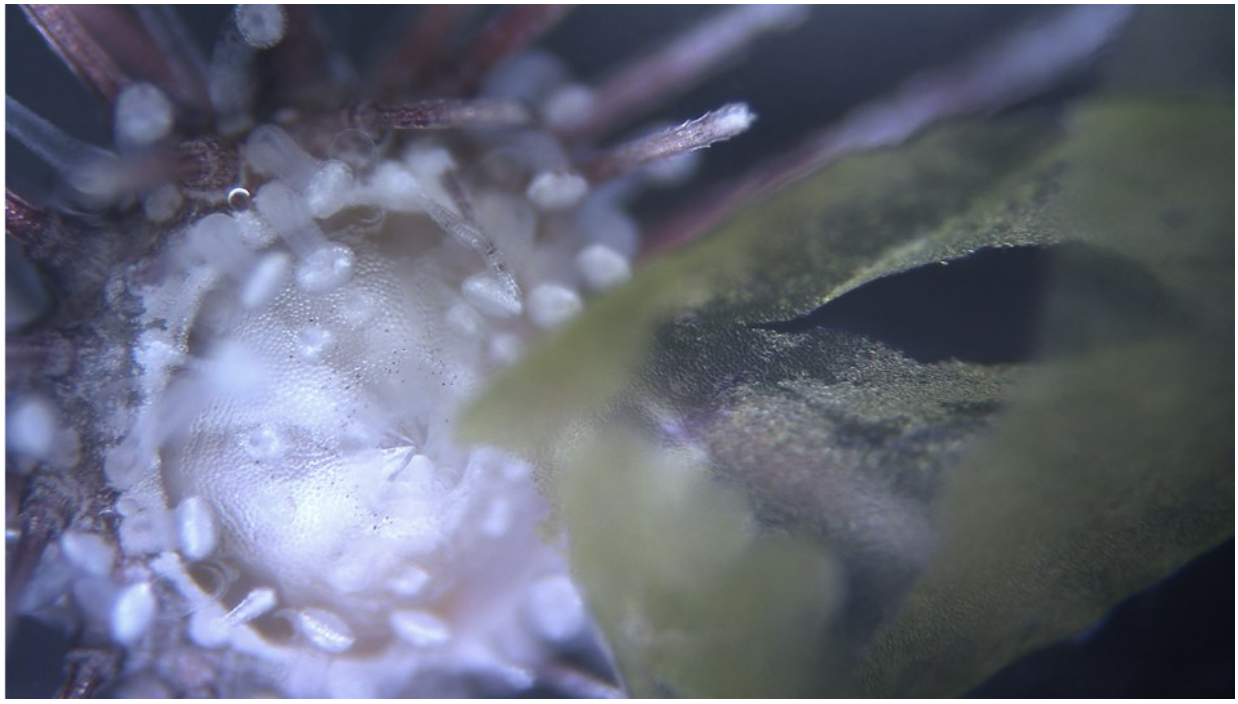
Improving early life survival of priority grazers



Improving early life survival of priority grazers

Final Report

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05/23/25

Completed in Fulfillment of PO# C44417 for

Florida Department of Environmental Protection
Coral Protection and Restoration Program
8000 N Ocean Dr.
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This report should be cited as follows:
{Patterson J and Spadaro J. 2025. Improving early life survival of priority grazers. Florida DEP. Miami, FL. 1-39}

This report was prepared for the Florida Department of Environmental Protection's (DEP) Coral Protection and Restoration Program by the above listed institutions. Funding was provided by the DEP Award No. C44417. The views, statements, findings, conclusions, and recommendations expressed herein are those of the authors and do not necessarily reflect the views of the State of Florida or any of its sub-agencies.

Acknowledgements

The authors wish to thank the staff of the collaborating institutions that assisted in executing experimental and data curation efforts for this project. The Florida Aquarium provided facilities and staff expertise for the *Diadema* aspects of the project. Mote Marine Laboratory provided facilities and staff experience for the *Maguimithrax* aspects of the project. Thanks also to the Florida DEP-facilitated Coral Restoration Team working group members who provided valuable input and feedback both before the start and after the conclusion of this project. Finally, special appreciation is extended to the Florida DEP Coral Protection and Restoration Program (CPR) whose efforts made this work possible, especially Brooke Robinson, Allison Holevoet, and Kylie Morgan. The DEP CPR provided guidance and administrative oversight that was vital to the success of this project.

Management Summary and Recommendations

This work was designed improve the ability to culture large, mobile invertebrate grazers that have demonstrated benefits for Florida's Coral Reef. The broad scale recommendation is to transfer information obtained on newly settled *D. antillarum* feeding protocols and *M. spinosissimus* settlement methods into production. Data obtained on *D. antillarum* from settlement to 12 weeks post metamorphosis suggests an initial feeding with the benthic diatom *Navicula perminuta,* followed by a transition to flocculated microalgae at around 3 weeks post metamorphosis or 0.8 mm test diameter. Growth rates obtained using these standardized diets appeared to be higher by ~50%, as previous work suggested it might take 8 weeks for *D. antillarum* to reach a test diameter of 1.0 mm. At around 1.5 mm test diameter (~8 weeks post metamorphosis), *D. antillarum* can be transitioned to a solid food diet of dried nori. In practice all of these diets will likely be supplemented by naturally occurring biofilms, turfs, and macroalgae that inevitably grow in the culture tank. In terms of *M. spinosissumus* settlement, provision of settlement substrates other than bare glass aquarium surfaces improved settlement and metamorphosis, but substrate orientation is likely a key component to improving post larval settlement and, ultimately, production of the species to support coral reef restoration efforts. Several candidate substrate types were tested against a control with substantially higher settlement on each substrate than on controls, however, vertical surfaces, in many cases outperformed candidate substrates in a horizontal orientation. There were no patterns in preference among substrate types when all were offered, except that vertical surfaces consistently outperformed any other substrate type, including controls. Ensuring substrates are oriented vertically, seasoned with natural biofilms or turf algae, and providing positive settlement cues is likely to substantially improve production of Caribbean king crabs.

Executive Summary

This report contains information on experiments conducted in FY 24-25 intended to address attenuation of production potential at critical life stages for the reef grazers *Diadema antillarum* and *Maguimithrax spinosissimus*. The sea urchin *Diadema antillarum* is a Western Atlantic coral reef herbivore important in maintaining productive reef community structure. Due to a die-off event in the 1980s, numbers of *D. antillarum* are exceedingly low on Florida's Coral Reef, but may be supplemented with aquacultured individuals. However, limitations in knowledge on culture methods hinders output. Understanding diet requirements for early post-metamorphosis juveniles may improve growth and survival of a culture. Current methods utilizing natural biofilm are less than ideal for rearing large numbers of urchins to a releasable size. Three experiments were run covering the first twelve weeks post-metamorphosis. Experiments 1 and 2 used replicable diets including a benthic diatom and flocculated live microalgae. The benthic diatom outperformed biofilms during the first three weeks post-metamorphosis in terms of growth, while flocc out performed biofilm during the 4-7th weeks post-metamorphosis. Mortality was consistent among treatments aside from the no-feed control in experiment 1, which experienced significant mortality by week three post-metamorphosis. Test diameter variation was highest in the treatments with the best growth. In Experiment 3, data strongly suggested that *D. antillarum* are unable to consume a macroalgae food source until ~1.5 mm test diameter and that they should remain on microalgae-based diets until this point. The Caribbean king crab, *Maguimithrax spinosissimus*, is the largest brachyuran crab in the western Atlantic. It is uncommon but ubiquitous on coral reefs and shallow hardbottom habitats throughout Florida, gulf waters, and the Caribbean region. It is an omnivore, but benthic algae, including chemically-defended and calcified algae that many herbivores tend to avoid, comprise the bulk of its natural diet. At sufficient densities, these crabs reduce benthic algal cover dramatically and lead to cascading effects of increased fish abundance and richness and significant increases in coral recruitment. The species has been identified as one targeted for stocking to facilitate improved coral reef restoration outcomes. Production of the species for this purpose is established in Florida and several other locations throughout the region, but post-larval settlement and metamorphosis, as with many aquacultured species, is a bottleneck to mass production. Two experiments were conducted to evaluate the effect of substrate type on settlement rates and settlement preference. Experiment 1 evaluated the effect of substrate type on settlement rate relative to no-substrate controls. All candidate substrates tested increased settlement over control treatments with marginal, if any, differences between treatments. Experiment 2 evaluated settlement preference for each of the candidate substrates in Experiment 1. Interestingly, there were no discernible differences in preference among candidate substrates or controls, but in an unanticipated result, vertical surfaces were a clear preference for *M. spinosissimus* post-larvae. Both experiments suggest that substrate(s) should be incorporated into *M. spinosissimus* production efforts but that those substrates should be oriented vertically in the culture vessel. By expanding the current understanding of these delicate life stages in aquaculture of these two important coral reef grazers, culture success can be improved to the benefit of restoration efforts for Florida's Coral Reef.

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Table 6. Table summarizing Manly's alpha values for settlement onto each candidate substrate at the 10 d time point with 'Other' substrate treatment included and expected settlement adjusted for observed mean larval mortality among trials. Note that, when corrected for larval mortality, 'Other' (vertical bare tank surfaces) and 500 um nylon mesh substrates were preferred settlement substrates.

1. DESCRIPTION

Project objectives fall under the goal of improved ability to propagate a suite of mobile invertebrate grazers and employ them in coral recruit co-culture. 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 priority 6.1 '*Propagation of Reef-Associated Species*'.

Coral propagation, both sexual and asexual, and outplanting represent an important facet of active restoration in response to stony coral tissue loss disease (SCTLD) and other stressors. These activities have the potential to return genetic diversity and biomass to reefs. However, outplanting corals alone does not address the stressors that cause their decline. To address the widely acknowledged impacts of macroalgal overgrowth, culture techniques for mobile invertebrate species that provide important grazing services have been developed. Both the long spined sea urchin *Diadema antillarum* and Caribbean king crab *Maguimithrax spinosissimus* have been documented in the refereed literature as having positive impacts on macroalgae coverage and reef health. The goal of this project is to improve propagation of these species, potentially for use in coral co-culture scenarios and primarily for use in stocking for restoration on Florida's Coral Reef.

1.1. Goal : Enhance settlement and/or early post-settlement survival in two important grazer species

Objective 1 – Identify a reproducible diet for newly settled *D. antillarum.*

Rationale: Current practice is allowing newly settled urchin to graze a naturally occurring and highly variable biofilm. Standardized diets have the potential to improve production and increase reliability.

Objective 2 – Evaluate substrate parameters affecting settlement rate and preference of *M. spinosissimus.*

Rationale: Anecdotally, the provision of complex and rugose substrate improves settlement of post-larval crabs. Optimizing the type of substrate to provide may improve production of crabs of the target stocking size for coral reef restoration efforts - a current bottleneck in the species' production.

1.2. 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 natural sexual coral recruitment in reef areas that have received grazer augmentation.
- Increased success of SCTLD-susceptible and other coral species outplants due to reduced algal competition.
- 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.

2. METHODS

The purpose and intended use of the data generated by the proposed 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 Department of Environmental Protection Coral Protection and Restoration Program (CPR). This will ensure that methodologies are not duplicated, best practices are employed, and project results are effectively communicated to all stakeholders. All required state and federal permits were obtained prior to the work beginning.

2.1. Task 1 – Methods and Quality Assurance

In partnership with staff of the CPR, a Quality Assurance plan was developed and eventually approved early in the project period. This plan outlined the purpose of the project, provided a brief historical overview and literature background, and provided a detailed description of the work to be accomplished and appropriate quality control methods. It also outlined planned methods for data acquisition, generation, and evaluation as well as documentation and data curation/storage.

2.2. Task 2 – Improve survival and growth of newly settled *Diadema antillarum* through to the "solid food" stage

Experimental system and replicate units - To accomplish this Task, three separate experiments were conducted to evaluate the potential of different standardized diets on *D. antillarum* growth and survival from immediately post metamorphosis (~0.6 mm test diameter) to about 12 weeks post metamorphosis (up to 3.0 mm test diameter). All three experiments were asynchronously run inside the same 355-L tray integrated into a recirculating aquaculture system. Natural seawater, sand-filtered and oxidized, was used for both experiments. A 20% water change was conducted weekly. Reverse osmosis freshwater was used as needed to compensate for evaporation. Total turnover within the tray was 5-6x hour^{-1}. Temperature and salinity were measured daily and were kept within ranges of 25.6-26.1°C and 34.5-35.5 ppt respectively. A water quality panel was taken weekly of ammonia, nitrite, nitrate, phosphorus, pH, alkalinity, and dissolved oxygen using standard measurement procedures. Five LED full-spectrum lights (Current USA 36-48" Satellite Freshwater LED Plus Full Spectrum RGB+W) were evenly spaced above 76 cm

above the tray. Replicate units were bins constructed from 12x12 cm plastic containers with 200 µm mesh sides and a water depth of 5 cm. Each bin received 125 ml/min water flow from a header tank in addition to flow through from the experimental tray. Experiments are described below in life-stage order. Chronologically, Experiment 2 was conducted first, followed by Experiment 3 and then Experiment 1.

Feeding treatments - Experiments 1 and 2 used the same three feeding treatments:
A naturally derived diatom-based biofilm (Treatment B), flocculated microalgae (5:2 V:V ratio of *Rhodomonas salina* and *Chaetoceros muelleri*, Treatment F), the benthic diatom *Navicula perminuta* (Treatment N), and a no-food control (Treatment C). For Experiment 1, treatments were given the title B1, F1, and N1 respectively, along with a no-feed control (C), while for Experiment 2, treatments were labeled B2, F2, and N2, respectively. For Experiment 3, two diets were tested in a factorial design with urchin size. Treatment F was successful in the first two experiments and repeated, and a macro-diet was prepared from dried nori. 1x1 cm squares were cut from sheets of pre dried and packaged nori (Two Little Fishes). Prior and post cutting, dried nori was stored in an enclosed container at 5ºC. Dried nori has been previously shown to be a viable diet for older *D. antillarum* juveniles (~6 mm TD).

Experiment 1 - This experiment ran from 28 January to 18 February 2025. A total of 20 replicate bins were used in Experiment 1. Due to the difficulty of settling *D. antillarum* larvae, replicate bins were overstocked with larvae in anticipation of low settlement rates. 400 competent larvae were stocked out randomly into 20 bins, with 20 larvae to a bin. While containing larvae, bins were all fed a mixture of live microalgae *R. salina* and *C. muelleri* in a 3:1 proportion twice daily. Juveniles were considered settled according to previously established metrics. Larvae began to settle one week after being added. Once each replicate bin contained four newly metamorphosed juveniles, excess larvae were removed. In the case of a bin having over four settled juveniles, excess urchins were moved to replicates that had not reached the desired number. In order to supplement replicates that did not end with at least four settled juveniles, early juveniles from the same cohort were transferred from a separate grow-out tank. Settled juveniles over 1 mm in TD were removed to ensure that the most recently metamorphosed urchins possible were used in the experiment. Any movement of juveniles among replicates or from the grow-out tank was done prior to the experiment start. The process of larval settlement required 13 days.

To initiate the experiment, each replicate bin was randomly assigned to one of the four treatments resulting in five replicates per-treatment. Each replicate was fed *ab libitum*, with biofilm grown on a 2.54 x 2.54 cm glass tile, and *N. perminuta* and flocculated microalgae fed directly to each respective bin. Replicates assigned to treatments N1 and F1 were given a clean glass tile to control for structure. Food was spatially constrained either to a glass tile or to an area covering ~3x3cm of benthic space. Live feed was removed and replaced when algae cells began to visibly die: floc was removed and replaced daily, whereas *N. perminuta* cells and biofilm glass tiles were replaced weekly. Replicates were managed such that an excess of food was available at all times.

Experiment 2 - While this experiment ran mostly prior to the start of the project period (29 May to 2 July 2024), significant effort was dedicated to generating data from photos and

subsequent analysis during the early stages of the project. A total of 18 replicate bins were used in Experiment 2. No unfed control was implemented as it was apparent urchins of this size class required food. 108 newly settled (~two weeks post-settlement) juvenile urchins were collected from the same grow-out system used in experiment 1. Six urchins from this population were placed randomly in each replicate bin. Each replicate was then randomly assigned to one of the three feeding treatments, resulting in six replicates per treatment. After the first TD data was collected, urchins were fasted for 24 hours before the first experimental feeding. Feeding followed the same experimental procedures as Experiment 1.

Experiment 3 - This experiment ran from 2 July to 7 August 2024. A total of 16 replicate bins were used in Experiment 3. Four treatments were examined in a factorial setup: two size classes, "large" and "small", along with two feed classes, the micro-sized diet (Treatment F) and the macro-sized diet (died nori). Small was defined as having a starting TD < 1.5 mm and large is defined as having a starting TD >1.5 mm. Previous pilot studies indicated that urchins at 2.0 mm TD were capable of eating macro food items like dried seaweed. Four urchins of the appropriate size class were placed in each replicate bin and feeding treatments were randomly assigned. Small urchins feeding on nori were labeled SN while those feeding on flocc treatment F were labeled SF. The same feeding treatments for large size class urchins were labeled LN and LF, respectively.

Data collection - Replicate bins were designed for removal from the RAS tray for photography without disruption to the juveniles. Individuals were photographed for test diameter (TD) measurement with a camera (MU-1000 HS) attached to an AmScope dissecting microscope using a calibration slide in each replicate. The TD of each juvenile was measured using a straight line through the center of the test with the image software analyzer ImageJ (version 1.54g). Survival was measured by counting the number of juveniles remaining in each replicate bin.

Statistical analysis - All data were analyzed using R statistical software, (version 4.3.3). Normality and homogeneity of variance were determined visually with a qqplot and residual plot, respectively. Growth data were log or square root transformed to meet normality assumptions and examined using a linear mixed-effects model (lmer) with TD as the dependent variable and time and treatment as fixed effects, with an interaction between time and treatment. Each replicate was treated as a random effect. When a significant main treatment or treatment*time interaction effect was noted, a pairwise post-hoc evaluating estimated marginal means was run to further analyze differences between individual treatments.

To test for variance in growth standardization among treatments, standard deviation around mean TD was analyzed with an ANCOVA with a covariate of standard deviation for all timepoints and a Levene's Test run for pairwise post-hoc comparisons when significance was noted. A Kendall rank correlation test was conducted to determine correlation between growth and variance. Binomial survival data were examined between treatments using a Pairwise Fisher Test. Significance was set at a critical value of $\alpha = 0.05$ for all analyses. Mean values are presented as mean ± standard error (SE).

2.3. Task 3 – Evaluate the effect of substrate and environmental conditions on settlement of Caribbean King Crab *Maguimithrax spinosissimus* larvae in culture

Experimental system and replicate units - To evaluate the effect of substrate on the settlement and metamorphosis of Caribbean king crab larvae in culture, the project team conducted a preliminary experiment to identify appropriate candidate substrate types and then two experiments to 1) measure substrate-specific settlement rates for each focal substrate independently and 2) evaluate preference of *M. spinosissimus* larvae between and among focal substrate types in culture. All three experiments were carried out within replicate 21L glass aquaria situated in 570L fiberglass raceway tanks. Water was supplied independently to each glass aquarium from the common recirculating source. Each aquarium was situated inside the 570L fiberglass raceway such that ~50% of the aquarium was always submerged in common system water to maintain constant and consistent temperature among and between experimental aquaria. The larger recirculating system features passive mechanical filtration by directing all water returning to the sump through a 5um filter sock and a moving bed biofiltration unit along with a large foam fractionation unit to process and export organic wastes. Temperature control is driven by a four-pipe heat exchange system wherein a reservoir is kept at 40C and another is kept at 13C; a thermistor is situated in the sump of the system and as system temperature deviates from the set point, solenoid controlling actuated valves either circulate the hot or cold loop water through an enclosed heat exchanger coil to bring system temperature back to target set point. Each recirculating system received an ~25% water change weekly and basic water quality parameters were monitored at least weekly. Replicate units were the independent 40cm x 20cm x 25.4cm glass aquaria fitted with an external standpipe cut to standardize a 23cm depth of water inside each aquarium. The internal standpipe was constructed of rigid aquaculture mesh (*e.g.*, "vexar") wrapped in 750um nylon mesh that was secured with a gel adhesive such that there were no openings in the standpipe larger than 750um below the waterline. This allowed for continuous water flow even with swimming larvae in the aquarium. Preliminary experiments were run before either Experiment 1 or Experiment 2 began and then Experiment 1 and Experiment 2 were both run concurrently in the same system.

Preliminary experiment/substrate selection - A series of nine candidate substrates were collected and assembled by the project team for preliminary screening. Fine nylon mesh of three different mesh sizes (150um, 500um, and 750um), a cast cement 'cookie', cut travertine/limestone tile, 800um polypropylene window screen mesh, a black high density aquarium filter sponge, a green low-density polypropylene aquaculture filtration mesh, and 0.64cm vexar mesh were all evaluated. For these preliminary trials (n = 7), candidate substrates of equal footprint were haphazardly placed inside of each experimental tank so that candidate substrates (and an area of bare horizontal tank bottom serving as a no-substrate control) covered 100% of the horizontal tank bottom surface. A haphazardly selected subset of 100 crab larvae were collected by staff 24 h after hatching using a modified plastic pipette and transferred to the experimental tank. Hatchery staff then exhaustively searched each candidate substrate and counted the number of settled post-larvae and juvenile crabs on each substrate. Substrates that consistently hosted very few or

no settled larvae were eliminated from the following experiments. The team ultimately selected two sizes of nylon mesh (500um and 750um), cut travertine/limestone tile, and the black high density aquarium filter sponge to evaluate in Experiment 1 and Experiment 2 below.

Experiment 1 - Substrate-specific settlement rates were measured in the same experimental aquaria described above. Focal substrates were re-cut such that they covered 50% of the horizontal tank bottom surface with bare tank surface serving as a no-substrate control in each trial. Treatment groups were randomly assigned to each experimental tank at the beginning of each trial. A single substrate was placed into each tank ~24h prior to each trial. Hatching was anticipated based on visual inspection of the maternal egg mass - when the eggs shifted from a deep maroon color to a translucent/tan color, the larvae's eyes have developed and this typically signifies imminent hatching. Upon hatching, a subset of 50 swimming larvae were haphazardly selected and moved into the experimental tank. Settlement was quantified by substrate type at 48h and 10d after the start of each experiment. The 48h time point is consistent with initial settlement of post-larvae and 10d is 2-3 days after the bulk of larvae have typically settled and after initial post-settlement mortality typically slows. The project team also quantified the number of larvae that settled on the vertical tank surfaces and standpipe.

Experiment 2 - Settlement substrate preference was measured in a similar design and the same apparatus as in Experiment 1 above, but substrates were again cut such that, along with a no-substrate control, 100% of the horizontal tank bottom surface was covered evenly with candidate substrates. Substrates were haphazardly placed at the beginning of each trial to control for any location bias within the aquaria. Trials of Experiment 2 were treated as a treatment group in Experiment 1 during the random assignment of treatments to experimental aquaria so that the individual aquaria used for Experiment 2 (and the treatments in Experiment 1) were randomized within each raceway during each trial to control for any location bias within the raceway(s). As in Experiment 1 above, substrates were placed haphazardly into each experimental aquarium ~24h prior to hatching of larvae for each trial. Upon hatching, a subset of 50 swimming larvae were haphazardly selected and transferred from the hatching tank into the experimental aquarium via a modified plastic pipette. Settlement was again measured visually at 48h and 10d after hatching with the number of settled larvae on each substrate type and horizontal bare tank surface (control) recorded at each time point. The project team also quantified the number of larvae that settled on the vertical tank surfaces and standpipe.

Statistical analysis - All analyses were performed in MatLab (2025a). Normality and heterogeneity of variance assumptions were evaluated in a residuals plot. Settlement rate data were log transformed to meet assumptions and were fit to a general linear model (fitrm) and analyzed in a repeated measures ANOVA (ranova). Settlement preference data were evaluated with a Manly's alpha matrix with $100/n$, where n represents the number of independent 'options' or choices available to larvae, representing isometric preference. Significance was set at $\alpha = 0.05$ for all analyses. Mean values are presented as mean ± standard deviation (SD).

3. RESULTS

3.1. Task 1 – Methods and Quality Assurance

The Quality Assurance plan successfully facilitated a rigorous scientific process and ensured usable data. The multiple experiments within the project went according to the plan and it was not necessary to modify the QA plan at any point during the project. All data will be made available as part of the final deliverables for this effort.

3.2. Task 2 – Improve survival and growth of newly settled *Diadema antillarum* through to the "solid food" stage

For improved interpretation, growth data over time and size variability histograms for Experiments 1 and 2 are combined in Figures 1 and 3, respectively.

Experiment 1 - Significant differences in the growth of newly settled juvenile urchins were detected among treatments (df = 3, p = <0.0001) with an interaction effect between treatment and timepoint (df = 9, p = <0.0001). Pairwise comparisons revealed that the N1 treatment had significantly surpassed the other treatments in terms of growth by week two and TD was significantly greater in the N1 treatment relative to the B1 or C treatments at the final timepoint. (Fig. 1).

Figure 2 contains survival data by treatment. The unfed C treatment had the lowest overall survival at 15.0 ± 6.1%, significantly lower than the B1 and N1 treatments. The F1 treatment had the next lowest survival at 45.0 ± 12.2% . The B1 treatment had 55.0 ± 14.6% , and the N1 treatment 60.0 ± 17.0% survival. Survival for the C treatment fell from 65.0% to 15.0% between weeks 2 and 3.

Variation in size was positively correlated with growth (Correlation coefficient = 0.67, p = 0.0001). Within treatment variability significantly differed among treatments (F = 4.86, p = 0.0415) and variation in final TD was higher in treatment F1 than in treatments B1 or C: F = 8.52, p = 0.0092 and F = 5.58, p = 0.0398, respectively (Fig. 3). At the conclusion of the experiment, mean TD for experimental treatments was 0.81 ± 0.04 mm.

Experiment 2 - At the beginning of the experiment, mean TD was 0.85 ± 0.02 mm. Continued differences in juvenile growth were detected among treatments with a significant treatment effect (df = 2, p = 0.0002) and interaction between time and treatment (df = 10, p = .0451), with the post hoc analysis revealing the F2 treatment having significantly higher growth than the B2 treatment at the final timepoint. Growth in the F2 treatment was significantly greater than the B2 treatment by week three (Fig. 1). No significant difference was noted between other treatments.

There were no significant differences among treatments regarding survival. Overall survival was relatively high at 64 ± 11% for the N2 treatment, 78 ± 7% for the B2 treatment, and 72 ± 10% for the F2 treatment. Survival data are not illustrated in this report as there were no significant differences.

There was a significant difference in variance of growth among treatments ($F_{2,71}$ = 7.2412, p = 0.0077), with the F2 treatment being significantly more variable than the B2 and the N2 treatments: F = 2.81, p = 0.0002 and F = 9.30, p = 0.0038, respectively (Fig.

3). Variation in size was again positively correlated with growth (Correlation coefficient = 0.75, $p < 0.0001$).

Experiment 3 - There was a significant difference in TD growth between feed classes, size classes, and time ($df = 1$, $p < 0.0001$; $df = 1$, $p < 0.0001$). There were also noted interaction effects between size classes and feed classes, as well as between feed classes and time ($df = 5$, $p < 0.0001$, $df = 5$, $p = 0.0006$). Post hoc analysis revealed a significant difference between the SN and SF treatments ($df = 119$, $p < 0.0001$, Fig. 4).

In terms of survival, the SN treatment had significantly lower survival than the SF, LF, or LN treatments at 31% (Fig. 5). Survival for the SF, LF, and LN treatments was high (94%, 100%, 94%, respectively). It was visually apparent within experimental replicates that small size class urchins were unable to consume the dried nori, while large size class animals readily consumed this food source (Fig. 6)

3.3. Task 3 – Evaluate the effect of substrate and environmental conditions on settlement of Caribbean King Crab *Maguimithrax spinosissimus* larvae in culture

Experiment 1 – A total of 3,200 larvae were used in 64 independent trials ($n = 16$) of Experiment 1 to evaluate settlement rates onto each of the four candidate substrates vs no substrate controls. Mean settlement across all treatments was 11.22% (+/- 1.19 SD) at 48 hours and 6.57% (+/- 0.75 SD) at 10 days (Table 1). The effect of substrate type and time on the settlement rate of *M. spinosissimus* larvae were evaluated by fitting a general linear model to the responses and then a repeated measures ANOVA was run on the coefficients of the model. While there were no significant differences (Table 2) in settlement with respect to treatment group over controls ($p = 0.28839$), nor an interaction between treatment group and time ($p = 0.76713$), visual inspection of the data suggests that 500um mesh seemingly "outperformed" other substrates in the 48 hour time point with a mean of 13% settlement and that 500um mesh and cut limestone tiles had the highest mean settlement rates of 7.00% and 7.38%, respectively, in the 10 day time point.

Experiment 2 – A total of 800 larvae were offered a selection of five focal candidate substrates in the settlement preference experiment and then settlement was measured at 48 h and 10 d into each trial. These data were analyzed using a modified Manly's alpha contingency table with a selectivity index value calculated for each substrate type based on the ratio of larvae observed on each substrate type during each sampling event to the number of larvae available. A selectivity index value of $100/n$, where n is the number of options/choices available, or higher represents a putative preference whereas an index value lower than $100/n$ indicates avoidance. No preference for any substrate was observed in the 48-h time period whether 'other' substrate (vertical bare tank surfaces – 6th effective treatment) was considered or not (Table 3). Likewise, no preference was observed for any substrate type at 10 d, whether 'Other' substrate was considered (Table 5) or not (Table 4). However, when expected settlement values and indices were corrected for observed mean larval mortality among trials (mean mortality of 46.875 larvae +/- 5.965 StDev), both vertical tank surfaces (*e.g.* 'Other') and the 500um nylon mesh treatments elicited a selectivity index value greater than $100/n$ indicating 'preference' (Table 6).

4. DISCUSSION

4.1. *Diadema* growth and survival

As the production of *D. antillarum* in aquaculture develops, ways to improve survival and growth in sensitive life stages becomes all the more important. Here we demonstrate that replicable, standardized diets can perform on par or better than the natural biofilms that are often used for sea urchin aquaculture. Monocultured *N. perminuta* worked well as a feed for juveniles shortly after settlement, despite heterogenous growth, whereas urchins that were at one mm TD or larger grew better on a flocculated diet, indicating a potential ontogenetic dietary preference. Despite the differences in the F and N treatments between experiments, their success compared to biofilm indicates that a more standardized and controllable diet did have positive effects on juvenile growth.

Between the two studies, similarities in growth trends in the alike treatments were evident. While the biofilm treatments kept to a narrow range, the *N. perminuta* and flocc treatments all had a wider spread centered at a higher median diameter. The urchin size heterogeneity in our study, specifically in the fastest growing treatments, indicates that individuals from the same cohort can have a wide range of growth performance. As size heterogeneity was most notable in treatments that had high growth, it may be that while genetic factors do influence the rate that juveniles grow, nutrition is the limiting factor for development when available diets are poor.

The early mortality experienced in these experiments was consistent across all feeding treatments despite improved growth rates when juveniles were fed replicable diets. Survival maintained a downward trend in Experiments 1 and 2 regardless of treatment, indicating some additional factor outside of diet was involved. However, roughly four weeks post settlement in Experiment 2, the rate of mortality decreased for all treatments. This is consistent with the threshold principle, which states that survival of many marine invertebrates stabilizes past a certain point in either size or age, and data from Experiment 3 indicate that in *D. antillarum* survival stabilizes around seven weeks post-settlement, or around 1.5 mm TD.

One factor that can influence juvenile condition is larval provisioning. Juvenile fitness is in part determined by larval condition due to nutrient transfer from larvae to juvenile. While the source of energy reserves for larval provisioning in *D.antillarum* is unknown, a similar mechanism is likely present. The C treatment in Experiment 1 had a dramatic fall in survival between 14 and 21 days, indicating larval provisions were adequate to sustain the urchins through metamorphosis and support basic functions for up to that time period before starvation began causing mortality. However, the lack of growth seen throughout the C treatment indicates that while larval provisions may aid in metamorphic success, and early juvenile survival, they are not adequate for TD growth. Therefore, even in the first week post-settlement, external nutrition is needed for juveniles to grow.

Experiment 3 provides strong evidence for a second threshold size at which urchins are able to make the switch from “soft” or “micro” benthic food sources to those that are more solid and larger in size. Survival and growth data indicate that this is around 1.5 mm test diameter, although the precise size was not determined and there is likely variability among individuals in this trait. From a production standpoint, more solid macro diets such

as dried nori and even easier, cheaper, and more standardizable than the N and F diets that were successful in Experiments 1 and 2. They do not require on-site culture of live microalgae and can be purchased in pre-packaged form. The ability to consume dried nori likely also indicated that urchins of ~1.5 mm test diameter or higher could graze on the living macroalgaes that commonly appear in culture systems. This also has ecological implications when considering the ontogeny of *D. antillarum* grazing at the reef scale.

In order for *D. antillarum* restoration to have a broad impact on Florida's Coral Reef, more individuals will need to be produced than one facility can manage. Methods of increasing survival are of great importance to *D. antillarum* aquaculture in particular due to the high effort and cost of rearing the species through its prolonged larval phase. Providing a standardized diet at a set quantity during the early juvenile stage will allow for not only faster development than what is achieved on a biofilm diet, but one that can be replicated across different production facilities. A standardized diet is one step towards a protocol that would enable broader application of aquaculture in this species. Other methods like on-shore grow-out of wild collected settlers bypass the larval phase in culture but still must rear early juveniles through a stage of high mortality. In both full aquaculture and on-shore grow-out, the benefit of a consistent, controllable diet that aids juvenile growth will improve overall production.

By addressing the gap in knowledge regarding early juvenile diets in sea urchins, increased growth, and with that increased survival, can be accomplished. The results of this study suggest the replicable diets provide a better, or perhaps supplementary, initial food source for *D. antillarum* than natural diatom biofilms. The benthic diatom *N. perminuta* performed well as an initial feed and flocculated microalgaes *Rhodomonas salina* and *Chaetoceros muelleri* performed well as a following feed roughly two weeks post settlement. A two-stage feeding regime may thus result in the highest levels of growth for *D. antillarum* cultures. Juvenile response to early diets in an aquaculture setting could also mirror wild settlement and survival. A nutritious benthic microalga available immediately at settlement may provide early juveniles with a better growth rate and higher chance of survival. While more work needs to be done to fully understand the needs of the early juvenile stage, elucidating early juvenile diets is critical to optimizing aquaculture of this important herbivore.

4.2. Settlement and metamorphosis of Caribbean king crabs

As the production of Caribbean king crabs to support coral reef restoration efforts in Florida and throughout the wider Caribbean region increases, identifying cost effective and efficient strategies to improve production yields as well as identifying bottlenecks in environmental and husbandry parameters that limit production is critical to scaling the method throughout the region. Given the scale at which grazer enhancement interventions are planned, production remains the most dramatic bottleneck to implementation and larval survival, settlement, and metamorphosis are likely the single biggest production limitation for restoration.

While survival and growth can, typically, be increased with intensive culture and care, the inherent increases in cost (*i.e.*, labor) quickly become strong limiting factors in scaling to mass production. When production scale is prioritized, the intensity of culture or care often suffers inherently or by design due to logistical and practical limitations without

dramatic operational cost increases. Thus, identifying cost-effective and efficient avenues to increase early life history success in mass production of target herbivore species, such as *M. spinosissimus*, are paramount to scaling the implementation of holistic coral reef restoration interventions.

The addition of settlement substrate is potentially one of the most cost-effective and scalable shifts in production methods that requires no, or minimal, increase in labor intensity. Anecdotally, the provision of complex microstructure (*e.g.*, nylon mesh, limestone rock) has resulted in substantially higher settlement and metamorphosis rates in culture. Here, the team evaluated several potential substrates that were all low-cost and potentially scalable in mass production efforts. While all of the structures out performed bare glass tank bottoms in Experiment 1, the lack of any significant differences between and among substrate types suggests that as long as the substratum features a complex surface geometry, settlement increases both early in larval and post-larval development (*i.e.*, within 48h of hatching) and during metamorphosis (*i.e.*, 10 days after hatching). Further, the strong, ubiquitous settlement response on "other" (*i.e.*, vertical) tank surfaces, in both experiments, suggests that, while substrate geometry likely plays a critical role in driving settlement success, substrate orientation offers a likely path to amplifying these processes. Indeed, at Mote's Florida Coral Reef Restoration Crab Hatchery Research Center, where Caribbean king crabs are currently in production, larvae are typically provided with hanging swatches of 500um and 750um nylon mesh in settlement systems. Experiment 1 results might suggest that finer mesh sizes are more conducive to early settlers with increased mean settlement during the 48-h time point, but substantially larger sample sizes (*e.g.*, number of clutches) are likely necessary to make a definitive determination on "ideal" settlement substrate(s) (and their orientation). However, in natural settings, it is likely that crab post-larvae seek out complex vertical and overhanging structures as refugia from benthic and demersal predators. It is also possible, as newly metamorphosed juvenile crabs have, often, exhausted their limited energy reserves (*i.e.*, yolk) and require feeds almost immediately, newly settled juvenile crabs seek out vertical structures as sediment and detritus are less likely to obscure or interfere with access to the turf algae resources that they likely rely on during early benthic life history stages.

While experiment 1 indicates that provision of substrate during settlement is likely to generally increase production and survival, Experiment 2 suggests that additional study is necessary to evaluate substrate orientation. As in Experiment 2, no differences were observed between or among settlement substrate types (until vertical tank surfaces were included in the analysis), but upon further investigation, high larval mortality rates had a strong effect on the results. While larval mortality was only able to be quantified in the 10-day time point, given that during the 48-hour visual assessment of settlement, swimming larvae were not counted, the effect on the results was dramatic, revealing a moderate to strong preference for two substrate types – vertical tank surfaces and 500um nylon mesh, which both agree with observations and results from Experiment 1. This analytical nuance highlights another strong bottleneck to production – larval survival. The project team have observed, as in many other aspects of the species' life history and behavior, variance between and among clutches in terms of larval survival is extremely high. The project team has identified this as a critical research priority and are actively working toward identifying avenues to increase larval settlement, survival, and metamorphosis in addition to addressing production bottlenecks at later life history stages.

Further, the project team is interested in evaluating the interaction of settlement substrate and chemical cues that may further amplify settlement responses and increase production. Many invertebrate larvae are capable of delaying settlement and metamorphosis in the absence of positive settlement cues or the presence of negative cues (*e.g.*, predators, competitors, indicators of degradation). A consequence of delayed settlement and metamorphosis in lecithotrophic larvae, such as *M. spinosissimus*, is metabolic exhaustion and potentially increased post-settlement mortality or molt failure. Identifying both positive and negative settlement cues and then capitalizing on these to induce settlement prior to yolk resources being exhausted could dramatically increase production capacity, early life history survival, growth, and fitness. Further, identifying and then culturing or providing appropriate post-settlement feeds or nutritional supplements is critical, combined with appropriate settlement cues and substratum to maximizing production, post-settlement survival, and growth.

The results of these experiments suggest that substrate, particularly vertically oriented substrates, are a first step. The project team will continue this work and evaluate seasoning these substrates with highly productive algal turf communities to optimize culture conditions for early life-stage *M. spinosissimus*. Ongoing research aims to identify both positive and negative settlement cues for the species as well as shelf stable feeds and nutritional supplements for these early life history stages as well as for broodstock. Addressing these gaps in our knowledge of the species' biology and larval ecology will be iteratively applied to their mass culture to continue to scale cost-effective production of crabs to support coral reef restoration efforts across Florida's Coral Reef.

5. TABLES AND FIGURES

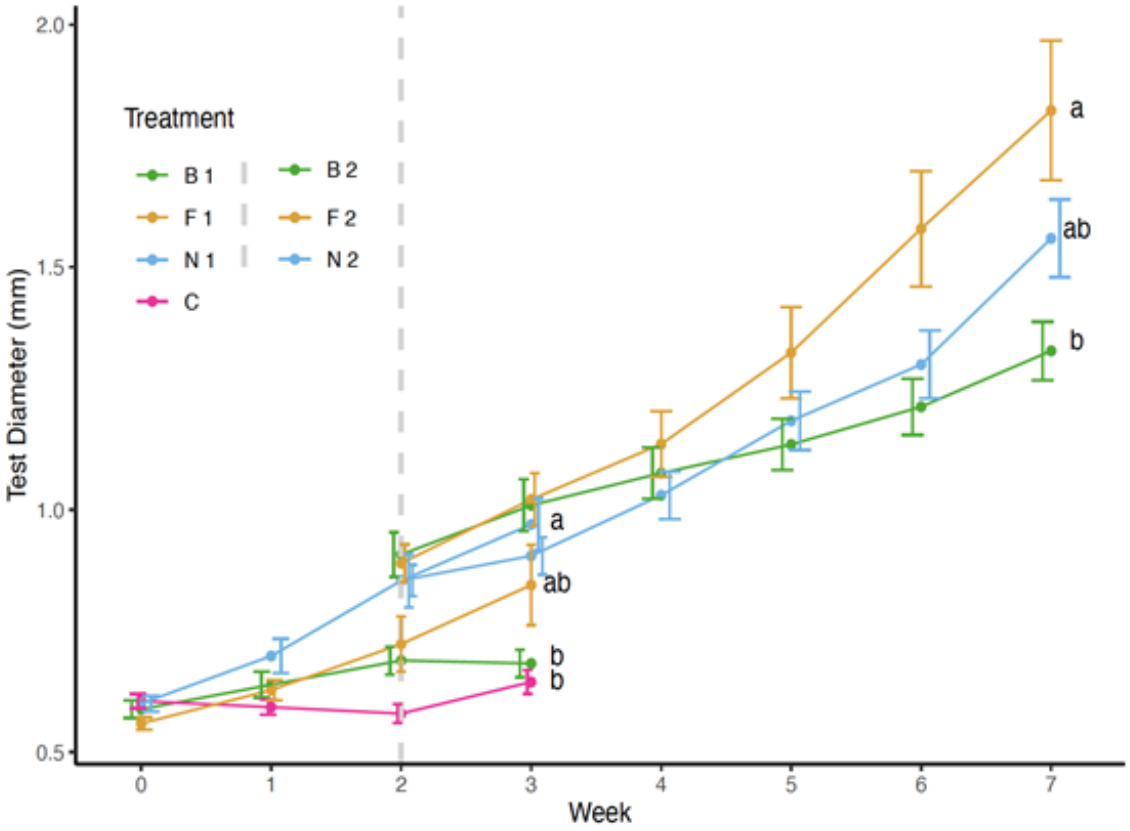

Figure 1. Growth of small juvenile *Diadema antillarum* over time when fed one of three experimental diets (B - biofilm, F - flocculated microalgae, N - *Navicula perminuta*). The dashed gray line separates Experiment 1 and Experiment 2, with an overlap of one week in terms of developmental time. C is a no feed control used exclusively in Experiment 1. Error bars represent mean ± SEM and letters denote significant differences among treatments with experiments.

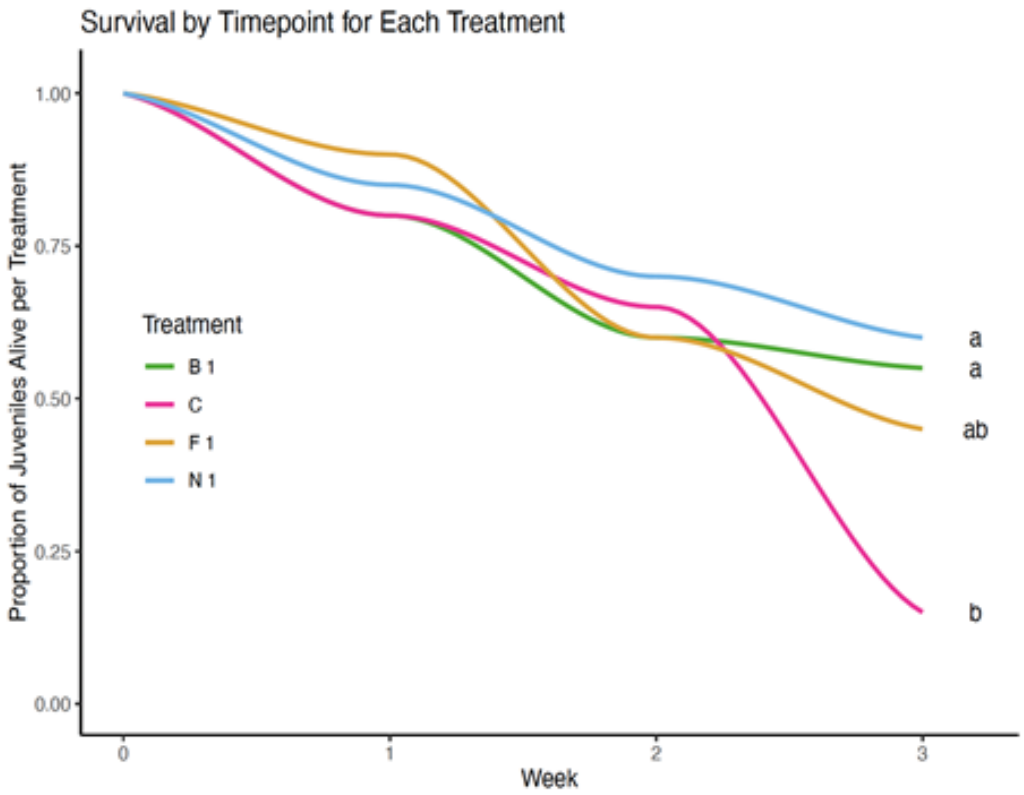

Figure 2. Proportional survival over time for newly metamorphosed *D. antillarum* in Experiment 1. Treatments were one of three experimental diets (B - biofilm, F - flocculated microalgae, N - *Navicula perminuta*) plus a no feed control (C). Lowercase letters denote significant differences among treatments.

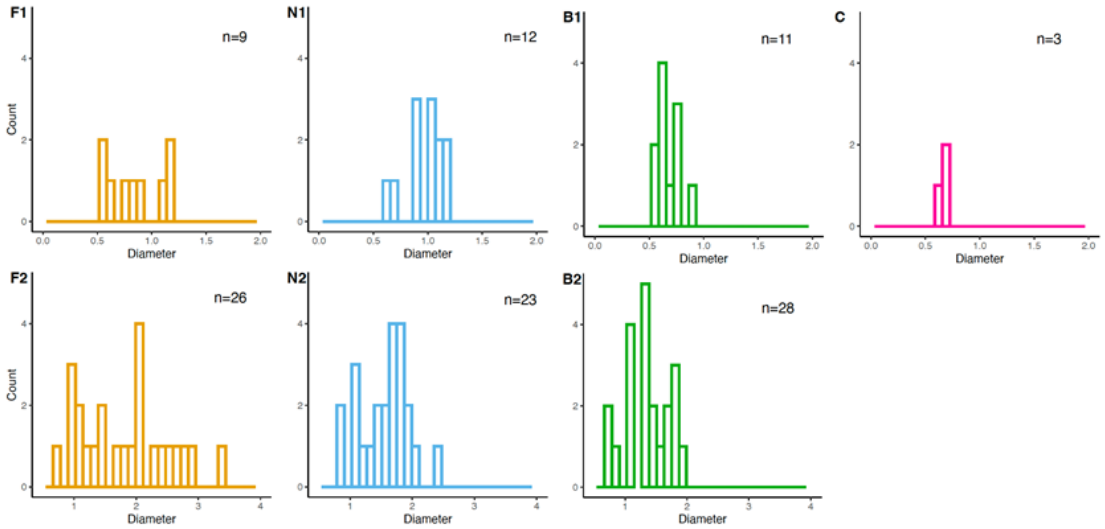

Figure 3. Histograms showing *D. antillarum* test diameter size frequency distributions for Experiment 1 (top row) and Experiment 2 (bottom row). Treatments were one of three experimental diets (B - biofilm, F - flocculated microalgae, N - *Navicula perminuta*) plus a no feed control (C), which was applied exclusively in Experiment 1. On each histogram, *n* is the total number of individual animals with a test diameter reported.

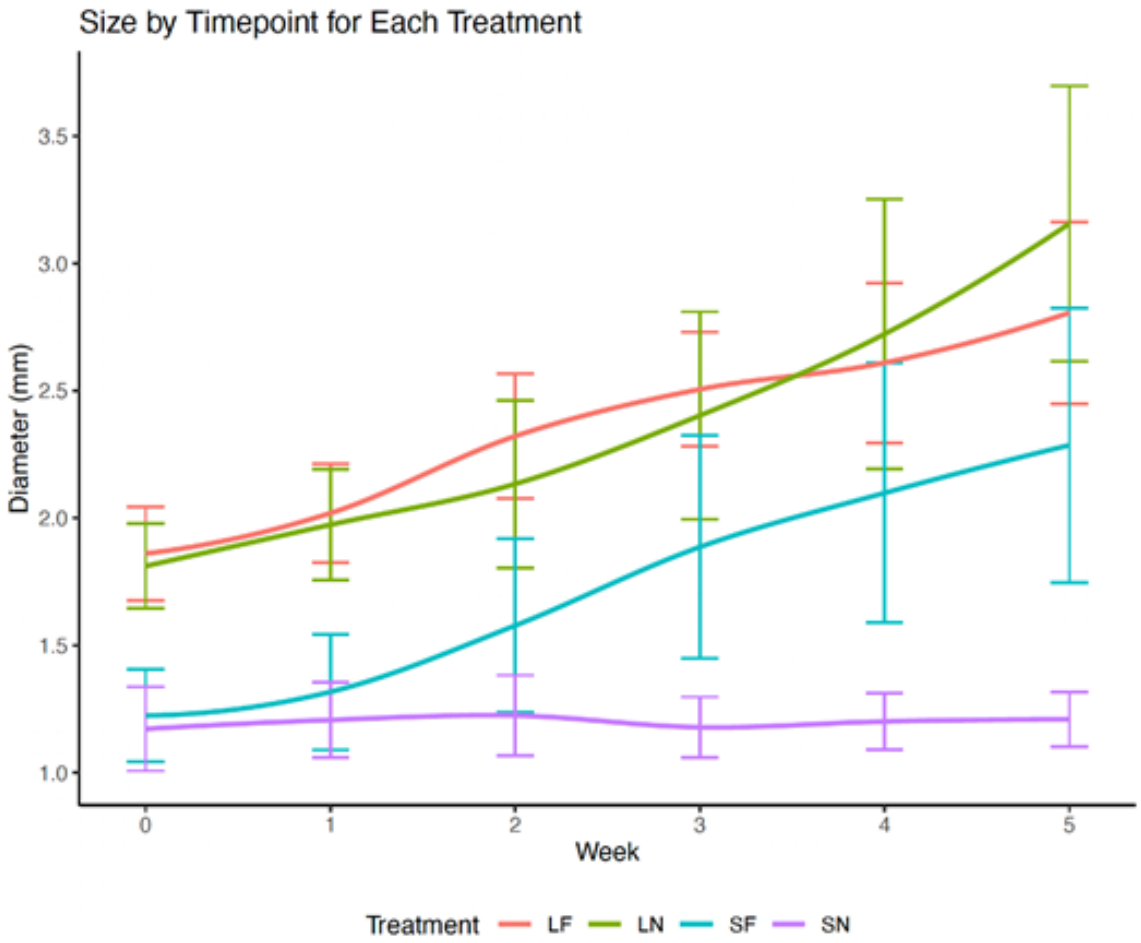

Figure 4. Growth of juvenile *Diadema antillarum* of two size classes (Large or Small - L or S) over time when fed one either flocculated microalgae (F) or dried nori (N).

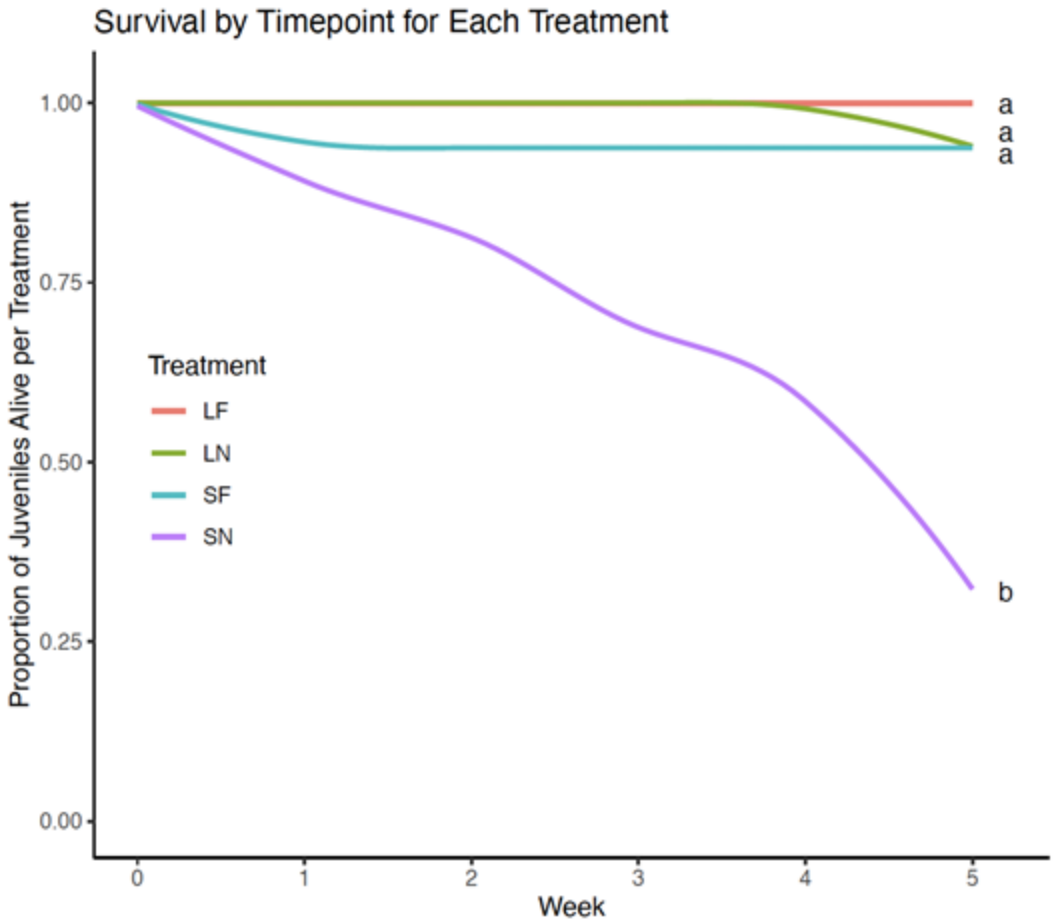

Figure 5. Proportional survival over time for small juvenile *D. antillarum* in Experiment 3. Treatments were one of two urchin size classes paired with one of two diets. LF - larger urchins fed flocculated microalgae, LN - larger urchins fed dried nori, SF - smaller urchins fed flocculated microalgae, SN - smaller urchins fed dried nori. Lowercase letters denote significant differences among treatments.

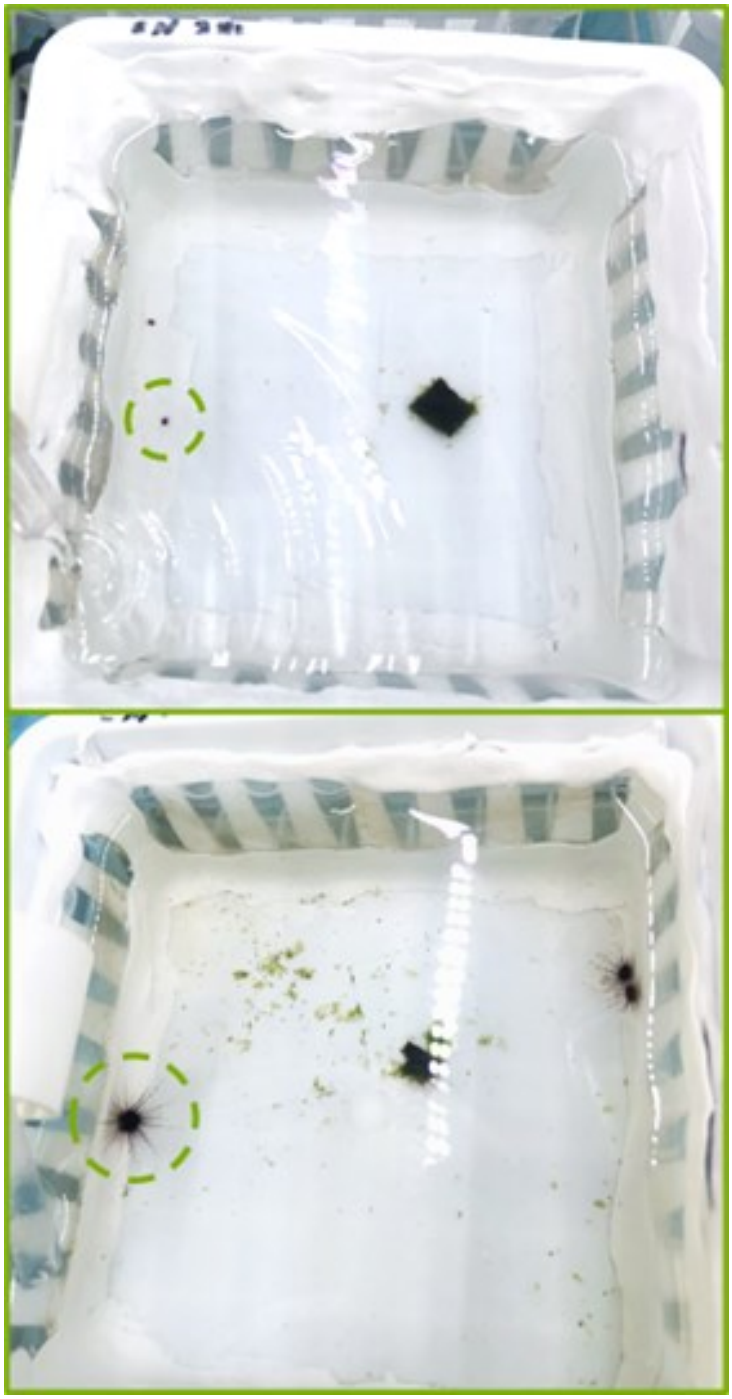

Figure 6. **-** Representative photographs from Experiment 3. Urchins in the SN treatment (circled, top photo) appeared to be too small to physically consume dried nori, while urchins in the LN treatment (circled, bottom photo) were able to macerate the food with growth the presence of fecal matter suggesting consumption and digestion. Both photos are from the end of the experiment at 5 weeks, which was 12 weeks post metamorphosis for the urchins.

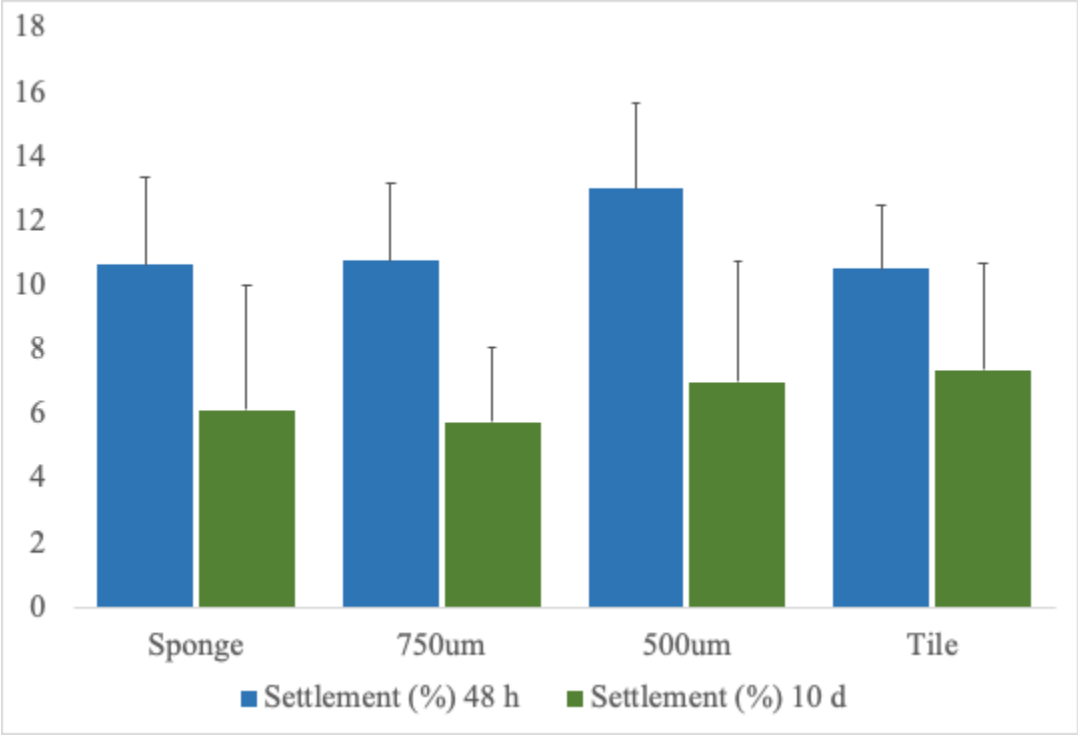

Figure 7. Cumulative settlement (# of settled juveniles on focal substrate, no-substrate control, and 'other'/vertical tank surfaces combined) by treatment. No apparent effect of treatment group on overall settlement rate in either 48 h or 10 d sampling time points. Error bars are standard deviation.

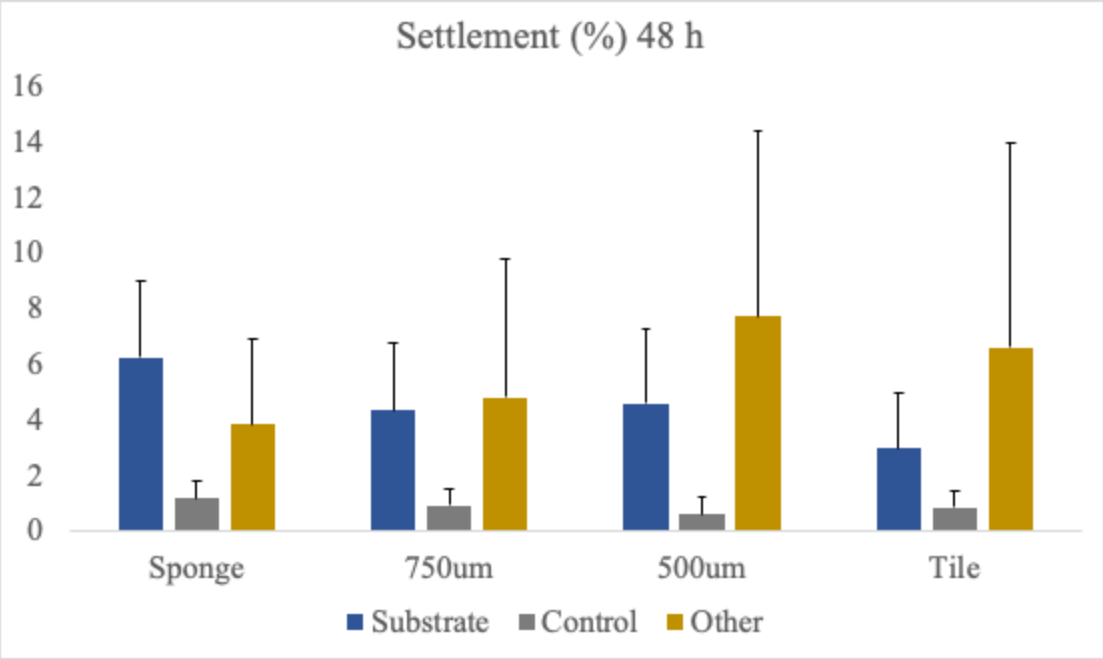

Figure 8. Summary of Experiment 1 results at 48 h time point. Mean number of settled juveniles and post-larvae by substrate type. Note the substantial settlement on 'other' surfaces among all treatment groups - these 'other' surfaces are all oriented vertically. Error bars are standard deviation.

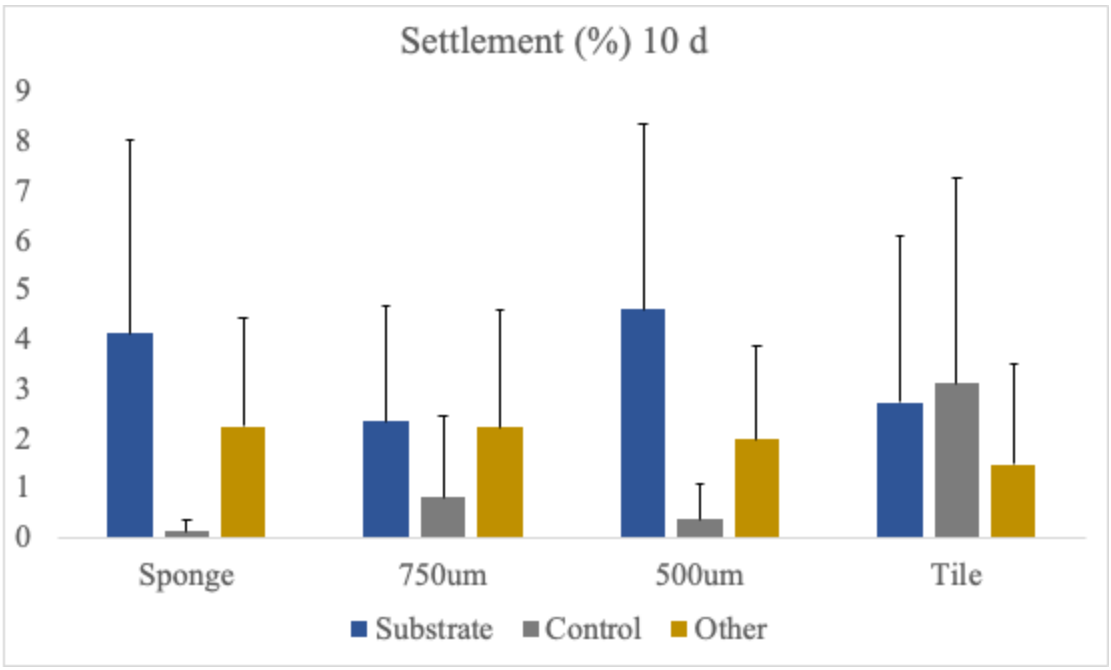

Figure 9. Summary of Experiment 1 results at 10 d time point. Mean number of settled juveniles and post-larvae by substrate type. Note the shift in relative settlement in the 'tile' (cut travertine/limestone tile) treatment group. Error bars are standard deviation.

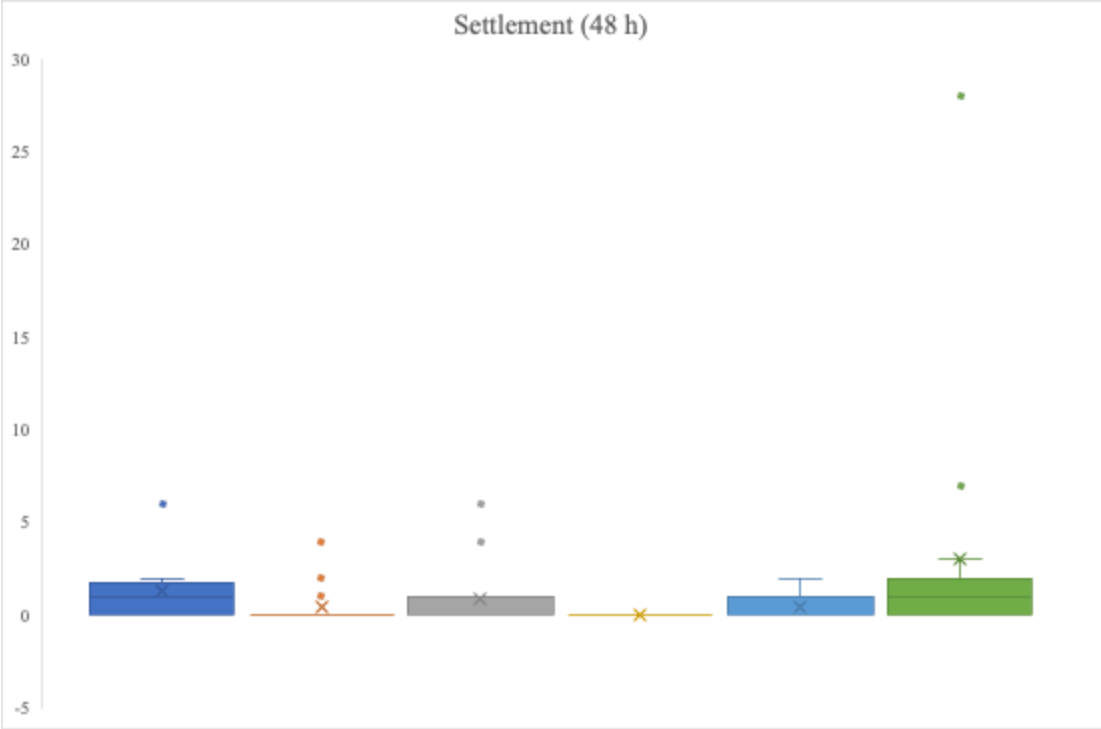

Figure 10. Summary of results of 48 h time point in Experiment 2. Note low mean settlement and extreme variance within and among substrates and trials. Error bars are standard deviation.

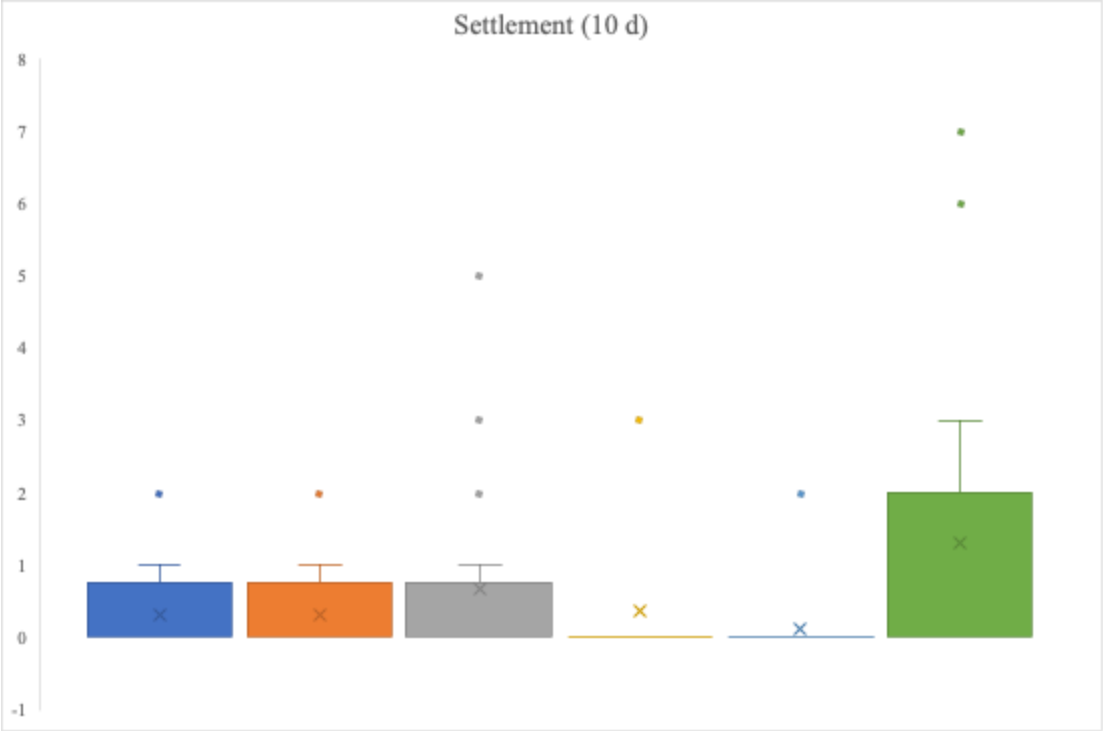

Figure 11. Summary of results of 10 d time point in Experiment 2. Note low mean settlement and extreme variance within and among substrates and trials. Error bars are standard deviation.

Figure 12. Candidate substrates selected for both Experiment 1 and Experiment 2. Left to Right: Black high density aquaculture filter sponge, cut travertine/limestone tile, 500um nylon mesh, 750um nylon mesh.

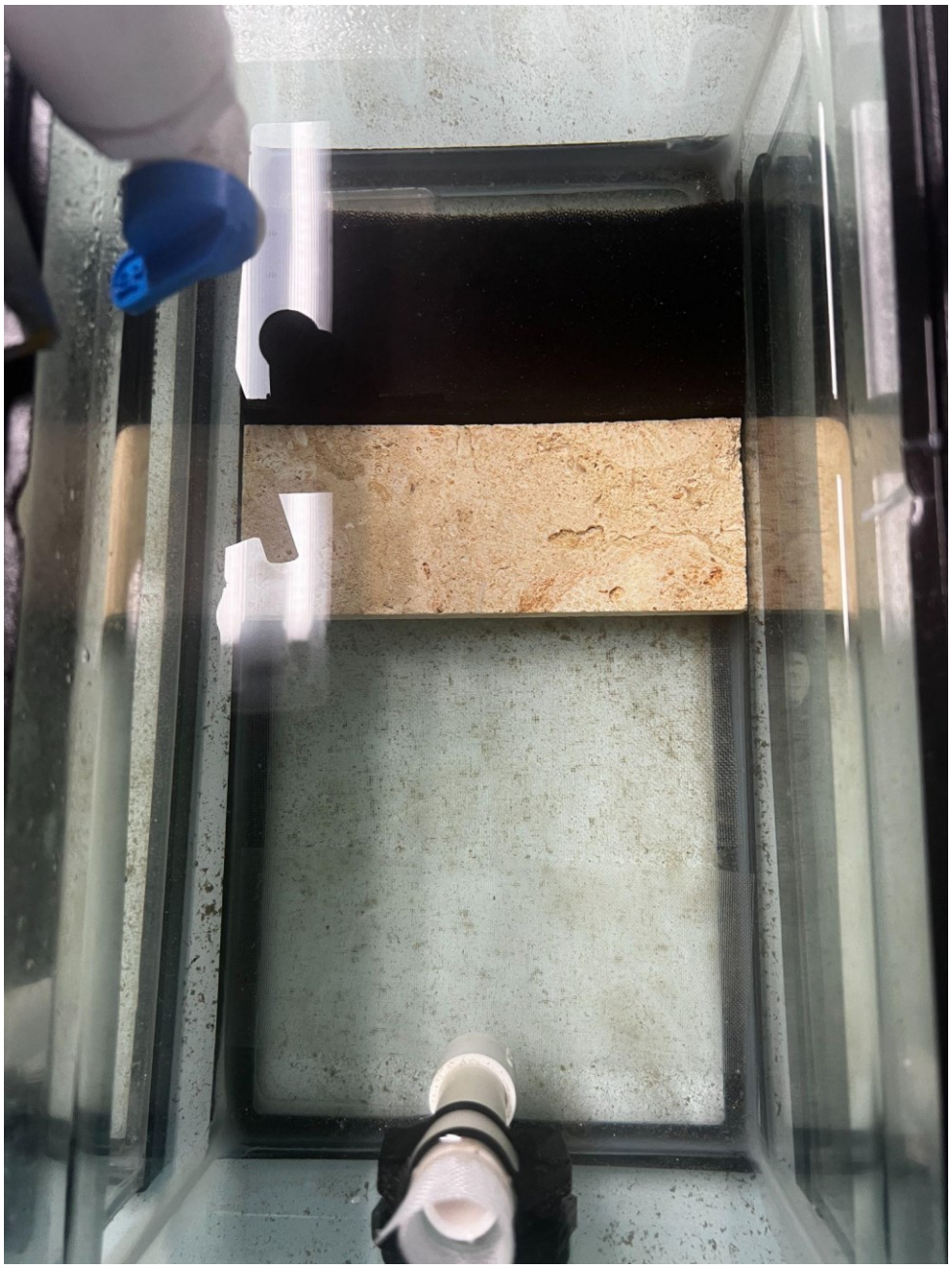

Figure 13. Representative photo of experimental setup in Experiment 2 - settlement preference experiment. Candidate substrates are laid horizontally on the bottom arranged top to bottom: Black high density aquaculture filter sponge, cut travertine/limestone tile, 750um nylon mesh, 500um nylon mesh.

Figure 14. Representative photo of a juvenile *M. spinosissimus* crab on the black high-density aquaculture filter sponge substrate at a 10 d time point during experiment 1.

Table 1. Summary of mean settlement (%) by substrate type and time point in Experiment 1. Mean settlement across all treatments was 11.22% (+/- 1.19 SD) at 48 hours and 6.57% (+/- 0.75 SD) at 10 days.

Experiment 1 - Settlement Rates

	Sponge	750um Mesh	500um Mesh	Tile	Mean
Settlement (%) 48 h	10.63	10.75	13	10.5	11.22
Settlement (%) 10 d	6.13	5.75	7	7.38	6.565

Table 2. Table summarizing the results of the repeated measures analysis of variance on the data fit with a general linear model using the fitrm and ranova functions in Matlab 2025a.

	SS	**DF**	**F**	**p**
Time	6.0563	1	1.1335	0.2889
Treatment:Time	0.46991	1	0.087945	0.76713
Error	1015.2	190		

Table 3. Table summarizing Manly's alpha values for settlement onto each candidate substrate at the 48-h time point. None of the candidate substrates exhibited a preference, in fact all, according to the model, all were avoided. Note that the cut limestone tile, the only natural substrate, did not have a single larval settler at the 48-h time point.

48 h Settlement Preference

Treatment	Mean Settlement	Expected Settlement	Manly's Alpha
Sponge	1.31	10	0.026
750 Mesh	0.44	10	0.009
500 Mesh	0.94	10	0.019
Tile	0.00	10	0.000
Control	0.50	10	0.010

Table 4. Table summarizing Manly's alpha values for settlement onto each candidate substrate at the 10 d time point. Again, in the absence of corrections for larval mortality, non of the candidate substrates were 'preferred' by *M. spinosissimus* larvae.

10 d Settlement Preference

Treatment	Mean Settlement	Expected Settlement	Manly's Alpha
Sponge	0.31	10	0.006
750 Mesh	0.31	10	0.006
500 Mesh	0.69	10	0.014
Tile	0.38	10	0.008
Control	0.13	10	0.003

Table 5. Table summarizing Manly's alpha values for settlement onto each candidate substrate at the 10 d time point with 'Other' substrate treatment included due to substantially higher settlement on vertical bare tank surfaces. Larvae did not exhibit a preference for any of the candidate substrates, including 'Other'.

10 d Settlement Preference

Treatment	Mean Settlement	Expected Settlement	Manly's Alpha
Sponge	0.31	8.33	0.006
750 Mesh	0.31	8.33	0.006
500 Mesh	0.69	8.33	0.014
Tile	0.38	8.33	0.008
Control	0.13	8.33	0.003
'Other'	1.31	8.33	0.026

Table 6. Table summarizing Manly's alpha values for settlement onto each candidate substrate at the 10 d time point with 'Other' substrate treatment included and expected settlement adjusted for observed mean larval mortality among trials. Note that, when corrected for larval mortality, 'Other' (vertical bare tank surfaces) and 500 um nylon mesh substrates were preferred settlement substrates.

10 d Settlement Preference

Treatment	Mean Settlement	Expected Settlement	Manly's Alpha
Sponge	0.31	0.52083	0.099
750 Mesh	0.31	0.52083	0.099
500 Mesh	0.69	0.52083	*0.221
Tile	0.38	0.52083	0.122
Control	0.13	0.52083	0.042
'Other'	1.31	0.52083	*0.419