Improving and expanding the portfolio of grazers available for coral co-culture and reef restoration

Improving and expanding the portfolio of grazers available for coral co-culture and reef restoration

Final Report

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Management Summary and Recommendations

Recommendations from this work are to begin incorporating *Cerithium* snails in concert with *Lithopoma* in co-culture of juvenile corals and coral recruits. *Batillaria* snails were effective as a minor component when used with *Cerithium*, but not in other combinations or alone. When small juvenile urchins are available, it may be possible to use *Tripneustes ventricosus* in coral co-culture even at relatively high densities to achieve positive results. Care should be taken when using an invertebrate grazer in coral co-culture, especially of very small coral recruits, so that overgrazing does not occur. This was likely observed with *Diadema antillarum* in the urchin co-culture experiment. To that end, next steps should include the collection of additional data on grazer size and growth rates to facilitate better prediction of overgrazing. Grazers that did not perform effectively within this project may do well at different sizes or densities and thus refinement of these parameters is a logical next step. Further development of spawning techniques for *Lithopoma* snails and *Tripneustes* urchins also appears warranted. Other effective grazer species also likely exist, and further expansion of this portfolio would be valuable. Finally, given the limitations of *Meandrina meandrites* larval production and post-settlement survival identified in this project, further rearing research – possibly including grazers – is needed before this species can be sexually propagated at scale.

Executive Summary

This project demonstrated the utility of invertebrate grazers in co-culture with sexually propagated SCTLD-susceptible corals. While almost all grazers tested resulted in reduced algal cover, specific grazers and assemblages resulted in increased growth of small *Pseudodiploria strigosa* (PSTR) colonies or *Diploria labyrinthiformis* (DLAB) recruits. Because every land-based system is different, one interesting outcome was that similar grazer assemblages (2/3 *Cerithium* snails in combination with 1/3 *Batillaria* or 1/3 *Lithopoma*) resulted in the best growth of DLAB recruits when identical experimental designs were applied in both Miami and the Lower Keys. Among other things these systems had major differences in water and light source, two of the primary parameters that affect coral and algae growth. This information provides hope that it may be possible to extrapolate species-specific differences in biological grazing activity outside the system in which an experiment is conducted. Beyond snails, a separate experiment using three different species of cultured sea urchins at similar densities in co-culture with *P. strigosa* corals revealed species-specific differences in their effects on coral growth rate but not survival. The current project also revealed some limitations in our current ability to produce certain grazers (*Lithopoma americanum* snails and *Tripneustes ventricosus* urchins) and *Meandrina meandrites* (MMEA) corals via aquaculture, as well as the need for further investigation to refine grazer species, sizes, biomass, and density used in coral co-culture. Ultimately, results contribute to coral reef conservation broadly and SCTLD response specifically because they advance the field of coral sexual propagation in land-based systems (in this case focused on SCTLD-susceptible corals), which represents an important and increasingly viable means of rebuilding levels of genetic diversity that have been lost in recent times. Significant resources have been dedicated to holding and spawning Florida corals in land-based systems, and this work improves the return on that investment.

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Table of Contents

List of Figures

Figure 8. *M. meandrites* recruits from September 2020 on February 25, 2021, just under 5 months post-settlement.

Figure 9. Mean *P. strigosa* individual colony size over time when coral plugs were co-cultured with three different species of juvenile urchins. Three replicate aquaria with 10 coral colonies each were used for each treatment and urchin densities were reduced from 25 to 15 individuals per replicate at day 42. Data are presented as mean ± SEM.

Figure 10. Survival of *P. strigosa* colonies over time when coral plugs were co-cultured with three different species of juvenile urchins plus a control. No significant differences existed among treatments (Kaplan-Meier $X^2 = 0.05$; d.f. = 3, $p = 0.9$).

Figure 11. Representative images of individual replicate tiles exposed to various snail grazer assemblages at days 0, 32, and 70 of the experiment.

Figure 12. (Left) Schematic of one complete replicate of experimental grazer assemblages established for experimental treatments. (Right) Mean change in live area (%) for colonies of *Diploria labyrinthiformis* colonies after ~70 days under different experimental grazer assemblage treatments. Larger solid dots represent means, smaller opaque dots represent raw data values. Error bars are ± SE.

Figure 13. (a) Algal biomass on each tile at the conclusion of the experiment regressed against grazer biomass present for that tile. Only tiles with > 0 g of algal biomass included. (b) Mean change in live area *D. labyrinthiformis* (cm^2) at the conclusion of the experiment regressed against algal biomass. (c) Mean Change in live area (cm^2) of *D. labyrinthiformis* regressed against total biomass of grazers present. Statistics from linear regression.

Figure 14. Representative images of individual replicate tiles exposed to various snail grazer assemblages at day 0 and day 70 of the experiment

Figure 15. Living tissue area increase of *Diploria labyrinthiformes* recruits exposed to various grazer assemblages and without grazers.

List of Tables

Table 4. Mean maximum diameter of *Meandrina meandrites* recruits reared from 2018-2021 spawning.

List of Acronyms

CCA – Crustose coralline algae

CIMAS – Cooperative Institute for Marine and Atmospheric Studies

CPRP – Coral Protection and Restoration Program

DCYL – *Dendrogyra cylindrus*; Pillar coral

DEP – Florida Department of Environmental Protection

DLAB – *Diplorida labyrinthiformis*; grooved brain coral

IC2R3 – Mote's International Center for Coral Reef Research & Restoration

KCl – Potassium chlorida

MMEA – *Meandrina meandrites*; maze coral

NAFM – Nights after full moon

NOAA – National Oceanographic and Atmospheric Administration

PSTR – *Pseudodiploria strigosa*; symmetrical brain coral

SCTLD – Stony Coral Tissue Loss Disease

TFA – The Florida Aquarium

1. DESCRIPTION

Project objectives fall under two goals: (1) build knowledge to develop scaled sexual propagation of grazers and *Meandrina meandrites* corals, and (2) evaluate the ability of grazers to cultivate a benthic community composition favorable to settlement, survival, and growth of SCTLD-susceptible corals. The outcomes of this project will be incorporated into an on-going coral disease response effort that seeks to identify management actions, remediate disease impacts, and **restore affected resources** among other outcomes.

This project is fulfilling priority recommendations highlighted in the "State of Florida Restoration Priorities for Florida's Coral Reef: 2021-2026" report. This project addresses both priority 3.5 '*Research and Development in Support of Land-based and In-water Coral Propagation and Rearing'* as well as priority 6.1 *'Propagation of Reef-Associated Species*'.

The majority of grazer/coral co-culture work to date has been conducted on Indo-Pacific species or with grazers obtained from wild collections. Here, we propose to gather critical information on the reproductive biology and spawn induction methods needed to further develop propagation programs for native grazers that are commonly used in Western Atlantic coral propagation. In addition, we will test the impact of grazers on post-settlement survival of two coral species identified as high-priority rescue species by the Coral Rescue Team. During the Coral Rescue and Propagation Workshop in October 2021, *Diploria labyrinthiformis* and *Pseudodiploria strigosa* were identified as a priority species for scaling-up sexual propagation, while *Meandrina meandrites* was identified as a priority species for additional research on propagation techniques before scale-up.

1.1. Goal 1: Build knowledge to develop scaled sexual propagation of grazers and *Meandrina meandrites* corals

Objective 1 – Describe the gametogenic cycle and reproductive biology of *Lithopoma* snails.

Rationale: Lithopoma snails are proven as a valuable grazer. However, small juveniles are ideal for new coral recruits and are difficult to collect from the wild.

Objective 2 – Test spawning methodologies on *Lithopoma* snails and *Tripneustes /Lytechinus* urchins.

Rationale: In addition to *Lithopoma* snails, juveniles of these two urchin species may help coral recruits. Previous anecdotal work suggests that larviculture of all three species is relatively straightforward, while reliable spawning has presented a challenge.

Objective 3 – Compile historical *Meandrina meandrites* spawning observations and produce spawning predictions for Florida; describe larval development, settlement, survival, and growth to date in ex situ culture.

Rationale: The reproductive timing of this species has only recently been described through ex situ spawning efforts at The Florida Aquarium and is not widely known by practitioners monitoring for spawning in the wild. There is currently no published information on fertilization, larval development settlement, early survival, or growth. This objective will gather critical biological information needed to expand larval rearing efforts in this species.

1.2. Goal 2 – Evaluate grazers' ability to cultivate a benthic community composition favorable to settlement, survival, and growth of SCTLD-susceptible corals

Objective 4 – Compare survival and growth of *Pdeudodiploria strigosa* recruits produced in 2022 and grown with or without invertebrate grazers.

Rationale: This high-priority species for scaled production has been a consistent spawner with relatively low post-settlement survival. We hypothesize that invertebrate grazers may improve outcomes.

Objective 5 – Assess the ability for different grazer assemblages to promote benthic communities that facilitate coral success across *ex situ* systems.

Rationale: Grazers may affect the benthos in many ways and every *ex situ* system is different. Measuring grazer assemblage effects across *ex situ* systems differing in light and water source (artificial lighting and UV-treated flow-through seawater sourced from Bear Cut, Miami at the UM facility vs. natural lighting and mechanically filtered, UV-treated flow-through seawater sourced from nearshore waters on Summerland Key, FL at Mote's IC2R3) may allow us to expand the inference space regarding general benthic algal community dynamics.

Objective 6 – Test various grazer assemblages' ability to increase survival and growth of *Diploria labyrinthiformis* recruits across *ex situ* systems.

Rationale: Complementary and/or antagonistic effects of grazer species and sizes is an understudied and emerging area of interest. Testing a common suite of grazer species with the same coral species across multiple ex situ systems is a robust means to address these questions.

1.3. Reef Management Application

Outcomes of this project have multiple potential applications for improved reef management. New knowledge, techniques, and capabilities generated by this project may aid restoration efforts and/or be applied to increase coral resilience through:

- Improved ability to propagate invertebrate grazers for *ex situ* production of coral recruits and understanding of which grazers to apply in which scenarios.
- Increased future availability of PSTR and DLAB sexual recruits to restoration practitioners.
- Algal mitigation at existing or future restoration sites where traditional outplanting or natural/induced spawning is targeted/anticipated.
- Methods that could be applied in the field to areas affected by acute disturbances that shift the phase dynamics of a reef in favor of algae - i.e., 2010 cold snap(s), bleaching events, coral disease - to avoid algal dominance.
- Herbivore assemblages that may be used to "prep" sites with appropriate settlement habitat for larval seeding or natural recruitment events to increase the rate of coral settlement and recruitment.

2. METHODS

The purpose and intended use of the data generated by the described activities are to inform regional and local management, specifically active restoration activities, aimed at improving the health and resilience of Florida's Coral Reef. Activities detailed herein were conducted under the advisement of relevant groups associated with, and staff of, the Florida DEP Coral Protection and Restoration Program. This was done to ensure that methodologies were not duplicated, best practices were employed, and project results were effectively communicated to all stakeholders. All required state and federal permits were obtained prior to the work beginning.

2.1. Task 1 – Describe reproductive biology of *Lithopoma* snails

For the period spanning July 2022 through May 2023, we worked with a marine life collector in the Florida Keys to obtain near-monthly overnight shipments of small lots (10 – 30 animals per lot) of recently- and locally-collected *L. americanum* snails. We were only unable to obtain shipments in September and March (February shipment was processed on March 1). Each month, snails were shipped directly to Dr. Stephen Geiger's molluscan fisheries lab at the Florida Fish and Wildlife Research Institute (FWRI) in St. Petersburg. Upon receipt, size and weight data were collected on each individual before dissection and fixation for histology. Histological sections were cut at FWRI and stained with hematoxylin and eosin to visualize cell nuclei, cytoplasm, and extracellular matrix.

Upon initial visual inspection of histology slides, gonads were located and a scoring system for the stages of gametogenesis was developed (**Figure 2**). Gonad color was also noted upon gross visual inspection following dissection. Based on the information collection, each individual snail was assigned a sex (or immature). These data were used to estimate sex ratio and size at sexual maturity for this species.

2.2. Task 2 – Test *Lithopoma* snail and urchin spawning induction

2.2.1. Attempted Spawning of Lithopoma americanum

270 total adult *L. americanum* (median shell width 2 cm), maintained in 450-L fiberglass tanks within a recirculating aquaculture system, were used as broodstock in spawning trials. Spawning was attempted on four occasions (10/18/22, 10/25/22, 11/2/22, and 03/30/23) using five different stimuli: 1) hourly increases in temperature, increasing from 21 to 32 ℃; 2) hourly decreases in salinity, decreasing from 35 to 25 ppt; 3) exposure to UV-irradiated seawater (irradiated for 180 minutes prior to trials); 4) desiccation (with one hour of emersion); and 5) addition of 8.2 mM hydrogen peroxide. Temperature and UV stimuli were effective in inducing spawning for *Lithopoma (Astraea) undosa* as described by Salas-Garza et al (2009). Desiccation, hydrogen peroxide, and UV exposure were used following methods Velasco and Barras (2017) for spawning in *Cittarium pica*. Spontaneous gamete release occurred in previous months and was often correlated with water changes involving exposure (desiccation) periods.

Three replicate 1.7 L containers were used for five treatments and a control. Containers were maintained with static, aerated 35 ± 1 ppt salinity and 23 ± 1℃ seawater (except for temperature and salinity treatments). Each container held 15 randomly selected individuals. Individuals were exposed to treatments for 3 h, after which time the water in containers was replaced with 35 ± 1 ppt salinity and 23 ± 1℃ seawater. Snails were observed for 3-4 hours following treatment, left in containers overnight, and returned to their tanks the following day.

2.2.2. Attempted Spawning of Lytechinus and Tripneustes urchins

A total of 9 *Lytechinus variegatus* and 8 *Tripnesutes ventricosus* were purchased from a marine life collector in the Florida Keys. The animals were accessioned into systems at The Florida Aquarium's Center for Conservation and maintained within recirculating systems housing corals and other urchins. Spawning was attempted on four occasions (04/19/23, 04/26/23, 05/03/23, and 05/10/23) using three different stimuli: 1) acute transfer to water 5°C warmer than ambient holding water (used effectively with *Diadema* and described in Pilnick et al. 2021); 2) coelomic injection with 0.5M KCl at 0.01mL per gram body weight, which is a standard method in urchin aquaculture; and 3) coelomic injection with 0.1M acetylcholine at 0.2mL per urchin as described for *T. ventricosus* in Guete-Salazar et al. 2021. Injections were administered by Florida Aquarium veterinarians. For thermal induction, 2-3 urchins of each species were placed in separate 5-gallon buckets of water brought to temperature with an immersion heater. Both buckets were closely monitored for spawning activity and urchins were returned to holding systems after 45 minutes. For injection trials, urchins were placed in individually labeled small buckets following injection and closely monitored for up to 45 minutes. When a spawn occurred, gamete identity (sperm or eggs) was determined and that animal was placed back in the holding system. To the greatest extent possible, we avoided repeating the same treatment on a given animal in consecutive weeks.

2.3. Task 3 – Describe *Meandrina meandrites* spawning and in land-based systems

Meandrina meandrites (MMEA) colonies collected from Florida's Coral Reef as part of the Florida Coral Rescue project were monitored for spawning starting in the fall of 2019 through the fall of 2022. These colonies were collected from the Lower Keys in October of 2018, and brought to The Florida Aquarium (TFA) Center for Conservation in Apollo Beach, Florida in December of 2018. This report summarizes observations of the spawning behavior of MMEA in ex-situ systems. Most of the spawning observations at TFA have been done in spawning systems with LED lights set to mimic the conditions found in the Florida Keys (Key Largo) as described in O'Neil et al. 2021. In greenhouse holding conditions, several male colonies have been observed to release sperm however no female colonies have been observed releasing eggs in greenhouse conditions and no larvae have been produced in the greenhouse. In addition to spawning observations, historical data on MMEA fertilization, larval production, and survival were compiled. Finally, a census of existing MMEA recruits on-hand at TFA was completed.

On nights when both male and female colonies spawn, we collect gametes to create larvae. Eggs of this species are not very buoyant and take a long time to float to the surface for collection. All water flow must be turned off and water not disturbed for eggs to come to the surface. During this time, eggs are exposed to sperm in the water column in the tank and fertilization occurs in the system. Eggs and sperm are left in the system for approximately 40 minutes to allow the eggs to fertilize and float to the surface. In normal spawning conditions for other species, the target sperm concentration is 1x10^6 cells/ml. However, MMEA usually do not release enough sperm into the system to reach that desired concentration.

Larvae are moved into settlement bins between 24 and 36 hours after spawning, after the swimming larvae begin to swim towards the bottom of the rearing container. Before they can be moved, we count the larvae so we can calculate the settlement percentage after the larvae settle. To count the larvae, we gently combine them into a sterile pitcher with a known volume of water. We gently homogenize the larvae in the pitcher and take a subset sample of a known volume that contains about 100 larvae, usually about 30mL. The subset is counted and the process repeated two more times to get an average number of larvae in the sample volume. The average is divided by the sample volume to get the number of larvae per mL, and then multiplied by the volume of the pitcher to get the number of larvae in the pitcher.

2.4. Task 4 – Evaluate *Pdeudodiploria strigosa* growth and survival with/without grazers

An experimental recirculating system was established in a TFA greenhouse. A total of 12 replicate tanks were randomly assigned one of four grazer treatments (n=3 replicates per treatment): *D. antillarium*, *T. ventricosus*, *L. variegatus*, or a no-urchin control. For each

treatment with grazers, 25 small, cultured juveniles of each species were included per replicate tank. Due to urchin growth, this density was reduced to 15 urchins per tank on day 42 of the experiment. Each replicate tank contained 10 separate coral plugs with individual ~5-month-old colonies of PSTR. The experiment was run for 105 days, with regular sampling intervals every 21 days. At each sampling interval, all tank surfaces except for the coral plugs were manually cleaned of algae and treatments were re-randomized to control for any potential tank effects due to differences in lighting. Each set of sea urchins and coral plugs were maintained as a paired grouping throughout the experiment. At the same time as tanks were re-randomized and manually cleaned, photographs of all individual coral plugs were taken on days 1, 21, 42, 63, 84, and 105. Image J was used to obtain coral colony size and survival data. Coral Point Count Estimate software was used to generate algal percent cover and species diversity.

2.5. Task 5 – Expose tiles to multiple grazer species and size classes at CIMAS

Plumbing for temperature control of flow-through seawater and setup (lights, probes, filtration, pumps, etc) of experimental tanks was completed for 24 experimental tanks. Tanks were partitioned into 12 equal-sized compartments; each was assigned 1 of the 11 experimental treatments in a pre-determined design to ensure all treatments were present in all tank locations (the 12th contained plumbing). Lids were constructed to allow for water flow but restrict snails from moving between compartments.

Individuals of the three target grazer snail species (*Batillaria minimia*, *Cerith literarum*/*floridanum,atratum*, and *L. americanum*) were obtained from co-PI Spadaro. Grazers were weighed (wet weight; g), measured (total length, mm), and then were placed into their randomly assigned tank and treatment (n=3 snails per compartment; n = 33 snails per tank; n = 792 snails total). Tiles were exposed to 10 different grazer assemblages (+ control tiles with no grazers) for a period of 69-70 days. At the conclusion of the experiment, all tiles were individually photographed. All snails were weighed and measured at the conclusion of the experiment. Each tile was then scraped of all fleshy algae, which was then dried to a constant weight and weighed to record final algal biomass on each tile. Photographs from all surveys were labeled, cropped, and imported into CoralNet for benthic community composition analysis.

2.6. Task 6 – Expose *Diploria labyrinthiformis* recruits to grazer assemblages at CIMAS

This experiment used a similar tank setup as Task 5and incorporated coral recruits. Colonies of *Diploria labyrinthiformis* (DLAB) ~5 months old were isolated (via bandsaw) from the ceramic plugs they were growing on and were mounted onto a clean ~4 x 4” ceramic tile using superglue. Each tile with DLAB was then photographed and placed into a compartment in every tank (n=11 tiles per tank) for a total of 264 tiles with DLAB.

Tiles were photographed on the following days of the experiment: 0, 20/21, 32, 41, 54, and 68/69. Partitions were cleaned, corals were fed, tanks were siphoned, and snails were

checked 2-3x/week. Water temperature was maintained at a target of 28°C. Final photos of tiles with colonies of DLAB were obtained on Day 68 and 69 at the termination of the experiment. Tiles were photographed with scale bars and these photographs were then analyzed in ImageJ to quantify live tissue area to calculate coral growth rates throughout the course of the experiment. Live tissue area was measured by calibrating ImageJ software to the scale bar in each photo and a freeform shape was digitally drawn around the perimeter of living coral tissue. Image J software then output the two-dimensional area of that shape. After photos were taken, all fleshy algal biomass was scraped from each tile and placed in an individually labeled tin weigh boat. Algal biomass samples were then dried at 60°C to a constant weight, and then weighed for a final dry weight of fleshy algal biomass. At the conclusion of the experiment, all snails were removed and re-weighed and measured. Tile photos were then processed using CoralNet software and a point-intercept method to estimate cover (%) of algae on each tile during each sampling event.

2.7. Task 7 – Expose tiles to multiple grazer species and size classes at Mote Marine Laboratory

Because different *ex situ* systems often have different characteristics that can restrict the sphere of inference on the results of a given experiment, Tasks 7 and 8 were intentionally designed to mimic the experimental design of Tasks 5 and 6, respectively. Conducting the same *ex situ* experiment in multiple settings offers the potential to produce corroborating results – thus improving replicability for practical implementation – or at least better understanding between-system variability. For the experiment at Mote, twenty 76-L tanks were acquired and a single 3.8 cm hole was cut into the end of each to allow for flow-through operation. Each tank was partitioned into 12 equal-sized compartments with perforated PVC sheet. Each compartment, except for the compartment containing the bulkhead fitting and standpipe, was randomly assigned one of the 11 experimental treatments.

Individuals of the three target grazer species (*B. minima, C. literarum*/*floridanum*/ *atratum,* and *L. americanum*) were collected from nearshore habitats in the Lower Florida Keys or purchased from local marine life collectors. Each snail was measured (total length; mm) and weighed (wet weight; g) before being randomly assigned to a treatment group and tank (n = 3 snails per compartment; n = 33 snails per tank; n = 792 snails total).

Tiles were exposed to 10 different herbivore assemblage treatments and a no-herbivore control treatment for a period of 70 days. All tiles were individually photographed initially and at 14-day intervals throughout the experiment. All snails were weighed and measured at the beginning of the experiment. Photographs from all surveys were labeled, cropped, and imported into ImageJ to measure coral living tissue area. These same images will be imported into CoralNet for benthic community composition analysis.

2.8. Task 8 – Expose *Diploria labyrinthiformis* recruits to grazer assemblages at Mote Marine Laboratory

A total of 222 experimental 4 x 4" tiles were prepared with either ~5 month old juvenile colonies of DLAB received from CIMAS or ~12 month old juvenile DLAB colonies settled and reared by Mote scientists. Each experimental tank was haphazardly assigned 11 tiles, one per compartment in all but the compartment containing the bulkhead fitting and standpipe. Tiles were photographed with a scale bar on days 0, 14, 28, 42, and 56. Additional photographs will be taken on days 70 and 84 in the next reporting period. Tank partitions and glass sides were cleaned, corals were fed, tanks were siphoned, and snails were checked for survival 2-3x/week. Temperature was maintained at ~28°C. Coral living tissue area (mm^2) and perimeter (mm) was measured with ImageJ during each sampling point using the digital images and growth rates were calculated for each colony through the duration of the experiment. Tile photos were then processed using CoralNet software and a point-intercept method to estimate cover (%) of algae on each tile during each sampling event.

3. RESULTS

3.1. Task 1 – Describe reproductive biology of *Lithopoma* snails

Sex ratios were nearly 50:50 balanced at 87 males:82 females. Based on observations of histological sections at various stages of gametogenesis and near complete emptying of the gonad following gametogenesis, we believe this species is a complete spawning species exhibiting synchronous gamete development. This means that all gametes develop concurrently and are completely released in a single or series of spawning events. Based on the size at sexual maturity, this species is likely iteroparous (completes multiple spawning cycles during a lifetime) rather than semelparous (spawns once and dies). Size at sexual maturity was smaller than expected, with females exhibiting 50% of the population mature at ~7.1mm shell length and males at ~4.9mm shell length (Figure 1).

3.2. Task 2 – Test *Lithopoma* snail and urchin spawning induction

For *Lithopoma* snails, no spawning occurred during treatment periods, observation periods, or in the overnight period after treatments. All raw data was submitted to DEP with this report (available upon request).

A total of 17 *L. variegatus* or *T. ventricosus* were induced to spawn using one of the injection methods (**Figure 3; Table 1).** Spawning generally occurred within 1-5 minutes following injection at rates and sex ratios like what we have historically observed for *Diadema* in temperature induced spawning. However, temperature induction was not effective for either *L. variegatus* or *T. ventricosus*. *L. variegatus* spawned in response to both potassium chloride (KCl) and acetylcholine, while only a single *T. ventricosus* female spawned following KCl injection. A potential problem with coelomic injection is that it can cause mortality in broodstock. Following four weeks of spawning induction trials, we observed mortality in one *T. ventricosus* individual and two *L. variegatus*, likely due to infection following needle puncture.

3.3. Task 3 – Describe *Meandrina meandrites* spawning and in land-based systems

A summary of gamete release in relation to the number of days after the full moon is shown in **Figure 4**. We have observed MMEA to be a gonochoric broadcast spawning species that spawns after either a late August full moon (2021) or a September full moon (2019 and 2020) at our facility. Reproductive strategy appears very similar to the closely related species *Dendrogyra cylindrus (*DCYL*)*, with some individual colonies switching sexes from year to year. Spawning was observed to occur between 15 and 20 nights after the full moon (NAFM), with males generally spawning over more nights than females. A summary of gamete release times in relation to sunset is shown in **Figure 5**. Sperm is released from male colonies in a series of pulses, and sperm release begins as early as one hour before egg release and as early as 22 minutes before sunset. Female colonies release eggs in a slow trickle, with no discernible visual "setting" or warning before release, and egg release occurs approximately 20 minutes after sunset (during the twilight period). This slow release, along with the fact that the eggs are not very buoyant and are relatively small (300-350 microns, **Figure 6**), can make catching the start of egg release difficult for this species. There are a couple instances during observations where we found gametes in the system but release time and colony could not be identified (this information was highlighted in yellow in a supplementary data table provided to DEP; available upon request).

Despite working with significantly less concentrated sperm than would be typically used for hermaphroditic broadcast spawning species, fertilization in the system water is consistently high, as high as 92% in 2021 (**Table 2**). As the eggs reach the surface, they are collected by either gently skimming the surface of the water with a clean cup, or by gently picking out the eggs from the system with a clean pipette.

Embryos develop quickly compared to other coral species, but are similar to DCYL. The first cell division occurs approximately 1 hour and 30 minutes after the female spawning (or approximately 50 minutes after we remove eggs from the tank). Cell divisions occur approximately every 45 minutes after that, with embryos recorded in 'prawn chip' phase as early as 7 hours after female spawning. By the following day, larvae are fully developed and swimming as early as 20 hours after spawning.

The total number of larvae produced each year and the total settlement and survival is shown in **Table 3**. Despite significant efforts to improve post-settlement survival, survival at one-year post-settlement remains at only 1% of initial recruits. Significant mortality occurs in the first 100 days (**Figure 7**). We attribute this to the very small size of the initial recruits, which are approximately 500 microns in diameter, and the very slow early growth. Recruits continue to be only 2-3 mm in diameter at 5-6 months post settlement (**Figure 8**). The small size makes them susceptible to damage when cleaning the settlement tiles and even to damage by grazers. However, after the first year, growth markedly improves for corals that are able to make it through the very difficult first six months. The mean size of recruits still under human care at TFA is shown in **Table 4**.

3.4. Task 4 – Evaluate *P. strigosa* growth and survival with/without grazers

The experiment was run for a total of 105 days. Image analysis to generate coral growth and survival data have been completed for five sampling points through day 84. Raw data for coral growth (**Figure 9**) and survival (**Figure 10**) were provided to DEP as separate supplemental files and are available upon request.

Survival of *P. strigosa* colonies was high (≥ 90%) and not different among treatments, despite elevated algae cover in no-grazer controls. This was likely due to the age (~5 months post settlement) and size (mean = 0.21 cm^2) of colonies at the beginning of the experiment as well as tightly maintained culture conditions that allowed the corals to persist despite overgrowth with macroalgae. Over 84 days, corals exhibited a seven-fold increase in area on average (mean = 1.47 cm^2 at day 84). *T. ventricosus* was the best performing urchin species in terms of coral growth, with *D. antillarum* producing a mean final coral size that was smaller numerically smaller than the control.

3.5. Task 5 – Expose tiles to multiple grazer species and size classes at CIMAS

The point intercept data from the tile photos have been processed with CoralNet and are still being analyzed at the time of this report. However, preliminary observations indicate a treatment effect on algal cover with the control and three *B. minima* treatments resulting in the highest algal cover (**Figure 11**). Treatments containing *Lithopoma* and/or *Cerithium* snails maintained relatively low cover of turf algae and CCA coverage began increasing in several treatments.

3.6. Task 6 – Expose *D. labyrinthiformis* recruits to grazer assemblages at CIMAS

Mean increases in live tissue area varied 4-fold among corals subjected to different grazer assemblage treatments during the course of our 10-week study (**Figure 12**). Corals exposed to grazer assemblages containing two *Cerith* sp. snails demonstrated the largest increase in live area. Specifically, corals exposed to two *Cerith* sp. snails and a single *L. americanum* (Treatment #8) increased in live area 324% ±119 (mean ±SE), and corals exposed to two *Cerith* sp. snails and a single *B. minima* (Treatment #5) increased in live area 261% ±90. Mean increases in live area were relatively similar for the remaining nine grazer assemblage treatments and ranged from a high of 161% ±61 (Treatment #7) to a low of 124% ±23 (Treatment #6). Corals on Control tiles with no grazers present (Treatment #11) had the lowest average increase in live area (83% ±25) and were the only corals that did not increase by at least 100% (i.e. double in size) during the course of the experiment.

We used linear regressions to investigate potential relationships between grazer biomass, algal biomass, and coral growth. We found a significant negative relationship between grazer biomass and the amount of algal biomass present on a tile at the end of the experiment ($F_{1, 77} = 41.7$, $p < 0.001$; **Figure 13a**). We also found a significant negative

relationship between the amount of algal biomass on a tile and change in live coral tissue area ($F_{1, 95} = 4.99$, p = 0.02; **Figure 13b**). Surprisingly, we found no relationship between the biomass of grazers influencing a tile and changes in live coral tissue area during the course of the experiment ($F_{1, 237} = 0.05$, p = 0.82; **Figure 13c**). We also did not detect a relationship between the biomass of grazers influencing a tile and changes in live area during the course of the experiment if we only included data from tiles were algae were present at the end of the experiment ($F_{1, 77} = 2.62$, p = 0.14).

3.7. Task 7 – Expose tiles to multiple grazer species and size classes at Mote Marine Laboratory

3.7.1. Snail Mortality

Over the course of the 70-day experiment a total of 60 snails died and were replaced with new living snails. Of the 60 snail mortalities, *L. americanum* accounted for 55 (92%), *Battalaria minima* accounted for 4 (7%), and *Cerithium* spp. accounted for 1 (2%).

3.7.2. Effect of Grazer Assemblage on Algal Community Development

The point intercept data from the tile photos have been processed with CoralNet but are still being analyzed at the time of this report. However, preliminary observations indicate relatively strong treatment effect on algal cover with both treatments including two *Cerithium* spp. snails appearing to result in the lowest algal cover and the control and three *B. minima* treatments resulting in the highest algal cover (see **Figure 14**). When the project team ended the experiment and removed the tiles from the experimental tanks, the algae growing on the tiles sloughed off precluding attempts to collect, dry, and weigh the algal biomass.

3.8. Task 8 – Expose *D. labyrinthiformis* recruits to grazer assemblages at Mote Marine Laboratory

A total of 14 of 220 DLAB recruits (6.4%) did not survive the experiment. In all of the treatments, the mean increase in living tissue area was >40% over the 70-day experiment (. The greatest mean change in living tissue area (100.39 %) was observed in the 2 *Cerithium* + 1 *Battalaria* treatment group while the smallest mean change in living tissue area (42.48%) was observed in the 3 *Lithopoma* treatment group (see **Figure 15**).

4. DISCUSSION

4.1. *Lithopoma* reproduction

The reproductive information obtained to date suggests that it may not be possible to spawn *L. americanum* snails on demand in land-based systems. Further spawning observation and analysis of the tremendous amount of histological data generated for this project will lead to greater understanding of the overall reproductive cycle in this species. While not conclusive, information currently on hand points to a potential annual as well

as lunar-based gametogenic and spawning cycle for *L. americanum*. For other grazers (i.e. urchins), spawning is achievable year round and while some weak lunar patterns have been identified it is also possible to induce spawn at any time of month. This may ultimately not be the case for *L. americanum* as the window for potential spawning may be more tightly regulated by environmental conditions. Nonetheless, observations of mass spawning in land-based tanks following water changes, pump adjustments, etc. seem to indicate that some exogenous trigger is required to induce final gamete maturation and spawning. Identifying any annual and lunar-based gametogenic cycles as well as triggers that might be effective during the spawning window may ultimately help in developing culture protocols for this species, much as they have recently been developed for corals held in land-based systems.

4.2. Induced spawning of *Lithopoma* snails and *Tripneustes/Lytechinus* urchins

Lithompoma snails have proven difficult to induce to spawn. Based on anecdotal observations and in part on data generated for Task 2, we believe that spawning trials may have taken place outside of the reproductive season for this species. This will be further explored through future work. Strategies used to induce spawning in this project would only be effective if mature gametes were present and ready for expulsion. Based on histological evidence obtained in Task 2, *L. americanum* may be a "complete" rather than "fractional" spawning species. This would mean that in each animal, all gametes develop as a single cohort and are released in a single spawning event. Long periods of time may exist where the animals are not capable of spawning because they are in earlier stages of gametogenesis. That said, given the synchronous volitional spawns we have observed, there is still likely some exogenous final spawning trigger, which would mean that animals must remain in a spawning capable phase for some period of time. Ultimately, management of *L. americanum* reproduction in land-based systems may prove to be more akin to coral spawning than sea urchins. To realize the goal of producing large numbers of small *L. americanum* for co-culture with newly settled corals, more work is required to fully understand the reproductive biology of the species and determine how to best optimize their reproduction.

Conversely, we were successful at inducing spawning in *L. variegatus* sea urchins through both injection methods tested. Acute transfer to warmer water, which has been very effective for *Diadema* over several years, did not work for either species tested in this project. Further, *T. ventricosus,* a species of increased interest for co-culture given the results of Task 3, was not reliably induced to spawn using any of the methods we tested. Possible explanations for the lack of spawning include nutrition and/or improper dosing of the injections. While the acetylcholine methods were adapted from techniques that were reported as effective on *T. ventricosus* (Guete-Salazar et al. 2021), urchins injected in that study were recently collected from the wild. Due to quarantine requirements and initial difficulty in obtaining urchins, our *T. ventricosus* were held in land-based systems for an extended period prior to injection. They were distributed among several systems, including those holding corals and *Diadema* broodstock, and were not target fed but rather grazed on primary productivity within the tank. It is possible that target feeding urchins commercial diets or live macroalgae would improve

gonad quality and spawning. Ultimately, we believe that this project has developed reliable spawning protocols for *L. variegatus* held in land-based systems, but more work is required to determine effective methods for *T. ventricosus*.

4.3. *Meandrina meandrites* reproduction

Spawning from MMEA in ex-situ induced spawning aquaria can be accomplished consistently by following protocols described in O'Neil et al 2021. However, due to the low number of eggs produced per female colony and the resulting low number of larvae produced, a large cohort of parent individuals would be necessary to produce larvae at scale. For example, in 2021 a total of 18,700 larvae were produced from 6 parent colonies. Using this ratio, in order to produce 100,000 larvae, a total of 32 parent broodstock would need to be spawned. This will require significantly more broodstock holding than is required for high-output hermaphroditic broadcast spawners, where up to 500,000 larvae or more can be produced from less than a dozen corals (i.e. *D. labyrinthiformis* or *C. natans*).

Despite consistent annual spawning activity, the species continues to be plagued by low post-settlement survival. This could possibly be improved by properly timed co-culture with juvenile snails or urchins, as was the original purpose of this project. We have also noticed the species is susceptible to high early mortality from what appears to be rapid tissue loss events on a few occasions, which were remedied by antiseptic dips such as in Lugol's iodide solution. Additional research into the early microbiome of coral recruits reared ex-situ and the potential cause for rapid mortality in these cases is warranted. It is possible that the rapid tissue loss events are related to bacterial community dynamics that are tied to algal overgrowth, as algae growing on the tile can release dissolved organic carbon sources that may impact the localized microbiome of the small juvenile corals. Considering the ease of spawning and larval rearing, and the previous high prevalence of the species on Florida's Coral Reef, we recommend that MMEA is a good candidate for continued research on how to improve post-settlement survival. However, in order to scale production of this species the post-settlement survival bottleneck will need to be primarily addressed.

It is likely that any scaled effort to produce MMEA for restoration will need to combine both sexual production of a limited number of new genets with asexual propagation in order to produce more material for restoration. We recommend that the juveniles of this species that are produced by TFA could be used for a micro-fragmentation study to determine the impacts of fragmentation on early growth.

4.4. *P. strigosa* co-culture with juvenile urchins

While grazer densities were reduced at day 42, the near complete lack of algae in grazer replicates in the final sampling points suggests that overgrazing may have been occurring. Further work to optimize grazer sizes and densities could help to strike a balance between algae control and inadvertent coral overgrazing. Targeted consumption of PSTR colonies by grazers was not observed during the experiment, and is supported

by high a similar coral survival among treatments despite the relative lack of algae. Overall, this experiment confirms the utility of certain urchin species for coral co-culture while suggesting areas for improvement in the specific methodologies used for implementation (i.e. urchin sizes and densities)

4.5. Grazer assemblage effect on benthic communities

While some data are still being analyzed, the benthic communities that resulted from the different grazer assemblage treatments appeared to differ substantially, and in some similar ways between experimental systems located at NOAA/CIMAS and Mote. This suggests that grazer identity and assemblage composition are likely to influence the foundation of benthic communities on novel structures in culture with an effect that may translate among different system designs.

4.6. Grazer assemblage effect on *D. labyrinthiformes* recruits

In both the NOAA/CIMAS and Mote studies, grazer species assemblage appeared more important than total grazer biomass, which did not equal more coral growth. Specific combinations of grazers demonstrated important consequences for coral growth and thus how efficiently practitioners can propagate corals. Interestingly, the analogous NOAA/CIMAS and Mote studies found that including two *Cerith* sp. snails and a heterospecific (either *L. americanum* or *B. minima*) allowed for the largest increase in live tissue of young DLAB colonies. Both studies also concluded that different grazer assemblages promote different benthic communities, and these benthic communities are likely key drivers to coral success and growth. While some results were broadly consistent between the studies, others such as the grazer assemblages leading to the lowest coral growth and specific composition of algal communities (e.g. by day 70 CCA was much more prevalent on NOAA/CIMAS tiles than those used at Mote) were different. This highlights our *a priori* understanding that different *ex situ* systems often have very different characteristics and the influences on benthic community succession are multifactorial. The present results demonstrate that grazer assemblages are one of the important factors in this succession and in the success of coral recruits. Ultimately, these grazers deserve increased consideration in coral co-culture.

5. TABLES AND FIGURES

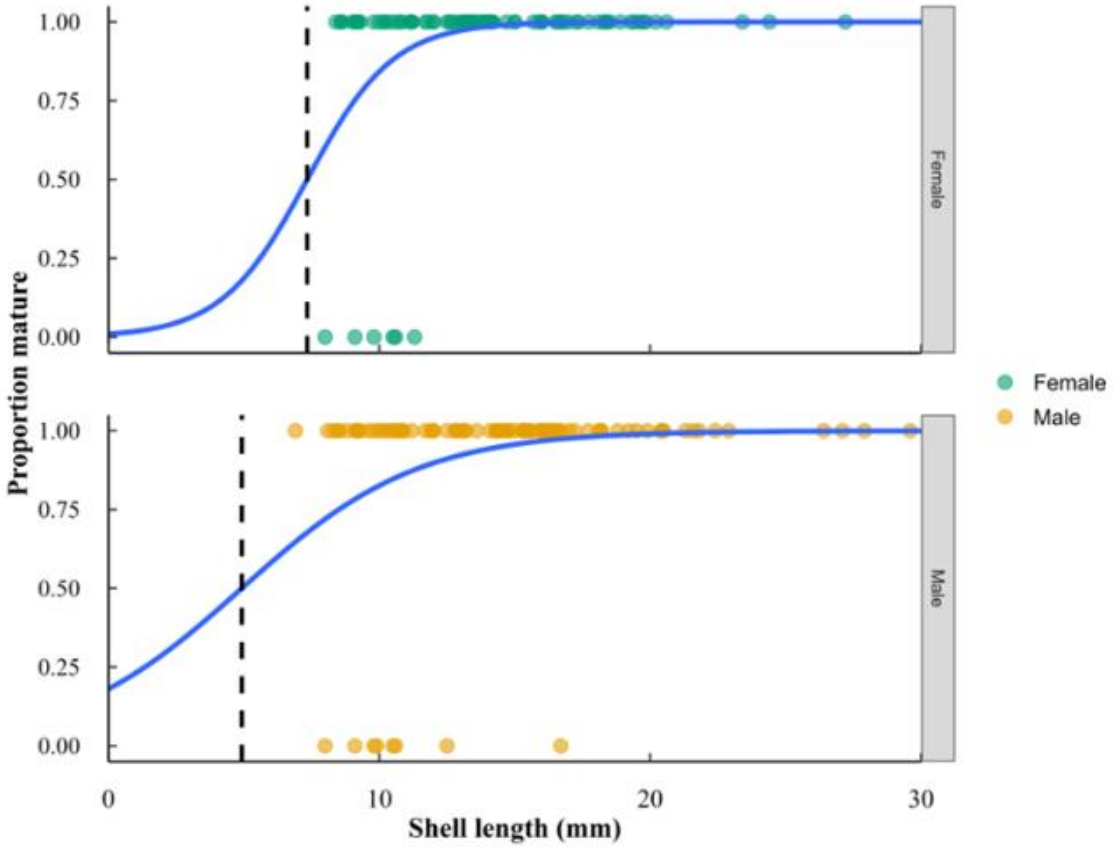


Figure 1. Size at 50% sexual maturity by shell length estimates for female and male *Lithopoma americanum*. Data points represent individual animals binomially classified as either mature or immature following dissection and histological gonad analysis (credit: FWRI)

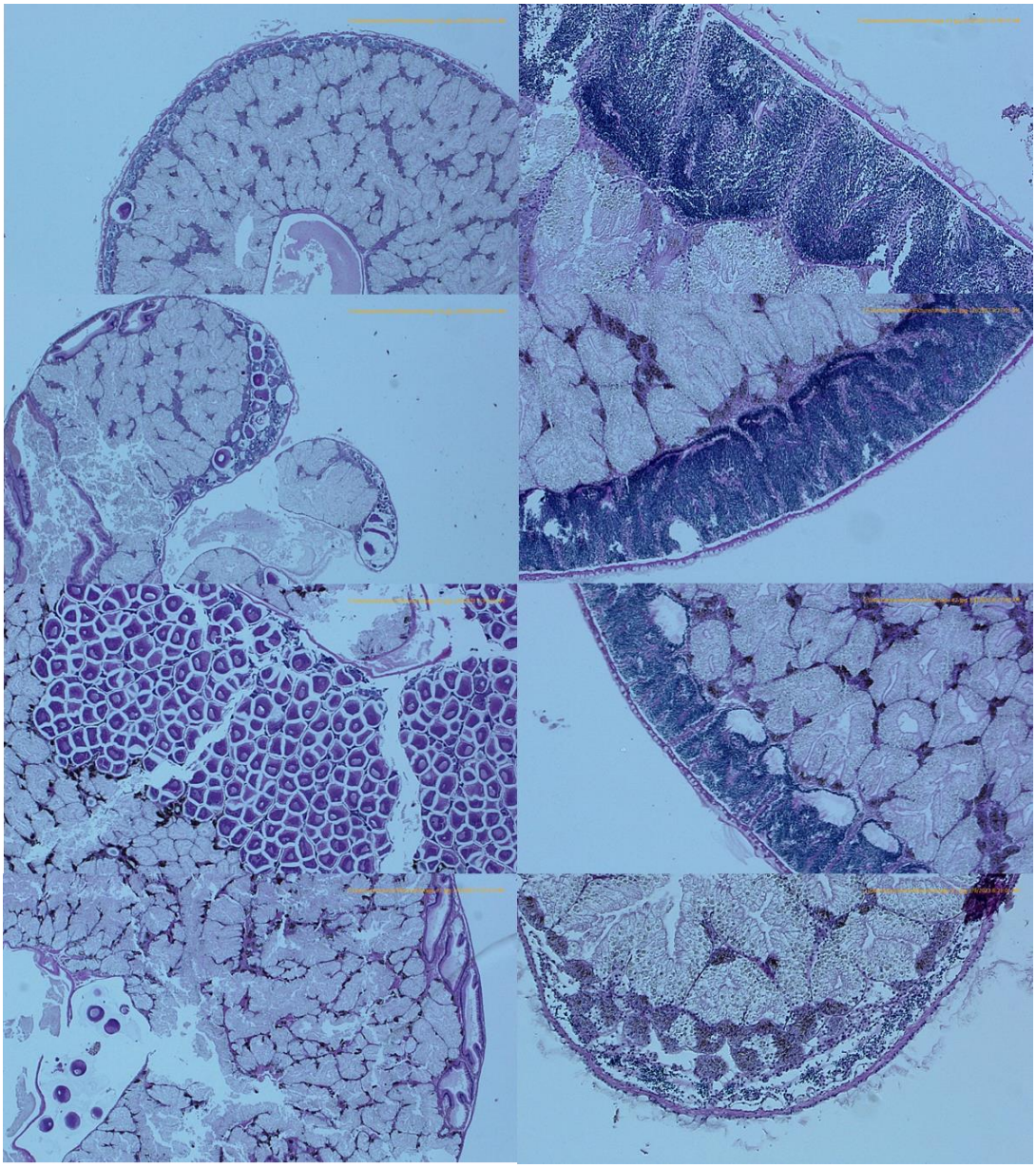


Figure 2. Representative images of *Lithopoma americanum* histological sections showing various stages of female (left column) and male (right column) gonad development. Assigned stages are as follows: 1) top row – early development; 2) second row – mid to late development; 3) third row – mature gametes; 4) bottom row – post spawn (credit: FWRI)

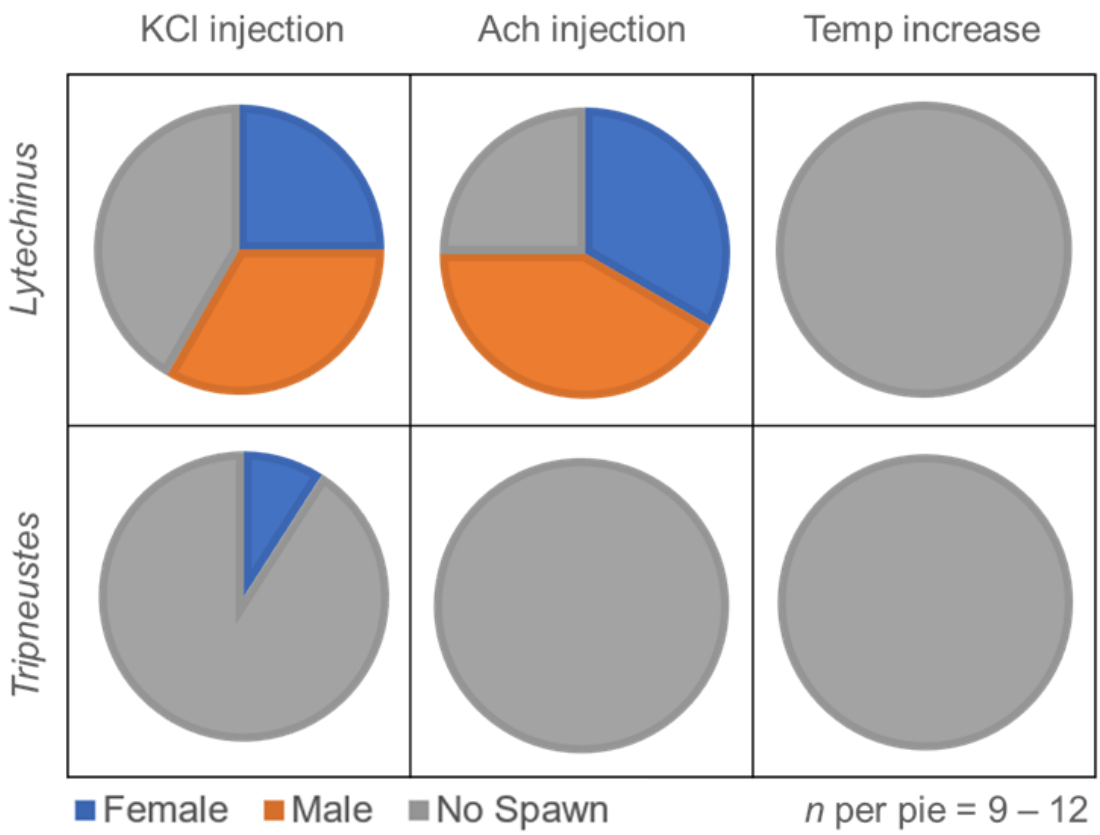


Figure 3. Summary data for all spawning trials using *T. ventricosis* and *L. variegatus* sea urchins injected with 0.5M KCl at 0.01ml per gram body weight, 0.1M acetylcholine at 0.2mL per animal or subjected to an acute 5°C temperature increase.

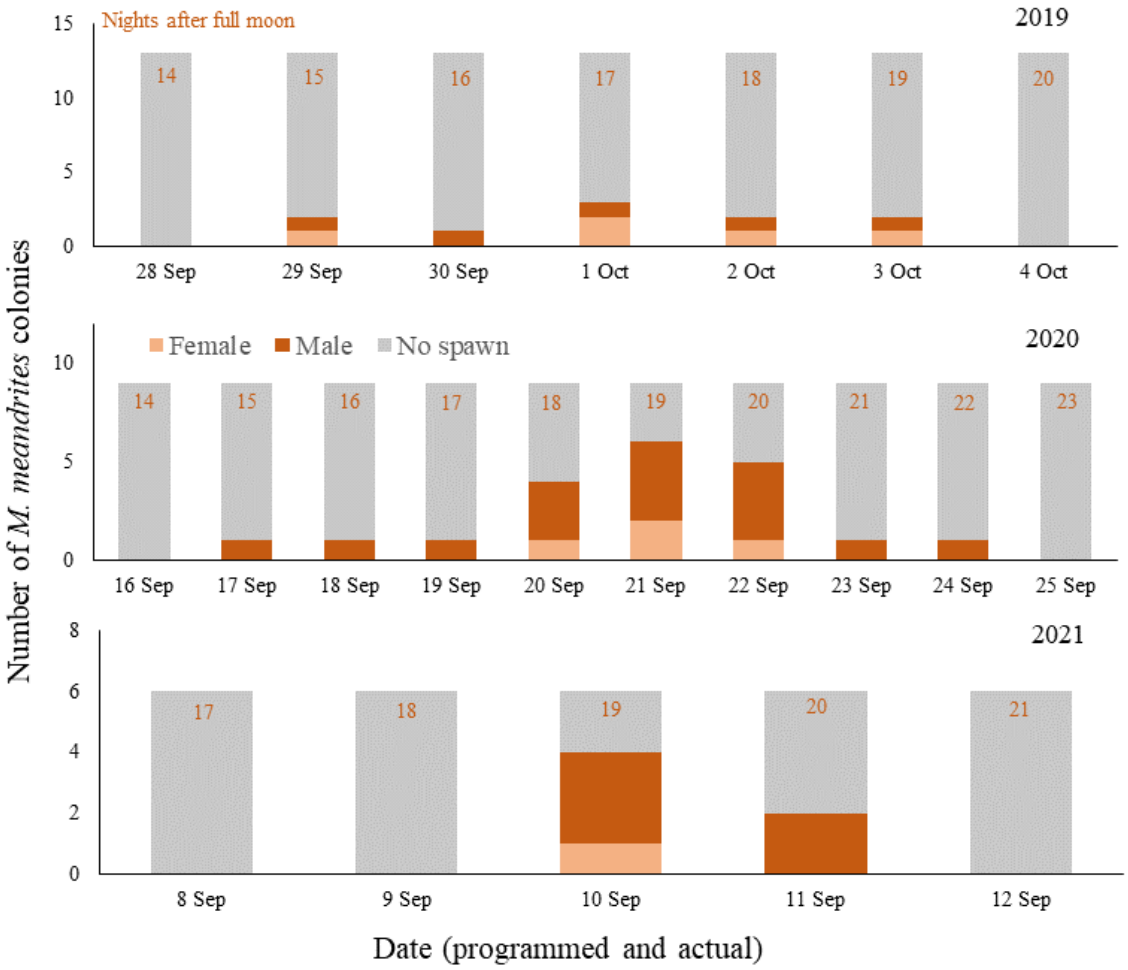


Figure 4. Date and nights after full moon during which egg and sperm release were observed in 2019 through 2021. Fully grey bars indicate that observations were made but no spawning was observed.

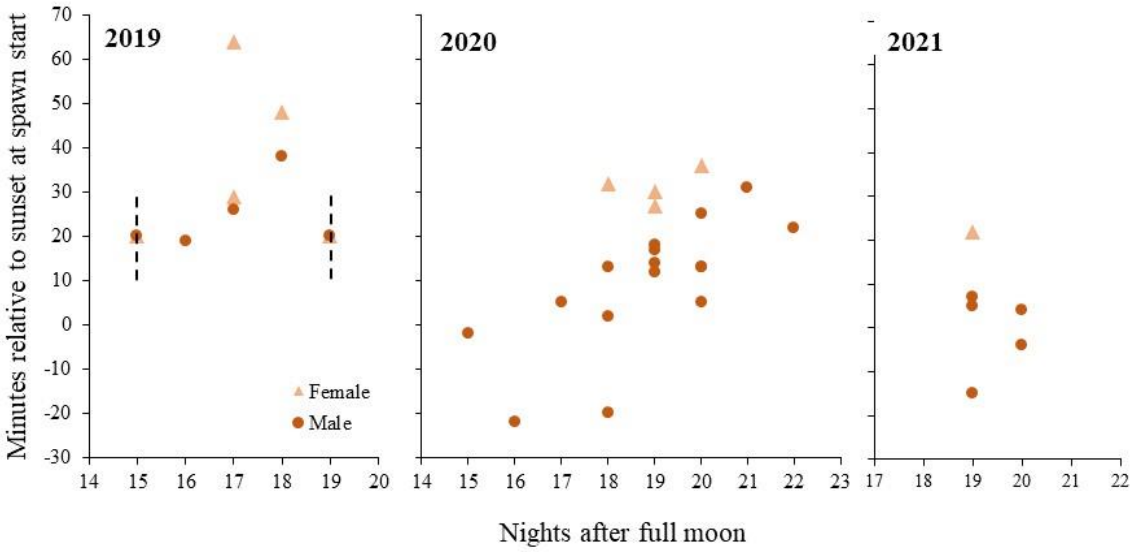


Figure 5. Time of male and female gamete release relative to sunset. Negative values indicate release occurred before sunset.

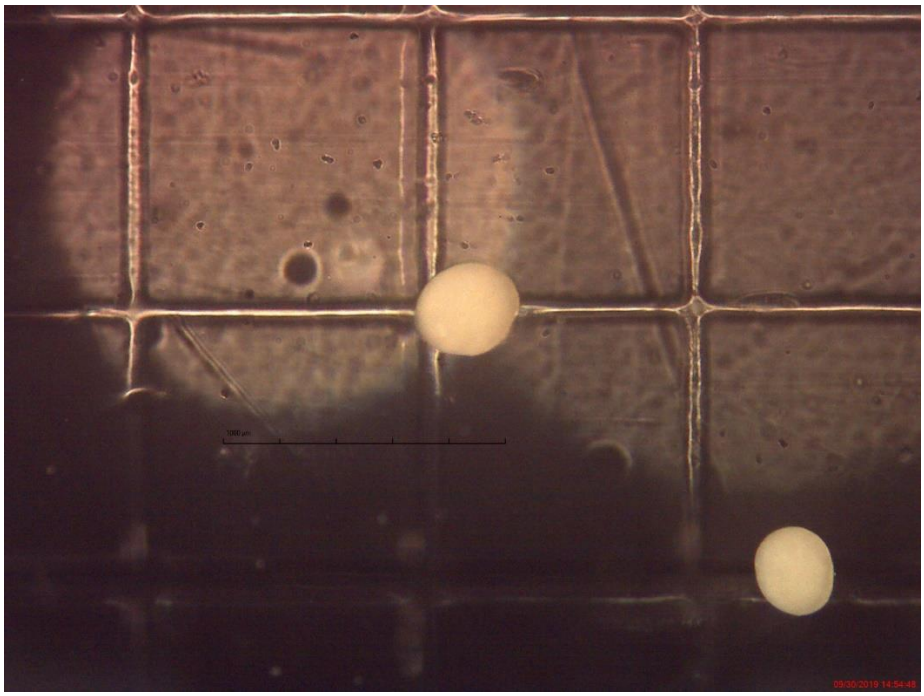


Figure 6. Eggs of *M. meandrites* photographed on a Sedgwick Rafter cell with 1 mm square lines shown.

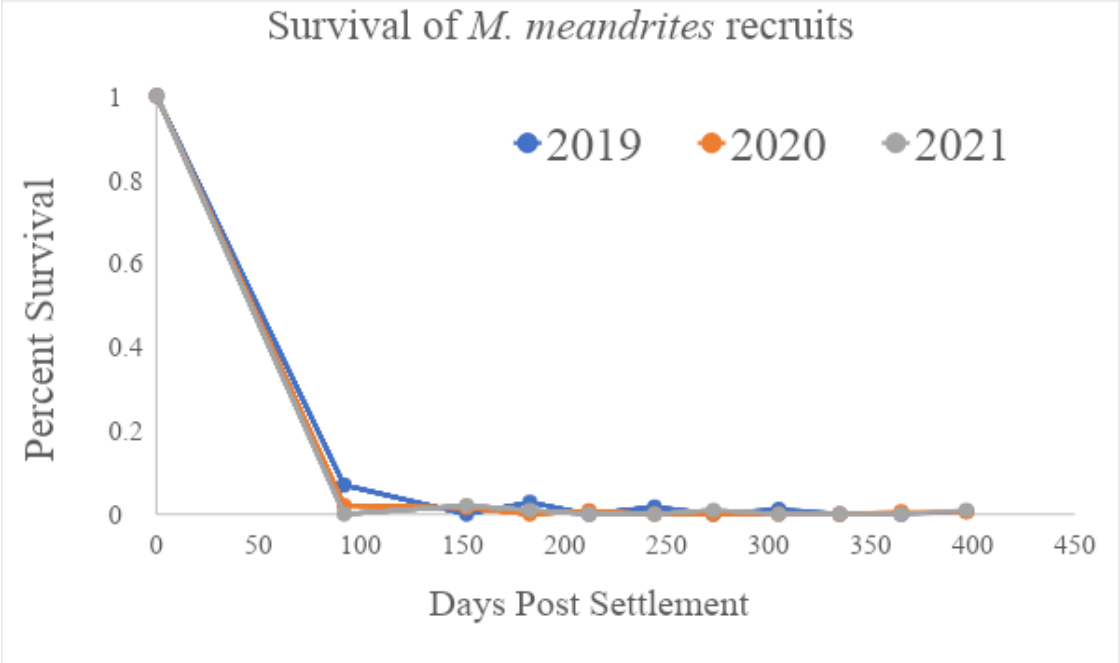


Figure 7. Post-settlement survival of *M. meandrites* recruits from 2019-2021

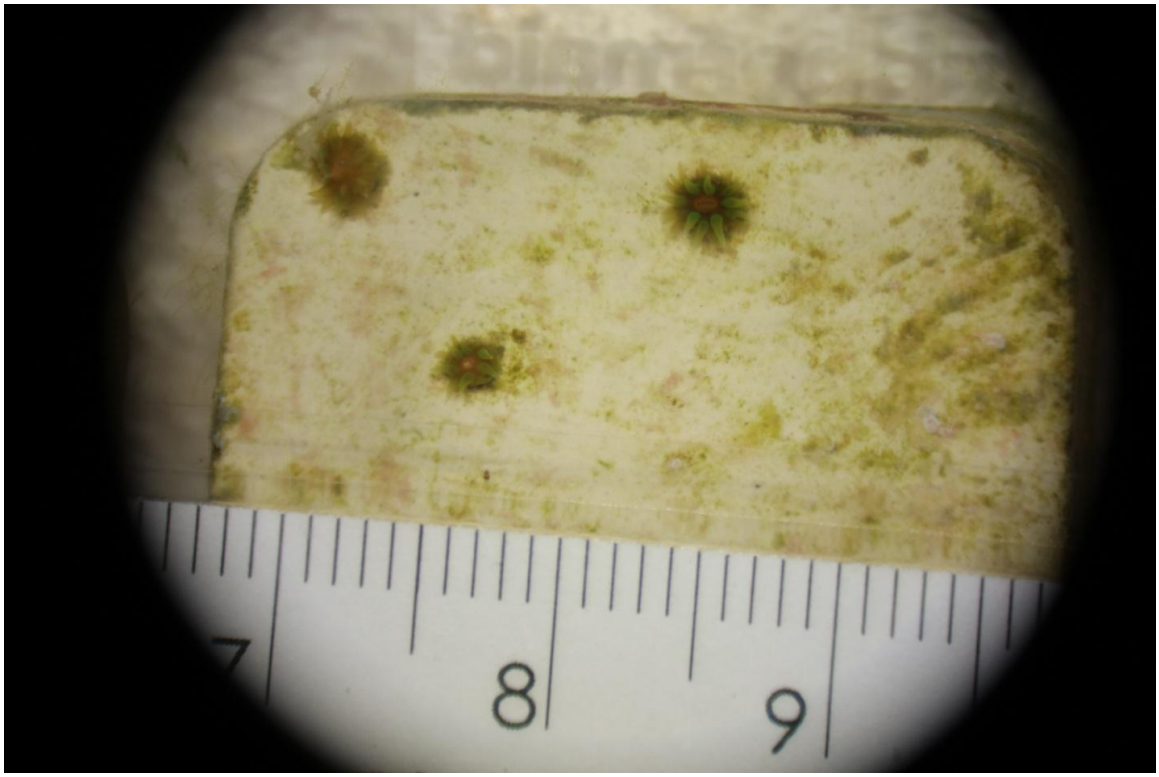


Figure 8. *M. meandrites* recruits from September 2020 on February 25, 2021, just under 5 months post-settlement.

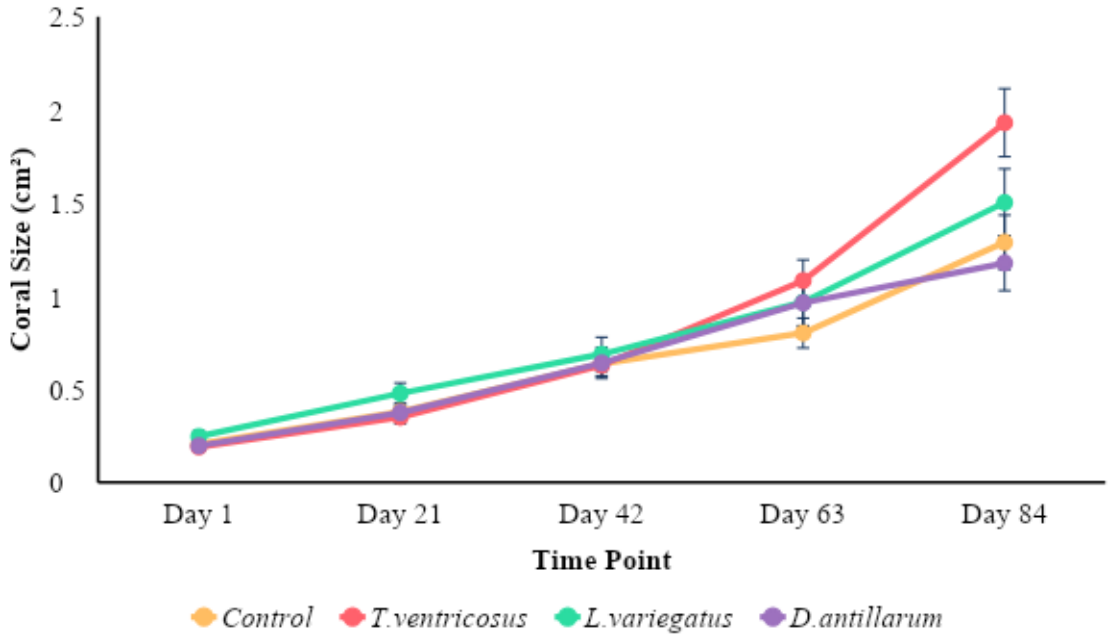


Figure 9. Mean *P. strigosa* individual colony size over time when coral plugs were co-cultured with three different species of juvenile urchins. Three replicate aquaria with 10 coral colonies each were used for each treatment and urchin densities were reduced from 25 to 15 individuals per replicate at day 42. Data are presented as mean ± SEM.

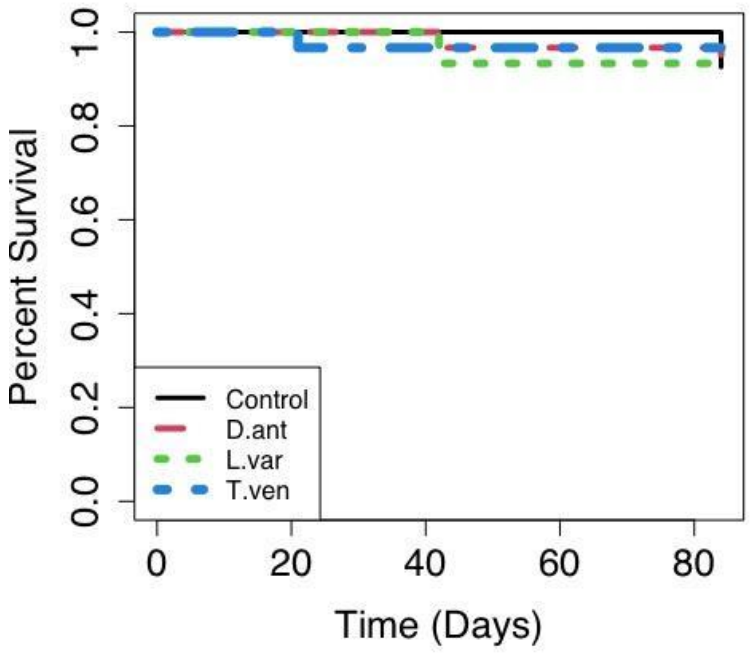


Figure 10. Survival of *P. strigosa* colonies over time when coral plugs were co-cultured with three different species of juvenile urchins plus a control. No significant differences existed among treatments (Kaplan-Meier $X^2 = 0.05$; d.f. = 3, $p = 0.9$).

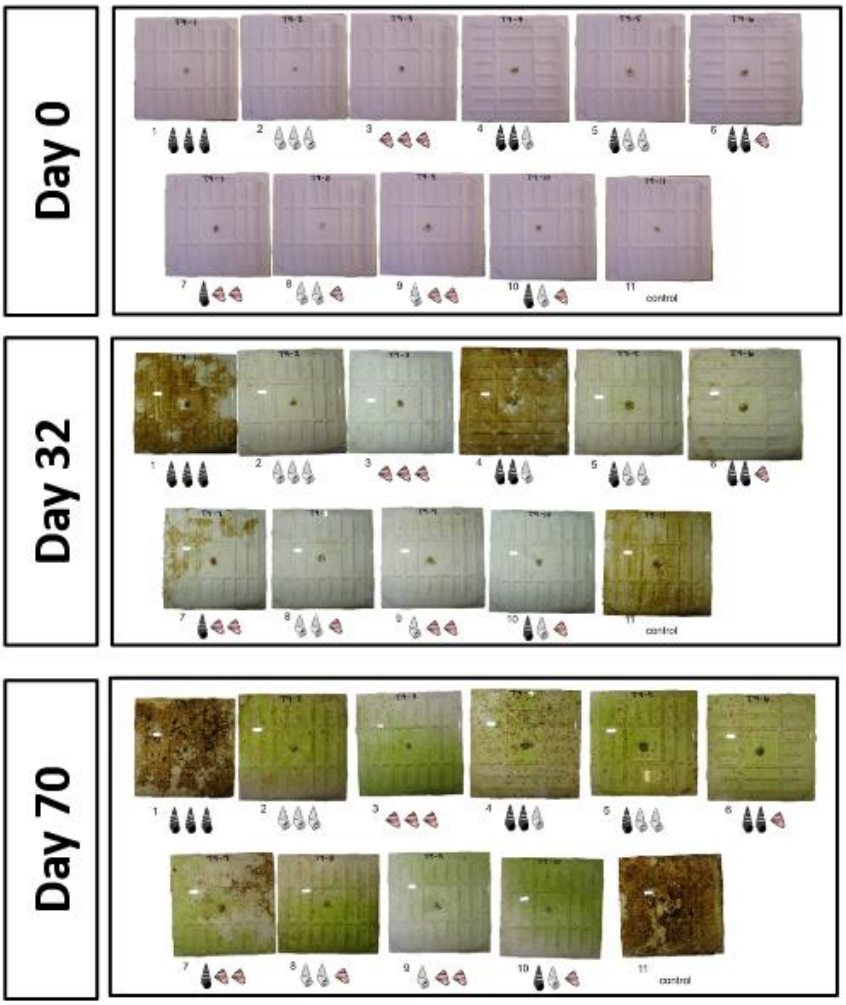


Figure 11. Representative images of individual replicate tiles exposed to various snail grazer assemblages at days 0, 32, and 70 of the experiment.

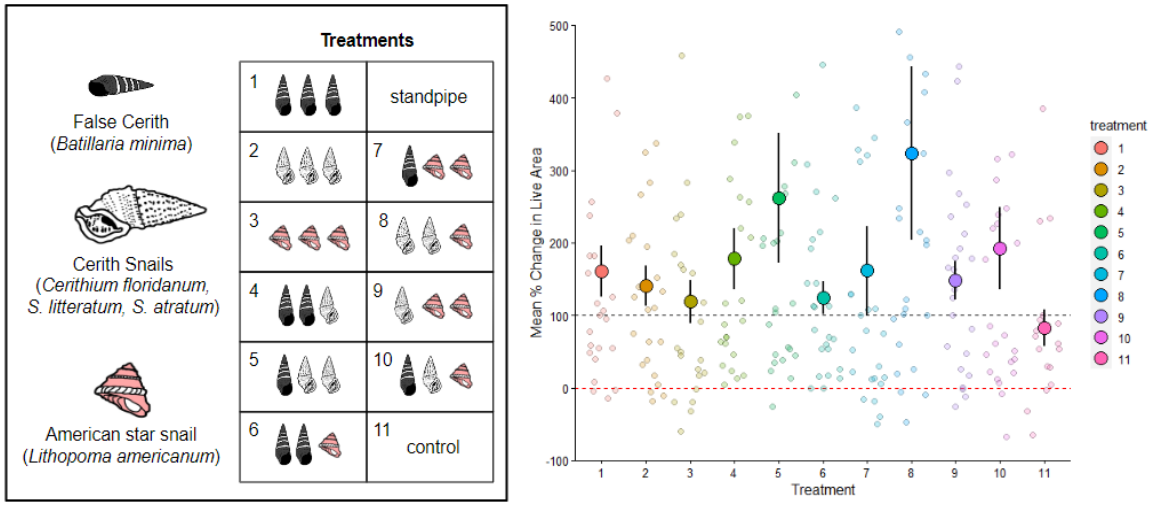


Figure 12. (Left) Schematic of one complete replicate of experimental grazer assemblages established for experimental treatments. (Right) Mean change in live area (%) for colonies of *Diploria labyrinthiformis* colonies after ~70 days under different experimental grazer assemblage treatments. Larger solid dots represent means, smaller opaque dots represent raw data values. Error bars are ± SE.

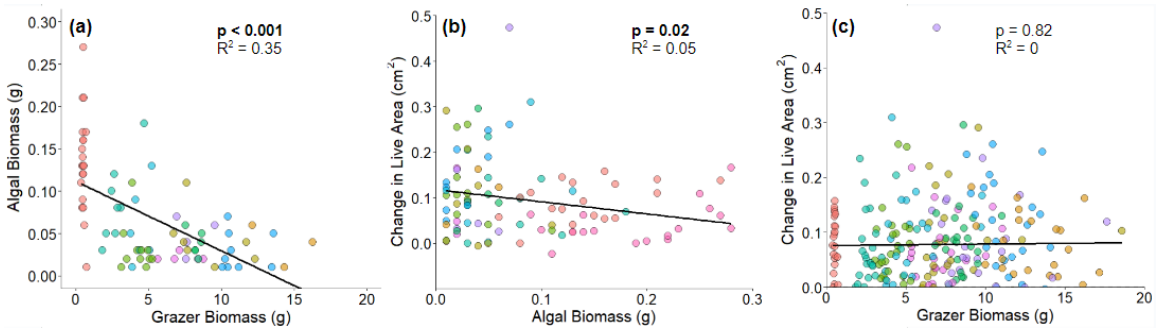
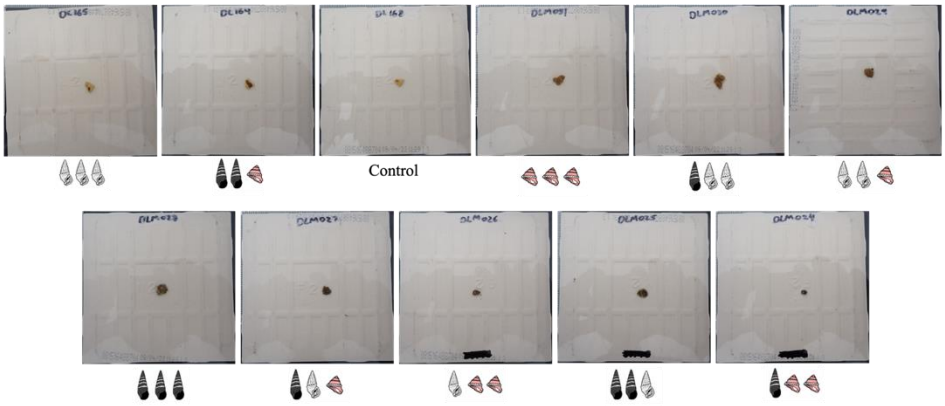


Figure 13. (a) Algal biomass on each tile at the conclusion of the experiment regressed against grazer biomass present for that tile. Only tiles with > 0 g of algal biomass included. (b) Mean change in live area *D. labyrinthiformis* (cm^2) at the conclusion of the experiment regressed against algal biomass. (c) Mean Change in live area (cm^2) of *D. labyrinthiformis* regressed against total biomass of grazers present. Statistics from linear regression.

Day 0



Day 70

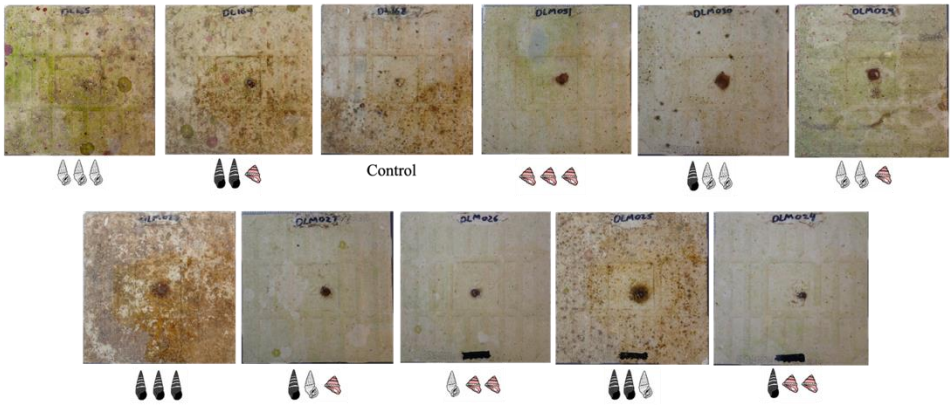


Figure 14. Representative images of individual replicate tiles exposed to various snail grazer assemblages at day 0 and day 70 of the experiment

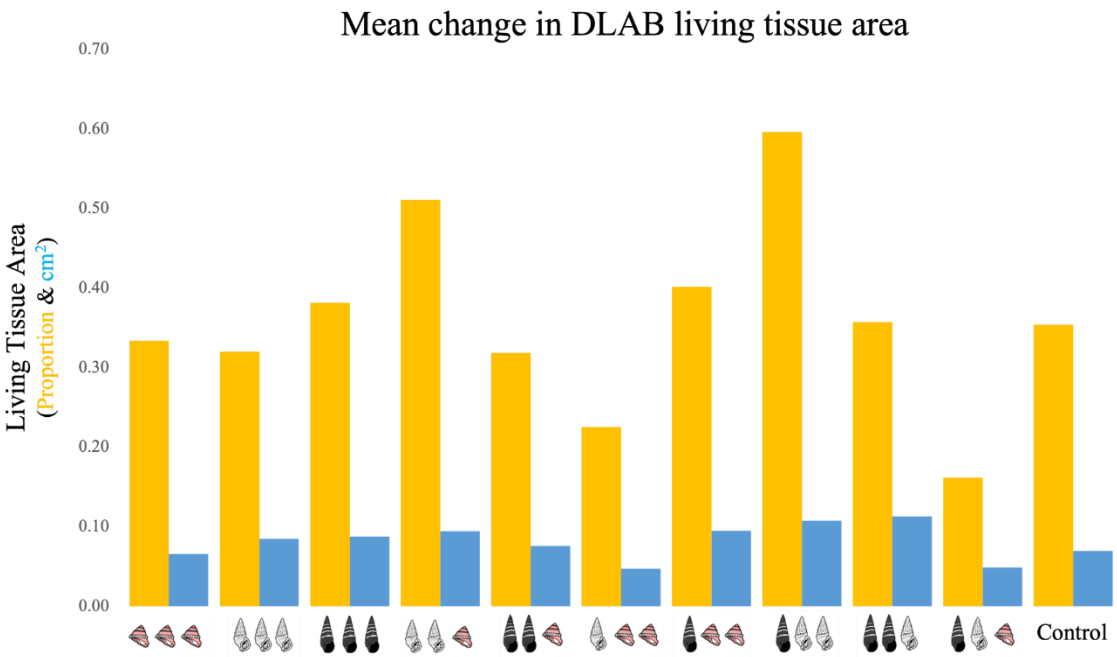


Figure 15. Living tissue area increase of *Diploria labyrinthiformes* recruits exposed to various grazer assemblages and without grazers.

Table 1: Total number of *Lytechinus variegatus* and *Tripneustes ventricosus* sea urchins by sex spawning in response to various potential induction agents.

Species	Treatment	Male	Female	No Spawn
L. variegatus	Acetylcholine	4	5	3
L. variegatus	KCl	3	4	5
L. variegatus	Warm water	0	0	12
T. ventricosus	Acetylcholine	0	0	11
T. ventricosus	KCl	0	1	10
T. ventricosus	Warm water	0	0	9

Table 2**:** *Meandrina meandrites* fertilization time and fertilization rate recorded during each spawning year.

Year	**Fertilization time (time-time)**	**Fertilization Duration (H:mm)**	**Fertilization Rate**	**# Larvae**
2019	19:08-19:48	0	83%	4,000
2020	18:59-19:39	0:40	>76%	14,600
2021	18:52-19:30	0:38	92%	18,700

Table 3. Number of *Meandrina meandrites* larvae produced, initial settlement and post-settlement survival at 1 year.

Year	**# Larvae**	**# Settled**	**Settlement %**	**# at 1 Year**	**Survival %**
2019	4000	419	10.5	3	0.7
2020	14,600	915	6.3	5	0.5
2021	18,700	1952	10.4	19	1.0

Table 4. Mean maximum diameter of *Meandrina meandrites* recruits reared from 2018-2021 spawning.

Year Spawned	# of Corals	Age at Measurement	Mean Max Diameter (cm)
2019	3	3 y 7 mo	7.2
2020	5	2 y 7 mo	4.6
2021	19	1 y 7 mo	2.6