelope (Yamano *et al.*, 2011) and replacing the typically algal-dominated communities. It has been suggested that these higher latitudes could represent a potential refuge for scleractinians.

Ocean acidification (OA) is a process which describes the changes in the ocean's carbonate system resulting from increasing anthropogenic CO<sub>2</sub> emissions, and is expected to play a strong role in structuring communities. Modelling and *in situ* observations suggest that the "tropicalisation" process of high latitudes could be halted by the ongoing declines of aragonite saturation state ( $\Omega_{arag}$ ) (due to ocean acidification), which may prevent the expansion of corals into the higher latitudes (Yara *et al.*, 2012; van Hooidonk *et al.*, 2014). By contrast, the biomass of seaweeds, which are able to exploit the fertilization effect of additional dissolved CO<sub>2</sub> being available, is expected to increase. At present, experimental evidence supporting such projected mechanisms are scarce and further research is needed, especially with focus on sensitive processes regulating early life stages.

Recruitment is a fundamental process for maintaining population replenishment and hence local persistence of a given species, or the poleward colonisation of novel environments to track thermal optima. Documented neurosensory disruptions of organisms under OA make early life stages unable to recognise olfactory, visual and auditory stimuli, decreasing their survival and settlement success. The downstream consequences of such impaired processes are expected to affect the ecological outcomes at community- and ecosystem-level (Nagelkerken & Munday 2016). While it is now well established that many calcifying taxa, including corals, are heavily threatened by OA, particularly at early life stages (Diaz-Pulido *et al.*, 2011; Kroeker *et al.*, 2013; Wittmann & Pörtner, 2013), non-calcified organisms (e.g. seaweeds which are able to exploit the fertilisation effect of CO<sub>2</sub>) and soft corals seem to benefit or at least adapt to OA (Connell *et al.*, 2013; Ruzicka *et al.*, 2013; Inoue *et al.*, 2013).

This likely allows seaweed to substitute calcifying reef species, leading to ecosystem shifts towards non-reef forming algal states over a coral state (Diaz-Pulido et al., 2011), reduced habitat complexity and biodiversity loss (Sunday et al., 2017), with unknown consequences for provisioning of ecosystem goods and services for humanity. Indeed, once seaweeds become dominant, coral recovery will be further suppressed as their larvae are repelled by algal chemical cues (Dixson et al. 2014).

Planulae larvae spend a variable amount of time in the water column before settling. Brooder coral species larvae (that directly release mature planula) are thought to both be able to rapidly settle or to travel long distance spending several days to weeks in the water column (Harriott, 1992). This provides an advantage for the settlement in isolated coral communities such as the high latitude coral communities of this study and the ability to survive long journey would especially facilitate the poleward shift of coral communities, allowing corals to find refuge in the cool higher latitudes. However this advantage will be lost if OA reduce the survival rates of brooded planulae. To our knowledge, no studies tested the effect of OA on brooded coral larvae and very few tested the effects of OA on the physiology of coral larvae. Nakamura et al. (2011) study on the larvae of *Acropora digitifera* (a broadcast spawner) suggests a minor depression of its oxygen consumption which was hypothesized to be a survival strategy against the stressful conditions that ocean acidification represents. This same study also showed an impairment of the metamorphosis process that could be attributed to energy depletion or abnormalities that could be related by different gene expression under OA. These early studies highlight the needs to assess the potential effects of OA on brooded planulae and to understand the physiological mechanisms involved.

Early life stages of corals, such as post settlement juvenile corals, are generally thought to be more susceptible to environmental change (Albright, 2011) and due to their unbalanced control of the calcification required for somatic growth (Higuchi *et al.*, 2014) could be highly sensitive to OA as it increases the energetic costs of calcification. However, the **effect of OA on juveniles** has only been tested in the laboratory where in addition to intrinsic bias (i.e.: tank), parameters such as overgrowth by algae cannot be tested. Research attempts to overcome this issue might be tackled using natural volcanic CO<sub>2</sub> seeps, which are considered a powerful natural analogue of future ocean conditions, and the most ecologically realistic tool for predicting responses of marine species to OA in their natural habitat.

In this project we used well-established CO<sub>2</sub> seep sites in Japan and Italy, to identify the direct and indirect effect of OA on coral recruitment at high latitude regions, which are considered thermal refugia for shifting coral species, and assess their competition with algal species, which are in turn boosted by anthropogenic CO<sub>2</sub> enrichment of the ocean.

## Materials and Methods

### Rationale of the project and hypotheses to be tested

Ocean acidification causes a reduction in crustose coralline algae (CCA) abundance and an increase in turf algae. Because the first provides positive chemical cues for coral larvae settlement while the second produce negative cues, we can expect a general decrease of positive cues under OA. Here we tested in the laboratory the effect of different cues (positive or negative) on coral larvae choice under OA in two distinct regions, the Mediterranean Sea and the Pacific Ocean. We predicted that coral larvae will select cues from control pH sites compared to those coming from low pH sites. Experiments were carried out using dual-choice chambers and a specific test was also constructed to verify the efficiency of the chamber to prevent external source of variations. In addition, coral larvae settlement was tested under natural and low pH conditions by placing larvae in the presence of recruiting tiles conditioned for one month at control and low pH sites. We predicted higher settlement rates for tiles conditioned at control sites, independent of the aquariums pH conditions. Finally, changes in the energetic demand and other physiological processes under OA could affect the survival of planula in the water column. We measured the respiration rates of planula under different conditions of pH and the presence of cues, and are analysing the gene expression of the planulae under ambient and elevated  $p\text{CO}_2$  to assess the possible direct effects of OA on the physiology of the planulae.

### Coral species

Two scleractinian species were used for this study: *Astroides calycularis* for the Italian side of the study and *Alveopora japonica* for the Japanese side.

#### *Astroides calycularis*

The orange coral, *Astroides calycularis* (Pallas 1766), is an azooxanthellate dendrophylliid coral endemic to the Mediterranean Sea. The species mostly occurs in the south-western part of the basin from the Gibraltar Strait to Sicily and in the south-western coast of the Italian peninsula (Terrón-Sigler et al., 2016a; Musco et al. 2016) where it is abundant from the surface to 15 m depth, but also observed down to 50 m. In shallow rocky areas it may form a belt (also referred to as “facies”) covering up to 90% of the colonised surface (Goffredo et al., 2011a). In the Adriatic Sea the species is present with sparse colonies along the Croatian coast only (Kružić et al., 2002; Grubelić et al., 2004), whilst sporadic records along the Italian side lack certified confirmation. The northernmost record along the Italian coast regards a dead colony found at Giglio Island (Bianchi and Morri, 1994). The northern limit of stable reef forming populations is at Ventotene (Pontine Islands), while the southern limit is in the Pelagie Islands (Musco et al. 2016).

*Astroides calycularis* is a thermophilic stenotherm form, consequently climate change may potentially have a positive effect to the species as the warming of the marine superficial waters could extend its distribution northward (Bianchi, 2007). However, the effects of temperature increase at the southern limits of their southern distribution should be carefully analysed. In fact, Prada et al. (2017) recently observed that *A. calycularis* is particularly sensitive to high summer temperatures, showing an increase in tissue mortality that in turn made the corals more susceptible to ocean acidification by affecting net calcification. Moreover, given its prevalent shallow distribution, this species is particularly subject to the increasing power of extreme sea storms expected under the climate change scenario in the Mediterranean Sea (Nissen et al., 2014).

### *Alveopora japonica*

*Alveopora japonica* is a hermatypic coral endemic to the coasts of Japan and Korea (Nishihira and Veron, 1995). It is a dominant species in the warm temperate areas of Japan and can be found up to Tateyama, Chiba, Japan (35°N) occupying spaces in the shallows (less than 10m) where other corals are typically seldom found. It can at times form dense carpets among kelp beds (Denis et al., 2013). This coral of the family Poritidae, forms small colonies (15 cm diameter) with long expanded polyps. It is a hermaphroditic brooding species, releasing planula containing zooxanthellae. The exact brooding season is unclear with reports of planula release having been shown from September to December in the Izu peninsula as well as in other temperate areas of Japan (Harii et al., 2001).

Living in high latitudes, this species is exposed to low temperature during winter (< 15 °C) which would typically be lethal to most hermatypic corals. While bleaching of *Alveopora japonica* associated to these low temperatures is observed, it can tolerate several months bleached and then recover during the warmer months (Higuchi et al., 2018). This high resilience to cold stress may be attributed to its capacity to feed heterotrophically (Jeong et al., 2012), or its peculiar association with zooxanthellae of the clade C and F (Chang et al., 2011; Rodriguez-Lanetty et al., 2000). Nevertheless, the bleaching of *A. japonica* due to cold stress leads to an absence of growth, and subsequently it could be expected that this species could increase in the future due to rising temperatures. However, the effects of ocean acidification on this species remain to be studied, particularly with regards to its effects on the early life stages, which are generally considered to be more sensitive.

### **Sampling sites**

Samples of different cues, water and coral larvae were collected from control and CO<sub>2</sub> coastal seep sites in two different regions: Vulcano Island (NE Sicily, Italy) and the Gulf of Castellammare (NW Sicily, Italy) in the Mediterranean Sea, and the Shikine Island (Izu archipelago, E-Japan) in the north-western Pacific ocean.

### *Mediterranean sites*

The collection of benthic samples for the cue experiment was carried out by two skin divers on May 2018 at two shallow sites (1-3 m depth) along a well-established pH/pCO<sub>2</sub> gradient in Levante Bay off Vulcano Island (38°22'53.27"N; 14°56'25.07"E; NE Sicily; Italy) (Boatta et al., 2013). The southern part of the bay is characterized by active shallow water CO<sub>2</sub> seeps generating a pH/pCO<sub>2</sub> gradient at depths ranging from 1 to 6 meters along its north-eastern rocky coast (Boatta et al., 2013) (Fig. 1). Hand collections of benthic samples were performed on rocky bottoms at distances of 300 meters (the high-CO<sub>2</sub> site with on average 7.8 pH units) and at 600 meters (ambient-CO<sub>2</sub> conditions at 8.1 pH units) from the main venting zone. *Astroides calycularis* samples and larvae were instead collected from the Gulf of Castellammare (38° 05' 01.75" N; 12° 48' 33.04" E; Western Sicily). Colonies were collected by detaching them from the vertical cliff where *A. calycularis* is abundant, while free swimming larvae were collected using a hand net.

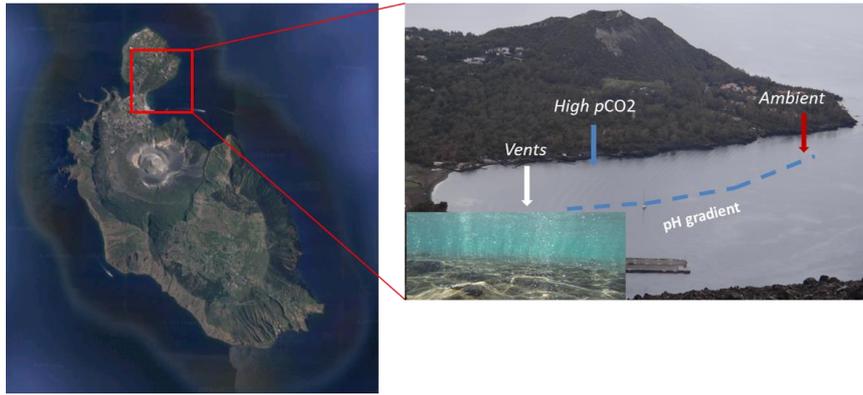


Figure 1. Satellite (left) and aerial (right) images of Levante Bay in Vulcano Island. The white arrow points to the main seep, from which the pH gradient originates moving outside the bay. The two sampling sites are marked by the blue and red arrows.

### *Pacific ocean sites*

All samples were collected at Shikine Island, a volcanic island east of the Izu peninsula in Japan ( $34^{\circ} 19' 9''$  N,  $139^{\circ} 12' 18''$  E) within  $\text{CO}_2$  seep sites in its shallow waters. Benthic samples of the different cues were collected in Sept 2018 at a high- $\text{CO}_2$  and ambient- $\text{CO}_2$  site located around Mikama Bay (Fig. 2). Both the geo- and the carbonate chemistry of these locations were extensively characterised by Agostini *et al.* (2015). The island coast is under the influence of the Kuroshio warm current, which promotes the presence of many different tropical and sub-tropical scleractinians in shallow waters. However, these are absent in the high- $\text{CO}_2$  site (Agostini *et al.*, 2018). Samples of macroalgae, turfs, and CCA were collected by scuba diving at depths ranging from 3 to 8 meters. *Alveopora japonica* colonies were collected from Hirizo-minamizu ( $34^{\circ} 36' 27''$  N,  $138^{\circ} 49' 29''$  E; Southern Izu Peninsula). *A. japonica* larvae were obtained from spawning colonies reared in tanks at the Shimoda Marine Research Center.



Figure 2. Satellite and aerial images of Mikama Bay in Shikine Island (Japan) where different seep areas originate pH/ $\text{pCO}_2$  gradients. The two collection sites are pointed out by the blue and red arrows

## Maintenance of the corals, planulae, and benthic samples

Corals in Italy were maintained in aquariums in a temperature-controlled room. The aquariums were filled with water freshly collected from the Gulf of Castellammare and the water was changed every day. Planula collected were separated in two aquariums, one was kept at ambient  $p\text{CO}_2$  and the other was enriched in  $\text{CO}_2$  by bubbling of pure  $\text{CO}_2$ . The  $p\text{CO}_2$  of the seawater was regulated with a pH-stat system (Aqua geek, Fukurow, Kawaguchi, Japan) controlling a solenoid valve on the  $\text{CO}_2$  gas line. The benthic samples providing the cues (outlined above) were kept in aquariums with the  $p\text{CO}_2$  maintained at the same level as the location they were sampled from using a similar system. In Japan, the corals were maintained in an outdoor aquarium with constant running seawater. The collected planulae were moved into two indoor aquariums with either an ambient  $\text{CO}_2$  or elevated  $\text{CO}_2$  level controlled with the same system as used in Italy. The benthic samples providing the cues were kept in separate indoor aquariums with running seawater and either ambient or elevated  $\text{CO}_2$  levels (as above), and artificial light on a 12:12 h cycle both in Italy and Japan. The temperature of the indoor aquariums was maintained at 20 °C by placing the aquariums in temperature-controlled water baths.

## Habitat choice experiments

### *Selection of the cues*

Coralline crustose algae (**CCA**), macroalgae (**Alg**), adult corals (*Astroides calycularis*, **AC**, for the Mediterranean and *Alveopora japonica*, **AJ**, for the Pacific) and control water (**Cwa**) were collected from **control sites** (i.e. pH ~8.0) while algal turf (**Tur**), macroalgae (**Alg\_Low**) and low pH water (**Lwa**) were collected from **low pH sites** (i.e. pH~7.8) in both regions to perform the choice experiments.

### *Choice experiments*

Three experiments were planned: 1) community choice, where larvae had to select between a group of species collected from the control sites and a group of species collected from low pH sites; 2) cue choice, where larvae had to select between different cues produced either by organism or water collected at control and low pH sites; 3) lateralization, where the two-choice system was tested to prevent any error deriving from the selection of one of the two sides of the chamber independently of the presence of chemical cues. Experiment 1-3 were performed in Japan while in Italy only experiment 2 was carried out. Experiments were performed in Italy in June and July and in Japan in September and October 2018.

### *Setting of the experimental two-choice chambers*

The two-choice chambers used in this study were made of a 50 cm long PVC pipe (50 mm in diameter) cut in half longitudinally to form a flume-like structure (Fig. 3a, b). At the two ends of the chambers, which were sealed, a 0.5 cm plastic hose was linked via a connector to a peristaltic pump able to provide a constant flux of water (Fig. 3a, b). A pre-survey made using colored water suggested 4 ml/min as a suitable flux to generate and keep constant a cues' gradient (left vs right) in the chambers (Fig. 4). The water overflow was guaranteed by cutting a little U-shaped crevice exactly at the top central portion of the chamber walls (Fig. 3a, b). In each experiment the larva/e was/were put exactly in the central part of the chamber while cues were located at each of the two ends of the chamber inside a mesh bag for the experiment performed in Japan or inside tanks from where the conditioned water was pumped inside the chamber for the experiment performed in Italy. For the purposes of the experiment it was considered as a choice when a larva moved

towards one of the two cues. The first 2.5 cm to the right and to the left of the chamber center were considered as a no choice and were not included in the analysis (Fig. 3a, b).

A set of six (for the experiment performed in Japan) or ten (for the experiment performed in Italy) two-choice chambers were used to assess how ocean acidification affects the habitat choice of the two selected species of scleractinian coral larvae. Larvae were exposed to different chemical cues represented by organisms and sea water collected at both control sites, where the two species usually live, and CO<sub>2</sub> coastal seep sites, in Japan and Italy.

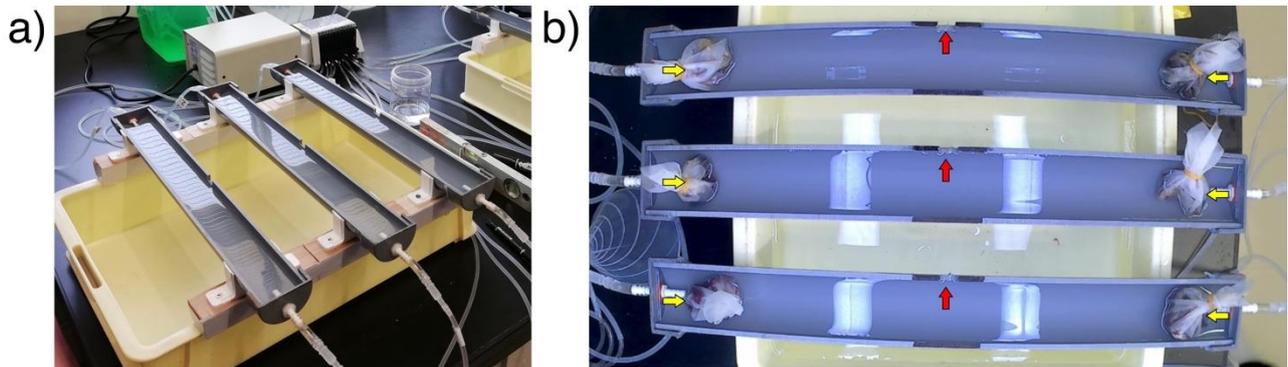


Figure 3 – a) The two-choice chambers used in this study were PVC pipe cut in half longitudinally to form a flume-like structure. The two ends of the chambers were sealed and a plastic hose was linked via a connector to a peristaltic pump. b) Details of two symmetric little u-shaped cuttings made at the top central portion of the chamber wall to allow water overflow (indicated by red arrows) and of the mesh bag used to contain the cues (indicated by yellow arrows).

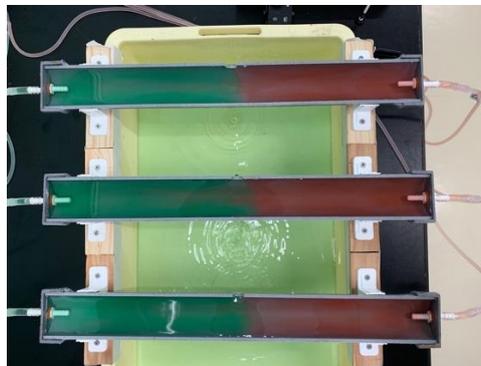


Figure 4 – Details of the pre-survey made using coloured water to allow fixing a flux to generate and keep constant a cues' gradient (left vs right) in the chambers.

### *Cue choice (in Italy)*

Ten dual-choice chambers were used for each run and 5 replicates were available for each of the 10 combinations from the 5 selected cues: CCA, Alg, AJ, Tur, Alg\_low. A volume of approximately 2 litres for the algal component and 1 litre for the coral was store in separate 40 L tanks supplied with air, and left for 48h under a photoperiod of 12:12 and at 19 °C. Turf and Alg low were kept under low pH conditions and the other cues at control pH. Four larvae were used each time, being placed at the center of each chamber at the start of each run. Water conditioned with the chosen cue was pumped in at the two ends of the chamber. Larvae were left for four minutes inside the pipe to acclimatise to conditions before the observation started.

After 20 minutes, larvae positions were recorded and the percentage of larvae in each side of the chamber was used to determine their choice. ~~In this case, the experiment was run using control water for all the cues.~~ Statistical analyses were performed using 10 different  $\chi^2$  tests, one for each cue combination.

#### *Community choice (in Japan)*

Larvae choice between control and low pH communities, i.e. organisms from control (CCA, Alg and AJ) and low pH sites (Tur and Alg\_Low), was evaluated by adding small samples of all of the "control cues" and all of the "low pH cues" available respectively into small mesh bags. Small tiles were added into the low pH community bags to simulate a solid substrate, represented in the ambient community by CCA and *A. japonica*.

Two experiments were performed to test for the larvae choice in presence of different communities. The first was run using two sources of water (control and low pH) running at the same time at the two ends of the chamber. In this experiment there were two fixed factors: 'Community', with two levels, control and low pH, and 'Side', with two levels, left and right. Each level of factor community was submitted to a water flux coming from the same site (i.e. low pH community was under the flux of low pH water and control community under that of control pH water).

The second experiment was run using two independent sources of water (control and low pH) at time. In this case there were 3 factors: 'pH', with two levels, control and low; 'Community', with two levels, control and low pH, and 'Side', with two levels, left and right. The number of replicates was seven. Data were analysed using 2-way and 3-way ANOVAs for the first and second experiment, respectively. Data were tested for homogeneity of variance and transformed when needed ( $\alpha = 0.05$ ). Post hoc comparisons were made using SNK tests.

#### *Cue choice (in Japan)*

Six dual-choice chambers were used two run the choice experiment under two pH conditions (control and low pH). Six cues were used for this experiment: CCA, Alg, AJ, Tur, Alg\_Low and water, either Cwa or Lwa according to the pH water flowing. Hence, the resulting 15 cue combinations were tested at control pH and low pH.

The planulae were given 2 minutes to acclimatize and 3 minutes to make their choice. The time that the larvae spent in the different portions of the chamber was recorded in seconds and it was used as response variable. Six replicates were made per treatment.

At the beginning of each experiment, one of the 15 combinations was randomly selected and each of the two cues involved was put at one of the two ends of the two-choice chamber under a constant water flow of either Cwa or Lwa. The selected cues were placed inside a mesh bag and placed systematically at one of the two ends of each chamber, making sure to alternate the position with respect to the side of each chamber to prevent the same cue from always being placed on the same side of the chamber. In the case where the cue was seawater (i.e. as a control blank), an empty mesh bag was included. This arrangement also allowed us to test for a choice of the chamber side.

For the statistical analyses, a total of fifteen 3-way ANOVAs were performed, one for each cue combination with the following factors: 'Cue', fixed with two levels (Cue A vs Cue B), 'Side', fixed with two levels, (Left and Right) and 'pH', fixed with two levels (Control and low pH). Data were tested for homogeneity of variance and transformed when needed ( $\alpha = 0.05$ ). *Post-hoc* comparisons were made using SNK tests.

### *Lateralisation (in Japan)*

Filtered seawater vs filtered seawater were tested to verify any preference of larvae for the right or left sides of the chambers. The experiment was performed using both control and low pH water. Data included two fixed factors: 'Side', with two levels, left and right, and 'pH', with two levels, control and low pH. Data were analysed by means of a 2-way ANOVA. There were seven replicates per each combination of the two factors. Data were tested for homogeneity of variance and transformed when needed ( $\alpha = 0.05$ ). *Post-hoc* comparisons were made using SNK tests.

### **Settlement**

The biofilm development on artificial substrates is a prerequisite for coral larvae settlement (Albright, 2011). Small marble tiles (3cm x 1cm x 1cm) were conditioned for one month under either control or low pH conditions to allow biofilm development, and to investigate for differences in the larval settlement rates of the respective corals under early stage biofilm conditions at the two pH levels. For Italy these tiles were conditioned in Vulcano island (Fig. 1) and for Japan, these tiles were conditioned in Shikine Island (Fig. 2). In both cases, three tiles from control and three from low pH sites were placed in an interspersed fashion into six-well plates (Fig. 5a). For Italy, three larvae of *A. calycularis* were placed in each well totalling 18 larvae per plate, with 14 plates used (sealed by parafilm). For Japan, five larvae of *A. japonica* were placed in each well totalling 30 larvae per plate, with 16 plates used (also sealed by parafilm). In both Italy and Japan, half of the wells were placed in a tank at control pH (~8.2) and the other half placed in a tank at low pH (~7.7). Larvae settlement (e.g. Fig. 5b) was checked every 2 days for 2 weeks in Italy, and every 2 weeks in Japan.

A survival analysis was used to analyse data from the larval settlement experiment. Here, larvae settled were no longer considered as part of the experiment (i.e. the distribution of remaining larvae followed a Kaplan–Meier survivor function) and were considered as "death" data. The survival analysis was performed using censored data, because not all of the available larvae resulted settled by the end of the experiment. The analysis was performed by means of the Survival R package (Therneau, 2012). The parametric Cox mixed-effects model function was fitted to data in order to estimate the total number of days a larva needed to settle by means of a GLMM (Generalized Linear Mixed Model) using an exponential error distribution. In this model the random variable 'well' was added to account for any uncontrolled variability inside each well.

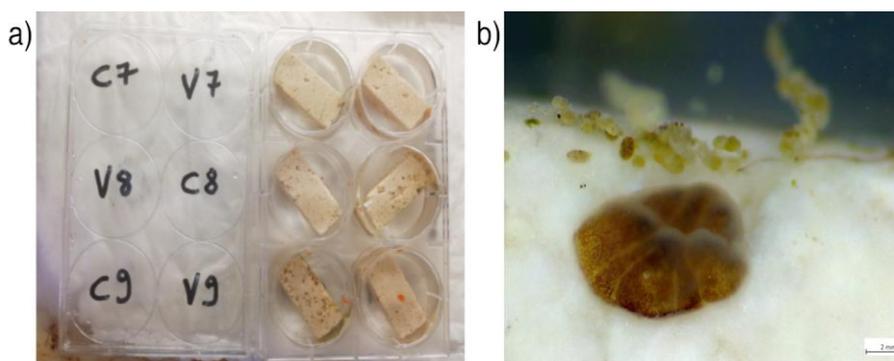


Figure 5. a) Details of the arrangement of the conditioned tiles in the six-wells plate tiles into six-wells plates. C = tiles conditioned at control pH and V = tiles conditioned at low pH. b) example of an *A. japonica* juvenile settled on the marble tile (after 9 weeks).

### Microrespiration

Planula respiration rates were measured using a microrespirometer equipped with clark type dissolved oxygen microsensors (Unisense GmbH, Denmark; Figure 6). The microsensors were calibrated against a zero oxygen solution and a 100 % oxygen solution as indicated by the manufacturer. The systems consisted in four 1 ml chamber in which the microsensor can be inserted. The whole system was immersed in a temperature bath to maintain the temperature at the same levels as the aquariums where the planula came from. The chamber contents were gently stirred using magnetic stirrers to avoid the build-up of an oxygen gradient but without hitting too vigorously the planulae.

Seawater at either ambient or elevated  $p\text{CO}_2$  was filtered on  $0.45\ \mu\text{m}$ . Chambers were filled and placed in the water bath for at least 5 min to temperature to equilibrate, a blank measurement (5 to 10 min) was then taken to check for contaminations or sensor faults. Planula were then inserted in the chambers and a first measurement was done for 10 min in each chamber and then repeated. This allow for 3 respiration measurements: two 10 min and one between both 10 minutes measurements. The average of this measurements was then used.

Titration of the respiration of the planula was performed to determine the most suitable number of planula. For *A. calycularis*, 2,4,6 and 8 planulas were placed and for *A. japonica* 2,4,8 and 12 planulas were placed. In both cases a linear relationship was found ( $R^2 > 0.94$ ) and the optimal number of planula was determined to be 3 for *A. calycularis* and 6 for *A. japonica* as they allowed for a sufficient decrease in oxygen over a 10 min incubation (around  $4\ \mu\text{mol l}^{-1}$  representing 2 % of initial value, which was around saturation).

Respiration rates of the planula were tested under the two levels of  $\text{CO}_2$  and under the presence of cues. In the case of *A. calycularis*, the effect of turf and crustose coralline algae originated cue was tested under both  $\text{CO}_2$  levels and for *A. japonica* only the presence of crustose coralline algae was tested under ambient level. In both case, a fragment of the algae was placed in a 50 ml falcon tube and shaken vigorously. The water containing the cue was then filtered on  $0.45\ \mu\text{m}$  just before the respiration measurement. New cue water was prepared for each sets of four measurements.



Figure 6. Setup used for the planula respirometry. The stirrers and chambers were immersed in a water bath to avoid temperature fluctuations during measurements.

### Planula motility and taxis

Five to 10 Planula larvae were transferred to high or low pH seawater in a 5 cm-diameter plastic petri dish and left overnight for acclimatization to each condition. The swimming movement of

planula larva was recorded under a stereomicroscope equipped with HAS-220 high-speed camera (DITECT Inc.). Ciliary movements were recorded under a phase-contrast microscope (Leica) with a 10x objective equipped with HAS-220 high-speed camera. For analysis of the tactic behaviour of planula larvae, a piece of CCA was put in the center of the petri dish. The swimming pathway of planula larva was recorded by a digital camera (Canon). All the images obtained above were processed for analysis of swimming velocity, ciliary beat frequency and swimming trajectories by a software Bohboh (Jin et al., 2013; Mizuno et al., 2017).

### **Transcriptomics**

To investigate the effects of Lower pH on coral larvae, we are planning to do RNA-sequencing using larvae incubated in normal and low pH (pH 7.8) conditions.

*Asteroides calycularis* and *Alveopora japonica* planula larvae were incubated in filtered seawater at control and low pH for 1 day in small containers. Then, samples were fixed in RNA-later (Therrmofisher Scientific). Total RNA was extracted by Trizol reagent (Therrmofisher Scientific), and Poly(A) RNA isolation with Dynabeads mRNA purification kit (Therrmofisher Scientific). The sequencing library was generated from poly (A) mRNA using NEBNext Ultra II Directional RNA Library prep kit for Illumina (NEB).

The incubation experiment of *A. calycularis* were conducted in Castellammare, Italy, and those of *A. japonica* and molecular experiments were conducted in Japan. For the experiment of *A. calycularis*, one experimental replicate of 10 larvae was prepared, and three experimental replicates of 30 larvae for *A. japonica* were prepared.



## Preliminary results

### Habitat choice experiments

#### *Cues choice (in Italy)*

Larvae choice varied greatly, ranging from 16.7% to 83.3% respectively for cues AC and Alg in the ACvsAlg combination. The  $\chi^2$  test results showed any statistical significant preference for the 10 different combinations but for the algae collected at control sites (Alg) in the ACvsAlg combination (Tab.1 and Fig. 7).

Main comparison	$\chi$ -square	df	p-value
ACvsCCA	2.00	1	0.1573
ACvsTur	3.2	1	0.073
ACvsAlg	8	1	<b>0.004</b>
ACvsAlg_Low	0.043	1	0.834
Alg_LowvsTur	0	1	1
Alg_LowvsCCA	0	1	1
Alg_LowvsAlg	0.142	1	0.7055
AlgvsTur	1.4706	1	0.225
AlgvsCCA	0.058	1	0.808
TurvsCCA	0.5294	1	0.466

Table 1. Results of the  $\chi^2$  tests performed on the 10 available cue combinations.

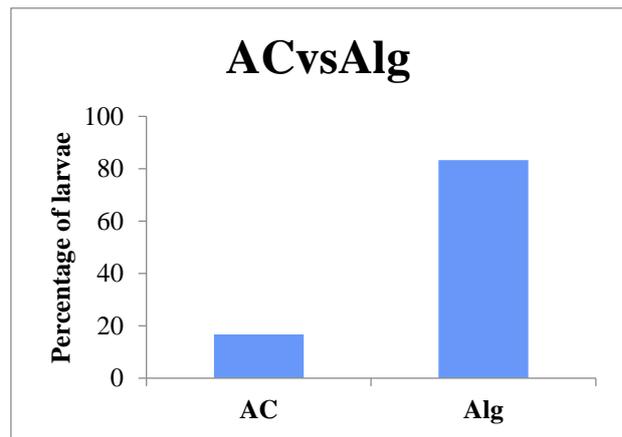


Figure 7. Details of the percentage of larvae selecting cue AC or Alg in the AC\_Al combination

#### *Community choice (in Japan)*

The ANOVAs performed on the community choice showed similar results for the two experiments, “2 pH at time” and “1 pH at time”, with only factor Community showing significant differences ( $P < 0.01$ ; Tab. 2).

In the “2 pH at time” experiment, when two sources of water, control and low pH, were running at the same time, larvae spent a significantly ( $P < 0.01$ ) greater amount of time ( $93.57 \pm 31.79$  sec SE) in the control community (CCA, Alg, AJ) than in the low pH community (Alg\_low, Tur) ( $34.29 \pm 26.26$  sec SE) (Fig 8.). In the “1 pH at time” experiment, when two sources of water, control and low pH, were running independently of each other, larvae spent a significantly ( $P < 0.05$ ) greater amount of time ( $100.61 \pm 32.91$  sec) in the control community than in the low pH community ( $38.18 \pm 25.34$  sec SE) (Fig. 9).

Source of variation	df	MS	F	P
<b>1 pH AT TIME</b>				
pH	1	4791.5	0.74	0.3951
Community (Co)	1	54562.57	8.39	<b>0.0057</b>
Side (Si)	1	2064.28	0.32	0.5759
pHXCo	1	1481.14	0.23	0.6354
pHXSi	1	103.143	0.02	0.9003
CoXSi	1	3363.5	0.52	0.4756
pHXCoXSi	1	2088.64	0.32	0.5736
Error	48	6505.89		
<b>2 pH AT TIME</b>				
Co	1	24603.57	4.46	<b>0.0452</b>
Si	1	1316.57	0.24	0.6295
CoXSi	1	21065.14	3.82	0.0624
Error	24	5513.36		

Table 2. Results of the two ANOVAs performed for the community experiment

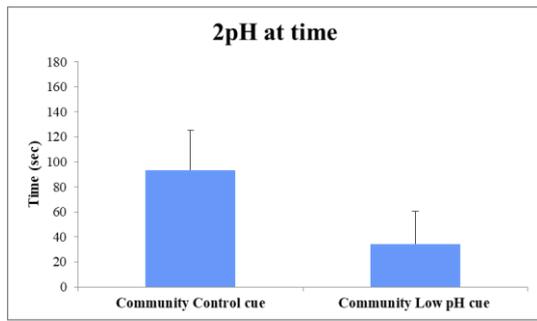


Figure 8. Average time (seconds) spent by larvae at both control and low pH community in the community choice experiment when two sources of water, control and low pH, were running at the same time. Error bars are means +SE .

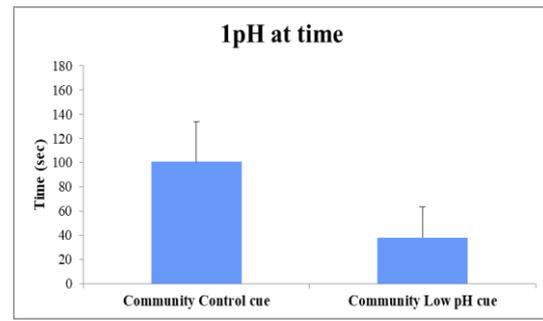


Figure 9. Average time (seconds) spent by larvae at both control and low pH community in the community choice experiment when two sources of water, control and low pH, were independently of each other. Error bars are means + SE.

### Cues choice (in Japan)

The ANOVAs revealed significant differences involving the factor cue only for five out of the 15 combinations. Such combinations always involved the level AJ of factor cue, that is the coral *A. japonica* (Tab. 3).

Source of variation	pH	Cues(Cu)	Side(Si)	pHXCu	pHXSi	CuXSi	pHXCuXSi
Factor level	Control, Low	AJ, Alg, Alg_low, CCA, pH, Turf	Left, right				
Combinations							
Alg_Low vs Alg	ns	ns	ns	ns	ns	ns	ns
pH vs Alg	ns	ns	ns	ns	ns	ns	ns
pH vs Alg_Low	*	ns	ns	ns	ns	ns	ns
pH vs CCA	*	ns	ns	ns	ns	ns	ns
pH vs Turf	ns	ns	ns	ns	ns	ns	ns
CCA vs Alg	ns	ns	ns	ns	ns	ns	ns
CCA vs Alg_Low	*	ns	ns	ns	ns	ns	ns
CCA vs Turf	ns	ns	ns	ns	ns	ns	ns
AJ vs Alg	*	*	ns	ns	ns	ns	*
AJ vs Alg_Low	ns	***	ns	ns	ns	ns	ns
AJ vs pH	**	*	**	***	ns	ns	ns
AJ vs CCA	*	***	ns	**	ns	ns	ns
AJ vs Turf	ns	*	ns	ns	ns	ns	ns
Turfvs Alg	ns	ns	ns	ns	ns	ns	ns
Turf vs Alg_Low	ns	ns	ns	ns	ns	ns	ns

Table 3. Synthesis of the results of the 15 ANOVAs performed for the cue choice experiments

### A. japonica vs Algae

The pHxCuXSi interaction was significant ( $F_{1,40}=5.84$ ;  $P<0.05$ ) for the AJvsAlg combination (Fig. 10). Larvae spent all the available time in cue AJ ( $180\text{sec} \pm 0.00 \text{ SE}$ ) when at the low pH condition and in the right side (SNK tests).

The AJvsAlg combination, showed significant differences ( $P < 0.05$ ) in the interaction between the three factors pHxCuXSi (Fig. 10). At low pH conditions and in the right side, the larvae spent all the available time to cue AJ ( $180\text{sec} \pm 0.00 \text{ SE}$ ).

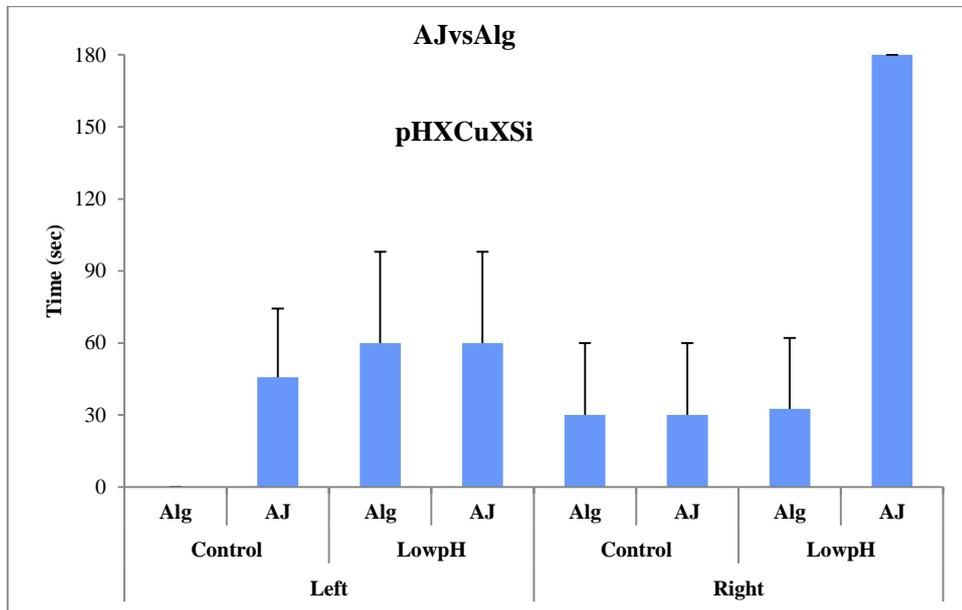


Figure 10. Average time (seconds) spent by larvae at different pH, side and cue (AJ and Alg) in the cue-choice experiment. Error bars are means + SE.

### A.japonica vs Algae low pH

The AJvsAlg\_Low combination, showed significant differences ( $F_{1,40}=19.08$ ;  $P < 0.001$ ) for the factor Cues(Cu). The larvae spent overall  $123.79\text{sec} (\pm 33.93 \text{ SE})$  on the AJ cue and  $29.08\text{sec} (\pm 25.46 \text{ SE})$  in the Alg\_Low cue (Fig.11).

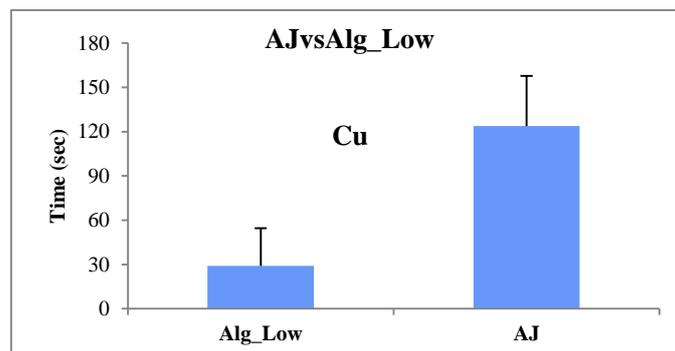


Figure 11. Average time (seconds) spent by larvae at cue AJ and Alg in the AJvsAlg\_Low combination of the cue-choice experiment. Error bars are means + SE.

## A.japonica vs Seawater

Significant differences ( $F_{1,40}=17.00$ ;  $P<0.001$ ) were found for the pHxCu interaction (Fig. 12a). At low pH conditions larvae spent significantly more time ( $162.08 \text{ sec} \pm 21.24 \text{ SE}$ ) on AJ than on Cwa ( $31.25 \pm 28.42 \text{ sec SE}$ ).

For this combination there was also a significant difference ( $F_{1,40}=5.02$ ;  $P<0.05$ ) for factor Side and larvae spent a greater amount of time on the right ( $89.08 \text{ sec} \pm 35.24 \text{ SE}$ ) than on the left side ( $48 \pm 3 2.26 \text{ sec}$ ) (Fig.12b).

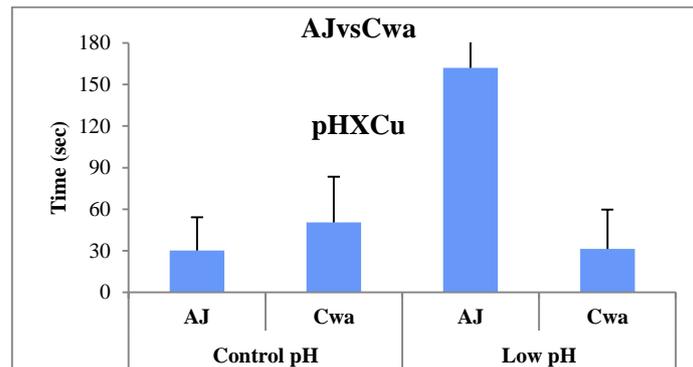


Figure 12a. Average time (seconds) spent by larvae at cue AJ and Cwa in the AJvsCwa combination of the cue-choice experiment. Error bars are means + SE.

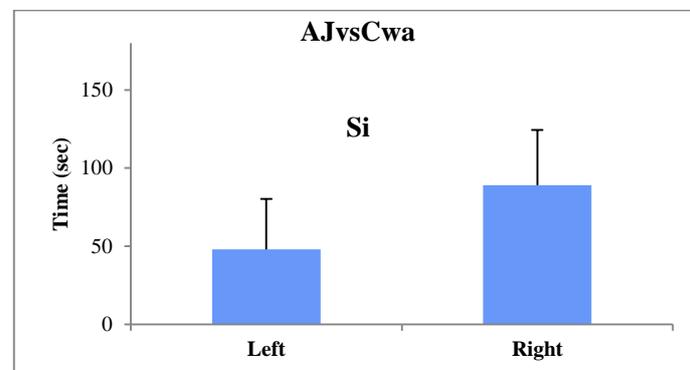


Figure 12b. Average time (seconds) spent by larvae at each side of the dual-cue chamber in the AJvsCwa combination of the cue-choice experiment. Error bars are means + SE.

### A. japonica vs CCA

Significant differences ( $F_{1,40}=8.57$ ;  $P<0.01$ ) were found for the pHxCu interaction (Fig. 13). At low pH conditions larvae spent significantly more time ( $161.08 \text{ sec} \pm 21.23 \text{ SE}$ ) on AJ than on CCA ( $37.92 \pm 28.53 \text{ sec SE}$ ).

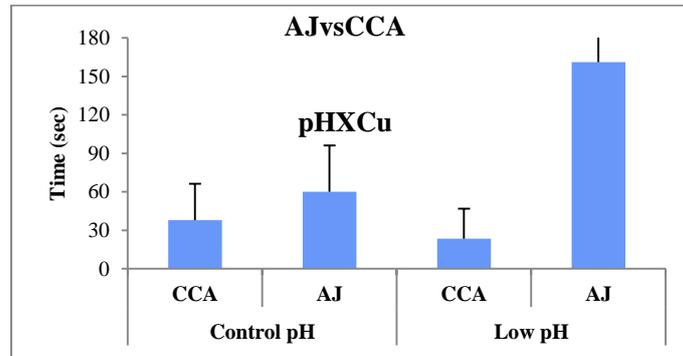


Figure 13. Average time (seconds) spent by larvae at cue AJ and CCA in the AJvsCCA combination of the cue-choice experiment. Error bars are means + 1SE.

### A. japonica vs Turf

The AJvsTur combination, showed a significant difference ( $F_{1,40}=6.26$  ;  $P<0.05$ ) in the Cues(Cu) factor. Larvae spent on average  $89.46 \text{ sec} (\pm 35.64 \text{ SE})$  on AJ and  $33.92 \text{ sec} (\pm 28.04 \text{ SE})$  on Tur (Fig.14).

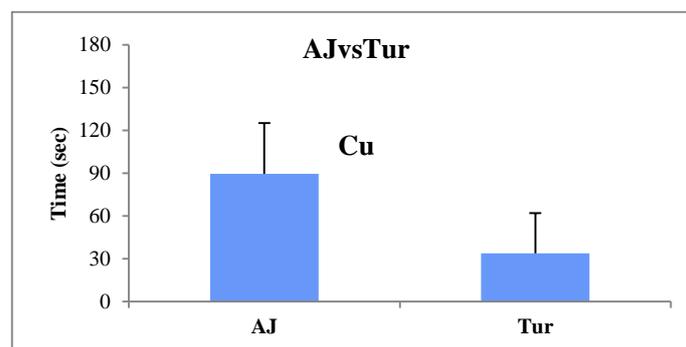


Figure 14. Average time (seconds) spent by larvae at cue AJ and CCA in the AJvsCCA combination of the cue-choice experiment. Error bars are means + SE.

### *Lateralization*

In the seawater vs seawater combination, the ANOVA does not show any larvae significant preference for the right or left sides of the chambers independently from pH or cue conditions.

## Settlement experiment

Survival analysis displayed significant differences (Tab. 4) between the two levels of factor pH (“Control” vs “Low”) and between the two levels of factor Site (“Control pH” vs “Low pH”).

Model: Surv(time, status)~pH Xsite + (1  well)					
Fixed coefficients					
	coef	Exp (coef)	Se (coef)	z	p
pH	-0.753	0.471	0.272	-2.77	5.60E-03
Site (Si)	-1.858	0.156	0.299	-6.21	5.30E-10
pHXS_i	0.677	1.968	0.412	1.64	1.00E-01

Table 4. Summary results for the survival analysis

Tiles conditioned at control pH sites recorded a significant higher larval settlement rate ( $P < 0.001$ ) compared to that of tiles conditioned at low pH site (Fig. 15), independently from the pH conditions. 50% of larvae settled within 6 days in tiles conditioned at control pH sites, while in those conditioned at low pH site the same percentage was achieved at the end of the experiment (15 days) (Fig. 15).

Overall, larvae inside the tank at control pH settled significantly faster ( $P < 0.001$ ) compared to those inside the tank at low pH (Fig. 16), independently from the site the conditioned tiles were from. In the tank at control pH 50% of settlement was reached within 6 days from the beginning of the experiment, while In the tank at low pH the same percentage of settlement was achieved at day 10 (Fig. 16).

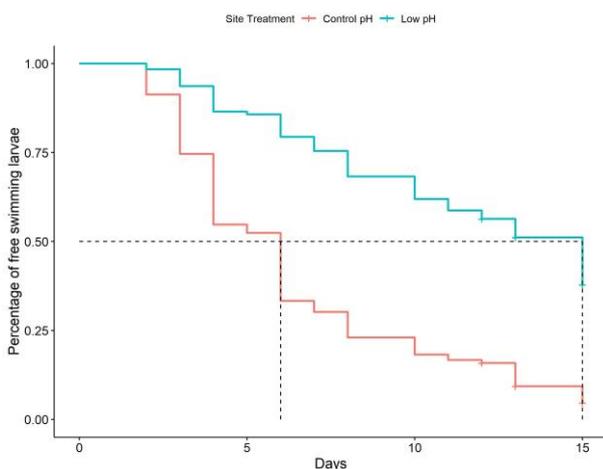


Figure 15. Diagram showing the percentage of planulae that settled during the two week long experiment on tiles conditioned at control and low pH conditions

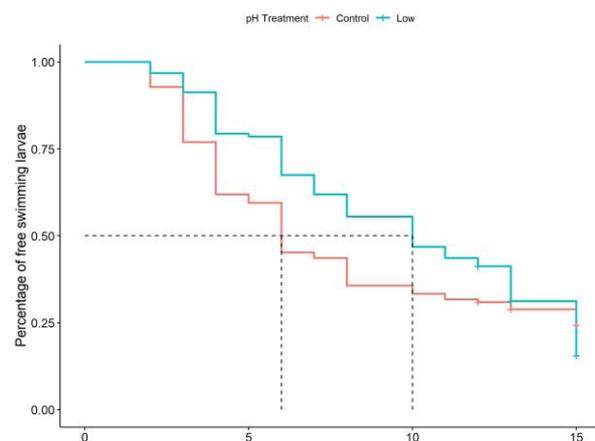


Figure 16. Diagram showing the percentage of planulae that settled during the two week long experiment in tiles deployed in tanks at control and low pH conditions

## Respiration rates of planulae

Globally, respiration rates *A. calycularis*' planulae ( $6.99 \pm 2.52 \mu\text{mol h}^{-1} \text{planula}^{-1}$ ) were higher than those of *A. japonica* ( $1.24 \pm 0.45 \mu\text{mol h}^{-1} \text{planula}^{-1}$ ), which can be expected considering the difference in size. Neither planulae from *A. calycularis* nor from *A. japonica* showed a significant change in respiration rates under the different pH levels (two-tailed Welch's t-test  $p > 0.05$  ; Figure 17)

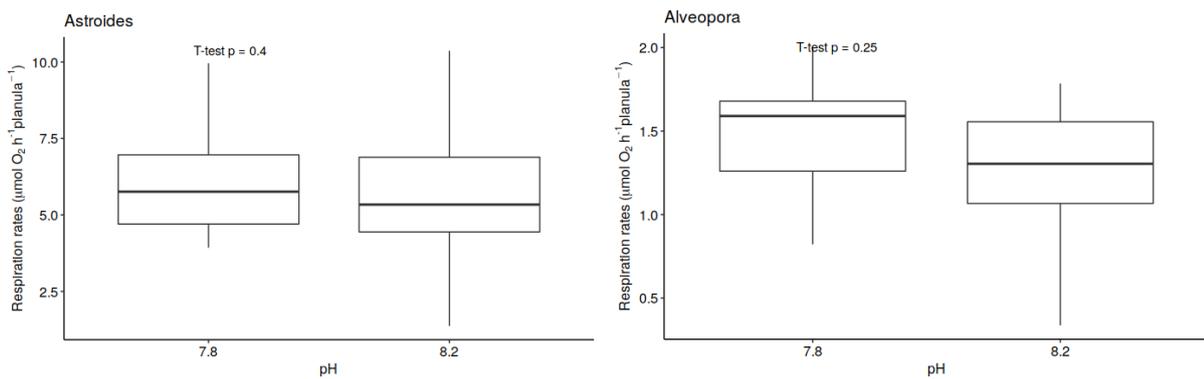


Figure 17: Respiration rates of the planula under the different pH condition.

The presence of cues significantly affected the respiration rates for both species. In the case of *A. calycularis* (Figure 18), as pH was previously found to not affect the respiration rates, only the effect of the presence of cues was tested. Both turf and cca cues lead to a decrease in the respiration rates (ANOVA,  $F_{2,47} = 22.31$  ;  $p < 0.01$ ).

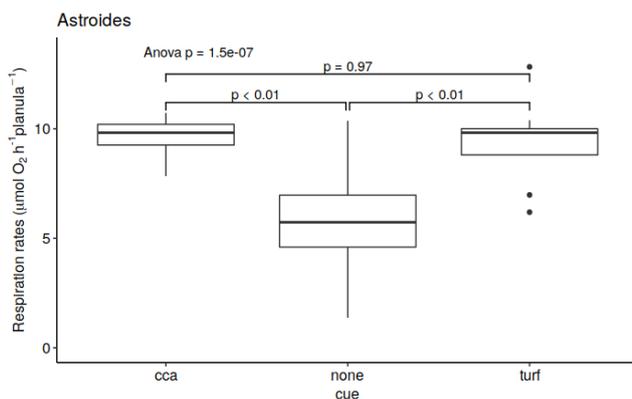


Figure 18: Respiration rates of planula in the presence of cues (cca, turf) compare to no cues for *A. calycularis*. The comparison show the results of TukeyHSD posthoc test.

In the case of *A. japonica*, the presence of cca cues lead to an increase in respiration rates (two-tailed Welch's t-test  $p < 0.01$  ; Figure 19).

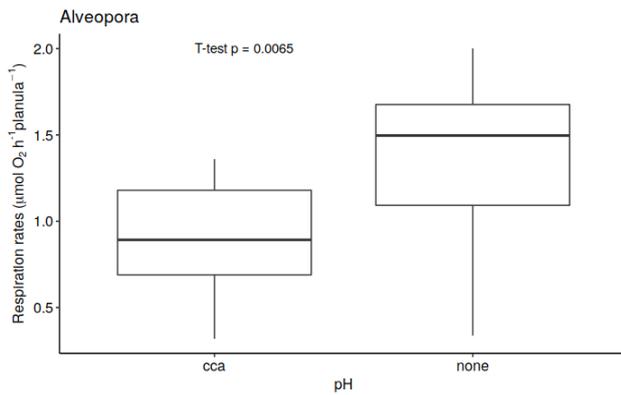


Figure 19: Respiration rates of planula in the presence of cues (cca) compare to no cues for *A. japonica*.

### Planula motility and taxis

Any differences in the swimming speed or ciliary beat frequency were observed between a low and high pH seawater in *Asteroides calycularis* and *Alveopora japonica* planula larvae. However, a clear difference in the swimming trajectory was observed in the response of Planula larvae to CCA. In *Asteroides calycularis*, the larva showed significant approaches to CCA in both low and high seawater conditions but only those in high pH condition persisted to stay on CCA. However, those in low pH showed temporal access to CCA but soon detached away from CCA. This tactic pattern was not observed in the Japanese species *Alveopora japonica*. Further setup of experimental design is necessary for more demonstration in the remaining research periods.

### Transcriptomics

Data are still in progress and RNA-seq using Hiseq 4000 in Macrogen will be bought for further analysis.

## **Future steps**

In 2019 the cues choice experiment carried out in Italy will be done again following the methods used in Japan. In both countries the skeleton and ultrastructure of coral polyps grown under control and acidified conditions will be analyzed and the growth performance assessed. The experiments on Planula motility and taxis and transcriptomics will be performed using more replicates and new variables. Transcriptomics data will be analysed and RNA-seq using Hiseq 4000 in Macrogen will be bought for further analysis.

Respiration rates of planula will be re-measured to confirm the result obtain and to test the effect of the age of the planulae. In addition, the respiration of newly settled juveniles and one-year-old juveniles will be measured using microsensors under control and acidified conditions. Finally tiles with newly recruited corals will be deployed in the field at the CO<sub>2</sub> seeps (Shikine and Vulcano) and respective control sites to follow their growth over the course of the year. The experiment on settlement will be run again in Japan using more replicates

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