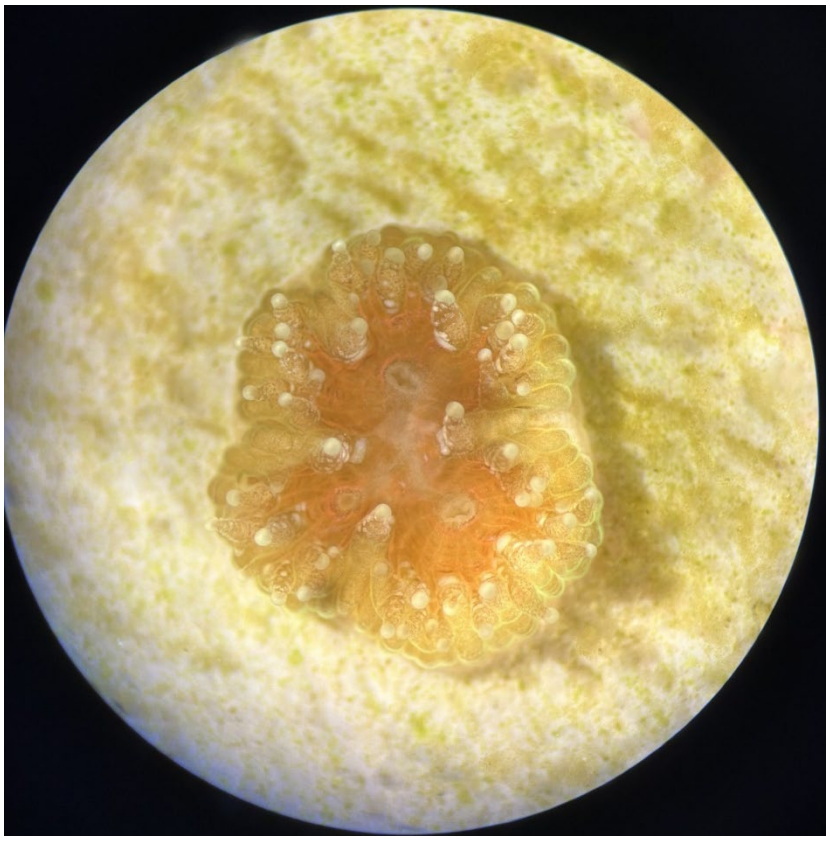
Coral spawning and optimization of coral propagation techniques in land-based nurseries



A 5 month-old Pseudodiploria clivosa recruit.

Coral spawning and optimization of coral propagation techniques in land-based nurseries

Final Report

Prepared By:

Nicole Fogarty

Krista Laforest

Ashley Weeks

Louis-Pierre Rich

University of North Carolina Wilmington Coral REEF Lab

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Florida Department of Environmental Protection
Coral Protection and Restoration Program
8000 N Ocean Dr.
Dania Beach, FL 33004

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Management Summary

The UNCW Coral REEF lab continues to pursue innovative research and development work regarding improving land-based coral spawning and fertilization as well as upscaling recruit rearing efforts through the optimization of probiotics. The use of probiotics to enhance aquaculture operations has been proposed; however, the application of bacterial isolates in the early life stages of corals is understudied. Previously the groups of bacterial strains have been isolated, tested, and showed promise for enhancing recruit health were reassessed, both individually and as combined isolate groups, to identify optimal strains for increasing the survival and growth of recruits. Acquiring the specialized skillset required to maintain and spawn corals in a land-based facility, along with the labor-intensive task of isolating, screening, and identifying beneficial probiotics requires a substantial amount of time. Despite these limitations, several strains of probiotics show preliminary evidence that can be utilized for enhancing survivorship and/or growth by over 15%, showing excellent promise for upscaling coral aquaculture for restoration efforts.

Executive Summary

Florida's Coral Reef has seen a drastic decline in coral coverage and a significant shift in its assemblage of coral species due to several stressors, namely a multi-year disease-related mortality event. Increasing genetic diversity on Florida's Coral Reef through sexual reproduction may improve resistance to disease and resilience to other stressors, such as increasing ocean temperatures, which is critical to the recovery of reefs and their persistence in the future. However, successful fertilization, larval development, and recruitment pose significant bottlenecks in the wild, preventing natural recovery from occurring. Furthermore, the logistical constraints of collecting coral gametes in the field and transporting them to a laboratory can impede research advances in coral reproduction and early life history stages. Large-scale innovation measures are therefore vital for the recovery of Florida's Coral Reef. The main objective of this research is to improve the methods of *ex situ* coral sexual propagation, with the aim of increasing genetic diversity of corals and upscaling restoration techniques. This was primarily accomplished through two research areas of focus: (1) enhancing methods in *ex situ* coral spawning, fertilization, and larval and recruit rearing and (2) screening bacterial isolates to test for their potential use as probiotics in coral aquaculture. This year spawning hours were shifted by six hours which has improved every aspect of the *ex situ* propagation methods. Additionally, we moved our broodstock to our four newly constructed spawning systems and collected thirteen *Diploria labryrinthiformis* for broodstock. Standard operating procedures were developed for future research quantifying sperm characteristics important for cryopreservation, artificial selection, and fertilization success in outplanted corals. Several bacterial treatments isolated from adults were identified to potentially enhance the survival and growth of *Pseudodiploria clivosa* recruits. These isolate groups are going to be further screened to identify if a single bacterial isolate is responsible for the enhanced health benefits. These findings have furthered the knowledge and understanding of various aspects of *ex situ* sexual reproduction and recruit grow-out techniques to upscale restoration efforts and enhance the genetic diversity of wild populations in the coming years.

Acknowledgements

We thank the Florida Department of Environmental Protection for their continued financial support of these projects, UNCW's Center for Marine Science (CMS) for providing the infrastructure for conducting this research, and aquaculture specialists Jimmy White and Ron Moore for their assistance. Furthermore, we thank Louis-Pierre Rich for system construction and the facilitation of several research projects and Danielle Norton for keeping the coral broodstock and recruits healthy. Additionally, we would like to thank Dr. Ushijima and his laboratory for their assistance with the bacterial isolation and identification (task 2). This research could not have been completed without the dedication of the following UNCW students: Meg Van Horn, Anna Edmundson, Annabel Burcham, Avry Regan, Ashley Weeks, Corinne Mickalites, Emily Ott, Grace Purcell, Hannah Garber, Jena Myers, Kayla Engle, Kayleen Bello, Kenna-Faith Hovey, Lahna McCann, Lauren Evans, Madison Proffitt, Maggie Fowler, Maureen Howard, Mazzy Cole, Nicolo Cohen, Paige Shackelford, Sam Heaton, Tara Hernandez, Tyler Pratt, Zoe Franzak. Finally, we thank the CMS leadership and staff for their support of our coral spawning facility.

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List of Acronyms

MAS	Minutes after sunset
DAFM	Days after full moon
GLMM	Generalized linear mixed model

1. AQUARIUM-BASED CORAL SEXUAL REPRODUCTION

1.1. Overview

Efforts to maintain and spawn corals in the land-based nursery at UNCW were continued. As this technology has only recently been applied to Caribbean corals, the methodologies as well as infrastructure are continually optimized to maximize spawning success and recruit survival. Four new spawning systems were constructed to increase species diversity and allow the manipulation of spawning time to facilitate gamete collection and experimentation. Additionally, a pilot project in sperm trait microscopy was conducted to better understand the nuances of fertilization for upscaling larval production for restoration and provide insight into optimal outplanting designs to maximize fertilization success. This has broad implications for *ex situ* coral reproduction and will help inform managers and stakeholders on how to optimize facility design and spawning protocols for upscaling restoration activities. Also, Dr. Fogarty continued to coordinate and facilitate the land-based assisted sexual reproduction (LASR) group consisting of experts in artificial light and indoor cue mimics. This group shares valuable information on land-based spawning techniques that advance knowledge in this emerging field.

1.2. Methods

Spawning and fertilization

Corals were collected off Broward County and were added to our spawning system between 2020-2021. This *ex situ* system mimics seasonal conditions experienced by corals in Broward County by altering temperature and light (solar and lunar) cycles. The facility houses *Orbicella faveolata* (n=11), *Pseudodiploria clivosa* (n=15), and *Diploria labyrinthiformis* (n=13) that have been identified as rescue priorities in the State of Florida, in addition to 11 genotypes of the critically endangered *Acropora cervicornis*. These species were monitored for spawning in August and September. To aid in research efforts, sunset time was shifted to occur 4 hours earlier in the day. Monitoring for spawning activity began three days after the full moon (DAFM), 90 minutes after sunset (MAS). Colonies were initially monitored every 10 minutes until setting occurred, after which corals were continuously monitored until spawning commenced. During spawning, data were recorded regarding the coral species, identifying number, time the colony set, proportion of colony setting, and spawn time. Once gamete bundles from a colony reached the surface, they were collected via pipette or petri dish. Bundles were then added to a gravy separator or, in the case of a smaller spawn (n<200 bundles), small cups. Bundles from multiple colonies were then mixed in equal portions and gently swirled by a shaker for approximately 90 minutes to encourage the bundles to break apart. Sperm concentrations were targeted at 10^6 by keeping a ratio of approximately 1 gamete bundle to 1 ml of seawater to optimize fertilization opportunities. Gravy separators were then placed in water baths set at (27^o C) for 1-2 hours until the first cell cleavage occurred.

Embryos were then rinsed a minimum of five times with clean seawater to avoid polyspermy.

Sperm Motility

During the 2023 spawning season, we learned an incredible amount about how to properly record sperm swimming traits with our high-speed video camera and microscope setup. We now understand how to maintain temperature, the appropriate chamber needed to record fast-moving sperm, the concentration at which sperm needs to be recorded, the type of microscope and objectives needed, and what external factors that can influence recording (i.e. vibrations from the HVAC and desktop computer).

Larvae rearing and settlement

Cleaned embryos were added to 70 L larval cones at a density of 1 larva/mL. Water was slowly added to the cones at a flow of 1mL/sec and aeration was added after advanced embryological development was apparent (24-48 hours). To maintain water quality within the cones, Saran wrap was used to remove lipids from the surface and dead, decaying embryos were pipetted from the cones. Additionally, cones were checked, cleaned, and banjo filters exchanged every 6 hours for the first 72 hours. Once larvae reached competency, conditioned tiles were placed in dish bins with Nitex mesh sides in raceways and directly in the larval rearing cones. Crustose coralline algae (CCA) were scraped from the sides of our raceways or provided by ArcReef and the Smithsonian Marine Station. The CCA was crushed into a fine powder with a mortar and pestle or coffee grinder and placed on tiles to serve as a settlement cue.

1.3. Results

Spawning and fertilization

The number of corals that spawn each year has varied by year and species since our facility was established (Table 1). Shortly after *O. faveolata* were collected and transferred to our facility in June of 2021, they showed signs of disease therefore only a few colonies spawned in 2021 and 2022. At least half of the *Pseudodiploria clivosa* colonies spawned in 2021 and 2022 (Table 1). The 2023 spawning season was the most successful in the past four years, which may be attributed to all the broodstock corals being held *ex situ* in our facility for 2-3 years under optimal conditions. For *P. clivosa*, 13 of 15 colonies spawned including one individual which likely finally reached size for sexual maturity (Table 1). For *O. faveolata*, 7 of 9 individuals spawned, indicating that the corals that survived the previous disease outbreak have fully recovered (Table 1).

Table 1 *Frequency of corals that spawned each year. ^ include colonies that were in our system for <3 months and *includes colonies that were diseased or recovering from disease.*

Species	**2020**	**2021**	**2022**	**2023**
O. faveolata	3 of 4^	3 of 11*^	4 of 11*	7 of 9
P. clivosa	4 of 6^	12 of 15^	7 of 15	13 of 15

Over the past four years, *O. faveolata* and *P. clivosa* spawned in our facility between 5-13 DAFM and between 157-462 MAS (Fig. 1). In 2023, after all corals had been in the spawning system for at least two years, many colonies spawned with a tighter synchrony, between 7-10 DAFM. More *P. clivosa* spawned in September than in August (Fig. 2), whereas spawning was equally split between the two months for *O. faveolata* (Fig. 3). Most *P. clivosa* spawned later in the evening between 240-444 MAS (Fig. 2), whereas most *O. faveolata* spawned between 196-264 MAS, which is consistent with field observations (Fig. 3). Our *Acropora cervicornis* broodstock did not spawn much this year, although on night 17 after the full moon we saw some bundles that were in the sump, so they spawned asynchronously. This loss of synchrony is likely due to light programming errors in the Mobius app which we could not control for. We have moved away from Mobius control and returned to season tables on the Neptune Apex to avoid this issue in future.

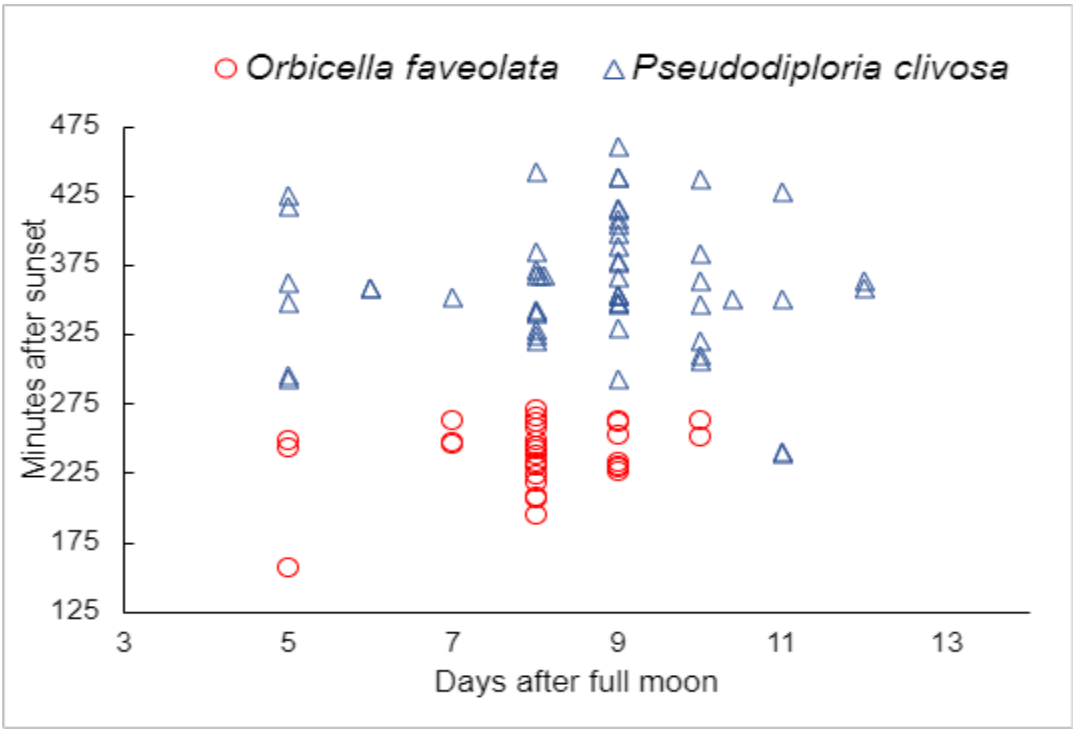

Figure 1*. Spawning times for Orbicella faveolata (red circles) and Pseudodiploria clivosa (blue triangles) colonies that spawned in the UNCW facility from 2020-2023.*

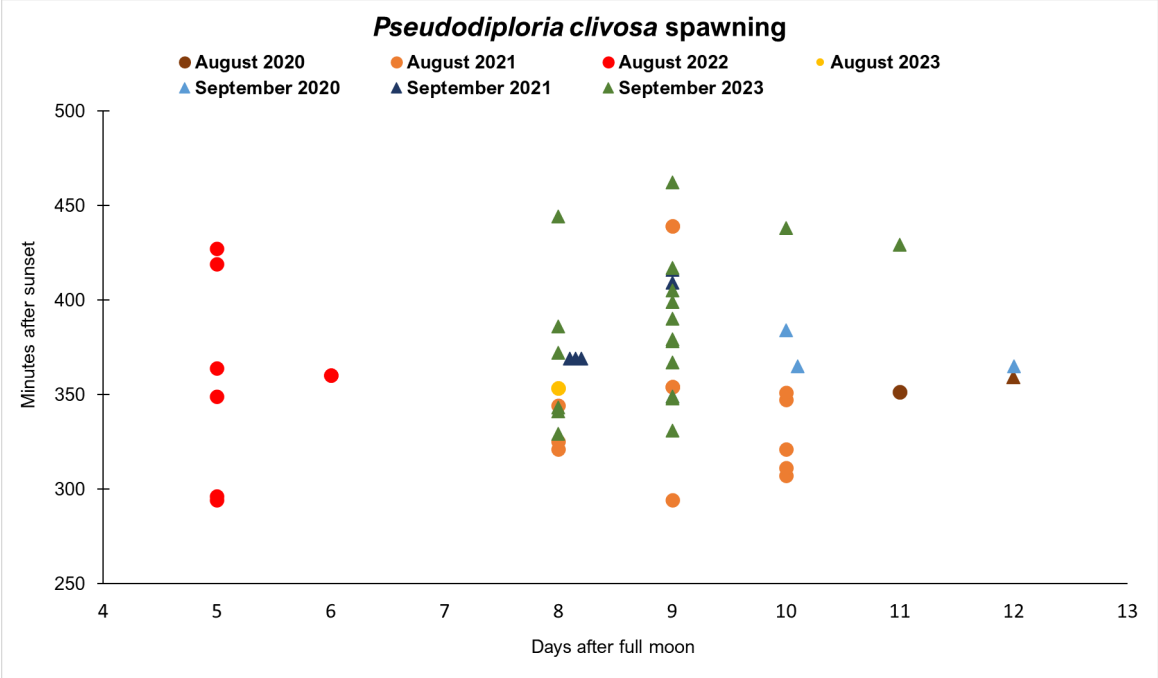

Figure 2. *Pseudodiploria clivosa spawning times in minutes after sunset (MAS) and days after the full moon (DAFM) in (circles) and September (triangles) from 2020-2023.*

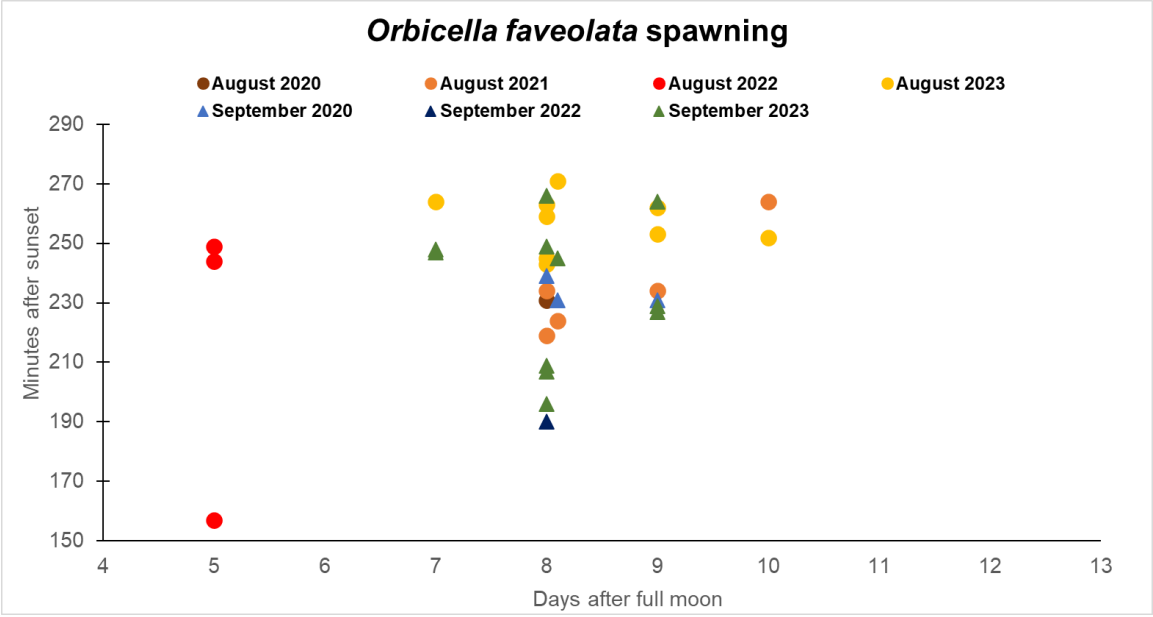

Figure 3. *Orbicella faveolata spawning times in minutes after sunset (MAS) and days after the full moon (DAFM) in August (circles) and September (triangles) from 2020-2023.*

Sperm Motility

We collected sperm from 4 *P. clivosa* and 7 *O. faveolata* colonies, two of which spawned in both August and September. We quickly realized that the sperm was getting too cool in the air conditioning for proper motility; therefore, we added a heated pad to maintain a consistent temperature. We are also looking into a stage warmer, which would also help the sperm stay at the appropriate temperature while recording videos. We tried several different chambers (i.e., deep-well slides, flat slides with cover slips on clay feed, and various glass bottom Petri dish). We found that when the water is too deep it affects the quality of the video. We also had to make considerable adjustments to the microscope, namely adjusting the condenser, trying phase and non-phase objectives, and using two different microscopes. To clearly see swimming sperm, you must have good contrast. To do this, the video camera took some troubleshooting as well. This was optimized by using 8-bit grey scale only and, most importantly, not compressing the file. To get accurate measurements it also became clear that we need to record the number of frames per second. The objectives and microscope must be very clean so particles do not interfere with sperm video. Finally, we discovered that slight air blowing and vibrations from the building HVAC cycling on and the computer tower running were enough to affect microscopy optics.

Larvae Rearing and Settlement

Over 500,000 embryos between *Orbicella faveolata* (n= 121,000) and *Pseudodiploria clivosa* (n=400,000+) were added into the four larval cones. Nearly 250,000 larvae were sent to collaborators and colleagues in Florida, South Carolina, and North Carolina. The remaining larvae were used for task 2 (probiotics experiment), an experiment finding the optimal light in the earliest life history stages, and to grow out for outplanting or experimentation. Settlement was largely successful for *P. clivosa* (average ~15%), whereas we continue to struggle to settle mass quantities of *O. faveolata* larvae, a species known to be challenging to rear. Currently the lab has 1,205 recruits from 2021-2023, with half of the recruits being reared this past year (Table 2).

Table 2 *Current number of corals recruits alive by year and species. * Indicates corals settled from donated larvae from Florida partners.*

Species	**2021**	**2022**	**2023**
O. faveolata	NA	NA	9
P. clivosa	187	323	523
P. strigosa	NA	NA	122*
A. cervicornis	NA	NA	2*
C. natans	NA	NA	39*

1.4. Discussion

Spawning and fertilization

Across all years, corals spawned consistently in terms of DAFM, with spawning becoming more synchronous in the past year, which increases the potential for successful fertilization. The timing of *Pseudodiploria clivosa* matched field observations in terms of DAFM, however, observations of spawning time in MAS were more prolonged *ex situ.* This may be a result of scarce field observations for this species, or this species may need more time to adjust to artificially shifting the sunset time earlier in the day. *Orbicella faveolata* spawning times in terms of DAFM and MAS closely matched that of wild spawning observations, which shows promise for shifting sunset times earlier in the day to facilitate research operations. Across three years, we observe consistent spawning times with repeated spawning in many colonies within our system. This indicates that the use of artificial environmental cues is reliable for signaling gametogenesis and spawning and that these corals are thriving in an *ex situ* system. These repeated observations allow us to better understand the optimal tank configurations and techniques for spawning and fertilization. As one of only a few laboratories in the country who are spawning Caribbean corals this provides us with the unique opportunity to train other land-based facilities to upscale restoration activities through sexual propagation

Sperm Motility

We have purchased many of the necessary supplies and equipment, and the microscopy facilities at UNCW has purchased a self-leveling stabilization table that will help with vibration from the HVAC, nearby equipment, or foot traffic while conducting this sensitive work. We look forward to continuing to make progress on this objective because understanding the general reproductive traits of each species and specific reproductive traits of broodstock individuals will help optimize crosses to enhance genetic diversity. Identifying individuals best suited to be sperm donors for future cryopreservation will also be important. Finally, understanding the sperm characteristics of rescue species will also influence outplanting design, particularly in regard to densities. For instance, there are tradeoffs between sperm longevity and velocity. Typically, species with high sperm velocities have lower sperm longevity and will need to be planted at higher densities to avoid sperm fatigue that results in fertilization failure. Likewise, species with higher sperm longevity are typically found at lower densities and more susceptible to polyspermy where more than one sperm fertilize an egg resulting in egg death. The susceptibility comes from the lack of need to evolve polyspermy blocks because they typically do not experience polyspermic conditions.

Larvae rearing and settlement

Larval rearing was greatly improved this year through the use of conical tanks and dish bins with Nitex mesh sides, however settlement still poses a significant bottleneck. While increased settlement occurs in optimal water flow conditions, the facility is limited in raceway space. Adding conditioned tiles to egg crates wedged in the bottom of conical tanks proved as a feasible alternative to raceways. In the following year, we will construct a new system for larval rearing and settlement that maintains the surface area of the conicals while reducing the space needed. Additionally, we hope that water quality will be greatly improved through the addition of critical life support systems, such as a UV filter and ATO. There was limited CCA available this year, which may have affected settlement success, particularly in *Orbicella faveolata.* This bottleneck will be greatly reduced through the establishment of a healthy population of CCA in our tank designed for culturing this vital settlement cue. Understanding species specific rearing and settlement methods will help to maximize recruit production and allow us to inform new facilities about best practices.

2. PROBIOTIC DOSING OF EARLY LIFE HISTORY STAGES

2.1. Overview

The use of probiotics is an understudied area for coral biology, with a particular focus on how probiotics can enhance coral reproduction, survival, and growth at the earliest life history stages. Collaborating with Dr. Blake Ushijima, the Fogarty lab conducted follow-up experiments to determine if dosing *Pseudodiploria clivosa* recruits with promising probiotic isolates tested in 2021 and 2022 can lead to enhanced survival and growth. In 2022, three isolate mixtures (15, 17, 19) were identified as the most promising probiotic candidates. These were further explored last year, both individually and in combinations to determine which enhanced both survivorship and growth of *P. clivosa*.

2.2. Methods

After the September full moon, the experiment began 17-18 days after spawning (9/8/23) and continued over 6 months after spawning. This project consisted of 21 treatments and one control, each of which initially contained 25 recruits and 5 replicates per treatment for a total of 550 recruits on 110 tiles. Four treatments were small mixtures that contained 3-4 individual bacterial strains/isolates; these 4 treatments showed some promise for enhanced survival and/or growth in our previous 2021 and 2022 pilot and screening projects (Table 3). Fourteen of the treatments consisted of the individual bacterial strains used in the mixtures tested in previous experiments. The remaining three treatments were different combinations containing 2 of the 4 mixtures containing 7-8 isolates.

Recruits were removed from the tile until there were no more than eight remaining on one side, with at least 5mm separation between them to prevent growth overlap over the course of the experiment. Once tiles were scored, an identification number was placed on the bottom of the tile, and tiles were randomly assigned to a treatment or the control before being placed on an eggcrate rack in a raceway; there were 5 racks, one for each replicate, containing one tile from each treatment group and the control.

Table 3. *Bacterial strain composition of the four treatment mixtures screened during previous experiments. PsH indicates these strains were cultured from healthy Pseudodiploria clivosa colonies in our system.*

Group 17-1 (G 17-1)	Group 17-2 (G 17-2)	Group 19-1 (G 19-1)	Group 19-2 (G 19-2)
PsH 17-2	PsH 17-1	PsH 19-2	PsH 19-1
PsH 17-3	PsH 17-6	PsH 19-4	PsH 19-3
PsH 17-4	PsH 17-10	PsH 19-6	PsH 19-8
PsH 17-5		PsH 19-10	

When the experiment commenced, recruits were approximately 2-weeks post-settlement (9/24-9/25/23) to ensure the basal plate and corallite was well formed. Inoculations occurred weekly for the first 5 weeks (weeks 0-4) by placing recruit tiles in jars for 24 hours then rinsed and placed in recirculating tanks at 26.44-26.89ºC. Additional inoculations occurred at week 8, and a final inoculation on week 16, for a total of 7 inoculations. Weekly survivorship counts and recruit care occurred throughout this 20-week experiment. Photogrammetry from 6 timepoints (weeks 0, 4, 8, 12, 16, 20) and subsequent measurements using cellSens Standard software tracked coral recruit growth.

2.3. Results

Survival

Over the 20-week experiment, survivorship varied among the 21 inoculant treatments and the seawater control (Log-rank analysis, p=0.0084; Table 4, Fig. 4). Four inoculants (2 individual strains, 1 group, and 1 group combination) showed promise with 4-16% higher survival over controls; however, these increases were not statistically significant (Log-rank survival analysis, p=0.3629 Table 4, Fig. 5). At the experiment's conclusion, Strain PsH 17-2 and group 17-1 each had 16% higher survival over control, while PsH 17-6 was 12% higher than control.

Table 4. *Log-rank survival analysis results for all 21 inoculants plus control and the top 4 inoculants plus control.*

Test	ChiSquare	DF	Prob>ChiSq
Log-Rank survival analysis between all inoculants	39.5634	21	**0.0084**
Log-Rank survival analysis between 4 top inoculants	4.3321	4	0.3629

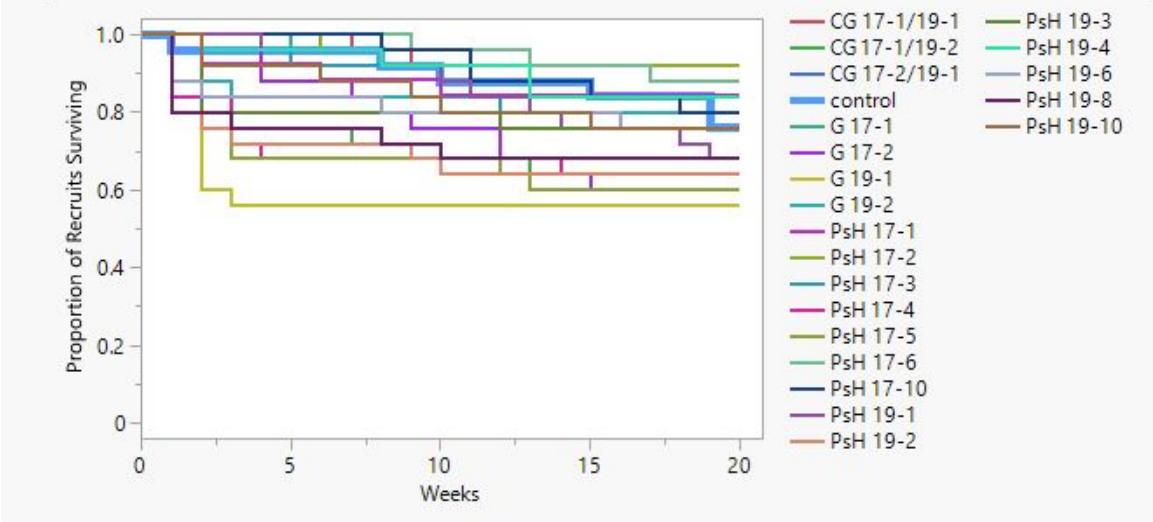

Figure 4: *Pseudodiploria clivosa recruit survival data over the 20-week experiment. The control is designated by the blue line.*

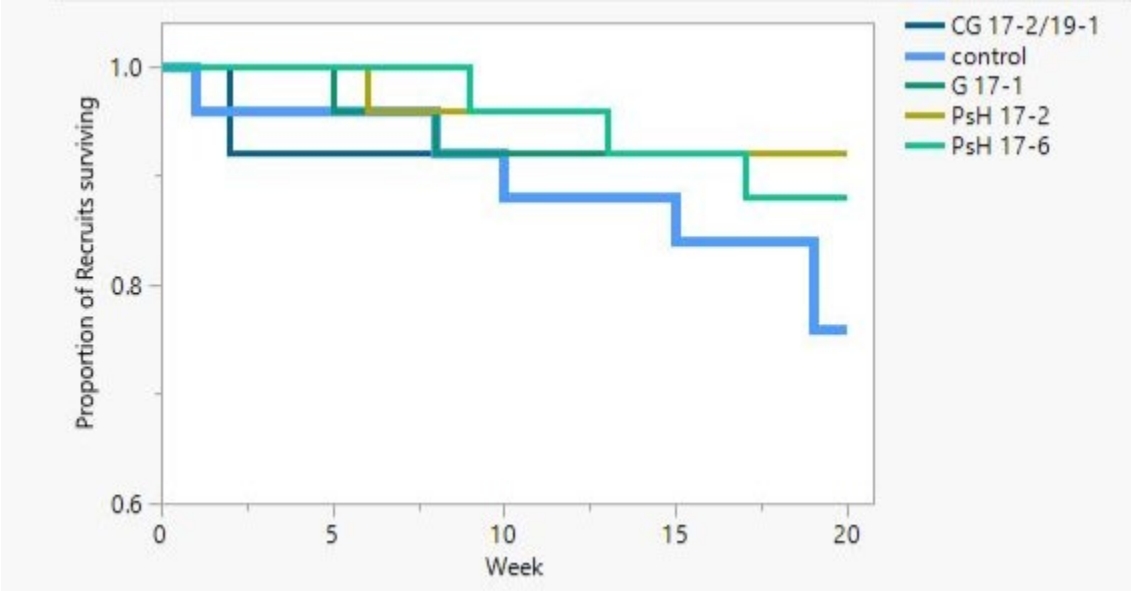

Figure 5: *Top four treatments with survivorship 12-16% greater than that of controls. Control is designated by the blue line. Based on log-rank survival test, no statistical significance was seen.*

Growth

Growth was also highly variable within treatments (Fig. 6), among treatments (GLMM $p<0.001$; Table 5), and throughout time (GLMM $p<0.001$; Table 5, Fig. 7). Although there was no difference in growth between the first 4 weeks of the experiment, growth increased significantly at each following time point (Tukey HSD, $p<0.05$; Fig. 7). One treatment, strain PsH 17-5, exhibited a statistically significant positive impact on coral recruit growth compared to the control (Fig. 6, 8). Although not statistically different, four other treatments (CG 17-1/19-2, PsH 17-4, CG 17-1/19-1, CG 17-2/19-1) showed promise by increasing growth on average of 11%, 14%, 20%, and 21%, respectively, over controls. Conversely, five treatments (G 19-2, PsH 19-1, 19-2, 19-6, and 19-8) demonstrated significantly lower growth rates than the control (Fig. 7, 8). The observed decreased growth compared to controls suggests that certain bacterial strains and mixtures may have adverse effects on coral recruits and warrants further investigation. The treatment mixture containing a combination of groups 17-2 and 19-1 has the potential to enhance both survival and growth. There was no significant difference among the 5 rack replicates (Table 5; Fig. 9)

Table 5. *The results of the GLMM with time and inoculant as the fixed factors and the rack as the random factor.*

Effects	**Source**	**Nparm**	**DFNum**	**DFDen**	**F Ratio**	**Prob > F**
Fixed Effects Tests	time	5	5	2622	1034.66	<.0001
	Inoculum	21	21	2622	3.19236	<.0001
	time*Inoculum	105	105	2622	0.46835	1
Random Effects	**Variance Component**	**Estimate**	**Std Error**	**95% Lower**	**95% Upper**	**Wald p-Value**
	Rack	0.1147652	0.0824146	-0.046764	0.2762949	0.1638

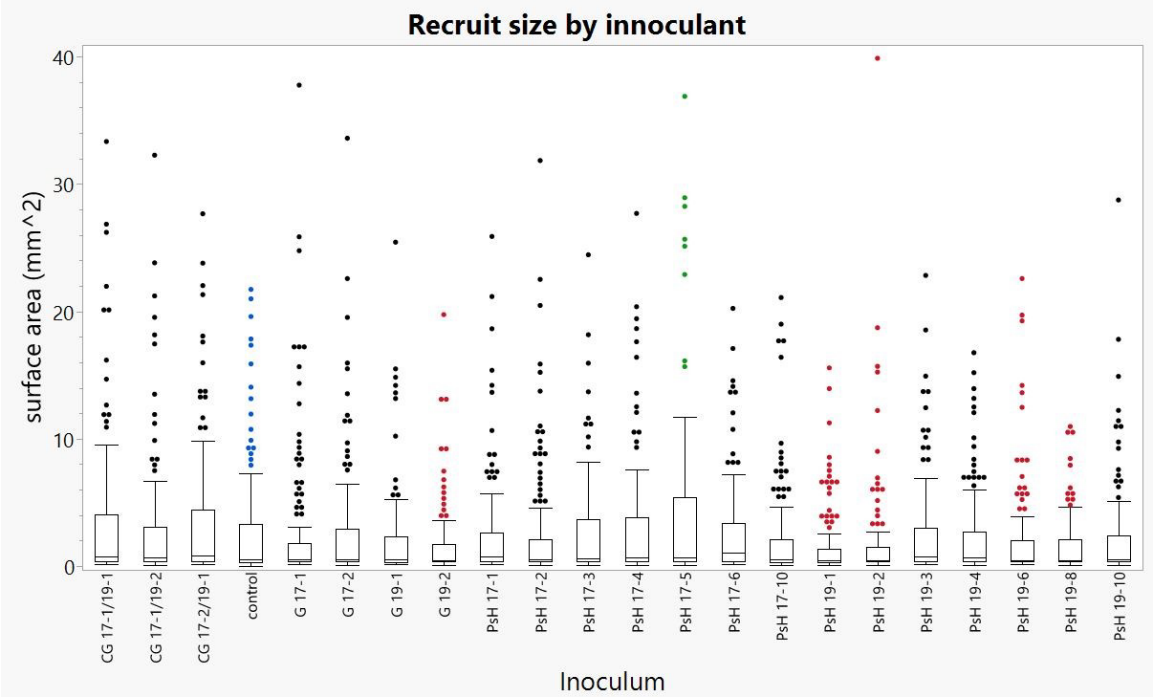

Figure 6: *Pseudodiploria clivosa recruit size across inoculants throughout the 20-week experiment (GLMM inoculant as a fixed effect (p<0.001). Different colors represent statistical differences in size (p<0.05; Tukey HSD). Inoculant PsH 17-5 (green data points) had recruits that were significantly larger than the control (blue data points). Those inoculants with recruits that were smaller than the control are designated in red.*

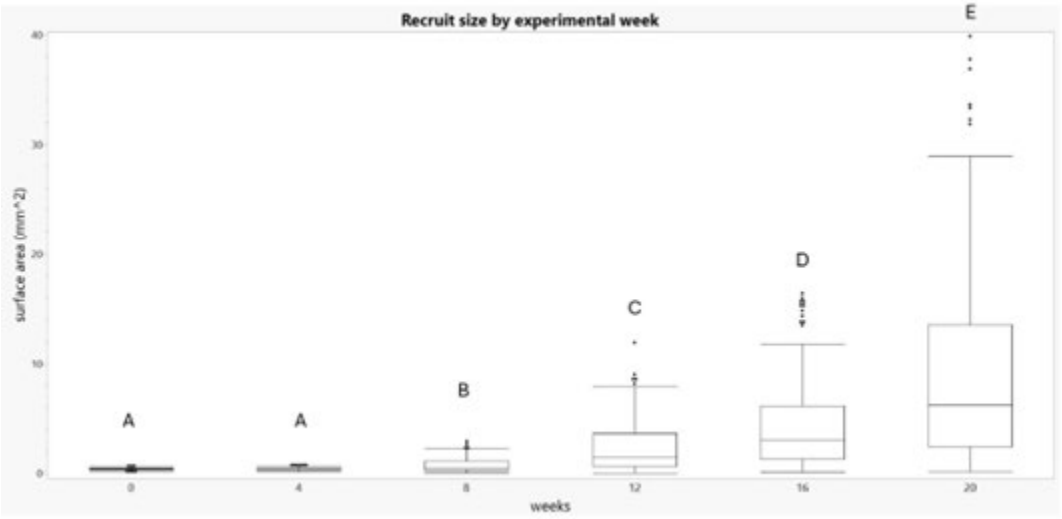

Figure 7: *Pseudodiploria clivosa recruit size across 6 time points over 20 weeks. Recruit size varied significantly across time in the GLMM model with time as a fixed effect (p<0.001). Different letters represent statistical differences (p<0.05; Tukey HSD).*

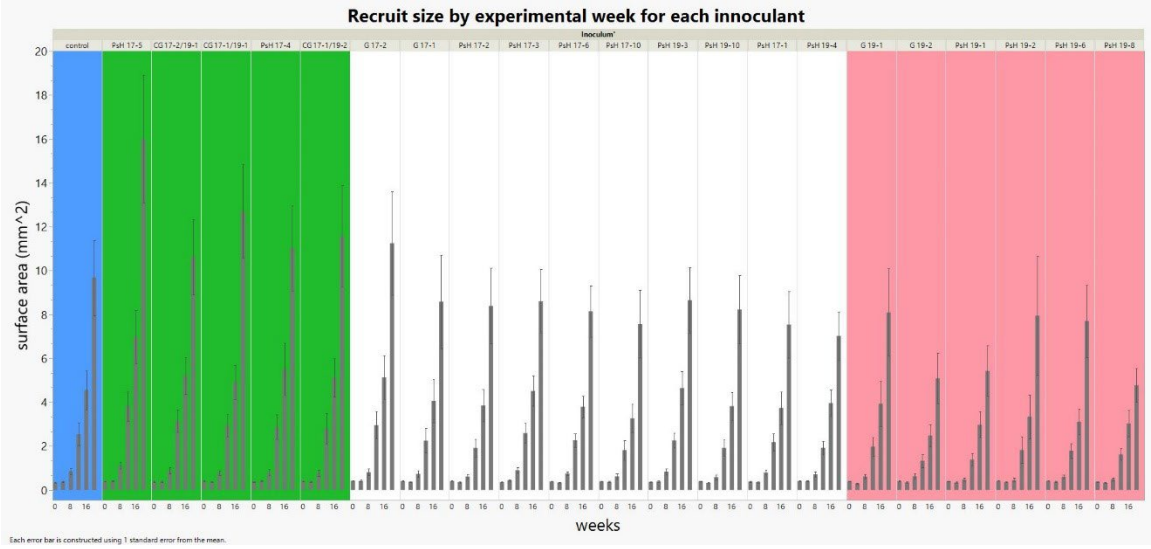

Figure 8: *Pseudodiploria clivosa recruit size across inoculants by time point. Inoculant PsH 17-5 recruits were significantly larger (p<0.05; Tukey HSD) than the control (blue); however, 4 other inoculants (*CG 17-1/19-2**,** PsH 17-4**,** CG 17-1/19-1, CG 17-2/19-1) also showed promise (green). *Those inoculants with recruits that were significantly smaller than the control are designated in red (p<0.05; Tukey HSD).*

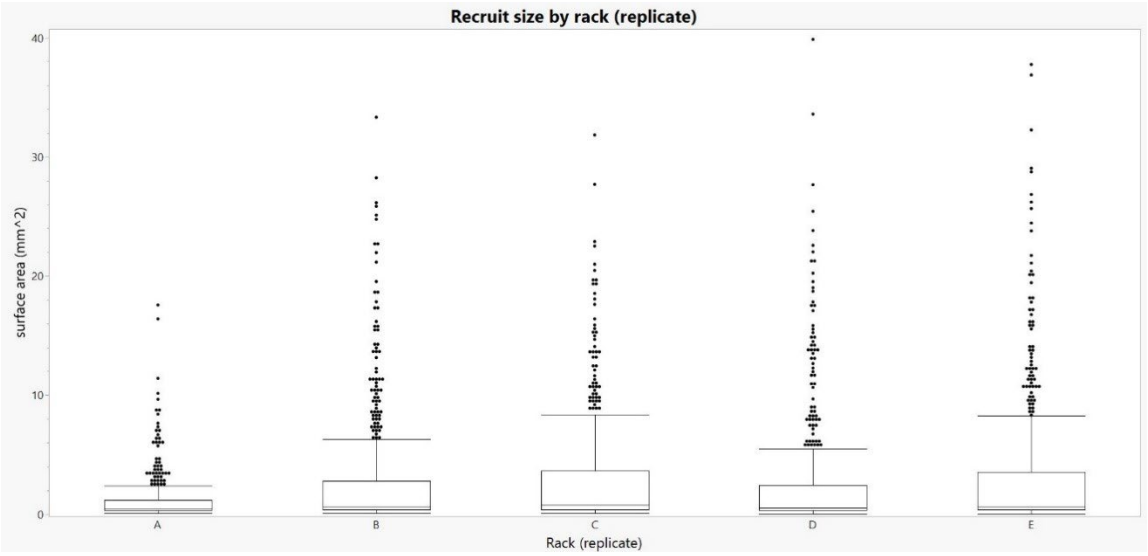

Figure 9: *Pseudodiploria clivosa recruit size across 5 replicate racks. No significant difference was seen across racks in the GLMM model with rack as a rack as a random factor (p = 0.1638).*

2.4. Discussion

The findings of this study reveal that while certain inoculants can positively influence coral recruit survival and/or growth, others may be detrimental. This underscores the necessity for careful selection and screening of bacterial treatments for use as probiotics for enhancing coral aquaculture. Identification of bacterial treatments that exhibit potential for increased survival or growth could enhance coral biomass production for restoration efforts. Future research should focus on understanding the mechanisms driving the differential effects of potential probiotic treatments and identifying the optimal probiotic for supporting coral health and resilience. For the upcoming 2024 spawning season, we will continue to further investigate bacterial strains and mixtures that showed promise for survival and growth for their potential use as probiotics. We will repeat the experiment with G17-1 with the current 4 isolates and swapping PsH 17-3 for PsH 17-6. We will also create new combinations within the top "17" isolates, as any benefit of the 17 & 19 combination group is likely driven by "17" isolates. However, we will retest the top performing combination groups. Finally, we may revisit dosing the larvae with the top performing groups.

3. FACILITY CAPACITY EXPANSION AND RESEARCH CAPABILITY INCREASE

Following a successful 2023 spawning season, we requested materials to build infrastructure improvements for task 1. We implement improvements that will allow for a better standard of care for corals in our care under task 1 and increase capacity for photogrammetry and data analysis for task 2 deliverables (Fig. 10). The larval system, quarantine, microscopy workstation, larval shipping coolers, and experimental rack upgrades are completed or acquired. The growout system is built and plumbing is being finalized prior to the addition of water, expected completion date is June 21, 2024. The sperm analysis improvements are purchased. We are moving the Center for Marine Science microscopy lab to a larger room. The CMS microscopes will be placed on the anti-vibration table, expected completion date is June 30, 2024.

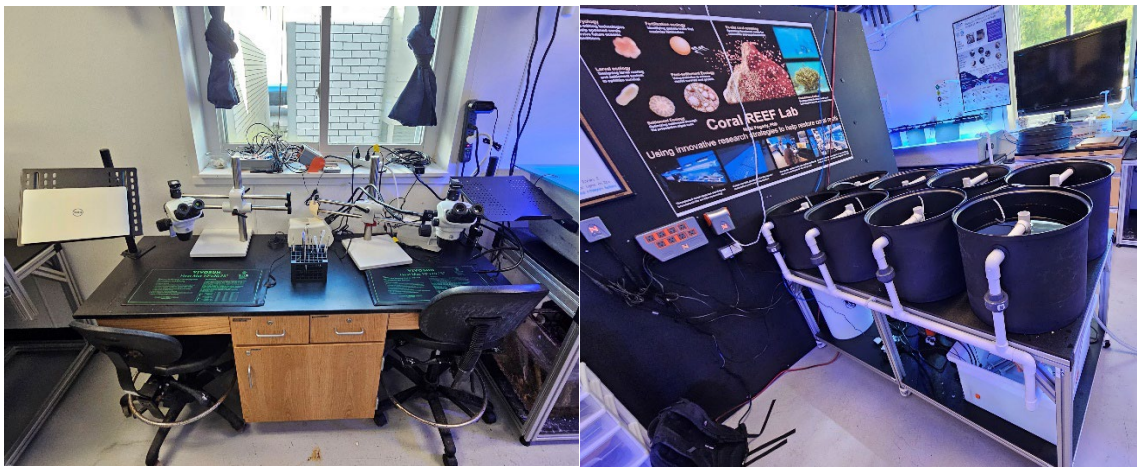

Figure 10: *Coral facility improvements for larval rearing (left) and photogrammetry (right).*