Supplementary Methods for:

Moore *et al.,* 2021
Rapid multi-generational acclimation of coralline algal reproductive structures to ocean acidification

Site Description

The sites studied here, Shell Island (16° 48' S, 123° 04' E) and Tallon Island (16° 40' S, 123° 14' E) are characterized by vastly different pH regimes (as displayed in Figure S2). The Tallon Island site is an intertidal reef platform located on the north eastern seaward margin of the island. This reef is dominated by seagrass and macroalgae which, in combination with large tidal ranges, creates strong diel pH variability of up to 1.06 units. Contrastingly, the intertidal Shell Island site is dominated by Acropora spp. corals which exhibit limited metabolic alteration of their local seawater carbonate chemistry [1,2]. Therefore, Shell Island exhibits low diel pH variability of less than 0.10 units. Long term monitoring of the pH regime at these sites was conducted between April–August 2016 [3], with the daily pH variability at each site displayed in Figure S2.

Collection and acclimation to aquaria

In October 2016, seventy *H.reinboldii* rhodoliths were collected from each site and transported in coolers to the Indian Ocean Marine Research Centre, Perth within seven hours. These individuals were cleaned thoroughly to remove epiphytes and randomly assigned to experimental tanks, with a roughly equal biomass of coralline algae assigned to each tank. All individuals were kept at pH 8.00 for one week, to allow for recovery from transport stress and acclimation to aquaria conditions. Following acclimation, pH was modified by 0.1 units each week until target pH conditions were achieved.

Experimental Design

The 48 experimental tanks were arranged in a block-type design, with 12 header tanks each supplying four experimental tanks with 30 ml min-1 of seawater via a spouting system of fine nozzles. Experimental tanks were rectangles of 3L plastic that each contained one submersible pump (2W Haliea, 0.5 m jet) providing greater than 1 m s^{-1} turbulent flow. Header tanks were rectangles of 25L plastic. Fresh seawater from Waterman's Bay, Perth was filtered to 25µm by a series of three sand filters and continuously supplied to these header tanks. pH manipulation occurred in the header tanks, with bubbling of CO_2 gas and CO_2 free air (created by pumping air through a sealed canister of soda lime) through air stones used to increase or decrease CO_2 concentrations. Both gasses were delivered using solenoids (TUNZE) that were connected to a pH controller (Apex Neptune) that monitored pH in each header tank (achieved treatment pH displayed in Table S1).

Experimental tanks were kept within plastic waterbaths which along with heaters (150 W Aqua One) in the header tanks, maintained temperature at 26–27°C (controlled via Apex Neptune temperature controllers). This temperature was selected to mimic the Kimberley region in winter [2] and was selected to minimize the influence of heat stress on the coralline algae. Light was provided by 150W LED lights (Malibu LED, Ledzeal) and followed a natural diurnal cycle of 0 µmol quanta m^{-2} s^{-1} from 18:00-6:00, increasing gradually to a peak of 150 µmol quanta m^{-2} s^{-1} at 10:00-14:00, then decreasing gradually to 0 µmol quanta m^{-2} s^{-1} at 18:00. These light levels were selected to mimic the sheltered understory habitats from which the coralline algae were collected and are therefore lower than those expected on open reef environments. See Anthony & Hoegh-Guldberg [4] for a discussion of varying light environments on coral reefs. Details of this experimental design are also described in [3].

Reciprocal Transplant Experiment

Using the same approach as described for the multi-generational experiment, generation six populations released spores which then settled onto the experimental tank walls to form generation seven recruits. Tank sections containing generation seven recruits were cut from the tanks and placed into new experimental tanks. However, unlike previous generations, half (24 plates) of the present-day treatment plates were interchanged with half of the ocean acidification treatment plates with the same level of pH variability. For example, half of the mean pH 8.00 high variability treatment replicates were placed into mean pH 7.70 high variability treatments and vice-versa. This method gave rise to the factors "transplant treatment mean pH" and "generation 2-6 treatment mean pH", with half of the coralline algae having the same mean pH for both factors (e.g. pH 8.00 and 8.00) and half having different mean pH's for these factors (e.g. pH 8.00 and 7.70). When randomly selecting the plates to be transferred, it was ensured that both sites of origin had an equal number of plates transferred per treatment combination. Treatment pH variability was kept constant during this transplant experiment as the aim was to separate transgenerational effects of mean pH from any transgenerational laboratory effects. 61 days after being placed in the new tanks, generation seven coralline algal plates were removed from the tank for conceptacle analysis. Measurements were completed after 61 days in the reciprocal transplant experiment (as opposed to 41-51 days for previous generations) as new recruits did not appear in the tanks until 10 days later than the previous generations.

Conceptacle Measurements

Microscopic images (Figure 1) were taken using a 10x magnification compound microscope (Leica DM 2500 LED) calibrated for diameter measurements using a 100 μm calibration slide. When imaging generation six plates the 10x objective lens of the compound

microscope stopped working, therefore half of generation six, and generation seven images were taken using a 4.5x magnification stereoscope (Olympus SN61) calibrated using a 1 mm slide. When analysing pictures taken using the 4.5x magnification microscope, owing to the greater area of the plate captured due to the reduced magnification, a random area of the image (matching the smaller area captured in the 10x magnification images) was used for conceptacle counts and diameter measurements. In order to check for any effect of magnification, multiple plates were photographed and analysed under both 4.5x and 10x magnification with the results showing consistency between the two different magnifications. Analysis of the ~1728 images taken was conducted using the image analysis software ImageJ, with a random selection of images analysed twice to check for consistency between multiple assessors. For each plate conceptacle abundance was measured by counting all the visible conceptacles in the area of analysis, then calculating the mean value of the three images for each plate. Conceptacles were only counted if the entire conceptacle could be seen in the area of analysis and they were undamaged, as only functioning conceptacles were deemed ecologically relevant. Counts of conceptacles were normalised by the area of the plate visible in the photograph from which they were counted, giving a final measure of number of conceptacles per mm^2 of plate (conceptacle abundance).

Individual conceptacle diameters were measured as the distance between the two most distant points of each conceptacle. Owing to the circular shape of conceptacles, measuring conceptacle diameter was deemed to be an efficient and accurate approach for characterising size. Mean conceptacle diameter for each plate was determined by calculating the mean of all diameter measurements across the three images taken per plate. This approach was used as some images had fewer conceptacles than others, therefore individual diameter measurements from images with low numbers would have had a heavy weighting on the overall mean if per

image means were taken first. It is worth noting that replicate numbers in this study differed from those reported in Cornwall *et al.,* [5] as following each generation coralline algal plates were sacrificed during geochemical analysis [5]. Therefore, varied replicate numbers (see tables S2-S5) exist for the different treatment and generation combinations in this study. Additionally, a higher number of plates were analysed in generation two because we deployed separate recruitment plates as well as cut sections from the tanks. However, owing to the success of both approaches we deemed that cutting sections from the tanks provided sufficient replication and therefore utilised only this method for the proceeding generations. A potential caveat of diameter measurements in this study, is that the age and developmental stage of individual recruiting crusts were unknown. Therefore, differences in conceptacle size between treatments/generations may represent differences in age, rather than an effect of the treatment or generation. However, as the appearance of the first visible recruits occurred roughly at the same time for any one generation, this ensured that the majority of recruits were of similar ages.

Growth and Recruitment Measurements

To measure growth, the two plates that were placed into a new experimental tank at the beginning of each generation were imaged every two weeks. Image analysis was then conducted in ImageJ in order to calculate the rate of linear extension of the coralline algae on these plates. Thus, the measurements of conceptacle abundances quantified in this study could be compared with the growth rates of the same populations (e.g., generation two linear extension rates vs generation two conceptacle abundances), in order to investigate whether any trade-offs between these two parameters were occurring. For example, a negative relationship between conceptacle abundance and growth rates would suggest that one of these performance measurements increases at the expense of the other, while a positive relationship

would suggest such a trade-off is not occurring. Additionally, at the end of each generation the total area of recruitment (the area of internal tank walls covered with next generation recruits) produced from the initial coralline algae on the two plates was measured by imaging the tank and using ImageJ to calculate the area covered. This allowed conceptacle measurements of coralline algae on the plates (e.g., generation two algae) to be compared to the total area of recruits (e.g., generation three recruits) that were produced from these seed crusts. Such an approach allowed us to investigate whether increasing conceptacle abundances within a population leads to a greater area of next generation recruitment. Thus, testing the link between our conceptacle measurements and fecundity.

References

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