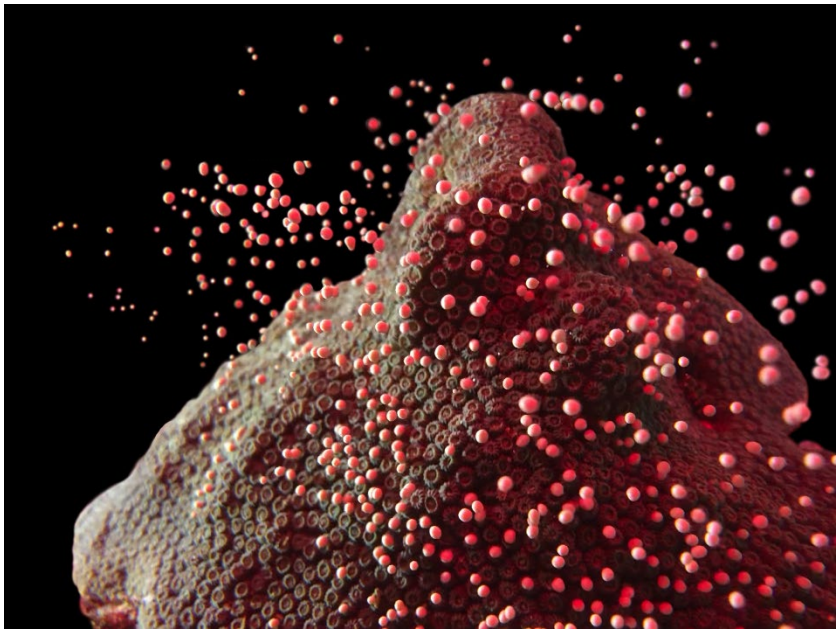
Supporting aquarium induced spawning and experiments to enhance coral propagation and restoration



Supporting aquarium induced spawning and experiments to enhance coral propagation and restoration

Final Report

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Supporting aquarium induced spawning and experiments to enhance coral propagation and restoration

Florida's Coral Reef is currently experiencing a multi-year disease-related mortality event that has resulted in massive die-offs in multiple coral species. Innovative and radical measures are needed to assist with the recovery of Florida's Coral Reef. The primary objective of our research is optimizing and upscaling ex-situ coral sexual propagation techniques, which ultimately serve to increase the genetic diversity of coral populations used for restoration. We focused on three promising areas of research to meet this objective: (1) enhancing methods in ex-situ coral spawning, larval rearing, and recruit grow out; (2) screening bacterial isolates and testing for their potential use as probiotics in the larval and recruit stages; and (3) attempting to create multigenotypic individuals (chimeras) in recruits and adults. This year with the addition of more colonies, larval rearing cones, and dedicated recruit rearing raceways, we improved every aspect of our ex-situ propagation methods. We have also improved methodologies and techniques to reduce the impact of ciliate predation and enhance coral recruit survivorship. We identified 3 bacterial groups isolated from adults that have the potential of doubling *Pseudodiploria clivosa* larval survival. Although none of the putative probiotics showed significant effects on recruit survival and growth, this is a promising and novel area of research we intend to pursue. The final task focused on creating chimeras with recruits and microfragmenting adults colonies. We were able to create chimeras with *P. clivosa* recruits for the 6 months after settlement. Attempting to create chimeras in adult *Orbicella faveolata* using microfragmenting techniques proved unsuccessful, though provided insight into the intergenotypic competition and steps toward creating universal terminology associated with chimeric formation and coral allorecognition. These three tasks furthered our knowledge and understanding of various aspects of ex-situ sexual reproduction and possible techniques to improve upscaling land-based restoration efforts in the coming years.

Task 1: Aquarium-based coral sexual reproduction (Spawn corals, larval rearing, settlement, recruit growout) (State of Florida Restoration Priority 3.3/3.5)

Coral husbandry

In the past three years we have built a seawater facility that is composed of 6 raceways, 2 large tanks, and 42 10 gal experimental tanks. Since the start of this grant in September 2021, we have been rearing 15 *Pseudodiploria clivosa*, 11 *Orbicella faveolata* (7 are reproductive size), 11 *Porites astreoides*, 2 *Pseudodiploria. strigosa*, and 11 genets of *Acropora cervicornis* (Fig. S1). In September and October, we had a disease outbreak that affected nearly all our *O. faveolata* and several *P. clivosa*. For weeks we battled the disease through various treatments (Lugol's, Koral MD, and antibiotics) and tried to bolster their health with amino acid supplements. We finally cut out the diseased areas and skeleton devoid of tissue (per Dr. Blake Ushijima's suggestion). Since then, they have been thriving and are overgrowing the cut margin. Some of the smaller pieces that were created during the cutting were microfragmented for use in the chimera experiment (Task 3). At the time of this report, all corals are growing and appear healthy.

Spawning and larval rearing

We had a successful 2021 spawning season and made great strides in understanding how to scale up larval production and settlement, and how to remedy nuisance organisms that predate upon coral recruits. The addition of larval rearing cones (similar to those previously used by Florida Aquarium and NSU), recirculating shallow trays for fertilization and tile cleaning, larval rearing/settlement bins with mesh windows (100µm), and a dedicated nursery were the greatest improvements made from last fiscal year.

Prior to the start of this grant, corals were monitored for spawning July 29-August 7 and August 27-September 3, 2021. No corals spawned after the July full moon, but 5 *Orbicella faveolata* (Fig. S2) and 14 *Pseudodiploria clivosa* spawning events (some colonies spawned on multiple nights) occurred in late August and early September. Only *P. clivosa* spawned (n=5) in September. Four of the corals that spawned last year in our system (Fig. S2), spawned again, while the others had been collected 2-3 months (n=10) prior to spawning. Spawning was remarkably similar among years and within a species (Fig. 1). The gamete crosses we conducted (8/30, 8/31, 9/1, 9/28, 9/29) yielded high fertilization rates of >85% (Fig. S3). In the larval rearing cones (Fig. S4), we were able to rear 100,000's of larvae with little effort and based on high resolution microscopy, the resulting embryos appeared extremely healthy. In August concentrations in the cones were approximately one larva/ml, while in September the larval concentration was approximately 0.6 larva/ml. In August, we also reared larvae in settlement bins with mesh windows that were floated in our nursery raceways. Between the two months, we created over 300,000 embryos. The *P. clivosa* larvae/recruits from September were used in the probiotic experiment (Task 2) and continue to be reared for various experiments.

Settlement and Recruit Rearing

Between August and September, we added ~100,000 larvae to the settlement bins (35cm x 30 cm x15cm) with mesh (100µm) windows that were placed in our nursery raceway (Fig. S5). In August the larvae concentrations were between 8,000-10,000 larvae per settlement bin, which proved to be too dense. On the bottom of each settlement bin there were 35-40 tiles (pre-conditioned for at least 1 month) sprinkled with crustose coralline algae (CCA) dust scraped from our tanks. In August, we had approximately 8,000 settlers. Not all tiles were scored for settlement before the ciliate invasion. Once the ciliates infested the tiles, they predated upon the recruits, leaving few survivors. For the September spawn, we used several different treatment methods (Lugol's, KoralMD, salinity alterations), implemented daily ciliate checks, and altered the water flow to the tanks by routing the

seawater directly from UV sterilizer through a 1-micron sediment filter into raceways. After implementing the treatments and infrastructure modifications, recruit mortality caused by the ciliates was drastically reduced.

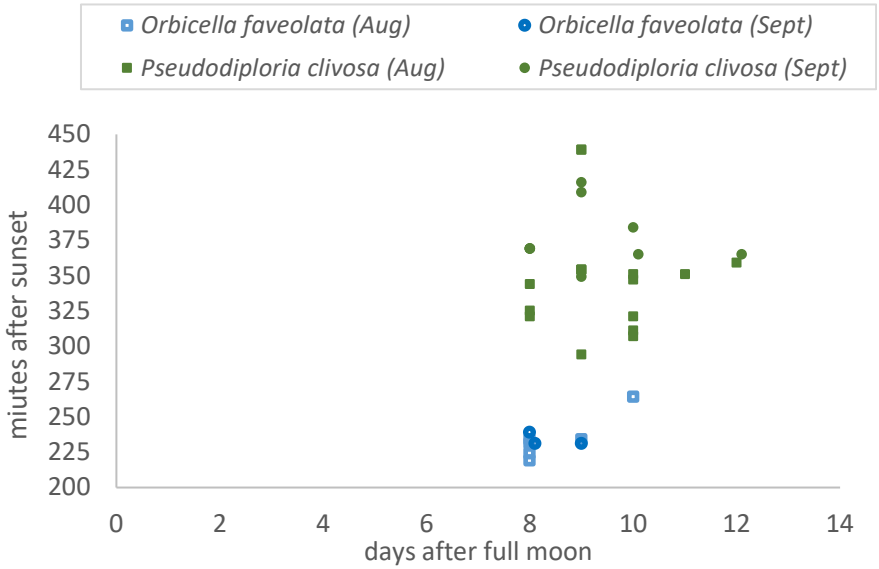

Figure 1. A comparison of spawning times from August and September 2020 and 2021.

In September, only *P. clivosa* spawned. On October 1 (2-3 days after spawning), we added 4,500 larvae to 7 settlement bins, for a total of ~31,500 larvae. We had 3,759 recruits (attachment and metamorphosed; Fig. S6) for 12% settlement, which is low and may be attributed to the short conditioning time or species of CCA we have in our tanks. Over the past 8 months, we had 11% survival (Fig. 2). Mortality can be attributed to several factors, such as ciliate predation, algal/diatom overgrowth, handling/cleaning, and trouble-shooting methodology for the chimera project as well as loss of recruits which settled on the bottom surface of tiles.

Land-based Assisted Sexual Reproduction (LASR) group

PI Fogarty has coordinated several aquaria based spawning meetings over the past 9 months: 7/29/21 (pre-spawn) and 12/3/21 (post-spawn debrief), 1/7/22 (funding brainstorm), 2/3/22 (budget/proposal), and several meetings to finalize grant submission and budget modifications. The key players in aquaria induced spawning attended these meetings (Figueiredo lab, Baker lab, Fogarty lab, Florida Aquarium, Mote, Jaime Craggs, and Michael Sweet). In July, we summarized the goals of our spawning efforts and reviewed protocols to enhance larval and recruit survivorship. In December, we debriefed on our spawning efforts and discussed some potential issues with the Neptune Apex system and how to remedy them. A subgroup met in January to discuss potential funding opportunities and discuss the most urgent needs to lab assisted spawning and reproduction. This subgroup (Florida Aquarium, NSU, Mote, UNCW) met and submitted a NOAA Ruth Gates grant in February. Additionally, we met several times in May to clarify and modify the budget and proposal. We received confirmation that the grant proposal will be recommended for funding.

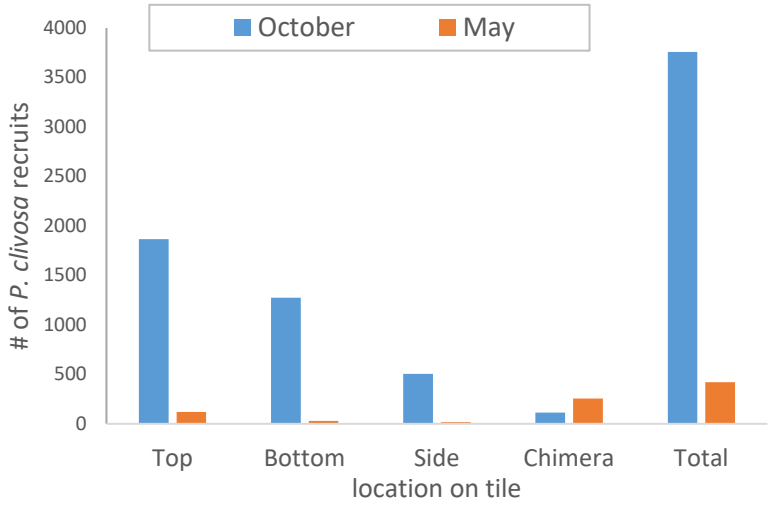

Figure 2. The location and total number of P. clivosa recruits quantified in October 2021 (within 3 weeks of spawning) and May 2022.

Summary and Recommendations

The learning curve of the past year was steep, and we are still identifying best practices. We were thrilled to have corals that had been in our system for over a year spawn again, and the level of precision was impressive. The larval rearing cones were an amazing addition that increased the number of larvae that we were able to rear, without sacrificing the quality of the larvae. We recommend institutions use this technique to upscale larval production. The ciliate invasion was frustrating, but we learned from the experience and were able to pivot by the time this grant commenced. The areas where we improved production and reduced labor has increased the percent settlement of corals and reduced ciliates and algae on recruit tiles.

Task 2- Probiotic dosing of larvae/recruits (State of Florida Restoration Priority 3.3/3.5):

In collaboration with Dr. Blake Ushijima at UNCW, bacterial cultures were collected, isolated, and grown from the microbiome of adult *O. faveolata* and *P. clivosa*. There were 25 groups of inoculums each with 10 bacterial strains, 250 strains total. After the August full moon, a pilot project was conducted to identify and remedy the challenges of conducting this experiment. This was extremely helpful and made for a successful experiment in September after the grant commenced. The aim of this task is to determine if probiotics can be identified to enhance larval and recruit survival and growth. Thus far, use of probiotics is an understudied area for coral biology, and little is known if probiotics can enhance coral survival and growth at the earliest life history stages.

Bacteria Culture Preparation and Bacteria Isolation

A mucus sample (5 mL) was collected from four land-based *P. clivosa* colonies (ID: 1, 2, 3, 4) using needle-less syringes and transported to Dr. Ushijima's microbiology lab at UNCW for microbiome analysis. A sterile environment was established using either a biosafety hood or an open Bunsen burner flame throughout the procedures of preparing bacterial isolate groups for coral recruit inoculations from the adult coral mucus samples. All mucus samples were homogenized and approximately half of the mucus samples were diluted 1:10 with artificial seawater (ASW). Ten aliquots (50 µL) of undiluted coral mucus and ten aliquots of diluted coral mucus (50 µL) were spread on marine seawater agar (MSWA) prepared plates using Rattler plating beads and incubated at 28.5°C for 48 hours. A total of 304 bacteria colonies were selected to maximize the diversity among

strains for isolation. Each bacterium was streaked and purified three times both by streaking on newly prepared MSWA plates and incubating at 28.5°C for 24 hours to ensure single strains of bacteria were isolated. Of those 304 isolated bacteria strains, 250 colonies were viable and well-developed and thus transferred to liquid cultures. Aliquots of 100 µL of minimal seawater broth (MSWB) were added to a 96-well plate and each selected bacterium strain was transferred to a well. The plates were incubated at 28.5°C for 24 hours on a shaker plate set at 160 rpm. The remaining cultures of bacterial isolates were cryopreserved at -80°C and stored as stocks for future inoculation.

Once the bacteria multiplied in the liquid cultures, 10 µL from 10 bacterial cultures were assigned to an isolate group and transferred to a microcentrifuge tube. A total of 25 isolate groups, with three replicates each, were centrifuged at room temperature for three minutes at 1600 rpm. Resulting bacterial pellets were used for inoculation, and the supernatant was discarded. Prepared inoculums in the form of bacterial pellets were stored at 4ºC for up to 10 days if not used immediately. The biosafety hood was used for preparing the liquid cultures, the 3 replicates of the 25 isolate groups, and the bacteria strain stocks for cryopreservation.

Spawning and Experimentation

In September, only *P. clivosa* spawned; therefore, we focused on this species for two experiments examining how the 25 isolate groups affected (a) larval survivorship over 9 days and (b) recruit survivorship and growth over 7 weeks.

a. **Larval experiment**

To ensure we were selecting viable embryos and not unfertilized eggs, we commenced the experiment on the second day after spawning. The larvae were reared in glass jars with 99ml of filtered seawater (0.2µ FSW) plus 100ul of each bacterial strain (1ml total) or 1ml of FSW for the control. Each isolate group was replicated 3 times and the control was replicated 5 times, for a total of 80 jars. Fifty larvae were initially added to each jar followed by daily counts and a 50% FSW change. Jars were inoculated on the 1st, 4th, and 7th day of the experiment. Jars were maintained in recirculating water baths at 27ºC.

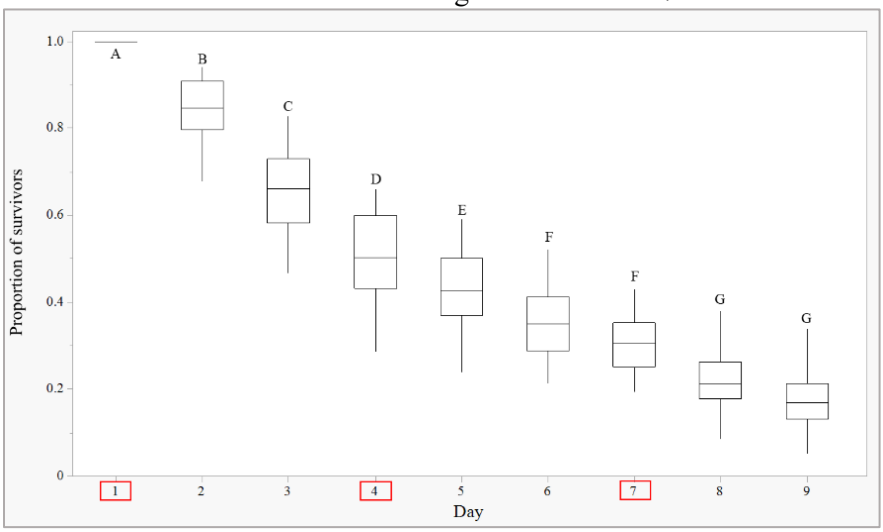

Figure 3.The cumulative change in survivorship of P. clivosa larvae over nine days. Boxes of box plots represent the 25th to 75th percentile, lines show medians, error bars represent smallest/largest values. Different letters denote significant differences across days.

As expected, larval survival declined over the experiment (Wilcoxon; X^2=207.28, p<0.001) and the day after inoculation showed a significant decrease in larval survival (Steel-Dwass, p<0.05; Fig. 3). There was no significant difference among the isolate groups using a nonparametric test (Wilcoxon test p>0.05, Fig. 4). However, the isolates that showed the

greatest survivorship (15, 17, 18, 19, 24) were examined further using a log-ranked test that showed isolate group 15 (p=0.0027), 17 (p=0.0491), and 19 (p=0.0331) survival curves were significantly higher than the control (Fig. 5). All of isolate 15 were Vibrio species, while half of 17 and 19 were Vibrios. Anecdotally, by day 6, most larvae had decreased in size, and a small portion of larvae settled at the bottom of several jars which were counted as survivors. The experiment was concluded on day 9 when survivors had either settled on the jar or reduced drastically in size.

This study provides the first screening of the effects of bacterial introduction on *P. clivosa* larvae survivorship in land-based aquaria. Significant differences were shown between the control group and three isolate group treatments. The findings suggest that certain bacteria may enhance the survivorship of coral larvae, but individual strains within groups need to be examined and replicated to determine if these isolates truly are probiotics.

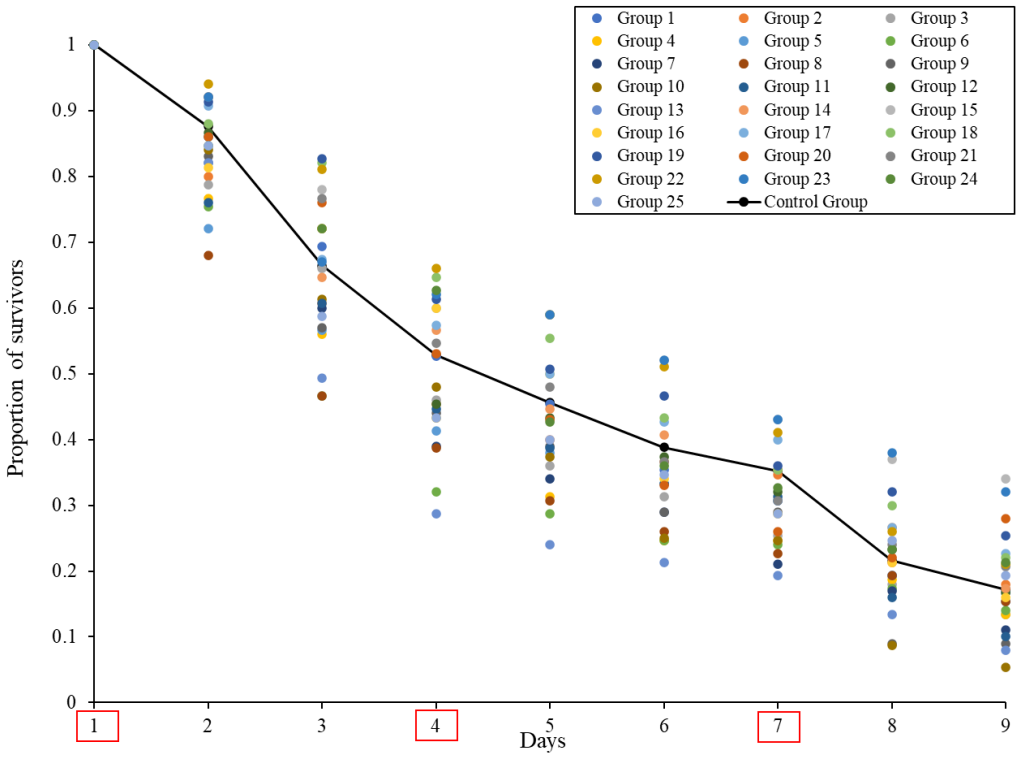

Figure 4. Larvae survivorship over nine days where bacterial inoculations occurred on days 1,4, and 7 (denoted with red boxes).

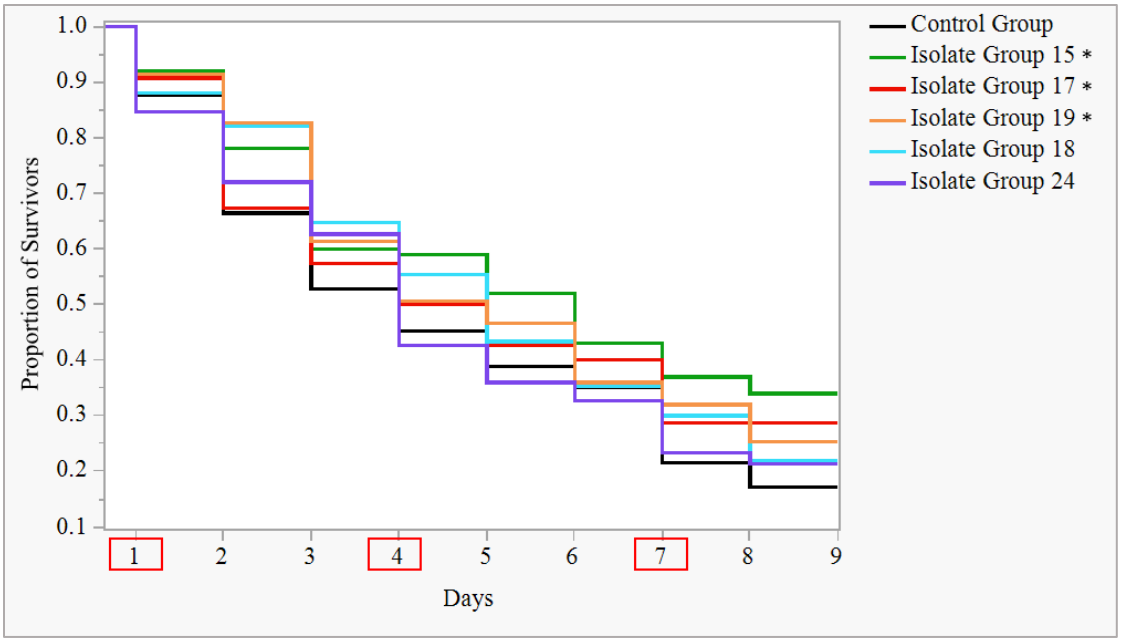

Figure 5. Pseudodiploria clivosa survival curves of putative probiotics. Asterisks in legend denote bacterial isolate groups (15,17,19) with significantly higher survivorship compared to the controls. Red boxes indicate the days of inoculation.

b. **Recruit experiment**

Recruits were settled on ceramic tiles that were conditioned in our land-based coral facility for at least 1 month prior to the experiment. Tiles with at least 3 recruits (17-19 days old) were selected for this experiment. Each isolate group was replicated 3 times and the control 5 times, for a total of 80 tiles. Bacterial inoculations and recruit counts were conducted weekly, and a subset of recruits were photographed using CellSens bi-weekly

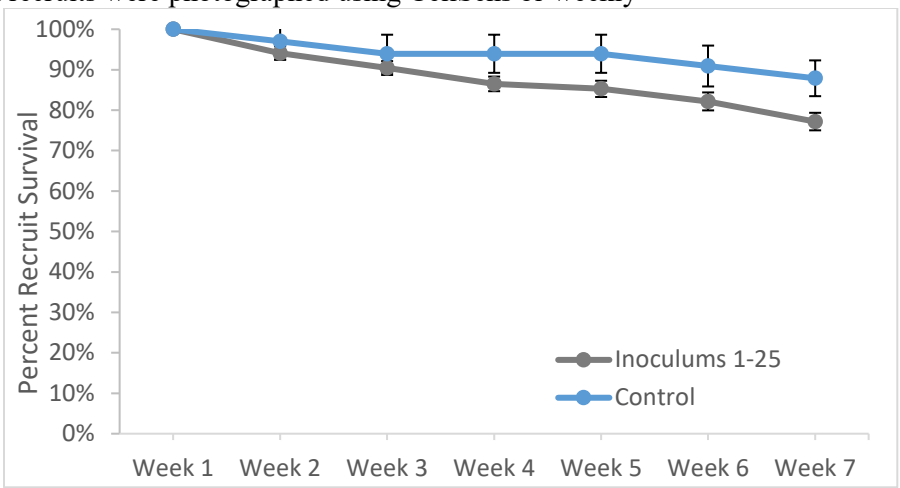

Figure 6. Pseudodiploria clivosa recruit survival for all bacterial treatments (Isolate Groups 1–25) pooled compared to the controls. There was no significant difference over time between the pooled bacterial treatments and the control (Mann-Whitney U/Wilcoxon Test, p>0.05)

Bacterial inoculation had a negative or no effect on recruit survival. Overall, there was no significant difference between the averaged survivorship for the controls and all 25 inoculum groups pooled together (Mann-Whitney U/Wilcoxon Test, p>0. 05; Fig. 6). Survivorship varied over the 7-week period and among the isolate groups and control (Fig. 8). In this study, none of the bacterial isolate groups enhanced survivorship over controls, thus providing little support for

these bacterial isolate groups to serve as an effective probiotic (ANOVA $p<0.05$). A survival analysis showed no significant difference in recruit survival between the controls and the four isolate groups with the highest survival (log-rank survival analysis, $p=0.9866$, Fig. 8).

These four groups were further analyzed for their potential impact on recruit growth over time. Growth was calculated as the average percent growth for each isolate group and the control using the following formula:

$$\text{Average Percent Growth} = \frac{\sum \frac{A_f - A_i}{A_i}}{n} \times 100$$

where A_f is the final recruit surface area, A_i is the initial recruit surface area, and n is the number of recruits per treatment group. A significant difference was seen among isolate groups with the highest recruit survival (9, 11, 17, 21) and the control (one-way ANOVA, $p=0.0172$). However, this was driven by isolate group 9 significantly reducing growth compared to the control (Tukey's post hoc, t-test, $t=-2.60$, $p=0.019$; Fig. 9). Additionally individual analyses for recruit growth demonstrated that there was no significant difference between the control and isolate groups with the highest survival and/or average percent growth (ID: 2, 10, 11, 17, 21, 25; t-test, $p>0.05$; Fig. 10). This study provides the first screening of the effects of bacterial introduction on *P. clivosa* recruit survivorship and growth in land-based aquaria and demonstrated inoculating recruits is unlikely to enhance survivorship and growth

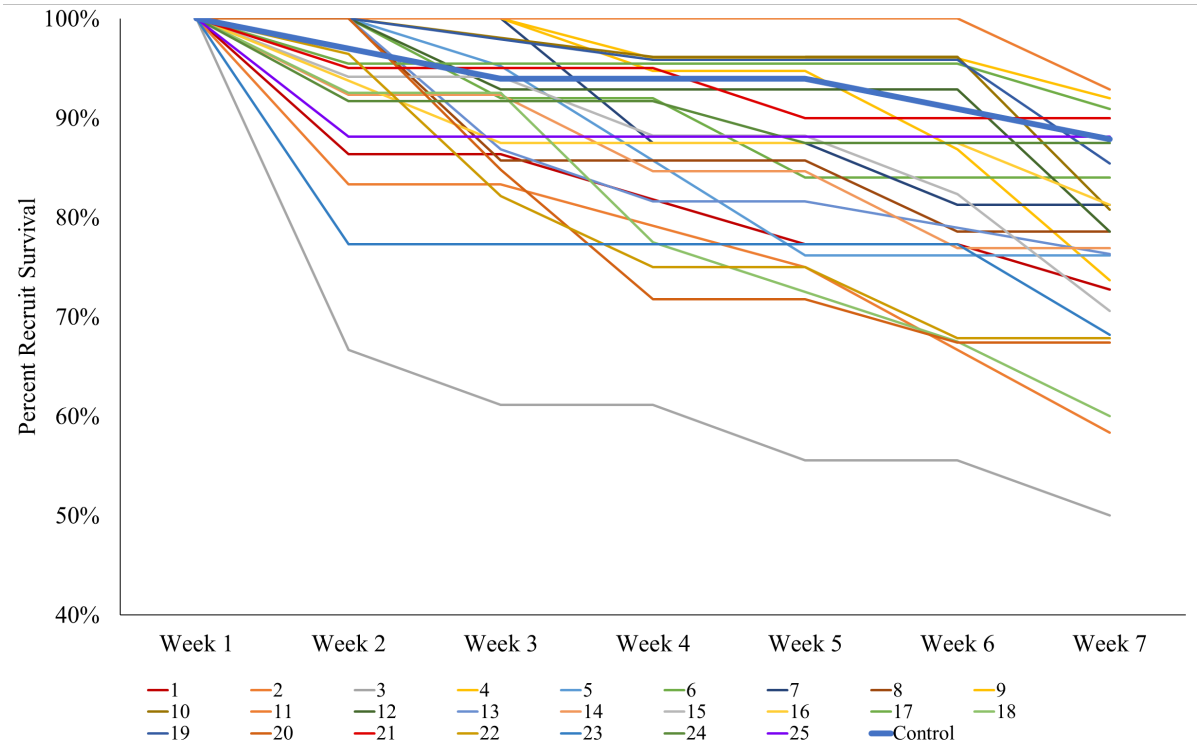

Figure 7. Pseudodiploria clivosa recruit survival over 7 weeks for 25 bacterial isolate groups and the control based on weekly survivorship counts beginning 17–19 days post-spawning.

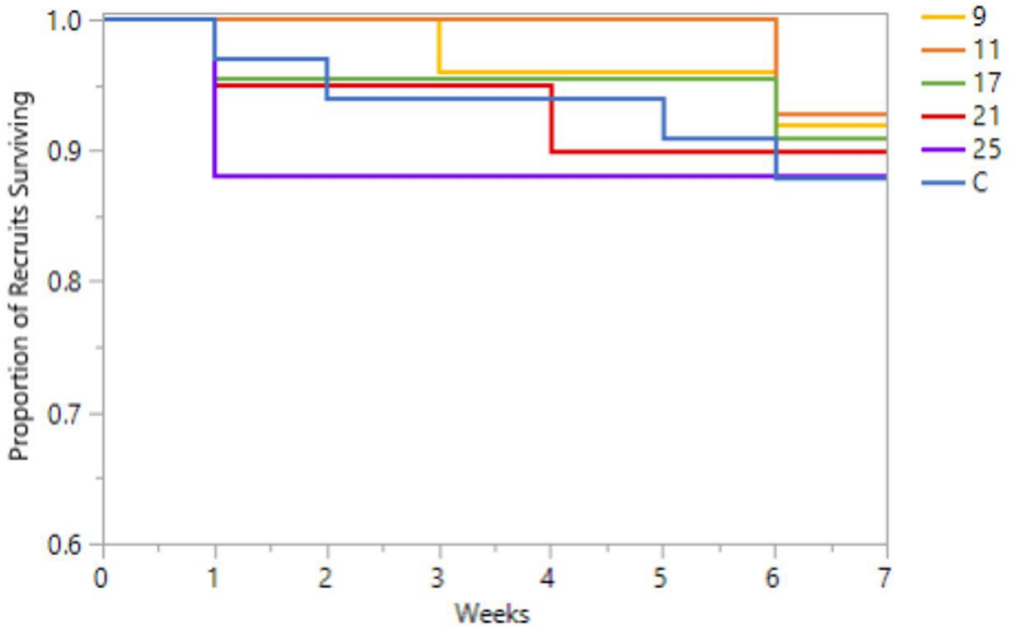

Figure 8. Pseudodiploria clivosa recruit survival log-rank survival analysis (p=0.9866) for the 5 bacterial isolate groups where survival lines were higher than or equivalent to the control based on weekly survivorship counts beginning 17–19 days post-spawning.

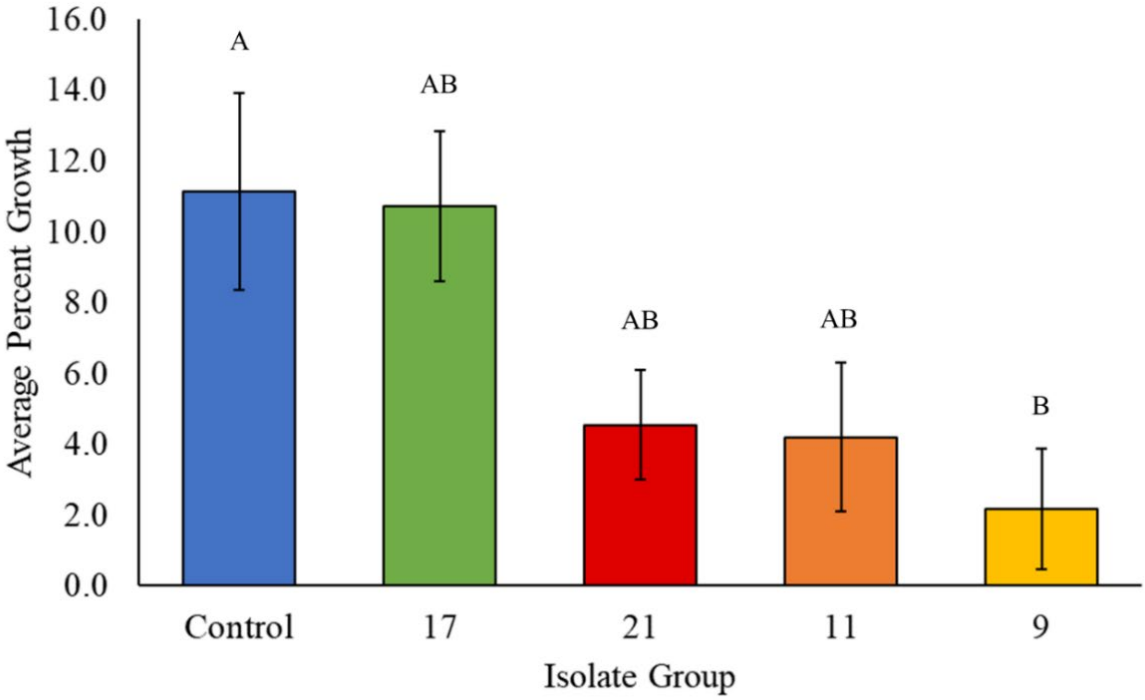

Figure 9. Pseudodiploria clivosa recruit percent growth after 7 weeks of bacteria inoculations for the 4 isolate groups with the highest survival. There were significant differences between the isolate group 9 and the control (One-way ANOVA, p=0.0172). Tukey's post hoc letters denote significant difference.

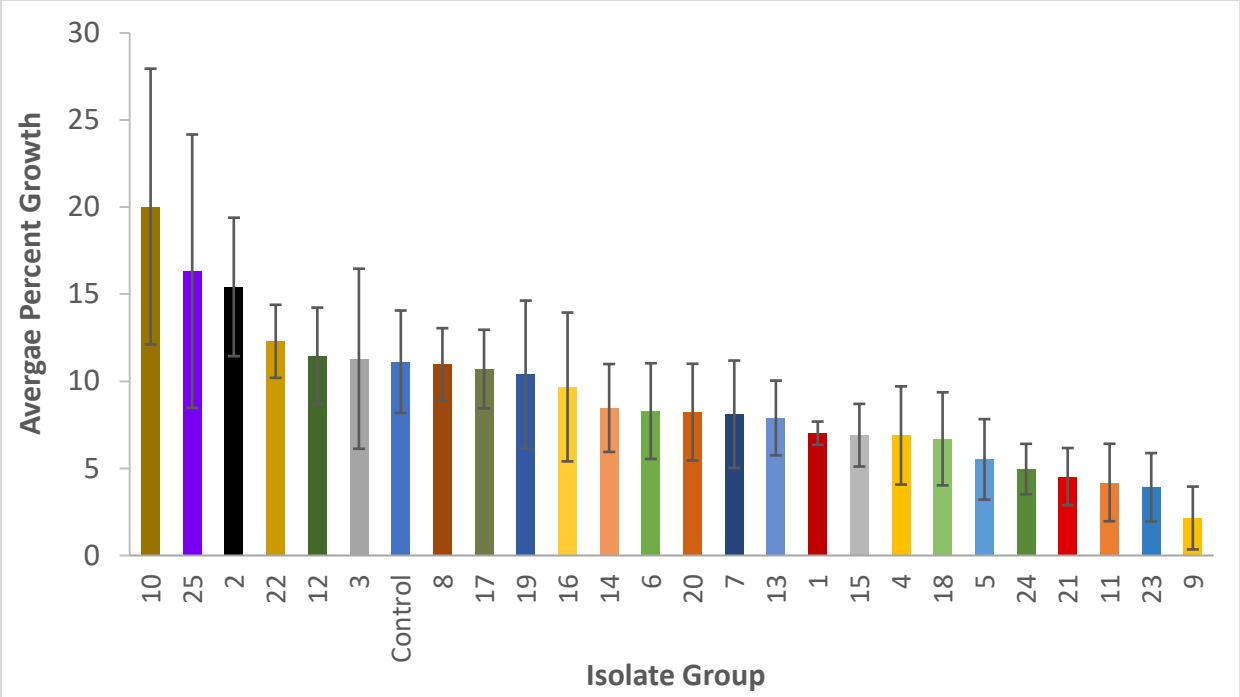

Figure 10. There was no significant difference between all isolate groups (ANOVA p>0.05). Likewise, each of the 3 highest growth isolate groups (2,10,25) were not significantly different than control.

Summary and Recommendations

In 2021 we isolated 250 bacterial strains of *O. faveolata* and *P. clivosa*. A total of 25 isolate groups each with 10 strains of bacteria were tested on *P. clivosa* larvae and recruits. We found 3 isolate groups that significantly increased larval survival over the control. None of the isolate groups enhance survival or growth of 2–9 week-old coral recruits (note, in this second experiment larvae were not inoculated). Based on these data, it seems that some bacterial isolates may be beneficial during the larval period; however, these putative probiotics may benefit recruit survival and growth as well, if inoculated as larvae prior to settlement. This initial screening allowed us to refine our methodology and identify potential probiotics, and we are poised to continue this work for this upcoming spawning season.

For spawning 2022, we will identify the 30 bacterial isolates acting as a putative probiotic. We also propose to inoculate *P. clivosa* larvae with more replicates of the 3 putative probiotic groups previously tested and test these 30 strains in smaller groups, if not individually to narrow which isolate might be driving the enhanced larval survival. We will also settle larvae dosed with the isolate groups to determine if early exposure to probiotics enhances recruit survival and growth. Corallite size (circumference converted to surface area) will be used for growth and measured using CellSens software. If other groups with the ability/expertise to conduct the microbiology and larval experiments want to pursue probiotics, we suggest using a coral with multiple spawning months, such as *Diploria labryrinthiformis*, to quickly test putative bacterial strains during consecutive months.

Task 3: Chimera formation during recruit and adult stages (State of Florida Restoration Priority 3.3)

Although genetic diversity is often viewed as the number of individuals with unique genotypes in a population, genetic diversity can occur within a single individual called a chimera. In corals, chimeras can form when 2 or more recruits or adults (each originating from a single zygote) fuse to create a colony with multiple genotypes. Fusing after settlement allows the colony to quickly increase in size and escape size-dependent predation and grazing.

Figure 11. Evidence of early (<10 weeks) chimeric formation in P. clivosa.

Chimeras have been suggested to aid coral resilience through a more varied response to stressors and offer a competitive edge. Multiple genotypes in a single colony through chimeric formation can be advantageous to combat future environmental changes and emerging disease, in addition to enhanced sexual reproductive success. For instance, if one of the genotypes within a chimera is resistant to a new disease or thermal stress, then it increases the likelihood that at least part of the colony will survive. Additionally, for sexual reproduction to be successful, more than one genotype is needed. Chimeras may enhance fertilization because unique genotypes are next to each other, which increases the probability of egg-sperm interactions and fertilization success through outbreeding. Despite the potential benefits there is much we do not know about chimeras. However, researchers are beginning to explore chimeric formation as an exciting restoration tool to enhance genetic diversity. The question of whether chimeras impart greater fitness at early life stages is also relatively understudied, although they show promising survivorship when challenged with disease (Williamson et al., preprint). By determining how to enhance the fusion of multiple genotypes, we can produce chimeras in the lab for the goal of outplanting these corals to the reef. The enhanced genetic diversity will likely lead to improved survival and possibly growth.

a. **Chimeric formation in recruits**

Chimera formation occurred naturally when recruits were <10 weeks old (Fig. 11). The question remains is if they can form chimeras as the recruit ages and their allorecognition system becomes more established. To examine this, we tried a technique previously used in *Porites* and *Acropora*, where settled *P. clivosa* would be extracted from the tile using a scalpel and glued onto a new substrate. Because of the fragility of *P. clivosa* skeletons, we were unable to use this extraction method without injuring the coral. Instead, we used a Dremel to cut the tile and glue the fragments next to each other. Although gluing 4 recruits in an area seemed promising at first, it was too difficult on a larger scale, so we used recruit pairs instead. For this recruit chimera formation experiment, we setup 39 recruit-pair arrays with 16-week old recruits

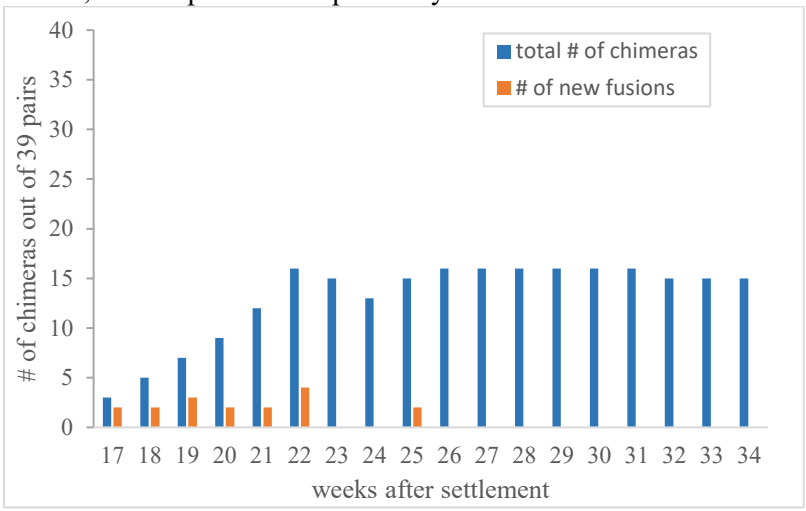

Figure 12. Chimeras formed throughout the experimental period, which was conducted for 18 weeks (17-34 weeks after corals settled).

Throughout the 18-week experiment, 7 of the 78 recruits died from ciliates or from being damaged while handling. All recruit pairs interacted at some point during the 18-week experiment. A total of 17 (44%) pairs exhibited seamless fusion of coral tissue between polyps (Fig. 12, 13). Most of the fusion occurred within the first 6 weeks of the experiment, while the latest fusion occurred at week 9 of the experiment, 25 weeks post-settlement. This supports the notion that *P. clivosa* recruits appear to have a long window of ontogeny (period in which allorecognition is incomplete and chimeric corals can form), thus potentially making them a promising candidate for chimeric coral formation as a restoration tool.

Figure 13. Progressive formation of chimeras in P. clivosa recruits throughout the experiment. Note in week 18 both primary polyps of the chimera are undergoing intratentacular budding and 4 mouths can be seen.

Two allorecognition responses have been observed: suture and overgrowth. Suture is defined as when two corals of differing genotypes secrete a skeletal barrier that separates differing individuals (Fig. 14). Overgrowth often follows suture as the faster growing individual grows over the skeletal barrier and begins to encroach on its neighbor (Fig. 15). This response follows that observed by Chadwick-Furman and Rinkevitch (1994) and Puill-Stephan et al., (2012). In this study, suture has also been observed preceding many fusion interactions. Until the remaining sutured corals have either overgrown each other or completed true tissue fusion, the outcome of the sutured corals is unknown. Currently, 18 pairs (46%) formed a permanent suture between the recruits and nearly all of those eventually had one recruit overgrow the other, yet most of the overgrown corals are still alive (Fig. 15).

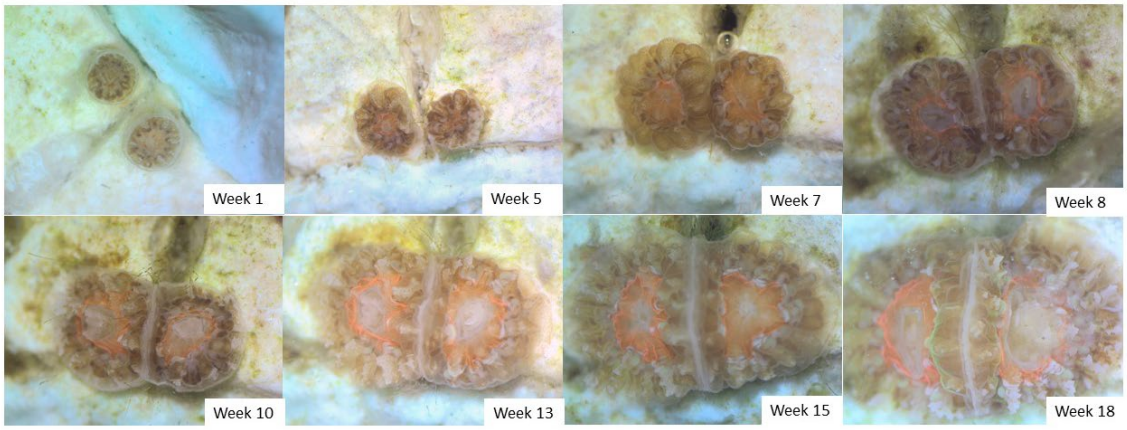

Figure 14. Example of recruit pair array where a suture was formed.

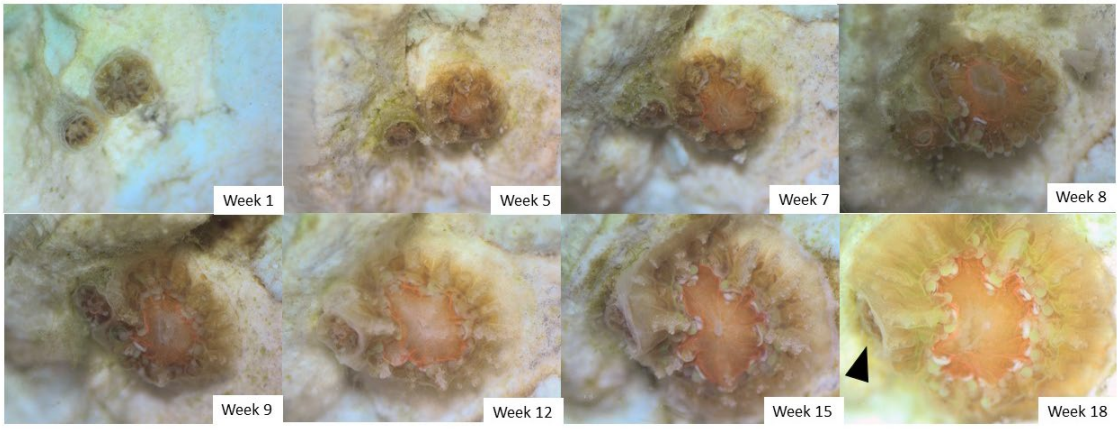

Figure 15. Example of recruit pair array where one larger recruit overgrew the smaller recruit, however it is still alive.

a. **Chimera formation in adults**

Studying chimeras in adults is more challenging because of their slow growth and competitive nature. Yet, a restoration technique, microfragmenting, may allow us to explore chimera formation in adult corals and determine if this deliberate chimera formation may be used in restoration efforts. The process of microfragmentation of adult corals reduces energy expenditure to sexual reproduction and instead allocates that energy to tissue and skeletal growth (Forsman et al. 2015). In this regard, microfragments are a useful way to replicate juvenile physiological behavior.

An adult chimera experiment was set up the first week of February using leftover fragments from the disease excision that occurred in October 2021 (see task 1). These fragments had not showed any signs of disease since being cut from the original diseased colony. There were two arrays in this experiment using 5 genotypes. In the "quad" array we glued four 1.5cm^2 microfrags next to each other. In this array, two of the microfrags were the same clone, while the other two microfrags were different genotypes (n=14 combinations, Fig. 16). This tested if different genotypes fuse, with the monoclonal pairing serving as a control. The second array was similar, but only two microfrags were paired (n=13 monoclonal and n=13 biclonal combinations Fig. 16). This array was designed to test if the potential stress of interacting with multiple genotypes would hinder monoclonal fusion or perhaps even chimeric fusion. Both arrays were examined over 11 weeks.

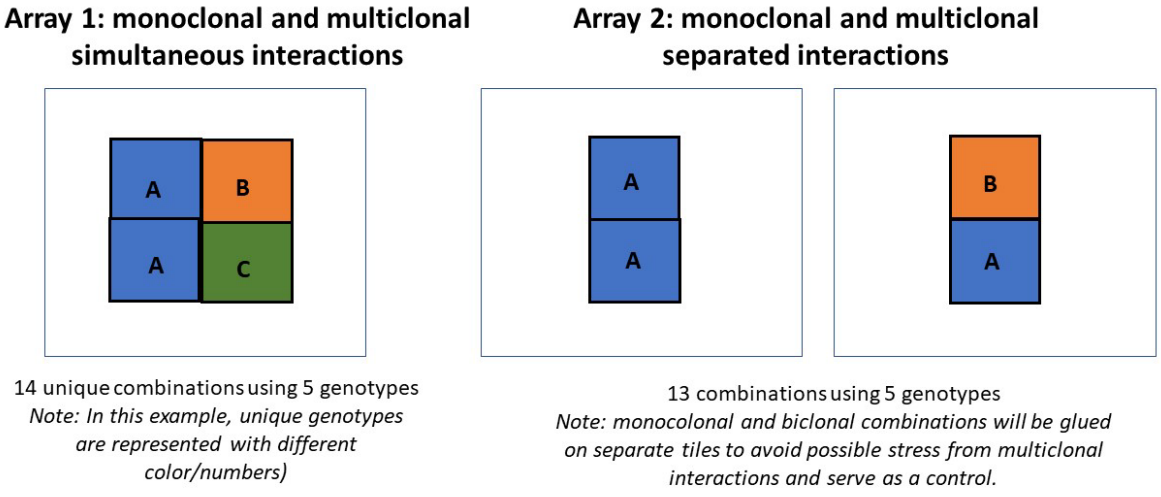

Figure 16. Adult chimera experimental setup with the microfragments. Array 1 is the quad array and array 2 is the separate monoclonal and biclonal pairings.

Figure 17. Aggressive interactions between genotype 10 (right) and 11 (left) using mesenterial filaments.

In both microfragment experiments, fusion occurred in nearly 100% of same genotype interactions. There were 7 outcomes for the adult microfragment arrays: (1) no interaction, (2) mesenterial filaments (no visible signs of aggression- Fig.17), (3) aggression (signs of tissue

recession-Fig.18), (4) tissue regression, (5) overgrowth by another genotype, (6) death, and (7) fusion (Fig. 19). For almost all multiclonal interactions, responses have been aggressive. More time is needed to determine the impacts of the different genotypic pairings, as mesenterial filament aggression has been observed frequently with accompanying mortality in the "losing" genotypes. *Orbicella faveolata* do not only appear to rely upon stinging the opposing coral's tissue, but rather their biomass. The aggressor directs large concentrations of mesenterial filaments into the openings of the opposing coral and seemingly extracts tissue and/or nutrients from inside the polyp using their mesenterial filaments (Fig. 17). This interesting observation should be a prime area for future research. Corals that have been the victims of this form of mesenterial filament attack have experienced tissue loss and recession as a result.

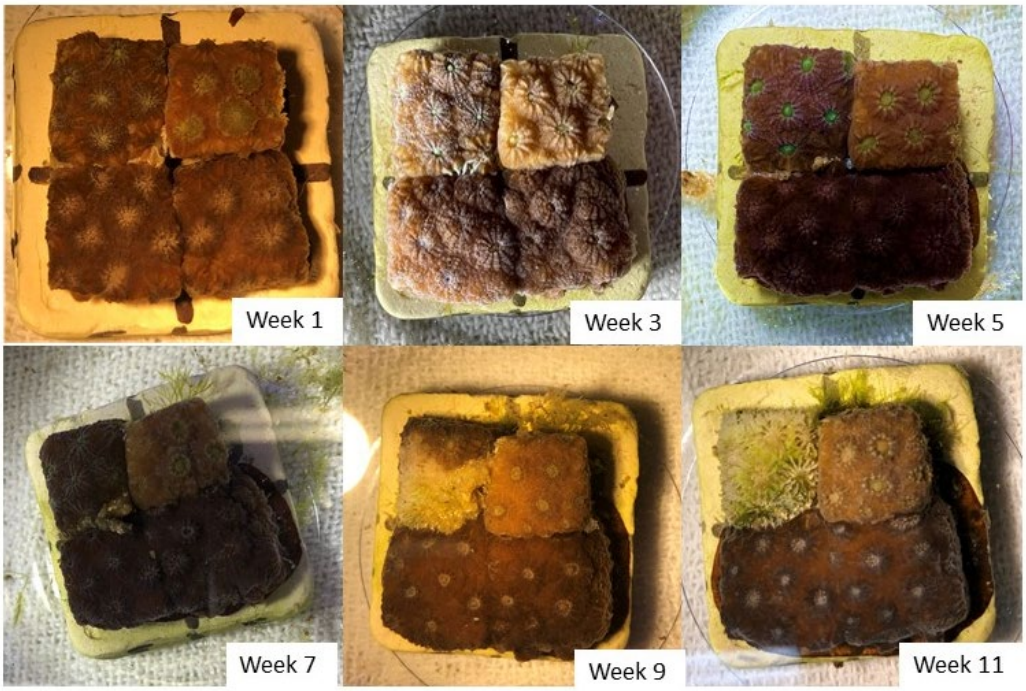

Figure 18. Quad array example of aggression between the monoclonal and the other 2 genotypes.

The results of the quad and pairing arrays often did not have a consistent outcomes or genotypic response. Of the 52 corals in the pairing array, fusion, regression, and aggression stayed consistent, while mesentaries waned and overgrowth and death increased (Fig. 20a). All 13 of the monoclonal pairs fused. Of the 56 corals in the quad arrays, 22 corals had fused, all of which were the monoclonal pairing. Genotype 11 is the only one which did not fuse with another clonemate; however, in the pairing array all 3 replicates of the monoclonal pairings fused. The frequency of individuals for each outcome varied over time. Like the recruits, most fusions occurred with the first 6 weeks of the experiment. Mesenterial filament, aggression, and tissue regression waned over time as the number of deaths and overgrowth increased. Overall, there were few times where the corals of different genotypes showed no interactions (Fig. 20b).

In the pairing arrays, genotypes 8 and 10 were the most aggressive, genotypes 5 and 9 were less aggressive, while the response of genotype 11 varied. In the quad array, the fused monoclonal pair often attacked the other 2 genotypes. This likely left little energy for the abutting biclonal pairs to fight each other. At the end of 11 weeks in quad array, most of the biclonal pairings were either dead or the tissue had receeded (Fig. 18). This suggests that an additional benefit of chimera formation is enhanced competitive ability. In our preliminary observations, a

well-developed allorecognition system appears to be developed in *Orbicella faveolata* adults with no chimeric formation occurring between microfragment genotypes. Monoclonal fusions are ubiquitous across genotypes. As aggressions are prolonged mortality increases.

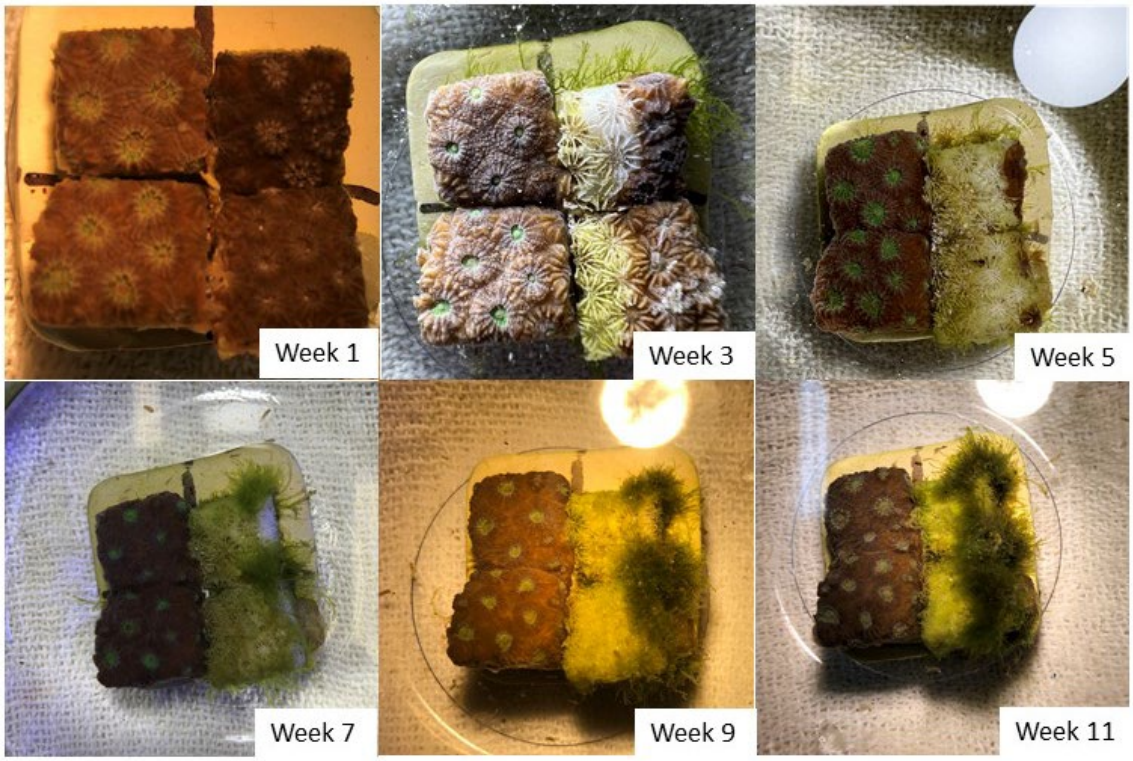

Figure 19. Example of fusion between fragments of the same genotype in a quad array.

Summary and Recommendations

This study found that *P. clivosa* recruits will continue to make chimeras until 6 months; presumably at this point their allorecognition systems have become fully developed. Chimeras can be created in recruits through dense settlement or positioning the recruits next to each other within the first 6 months. Creating chimeras for restoration may create more robust outplants, where at least partial survival is more likely after a disturbance (heat stress, cold stress, disease, etc.). Studying chimeras in adults is more challenging because of the slow growth and competitive nature from a fully developed allorecognition system. We did not successfully create chimeras using microfragmenting techniques; however, our "controls" of monoclonal pairs did fuse. Exposure of the arrays to various stressors which can be easily replicated in aquaria, such as thermal stress, may inhibit or at least weaken the allorecognition responses and therefore promote or accelerate chimeric fusion between adult microfragments. *We plan to pursue a pilot experiment subjecting chimeric arrays created through microfragmentation to thermal stress to determine if allorecognition can be subdued until chimeric fusion. Once (if) fused, the stressor will be slowly returned to ambient temperature.*

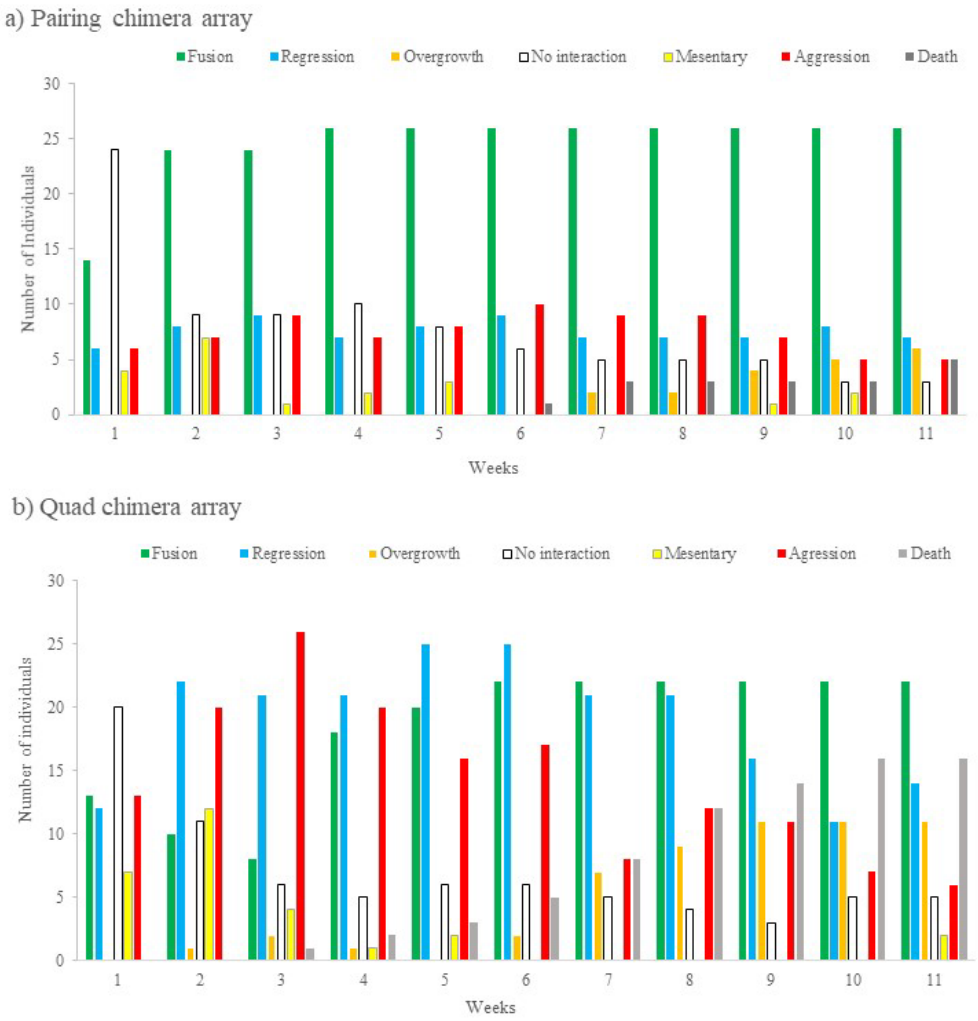

Figure 20. The number of individuals that display each of the potential outcomes in the (a) paired chimera array and (b) quad chimera array.

Social Media Information

Dr. Fogarty and the Coral REEF lab at the University of North Carolina Wilmington have been busy over the past year with aquaria-based coral spawning. They successfully spawned 2 species of corals, *Orbicella faveolata* and *Pseudodiploria clivosa*, that were maintained in their system for over a year. This is exciting because it means the corals completed their gametogenic cycle in their laboratory! The resulting gametes were fertilized and over 300,000 larvae were reared. Larvae and recruits from these efforts were inoculated with bacterial isolates to screen for potential probiotics. They found that 3 bacterial isolate groups increased larval survivorshiop. Additionally, the Coral REEF lab tested if chimeras (where more than one genotype fuses to form a single colony) can be created in the laboratory. If a colony consists of many traits, such as disease resistance or thermal stress, then it will be more likely to survive. They found that *Pseudodiploria clivosa* recruits can form chimeras for 6 months after settlement. These research projects have exciting implications for restoring Florida's coral reefs.

- Social media photos can be found below and downloaded here: https://uncw4-my.sharepoint.com/:f:/g/personal/fogartyn_uncw_edu/EvcZB-rmnzBKluZ_NskQDSUBxOWtC4fp24YiEcNV1r0WKQ?e=a1vB56

Supplemental Images

Figure 21. Raceway with corals in the UNCW SEAS (Spawning and Experimentation of Anthropogenic Stressors) facility

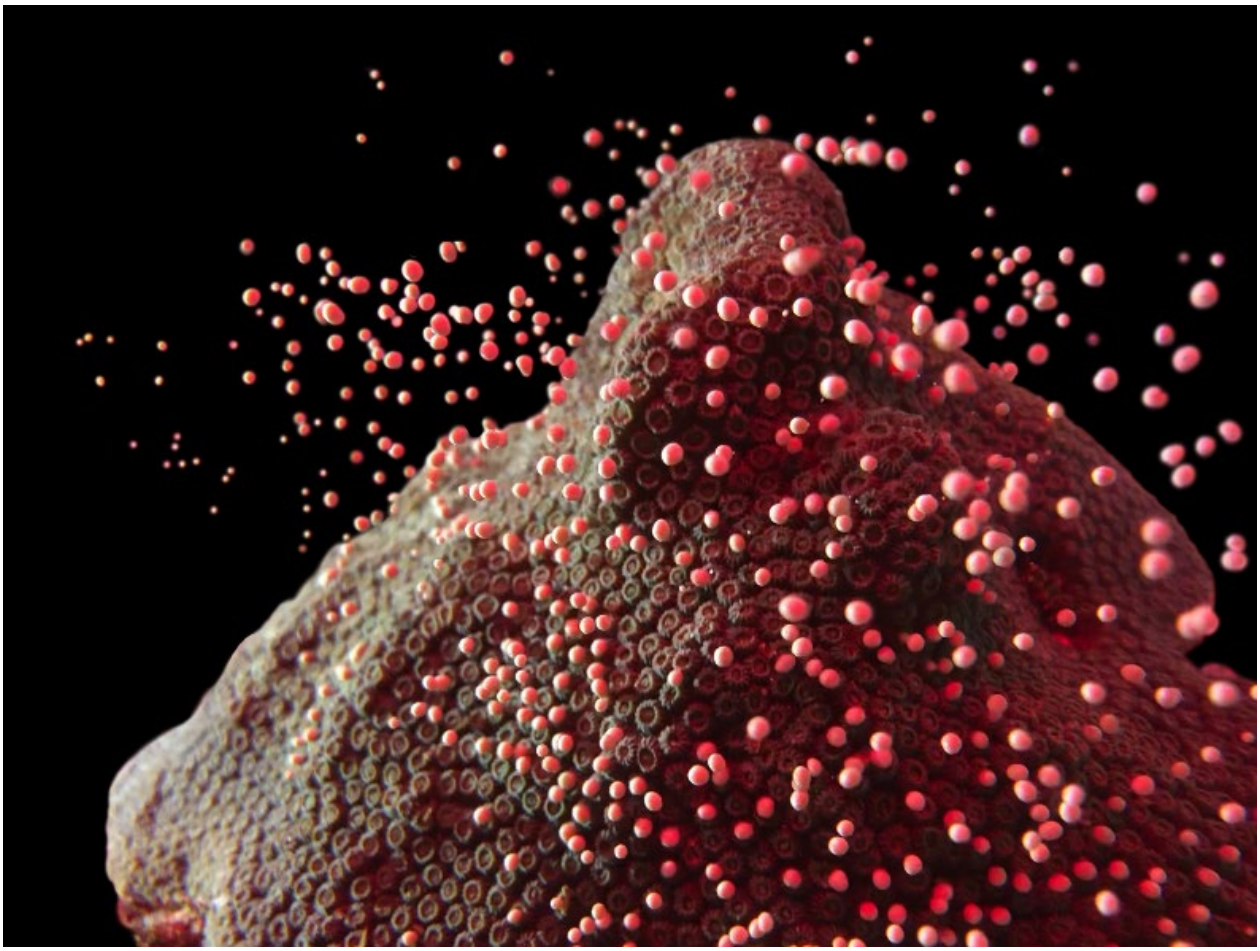

Figure S2. Orbicella faveolata spawning in the Fogarty lab.

Figure S3. Recirculating trays used to keep temperature constant during fertilization and settlement tiles cleaning and scoring.

Figure S4. Larval cones that recirculate in our sump.

Figure S5. Bins with mesh windows (100μ) used for larval rearing and settlement.

Figure S6. Pseudodiploria clivosa recruit on our settlement tile.

This report is submitted in accordance with the reporting requirements of the above DEP Agreement number and accurately reflects the activities associated with the project.