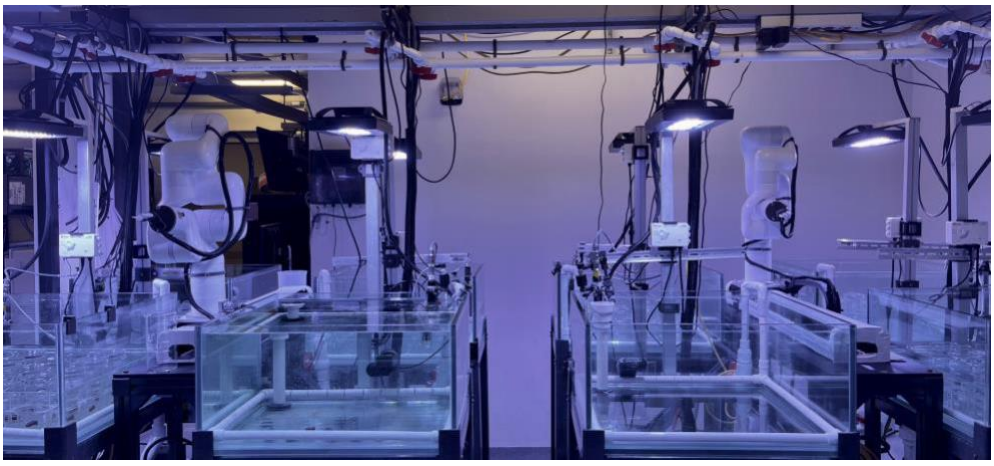
Assessing the effects of environmental cofactors on Stony Coral Tissue Loss Disease (SCTLD) transmission and progression rates Stony Coral Tissue Loss Disease



Two robot arms used at the Experimental Reef Lab to dose nutrients and disease into independent coral vessels.

Assessing the effects of environmental cofactors on Stony Coral Tissue Loss Disease (SCTLD) transmission and progression rates Stony Coral Tissue Loss Disease

Final Report

Prepared By:

Ana Palacio Castro [1,2]
Michael S. Studivan [1,2]
Ian C. Enochs [2]

[1] Cooperative Institute For Marine And Atmospheric Studies
[2] Atlantic Oceanographic and Meteorological Laboratory

6/15/2023

Completed in Fulfillment of Grant SA00002041; PO C01B6 for

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

This report should be cited as follows:
Palacio-Castro AM, Studivan M.S., Enochs I.C. 2023. Assessing the effects of environmental cofactors on Stony Coral Tissue Loss Disease (SCTLD) transmission and progression rates Stony Coral Tissue Loss Disease. DEP. Miami, FL. 1-19

This report was prepared for the Florida Department of Environmental Protection's (DEP) Coral Protection and Restoration Program by the University of Miami. Funding was provided by the DEP Award No. C01B6. 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.

Management Summary

SCTLD has caused substantial coral die-offs on Florida's Coral Reef and throughout the Caribbean. Disease mitigation efforts can benefit from understanding the environmental conditions that increase host susceptibility to the disease as well as pathogen virulence. However, previous field-based monitoring studies have been inconclusive in identifying environmental conditions that favor SCTLD incidence and prevalence. We built and used a new high-replication multi-stressor system to investigate the role of environmental cofactors, specifically ammonium input and high temperature, in *Orbicella faveolata*'s susceptibility to stony coral tissue loss disease (SCTLD). In this project, we (1) incorporated the use of artificial seawater in the Experimental Reef Lab in order to control water quality and reduce external natural seawater fluctuations (e.g., variable nutrient levels, potential pathogen inputs); (2) developed the Sequential Treatment Application Robot (STAR) system for high-replication multi-stressor experiments, capable of running independent disease transmission assays while at the same time incorporating high- precision dosing of stressors (e.g., nutrients, sediments, and disease doses); (3) used this system to expose *O. faveolata* fragments to elevated ammonia and temperature for two weeks, and (4) evaluated if pre-exposure to these environmental conditions increase *O. faveolata* diseased susceptibility when exposed to diseased water. We found that corals exposed to higher temperatures (31 °C vs 28 °C) experienced lower disease transmission rates, independently of the nutrient treatment. Our experimental results support previous field studies that have found a reduction in SCTLD activity during the summer. Future work should test the effect of temperature on SCTLD using multiple corals species, assess additional temperature levels, and address the potential mechanisms by which temperature impact SCTLD dynamics.

Executive Summary

Since 2014, Florida's Coral Reef has experienced unprecedented coral losses due to stony coral tissue loss disease (SCTLD). Field reports have indicated seasonal differences in the disease incidence and prevalence, suggesting that SCTLD could respond to environmental variables that can exacerbate or mitigate transmission and lesion progression rates. However, there is no consensus yet about the potential conditions that can exacerbate SCTLD virulence or corals' susceptibility to it. For example, while some field studies suggest that elevated temperature could reduce SCTLD progression and incidence (Williams et al. 2021; Meiling et al. 2020), others have found that neither lower nor higher temperature affect SCTLD dynamics (Aeby et al. 2019, Alvarez-Filip et al. 2019, Estrada-Saldivar 2021, Muller et al. 2020), and one study has found that warmer temperatures promote faster tissue loss at least in one coral species (Paul et al. 2022). Similarly, while one ex-situ study found that nitrogen enrichment (NO_3) could increase SCTLD progression rates in at least one of two species tested (Aeby et al 2021), a field study that manipulated nutrient concentrations using Osmocote fertilizer found no differences in SCTLD progressions or surrounding prevalence near the nutrient-enriched locations (Carreiro 2022). Discrepancies between these results could arise from the fact that multiple environmental conditions often covariate in the field (e.g., seasonal changes in light, temperature, and nutrient levels), making it difficult to distinguish what specific factors or combination of factors shape SCTLD incidence and prevalence. Ex-situ manipulations of the specific environmental conditions can help to establish these relationships by precisely manipulating individual and combined factors (e.g., nutrients alone, temperature alone, and combined nutrients and temperature) and measuring corals' response to disease exposure.

In this project, we built and used a new high-replication multi-stressor system to investigate the role of environmental cofactors, specifically NH_4 input and temperature (spring vs summer values), in *O. faveolata*'s susceptibility to (SCTLD). We incorporated the use of artificial seawater in the Experimental Reef Lab (ERL) in order to control water quality and reduce external natural seawater fluctuations (e.g., variable nutrients and potential pathogen inputs). This was especially needed since previous nutrient data show high nutrient fluctuations in the water coming from Biscayne Bay. We then developed the Sequential Treatment Application Robot (STAR) system for high-replication multi-stressor experiments, capable of running independent disease transmission assays, while at the same time incorporating high-precision dosing of stressors. Finally, we used this system to expose *O. faveolata* fragments to nutrients and temperature conditions for two weeks, and then evaluated if pre-exposure to these environmental treatments increased *O. faveolata* disease susceptibility when exposed to diseased water. We found that corals exposed to 31 °C experienced lower disease transmission rates compared to corals maintained at 28 °C, regardless of the nutrient treatment. Our experimental results support previous field studies that have found a reduction in SCTLD activity during the summer. Future work assessing the role of temperature impact SCTLD dynamics should include multiple corals species, additional temperature levels, and address the potential mechanisms shaping these dynamics.

Acknowledgments

We would like to thank Dr. Chris Kelble, Tyler Christian, Alexandra Fine, and Ian Smith from NOAA/AOML for their assistance in processing the nutrient samples. Jocelyn Karazsia and Lisa Gregg were critical to obtaining the collection permits for the colonies of opportunity used in the experimental work. The first two rounds of disease donor corals were collected under FWC SAL-22-2116A-SCRP to Ian Enochs, and the third round of collections was conducted by Karen Neely under FWC SAL-22-2443-SCRP and FKNMS-2021-134-A1 to Andrew Baker and Carly Dennison.

Table of Contents

List of Figures

List of Tables

List of Acronyms

SCTLD - Stony Coral Tissue Loss Disease
STAR - Sequential Treatment Application Robot System
NOAA/AOML - National Oceanic and Atmospheric Administration Atlantic Oceanographic and Meteorological Laboratory
UM/AOML - University of Miami Atlantic Oceanographic and Meteorological Laboratory
ERL - Experimental Reef Laboratory
DEP - Department of Environmental Protection
qPCR - Quantitative Polymerase Chain Reaction
HN - High Nutrients
LN - Low Nutrients
RO - Reverse Osmosis

1. EXECUTIVE SUMMARY

Since 2014, Florida's Coral Reef has experienced unprecedented coral losses due to stony coral tissue loss disease (SCTLD). Field reports have indicated seasonal differences in the disease incidence and prevalence, suggesting that SCTLD could respond to environmental variables that can exacerbate or mitigate transmission and lesion progression rates. However, there is no consensus yet about the potential conditions that can exacerbate SCTLD virulence or corals' susceptibility to it. For example, while some field studies suggest that elevated temperature could reduce SCTLD progression and incidence (Williams et al. 2021; Meiling et al. 2020), others have found that neither lower nor higher temperature affect SCTLD dynamics (Aeby et al. 2019, Alvarez-Filip et al. 2019, Estrada-Saldivar 2021, Muller et al. 2020), and one study has found that warmer temperatures promote faster tissue loss at least in one coral species (Paul et al. 2022). Similarly, while one ex-situ study found that nitrogen enrichment (NO_3) could increase SCTLD progression rates in at least one of two species tested (Aeby et al 2021), a field study that manipulated nutrient concentrations using Osmocote fertilizer found no differences in SCTLD progressions or surrounding prevalence near the nutrient-enriched locations (Carreiro 2022). Discrepancies between these results could arise from the fact that multiple environmental conditions often covariate in the field (e.g., seasonal changes in light, temperature, and nutrient levels), making it difficult to distinguish what specific factors or combination of factors shape SCTLD incidence and prevalence. Ex-situ manipulations of the specific environmental conditions can help to establish these relationships by precisely manipulating individual and combined factors (e.g., nutrients alone, temperature alone, and combined nutrients and temperature) and measuring corals' response to disease exposure.

In this project, we built and used a new high-replication multi-stressor system to investigate the role of environmental cofactors, specifically NH_4 input and temperature (spring vs summer values), in *O. faveolata*'s susceptibility to (SCTLD). We incorporated the use of artificial seawater in the Experimental Reef Lab (ERL) in order to control water quality and reduce external natural seawater fluctuations (e.g., variable nutrients and potential pathogen inputs). This was especially needed since previous nutrient data show high nutrient fluctuations in the water coming from Biscayne Bay. We then developed the Sequential Treatment Application Robot (STAR) system for high-replication multi-stressor experiments, capable of running independent disease transmission assays, while at the same time incorporating high-precision dosing of stressors. Finally, we used this system to expose *O. faveolata* fragments to nutrients and temperature conditions for two weeks, and then evaluated if pre-exposure to these environmental treatments increased *O. faveolata* disease susceptibility when exposed to diseased water. We found that corals exposed to 31 °C experienced lower disease transmission rates compared to corals maintained at 28 °C, regardless of the nutrient treatment. Our experimental results support previous field studies that have found a reduction in SCTLD activity during the summer. Future work assessing the role of temperature impact SCTLD dynamics should include multiple corals species, additional temperature levels, and address the potential mechanisms shaping these dynamics.

2. INTRODUCTION

Florida's coral reefs are currently experiencing a multi-year disease-related mortality event that has resulted in massive die-offs in multiple coral species. Approximately 21 species of coral, including both Endangered Species Act-listed and the primary reef-building species, are experiencing tissue loss lesions, and some of them whole colony mortality. First observed near Virginia Key in late 2014, the disease has since spread to the northernmost extent of Florida's Coral Reef, and south to the Dry Tortugas. The best available information indicates that the disease outbreak is continuing to spread southwest and throughout the Caribbean. Coastal coral communities in Florida are also experiencing environmental stressors associated with climate change and anthropogenic activities, including coastal development, construction, and land-based sources of pollution. These factors can lead to additive or synergistic impacts on coral health as they often entail simultaneous exposures to high temperatures, low pH, elevated nutrients, and sedimentation stress.

The impacts of environmental cofactors on SCTLD susceptibility and prevalence are still relatively unknown. However, other coral diseases have been found to be exacerbated under elevated nutrients or temperature. High nutrients, increased the prevalence and severity of diseases in *Siderastrea siderea* (Voss and Richardson 2006, Vega Thurber et al. 2013) and *Orbicella* spp. (Bruno et al. 2003), and both elevated nutrients and temperature can disrupt corals' associated microbial communities, promoting the proliferation of opportunistic or pathogenic microbes (Vega Thurber et al. 2009, McDevitt-Irwin et al. 2017). In some coral species, genetic variability results in differential genotypic susceptibility to diseases (Vollmer and Kline 2008; Miller et al. 2019), but even disease-resistant genotypes can become disease-susceptible when they are exposed to additional stressors such as coral bleaching (Muller et al. 2018). Interestingly, for SCTLD in particular, field data has shown a negative correlation between the disease and thermal stress. These studies suggest that elevated temperature could reduce SCTLD progression and incidence (Meiling et al. 2020, Sharp et al. 2020, Williams et al. 2021). However, this hypothesis needs to be further tested as confounding environmental factors, that can also modify disease dynamics, are often correlated with temperature in the field (e.g., seasonal changes in light and nutrient levels). To our knowledge, there are no published studies addressing the effects of elevated nutrients on SCTLD, but preliminary experiments have suggested an increase in progression rates associated with higher nitrates in *Montastrea cavernosa* (Aeby et al. 2021).

To better understand the roles of different environmental cofactors on SCTLD dynamics, field studies need to be complemented with *ex-situ* experiments, where some environmental conditions can be precisely manipulated while maintaining other conditions constant among the treatments. These experimental designs are required to determine the effects of individual stressors on coral susceptibility to disease, as well as the potential interactive and/or synergistic impacts of multiple stressors. *Ex-situ* approaches will provide critical data to resource managers to evaluate risks associated with covarying environmental impacts and the persistence of the SCTLD outbreak and

will support improved regulations regarding water quality management and environmental impact mitigation from coastal development activities.

One critical limitation for performing multi-stressor experiments in combination with SCTLD exposures is the complexity of the *ex-situ* lab conditions required to allow adequate replication, precision, and independence of the treatments. The Experimental Reef Lab (ERL; https://www.aoml.noaa.gov/experimental-reef-lab/) at the University of Miami's Cooperative Institute for Marine and Atmospheric Studies (CIMAS) was designed and constructed by the Coral Program at NOAA's Atlantic Oceanographic and Meteorological Laboratory (AOML) to simulate end-of-century climate change scenarios for high-precision coral stress challenges. This state-of-the-art lab features two aquarium systems: (1) A system with 16 independent, recirculating 150 L aquaria, with a suite of instrumentation, gas flow controllers, and software to allow rapid modulation, feedback, and data collection of temperature, pH, and light parameters, and (2) A system with four 250 L flow-through raceways modified to support up to 160 0.5 L coral vessels with independent water sources for disease transmission assays and in-line disease treatments (full details in Studivan et. al 2022). Although the latter system is particularly well-suited for assessments of disease vectors and evaluations of treatment approaches, the current infrastructure does not have the capability to conduct high-replication disease challenges, while at the same time incorporating high-precision monitoring and control of environmental cofactors such as temperature, light, pH, nutrients, and sedimentation. This limits the implementation of experimental designs that incorporate disease challenges in combination with other stressors.

In this proposal, we built on our previous experience with *ex-situ* exposure of corals to multi-stressors and diseases (Young et al. 2020, Palacio-Castro et al. 2021, 2022, Studivan et al. 2021, 2022, 2023, Studivan et al. in review) to further test the interactions among pre-exposure to environmental stressors and coral responses to SCTLD. The overall goal of the proposal was to test, under controlled *ex-situ* conditions, if environmental co-stressors can exacerbate or mitigate SCTLD transmission and progression. To accomplish this, we developed the infrastructure required to conduct highly controlled and replicated experiments with the capacity of manipulating multiple environmental co-factors (e.g., light, temperature, nutrients, sediments), exposing the corals to consistent doses and pathogen loads of SCTLD, and monitoring the resulting SCTLD dynamics (transmission and lesion progression rates). Our specific objectives were to:

- **Increase the experimental replication capability for disease transmission experiments** in highly controlled aquaria by incorporating the individual coral vessels system previously used in disease transmission assays (e.g., Studivan et. al 2022).
- **Build the capabilities required to test multi-stressor effects on SCTLD**. This includes (1) an artificial seawater system that will not be subjected to external natural seawater fluctuations in water quality (e.g., variable nutrient levels, potential pathogen inputs) and (2) an automated high-precision dosing system to

achieve target levels of exposure to stressors (e.g., nutrients, sediments, and disease doses).

- **Experimentally test if environmental co-factors modify the transmission and progression rates of SCTLD** in ecologically important and/or ESA-listed coral species by pre-exposing coral fragments to elevated nutrients and/or temperature in a fully independent factorial design followed by SCTLD exposure.

3. METHODS

3.1. Description of the built STAR system

The STAR system (Fig. 1) implemented in this project consisted of (1) eight glass aquaria with temperature control (2). From these, six tanks contained (3) 16 beakers each, which hosted individual coral fragments subjected to a combination of temperature, nutrients, and disease treatment; and (4) two tanks held either diseased or healthy fragments which acted as donor colonies for the experiment. Two robotic arms (5), each one affixed to a linear track (6) allowed a robot to access and dose beakers in three of the large aquaria while sourcing the water from both, the diseased and healthy donor tanks. A dosing system (7) composed of a syringe pump that dosed nutrients, and two peristaltic pumps that dosed either healthy or diseased water, were attached to each arm and delivered specific treatments to the beakers. All pumps were connected to tubing (8) that was routed from the healthy and diseased tanks, or the nutrient stock reservoir (9), along the arm and track, to an end effector (10). Two vats (11) were used to mix and supply artificial seawater to the system. At a given time one vat was providing water to the two tanks containing the healthy and diseased donors, while the second vat was getting RO water (12) and then mixing the next batch of artificial seawater.

When an arm reached a beaker, it sequentially applied the treatments preprogrammed in a graphical interface (13) for that specific coral (e.g., either healthy or diseased water, and either nutrients or not nutrients). Finally, each beaker inside the large aquaria contained a stir bar to ensure gas exchange, water flow, and nutrient mixing, which were controlled through a stir plate system located under the six experimental glass tanks (14). Data on the arm movement and status were logged and uploaded online using a cellular-enabled watchdog device (15), which also alerted users if any errors were encountered.

Although this design was specifically targeting a multi-stressor waterborne disease transmission experiment, each of the subsystems with STAR is customizable for different applications, including tank size and numbers of replicates, as well as treatment types including stressor combinations, water sources, and volumes.

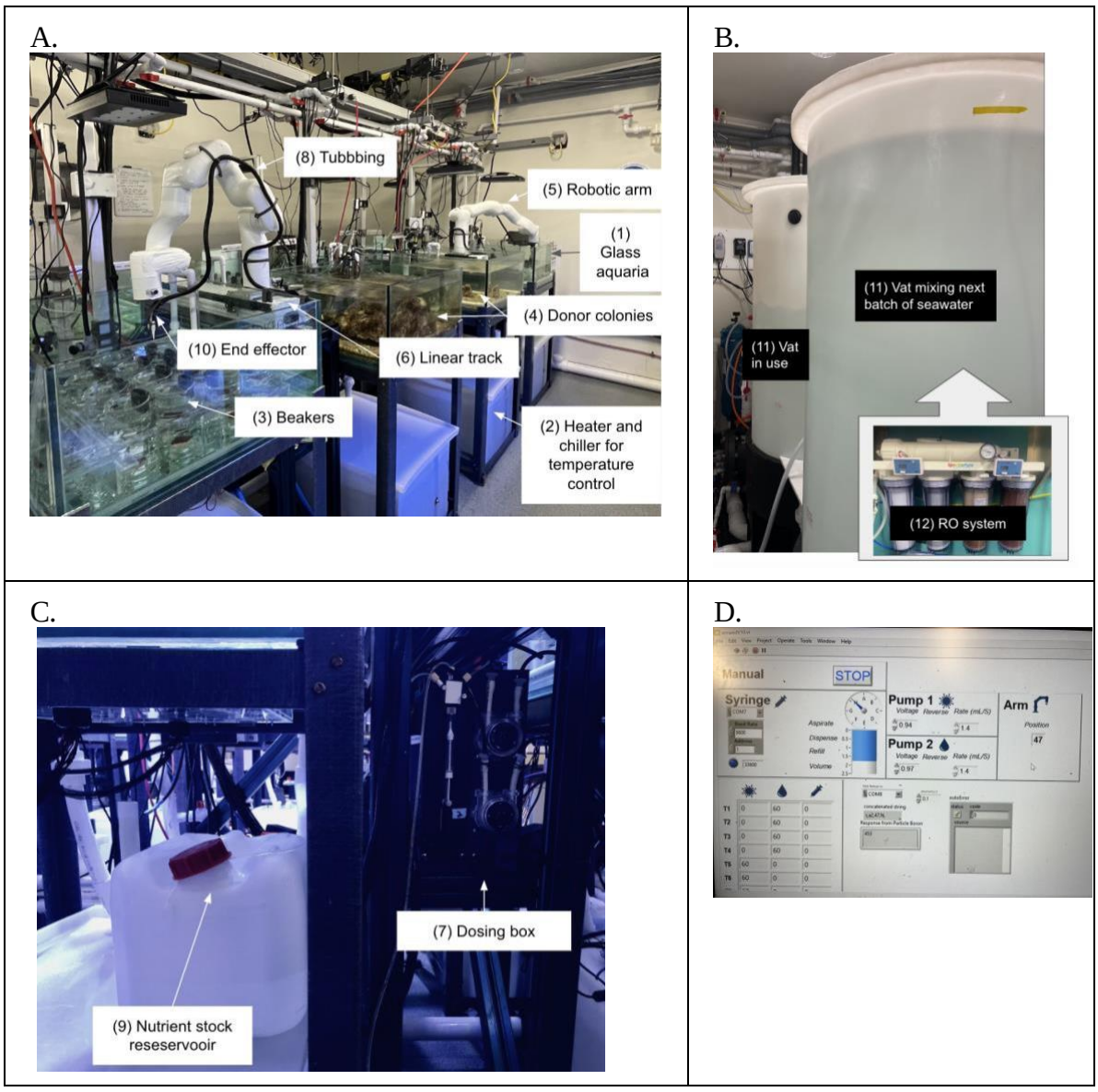


Figure 1*: STAR system set up in the University of Miami and NOAA Experimental Reef Lab. A. Integrated tanks and arms system, B. Artificial seawater system, C. Dosing box for water and nutrient delivery, and D. LabView graphic interface.*

3.1.1. STAR system physical components

Two 6-axis robot arms (xArm 6) and two linear tracks were purchased from UFACTORY. Each arm has a 6kg potential payload and a 700mm reach, which was further extended with a linear track that provided an additional 700mm of travel. A custom end effector was built and mounted to the distal surface of the arm, directing three

nozzles that dosed the different solutions to each beaker (seawater and nutrient stock). Tubes extend back from the nozzles and are routed along the arm using 3D-printed guides. Communication with the arm was provided through an AC-powered control box, which connects to a PC via an ethernet connection.

Additionally, two dosing boxes were built in-house. These included a syringe pump (Kloehn v6, Norgren) to dose small volumes of any solution (e.g., nutrients) and two brushless peristaltic pumps (A201BX, Anko) to dose bigger volumes (e.g., to provide seawater to the experimental units). Finally, the Stir plate system consisted of an array of 16 fan motors, affixed to laser-cut acrylic armature. A 3D-printed spacer was designed to hold two magnets in place with opposite poles facing upward on the top of each motor, which spin a stir bar placed in the overlying glass beaker when moving. The stir plate system ensures water mixing and gas exchange to maintain a healthy environment for the corals.

3.1.2. STAR system software

The STAR system and graphical user interface were programmed in LabView, and the control of the arm movement was achieved by calling Python functions for each movement step which targeted a specific coral vessel or beaker.

The LabView code includes eight different virtual instrument programs (VI's) that are organized in a single LabView project. These VI.s control the main user interface and loop of functions, including the initial startup, dosing treatments, arm steps, peristaltic pumps speeds, direction, and duration, and syringe pump volume, speed, and direction used in each step (beaker position; Fig. 1D).

The Python code was built using the xArm Python Software Development Kit. This code controls the arm position using a coordinate system that can be specified for each of the 48 beakers dosed by an arm. Each position is defined in cartesian coordinates (x,y,z), followed by a roll, pitch, yaw, and radius parameter for the arm.

The stir plate code was written in Arduino and uploaded to Teensy 3.5's using Teensyduino. The stir plate program has two different modes, one containing the main menu where the user can edit each of the motors' voltages individually to change the rotation speed, and an active mode that is displayed when the stir plate is in use and the controller is sending the user-input voltages to each motor.

Finally, a cellular watchdog coded using Particle.io sends error and treatment analytics to a Particle.io Boron device via serial communication. This information is published to ThingSpeak.com via a cellular modem. While this component is not critical to the operation of the STAR system, it is useful for collecting system statistics (e.g., time spent from one beaker to the next one) and quickly flagging and diagnosing potential issues.

3.2. Environmental co-factors experiment.

3.2.1. Experimental coral collection: Three *O. faveolata* and three *Pseudodiploria clivosa* colonies were collected as corals of opportunity in July 2022. The colonies were rescued after the collapse of the Star Island seawall at the Port of Miami and transported to the Experimental Reef Lab at the University of Miami Rosenstiel School. The colonies were maintained in touch tank systems at 28 °C until February 2023 when they were fragmented and labeled in preparation for the experiment.

Figure 2: *Divers recovering a coral colony off Star Island, Miami Beach, FL. (from left to right) C. Dennison and R. Karp from UM Miami's Baker Lab, J. Unsworth from the UM Lirman Lab, and K. Macartney, a NOAA CCME/University of Texas Post-Doctoral Fellow*

3.2.2. Pilot test of coral health in the STAR system: To ensure that the new system provided optimal conditions for coral maintenance, 15 coral fragments from the three collected colonies (including *O. faveolata* and *P. clivosa* fragments) were maintained in the STAR system for a 5-week period. The fragments were observed daily without detecting any signs of stress or tissue loss that can mislead the transmission experiment results. After this test, we proceed to run the experiment to test the effects of temperature and nutrients on corals' susceptibility to SCTLD.

3.2.3. Selection of experimental fragments: A subset of tissue samples were collected and preserved from each experimental colony (n=3 per colony) in October 2022 to assess their Symbiodiniaceae communities. Total DNA was extracted following standard protocols and analyzed using quantitative PCR (qPCR). These preliminary results showed that while *O. faveolata* colonies

were heavily dominated by the genus *Durusdinium*, *P. clivosa* colonies had more diverse Symbiodiniaceae communities with variable abundances of multiple genera. To remove the potential effects of symbiont identity from our study, we selected the *O. faveolata* colonies (n=80 fragments; 24-30 fragments per colony) to be used as the experimental corals (Table 1). Including corals with variable symbiont communities would have complicated an already multifactorial experiment (two nutrient levels and two temperature levels) and might add confounding effects.

3.2.4. Water source for the experiment: Artificial seawater was used throughout the duration of the experiment to avoid unwanted contamination from nutrients or other photogenic microorganisms present in Biscayne Bay seawater. Artificial seawater was made by mixing reverse osmosis (RO) water and Instant Ocean sea salt to 35 ppt salinity. Two ~ 750 L vats were used in the experiment (Fig. 1B). While one was supplying water to the experiment, the second one was being filled with RO and mixing the new batch of salts.

3.2.5. Temperature treatments: All corals by colony were haphazardly and equally allocated to two temperature treatments (28 °C and 31 °C). These treatments correspond to seasonal temperature conditions experienced on nearshore coral reefs in Florida and represent local ambient seasonal values (e.g., spring and summer). On day one of exposure to the environmental cofactors, corals assigned to 28 °C were directly transferred to beakers allocated to tanks set at this temperature. For the corals assigned to 31 °C, the initial temperature of 28 °C was gradually increased 0.4 °C twice a day (morning and afternoon) until the target temperature was reached (Fig. 2).

Temperature in each one of the eight tanks (six experimental tanks and two holding the donor colonies) was measured and recorded every 5 min with a high-accuracy RTD sensor (TTD25C, ProSense). Based on the temperature reading, the temperature was manipulated with a 300 W aquarium heater (TH300, Finnex) and a titanium chiller coil (Hotspot Energy) as described in Enochs et al. (2018).

3.2.6. Nutrient treatments: On day one of exposure to the environmental cofactors, corals assigned to the control ammonia treatment (LN) were placed inside beakers which were sequentially dosed with 60 mL of artificial seawater. Corals assigned to the high ammonia treatment (HN) were placed inside beakers which were sequentially dosed with 60mL and 0.5 mL of an ammonia solution [600uM] intended to replenish the ammonia loss in the water flowing out of the beakers. A subset of beakers (n=16) was assigned to be nutrient “blanks’. These were maintained under the different nutrient and temperature treatments but did not contain corals, in order to assess the potential effects of the biological activity (e.g., nutrient uptake) on the

nutrient concentrations. All nutrient treatments were evenly allocated among the two temperature treatments and coral colonies.

Nutrient samples (30-40 mL) were collected two to five times a week to monitor treatment levels using cleaned 50 mL syringes. The samples included each experimental beaker, the vats providing and mixing artificial seawater, the healthy and diseased donor tanks, and the nutrient stocks used to dose NH_4. Nutrient concentrations were measured using a Seal Analytical AA3 Analyzer. The instrument was calibrated before each run following the Standard Operating Procedure for the Calibration of the AA3 Nutrient Analyzer protocol (AOML-NL-SOP-031) using standard solutions.

3.2.7. Disease donors: Two *M. cavernosa* colonies showing signs of SCTLD were collected and transported to the ERL in order to inoculate the water used for disease transmission (Fig. 3). The colonies were located ~100m south of Broward County monitoring site BC1 (26.14758, -80.09610) at 21-23 ft depth. These colonies were maintained in a closed system for approximately two weeks until the disease transmission phase started. On day one of disease transmission, their holding tank started to provide water to the experimental beakers assigned to the disease treatment, and more artificial seawater was constantly added to the disease holding tank to replenish the water that was used.

Figure 3: One of the two M. cavernosa *colonies collected in Broward County to serve as a diseased donor.*

A second batch of disease donors (two *O. faveolata* and one *Colpophyllia natans*) were collected two weeks after the first donor colonies did not transmit disease and the tissue loss on them stopped. These new colonies were collected from Marker 48-6 off Marathon (24.68510, -81.04293) by Karen Neely. Lesions on these donors are progressing very quickly, suggesting active SCTLD.

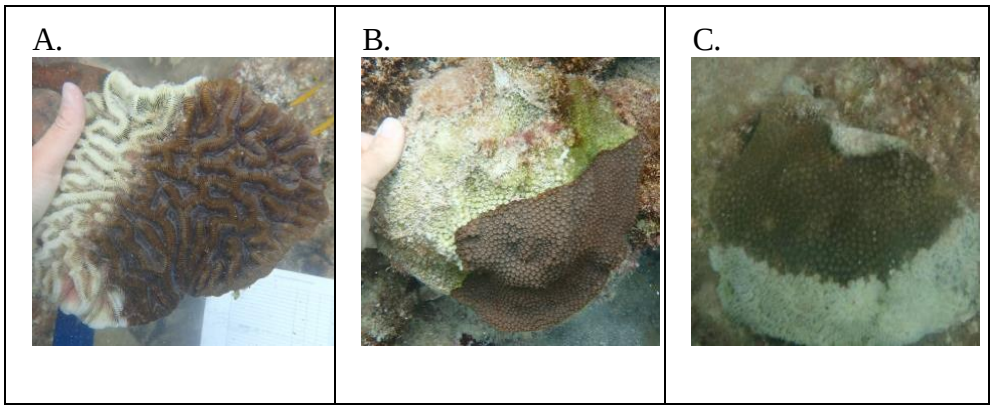


Figure 4: *Pictures of the second batch of disease donors collected near Marathon Key by Karen Neely and collaborators. A. Diseased* C. natans *donor, B. and C. Diseased* O. faveolata *donors.*

3.2.8. Disease treatments: After 16 days of exposure to the environmental co-factors, most experimental beakers (n = 66; Table 1) started to be exposed to the disease treatment, consisting of the addition of artificial seawater that came from the tank holding the disease donor colonies. Fewer fragments (n = 14; Table 1) were maintained under a placebo treatment and continued to be dosed with artificial seawater coming from the tank holding the healthy donor colony (Fig. 1). These last acted as negative controls for disease transmission to ensure that tissue loss was not present for other reasons not related to exposure to the pathogen.

Before starting the application of diseased water, the settings on the peristaltic water pumps were changed to slow down the seawater application. This was done to reduce the risk of cross-contamination among the beakers because of the occasional splashing observed using the original pump speed.

After two weeks of exposure to water coming from the first disease donor, none of the experimental fragments showed signs of SCTLD, and lesion progression on the donor colony had stopped. To adjust to this, a second batch of SCTLD donors was introduced in the disease donor tank (Fig. 4). These new donors showed continued active lesion progression (Fig. 5).

Table 1: *Summary of the experimental fragments used in the environmental cofactors experiment and the experimental conditions that they were exposed to.*

Colony ID	Environmental cofactors treatment	28 °C + HN	28 °C + LN	31 °C + HN	31 °C + LN	Total
	Disease treatment					
A	Placebo	1	2	1	2	**6**
C	Placebo	1	1	0	1	**3**
D	Placebo	1	1	2	1	**5**
A	SCTLD	6	6	6	6	**24**
C	SCTLD	5	6	5	5	**21**
D	SCTLD	5	5	6	5	**21**
Total		**19**	**21**	**20**	**20**	**80**

3.2.9. Disease monitoring: Daily observations and pictures were collected once the corals started to be exposed to the diseased water. Observations stopped when three consecutive days with no new lesions were recorded.

A.

B.

Figure 5*: Disease progression in the donor tank. A. disease donors seven days after collection. B. disease donors nine days after collection.*

4. RESULTS

4.1. STAR dosing system performance during the environmental co-factors experiment.

The STAR system has been continuously running for 41 consecutive days. During the first 16 days, the system was dosing artificial seawater from the healthy donor tank to all the beakers (60 mL each dose, n = 96 beakers), and a solution of ammonia at 600uM concentration to the beakers assigned to the HN treatments (0.5 mL per dose, n = 43 beakers). These dosing conditions characterized phase 1 of the experiment, when the corals were preconditioned to the environmental co-factors (nutrients and temperature) without being exposed to SCTLD. In Phase 1, the peristaltic pumps were set to dose seawater at a speed of 2.6 mL s^{-1}, resulting in an average dose duration of ~ 39 s per beaker (Table 2; Fig. 6A). However, individual dose durations were shorter for the beakers that were only dosed with seawater, compared to the ones dosed with water and nutrients (Fig. 6). Under these dosing conditions, the duration of an Arm round (the time needed for the arm to come back to a beaker) increased to ~39 min, resulting in ~ 4.6 full volume seawater exchanges in each beaker per day, and a total addition of nutrients of ~14 u mol NH_4. Since occasional splashing between beakers was observed under this design speed, the voltage of the peristaltic pump was reduced for the next experimental phase when splashing could cause cross-contamination between the disease and placebo treatments.

Table 2: *Statistics of the STAR system while exposing the corals to the environmental co-factors (Phase 1) and while exposing the corals to the environmental co-factors and disease (Phase 2). Arm describes the two robot arms in the STAR system.*

Phase	Arm	Mean duration/ beaker (s)	Mean duration round (min)	Doses/ day	Vol SW / day (mL)	Full SW changes / day	Mean NH_4 / day (umol)
1	Armand	38.7	31.0	46.5	2789.8	4.6	13.9
1	Armanda	39.1	31.3	46.0	2761.3	4.6	13.8
2	Armand	61.4	49.2	29.3	1757.9	2.9	13.2
2	Armanda	61.7	49.3	29.2	1751.1	2.9	13.1

During the following 28 days of the experiment (days 17-41), the START system dosed artificial seawater from the disease donor tank to 66 of the 80 beakers in the system, and water from the healthy donor tank to the remaining 14. This was accomplished while also maintaining the nutrient dosing in the beakers allocated to HN, regardless of the disease treatment. These dosing conditions characterized phase 2 of the experiment, when the

corals continue to be exposed to the environmental co-factors while at the same time being exposed to SCTLD. For Phase 2, peristaltic pump speeds were reduced to 1.4 mL s^{-1}, resulting in an average dose duration of ~ 61 s^{-1} per beaker (Table 2; Fig. 6B). Under these dosing conditions, the duration of an Arm round increased to ~49 min, resulting in ~ 3 full volume seawater exchanges in each beaker per day. To maintain a similar daily nutrient addition, the nutrient dose was increased from 0.5 mL to 0.75 mL per dose, resulting in a total addition of nutrients of ~13 u mol NH_4 per day.

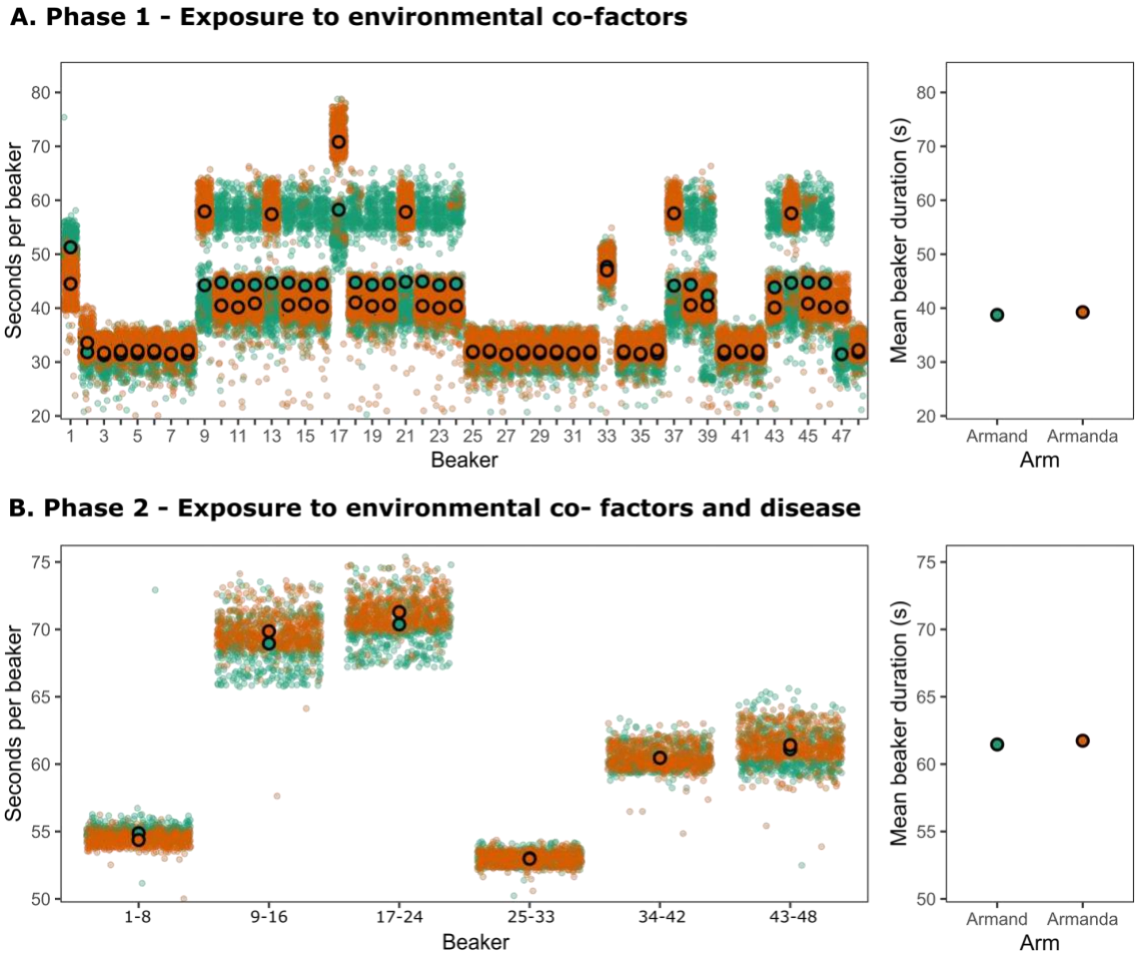


Figure 6: *Duration of an arm step reported using the watchdogs and using Particle.io software. The x-axis represents the beaker number and the y-axis the seconds spent by the arm dosing that beaker. The colors represent the two different arms being used. A. duration of the dosing steps during Phase 1 of the experiment. B. duration of the dosing steps during Phase 1 of the experiment when arms when the dosing was slower. Data collection was pooled every eight beakers for Phase 2 of the experiment since individual beaker statistics were unnecessary and expensive.*

4.2. Temperature conditions

The temperature treatments have been maintained for the 41 days of the experiment, during which more than 89,000 temperature measurements were collected (Fig. 7). Three experimental tanks and two tanks holding the donor colonies have been maintained at 28 °C since day one of the experiment. There was no ramp-up for these corals since they were already acclimated to this temperature. For the three experimental tanks assigned to 31 °C, the temperature was ramped up from 28 °C to 31 °C during a 4-day period by increasing 0.4 °C in the morning and in the afternoon. We did not observe bleaching, paling, or other stress signs in the fragments maintained under elevated temperature.

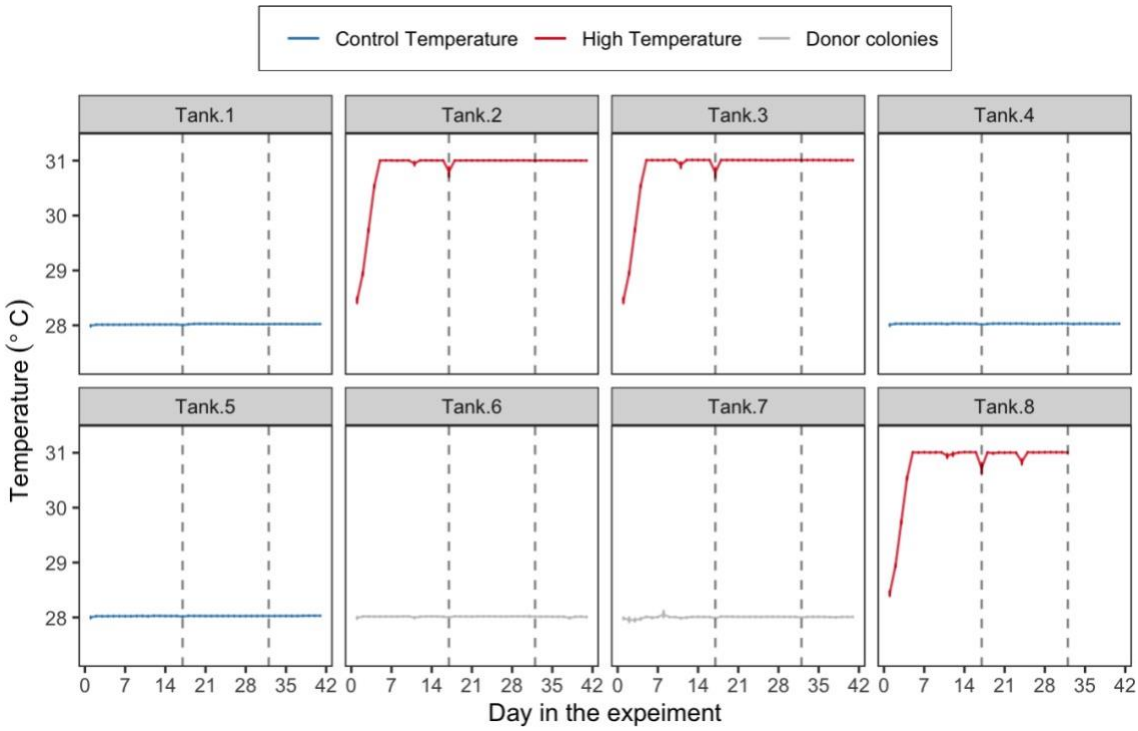


Figure 7: *Temperature conditions in each one of the tanks used in the experiment. Tanks 1-5 and 8 hosted the experimental beakers. Tanks 6 and 7 hosted the diseased and healthy donor colonies, respectively. The vertical dashed lines show the days when the first and second batches of disease donors were introduced to the experiment.*

4.3. Nutrient conditions

The nutrient treatments have been applied to the beakers for the 41 days of the experiment. This represents approximately 1,500 nutrient doses added to each beaker assigned to the HN treatment. During the experiment, more than 2,000 nutrient samples have been collected and analyzed for NH_4 concentration (Fig. 8). We found that ammonium concentrations in the LN treatments were low, suggesting that artificial seawater is a good option for nutrient-related experiments. The ammonium concentrations in the HN treatment were variable, showing a strong nutrient uptake in the beakers, especially during the first three weeks of the experiment. We also found that corals being dosed with the disease water were exposed to higher nutrient concentrations

than the corals exposed to the healthy water. This is not unexpected since the tissue released in the tank containing the disease donor was providing additional ammonium to the corals exposed to this water source.

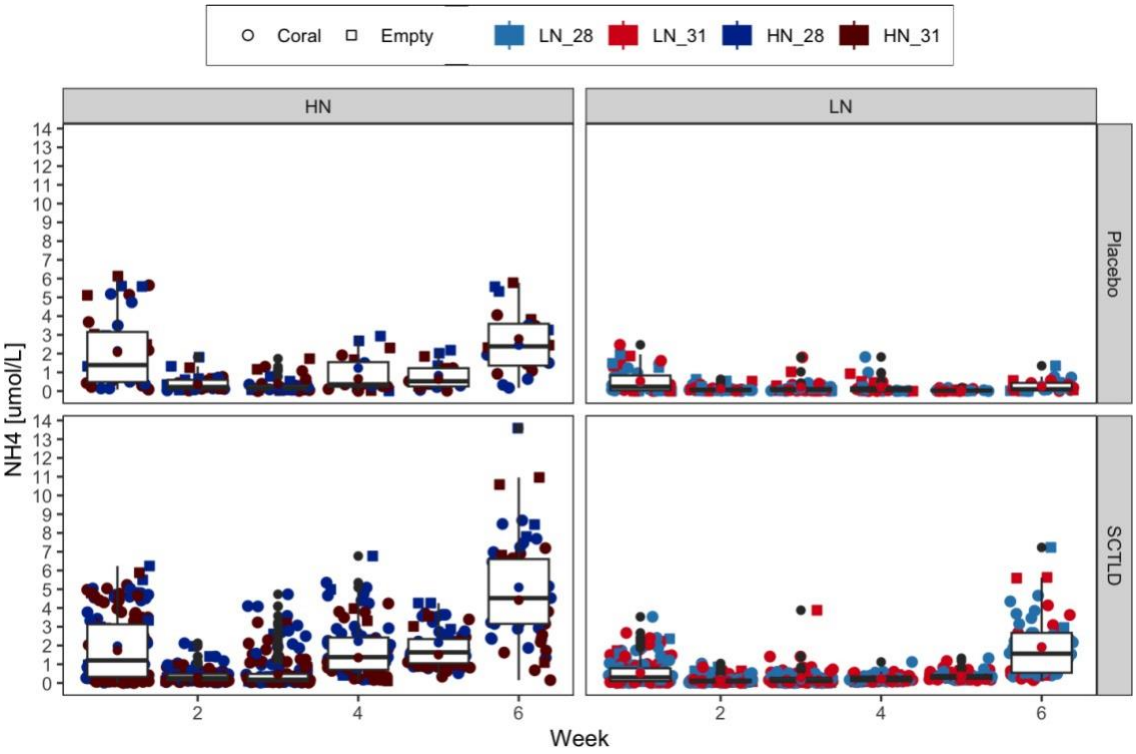


Figure 8: *Weekly ammonium concentrations in the experimental beakers. The colors represent the environmental conditions of each beaker as a combination of nutrient and temperature treatments. The shapes of the individual data points denote if the beaker contained an experimental coral (circles) or if it was a blank (squares).*

4.4. Disease lesions

A confirmed (progressing) disease lesion appeared in one fragment after 12 days of exposure to the second batch of donors (Fig. 9). New diseases lesions were recorded daily by monitoring all corals in the morning and the afternoon. After three consecutive days without recording new lesions (days 21, 22 and 23 after exposure to the second batch of donors), the diseased trial was ended. A total of 26 fragments developed progressing lesions during the disease trail, most of them in the 28 °C temperature treatment (23/26) with only three lesions being developed in the 31 °C temperature treatment (Table 3). The nutrient enrichment did not affect disease transmission, with 14 lesions occurring in the LN treatments and 12 lesions in the HN treatment (Table 3).

Table 3: *Number of fragments showing tissue loss in each one of the combined nutrients, temperature, and disease exposure treatments.*

	Genotype			
Treatment	**A**	**C**	**D**	**Grand Total**
28 °C + LN + Placebo	0	0	0	**0**
28 °C + HN + Placebo	0	0	0	**0**
31 °C + LN + Placebo	0	0	0	**0**
31 °C + HN + Placebo	0	0	0	**0**
28 °C + LN + SCTLD	4	4	4	**12**
28 °C + HN + SCTLD	5	2	4	**11**
31 °C + LN + SCTLD	2	0	0	**2**
31 °C + HN + SCTLD	1	0	0	**1**
Total	**12**	**6**	**8**	**26**

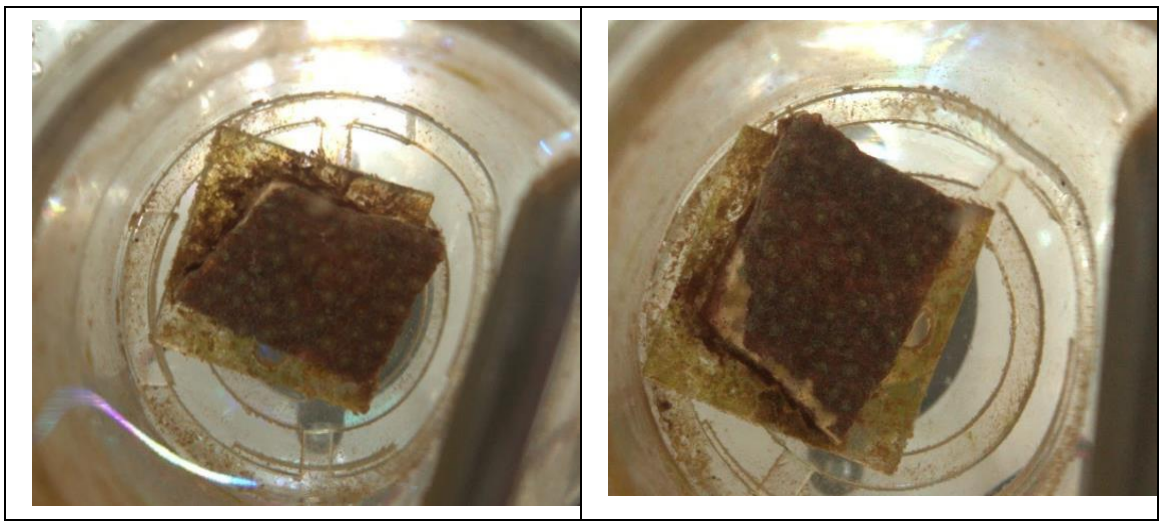


Figure 9: *Same coral fragment photographed within a day difference showing progressing tissue loss.*

5. DISCUSSION

5.1. Implementation of the STAR system for multi-stressor and disease experiments

The use of robotic arms offered a promising avenue for improving the study of multi-stressor effects on coral health and their susceptibility to additional threats such as warming or diseases. In general, multi-stressor studies are complex since the replication needed to test the effects of individual and combined stressors increases exponentially with the addition of one stress source. This, in turn, increases the costs, potential complications, and potential issues when carrying out the experiments. Here we implemented robotic automation to overcome these challenges by developing the Sequential Treatment Application Robot (STAR) system and used this system to study the effects of temperature and nutrients on coral susceptibility to SCTLD.

The STAR system allowed unsupervised constant treatment application, during day and night, with high precision. In combination with the current capabilities to manipulate temperature and light conditions at the Experimental Reef Laboratory, the STAR system allows to (1) combine controlled temperature and light conditions with precise doses of nutrients, (2) control the volumes of disease water applied to a given coral and build dose/response curves, (3) combine the application of multiple stressors such as disease, nutrients, acidified water, or sediments, and (4) compare potential disease treatments (e.g., multiple antibiotics and probiotics) and find effective doses.

In our specific experiment, the robotic arms were programmed to dispense nutrients (NH_4) and diseased water on specific beakers and time intervals, ensuring uniform nutrient and disease exposure across experimental samples. This facilitated investigating the impacts of ammonium enrichment on corals susceptibility to SCTLD. The use of the STAR system, in combination with constant monitoring of the ammonia concentration in the stock solutions minimized human error and variability in nutrient dosing. Additionally, the sequential dosing of the disease water combined with the high replication and independence of each beaker ($n = 96$) ensured that the disease transmission results were not affected by variability in the levels of disease dose applied to each beaker, or by cross-contamination among the experimental corals.

Future improvements can be incorporated into the STAR system such as implementing advanced sensing technologies to monitor and control environmental parameters in real-time. For example, sensors can be attached to the robotic arm to measure nutrient concentrations, pH levels, temperature, and other relevant variables, allowing for automated adjustments and maintaining stable experimental conditions throughout the duration of the study. Additional experimental capabilities also include the application of stressors on a range of continuum values, rather than in categorical stress levels (e.g., presence vs absence, or low vs high). This will allow testing for non-linear responses or tipping points, which are critically relevant to management targets and policy.

5.2. Effects of the environmental cofactors on SCTLD transmission

In this project, we assessed the effects of two environmental co-factors cofactors, temperature and elevated nutrients, on SCTLD transmission rates. While pre-exposure to elevated ammonium did not influence *O. faveolata* susceptibility to SCTLD, elevated temperature (31 °C) reduced the risk of SCTLD transmission compared to lower temperatures (28 °C). These *ex-situ* results support other studies that have found a negative association between SCTLD activity and sea surface temperature in the field (e.g., Meiling et al. 2020, Williams et al. 2021). Interestingly, a previous study that tested the effects of temperature in SCTLD progression rates found faster progressions in *C. natans* when the corals were maintained at 31 °C, compared to 21 or 26 °C, but this was not the case for *M. cavernosa* corals (Paul et al. 2022). Combined, these results suggest that temperature might have opposite effects on SCTLD transmission and progression rates, or that the effects could be species specific. Future research should include *ex-situ* evaluation of a variety of coral species to test if elevated temperature has a similar effect mitigating SCTLD transmission across multiple holobionts (coral host and their specific symbiotic communities) and use bigger fragments that allow better track lesion progressing overtime.

Although some studies have suggested that the reduction in SCTLD activity during periods of elevated temperature might be related with the loss of the algal symbionts (family Symbiodiniaceae) during heat stress (Meiling et al. 2020), in our study, the temperature conditions in the high temperature treatment did not cause visual signs of coral paling or bleaching. Our preliminary assessment of the algal symbiont communities revealed that all three experimental colonies predominantly hosted thermotolerant algal symbionts in the genus *Durusdinium*, which might explain the lack of signs of heat stress in the experimental fragments.

Finally, pre-exposure to elevated ammonium did not influence disease transmission in either of the two temperature treatments tested in this experiment. However, these results might be interpreted with caution since the addition of the disease source increased nutrient concentrations to similar levels to out HN treatment (Fig. 8). Because the diseased tissue being released by the donor colonies adds nitrogen and phosphates to the corals dosed with SCTLD, it is difficult to maintain a disease treatment under low nutrient levels. Field data could be used to assess if these elevated nutrient concentrations persist near disease colonies in the reef environment as well.

5.3. Next steps and management recommendations

Our experiment supported the reduction of SCTLD transmission rates under higher temperature levels using ex-situ experimentation. The use of the STAR system allowed running a disease transmission experiment where each fragment was exposed to the same volumes of disease dose, while at the same time guaranteeing that transmission in each fragment (beaker) was independent of the transmission in other fragments. Furthermore, our

results suggest that the reduction of SCTLD activity during warmer periods is not necessarily related to the bleaching of the coral, nor to the reduction of tissue sloughing off from the diseased corals (Williams et al. 2021). In our experiment, both temperature treatments were dosed with the same volumes of disease water and therefore reductions in SCTLD transmissions might be related to the physiological state of the coral holobiont or the pathogen(s), and not to the availability of disease source.

These results can help managers to design monitoring and intervention strategies for SCTLD in order to prioritize the use of resources when they are most needed or more effective. For example, SCTLD treatment efforts can be concentrated during the high-temperature peaks, since fewer active disease lesions can be treated more effectively (Williams et al. 2021). This can be accompanied by constant monitoring once temperatures start decreasing again to treat the remaining active lesions before cooler temperatures might accelerate SCTLD activity.

Future ex-situ experiments testing the role of temperature on SCTLD dynamics should assess if the mechanisms involved in these dynamics are related to changes in the coral holobiont (e.g., coral health, algal and prokaryotic communities' composition) or with changes in the pathogen(s) virulence. It is possible that the dominance of Durusdinium symbionts further increased in the corals at high temperatures, which could have reduced their susceptibility to SCTLD compared to other fragments with higher abundances of other algal symbiont types (Dennison et al. 2021). Similar experiments should also test the effect of lower temperatures on the transmission and progression rates (i.e., 23-25 °C), and try to determine if there is a threshold temperature under which SCTLD transmission and progression rates start declining.

Finally, the use of artificial seawater was an effective solution to control the nutrient levels in this experiment and therefore could be considered as a good option in future experiments performed in locations that might not have optimum natural seawater quality (e.g., coastal locations exposed to strong nutrient fluctuations). However, for disease transmission experiments, in particular, it should be considered that the use of disease donors as the pathogen source increases nutrient concentrations in the corals exposed to disease, making it difficult to maintain low nutrient levels and thus test if higher nitrogen or phosphate availability modulates SCTLD activity.

6. CITED LITERATURE

Bruno, John F., Laura E. Petes, C. Drew Harvell, and Anneliese Hettinger. 2003. "Nutrient Enrichment Can Increase the Severity of Coral Diseases." *Ecology Letter*s 6 (12): 1056–61.

Dennison CE, Karp RF, Weiler B, Goncalves A, del Campo J, Rosales S, Traylor-Knowles N, Baker AC. 2021. The role of algal symbionts (genus *Breviolum*) in the susceptibility of corals to Stony Coral Tissue Loss Disease in South Florida. Florida DEP. Miami FL. 1-23.

Enochs, I. C., D. P. Manzello, P. J. Jones, C. Aguilar, K. Cohen, L. Valentino, S. Schopmeyer, G.Kolodziej, M. Jankulak, and D. Lirman. 2018. "The Influence of Diel Carbonate Chemistry Fluctuations on the Calcification Rate of Acropora Cervicornis under Present Day and Future Acidification Conditions." *Journal of Experimental Marine Biology and Ecology* 506 (September): 135–43.

Estrada-Saldívar, Nuria, Blanca A. Quiroga-García, Esmeralda Pérez-Cervantes, Omar O. Rivera-Garibay, and Lorenzo Alvarez-Filip. 2021. "Effects of the Stony Coral Tissue Loss Disease Outbreak on Coral Communities and the Benthic Composition of Cozumel Reefs." *Frontiers in Marine Science* 8.

McDevitt-Irwin, Jamie M., Julia K. Baum, Melissa Garren, and Rebecca Vega Thurber. 2017. "Responses of Coral-Associated Bacterial Communities to Local and Global Stressors." *Frontiers in Marine Science* 4 (August): 1–16.

Meiling, Sonora, Erinn M. Muller, Tyler B. Smith, and Marilyn E. Brandt. 2020. "3D Photogrammetry Reveals Dynamics of Stony Coral Tissue Loss Disease (SCTLD) Lesion Progression Across a Thermal Stress Event." *Frontiers in Marine Science* 7.

Miller, Margaret W., Philip J. Colburn, Emma Pontes, Dana E. Williams, Allan J. Bright, Xaymara M. Serrano, and Esther C. Peters. 2019. "Genotypic Variation in Disease Susceptibility among Cultured Stocks of Elkhorn and Staghorn Corals." *PeerJ* 7: e6751.

Muller, Erinn M., Erich Bartels, and Iliana B. Baums. 2018. "Bleaching Causes Loss of Disease Resistance within the Threatened Coral Species Acropora Cervicornis." *eLife* 7 (September). https://doi.org/10.7554/eLife.35066.

Paul V, Lefcheck J, Pitts K, Scheibler S, Vekich T (2022) Investigation of temperature as a driver of stony coral tissue loss disease dynamics. Florida DEP. Miami FL. 1-19.

Palacio-Castro AM, CE Dennison, SM Rosales, and AC Baker (2021) Variation in susceptibility among three Caribbean coral species and their algal symbionts indicates the threatened staghorn coral, *Acropora cervicornis*, is particularly susceptible to elevated nutrients and heat stress. Coral Reefs. 40: 1601–1613

Palacio-Castro AM, Rosales SM, Dennison CE, Baker AC (2022) Microbiome signatures in *Acropora cervicornis*, are associated with genotypic resilience to elevated nutrients and heat stress. Coral Reefs. doi:10.1007/s00338-022-02289-w/.

Palacio-Castro AM, CE Dennison, SM Rosales, and AC Baker (2021) Variation in susceptibility among three Caribbean coral species and their algal symbionts indicates the threatened staghorn coral, *Acropora cervicornis*, is particularly susceptible to elevated nutrients and heat stress. Coral Reefs. 40: 1601–1613

Sharp, W, Maxwell K, Smith K, Hunt J (2020) Investigating the ongoing coral disease outbreak in the Florida Keys: continued SCTLD monitoring at middle and lower Florida Keys, experimental coral restoration of SCTLD-susceptible coral species, and assessing the prevalence of SCTLD on intermediate reef habitat. Florida Fish and Wildlife Conservation Commission.

Studivan MS, A Shatters, DL Dodge, JL Beal, JD Voss (2021) Synergistic effects of thermal stress and estuarine discharge on transcriptomic variation of *Montastraea cavernosa* corals

in southeast Florida. Frontiers in Marine Science 8:662220
Studivan MS, AM Rossin, E Rubin, N Soderberg, DM Holstein, IC Enochs (2022) Reef sediments can act as a Stony Coral Tissue Loss Disease (SCTLD) vector. Frontiers in Marine Science 8:815698
Studivan MS, Baptist M, Molina V, Riley S, First M, Soderberg N, Rubin E, Rossin A, Holstein DM, Enochs IC (2022) Transmission of stony coral tissue loss disease (SCTLD) in simulated ballast water confirms the potential for ship-born spread. Sci Rep. 12(1):19248.
Studivan MS, Eckert R, Shilling E, Soderberg N, Enochs IC, Voss JD (in prep) Transcriptomic examination of corals exposed to stony coral tissue loss disease (SCTLD) and antibiotic intervention indicates potential for recovery.
Vega-Thurber, RL., Deron EB, C Fuchs, AA Shantz, R Mcminds, and JR Zaneveld (2013) Chronic Nutrient Enrichment Increases Prevalence and Severity of Coral Disease and Bleaching. Global Change Biology 20 (2): 1–11.
Vega-Thurber R, DD Willner-Hall, B Rodriguez-Mueller, C Desnues, RA Edwards, F Angly, E Dinsdale, L Kelly, and F Rohwer (2009) Metagenomic analysis of stressed coral Holobionts. Environmental Microbiology 11 (8): 2148–63.
Vollmer, SV, and DI Kline (2008) Natural disease resistance in threatened staghorn corals. PloS One 3(11): 1–5.
Voss JD, LL Richardson (2006) Nutrient enrichment enhances black band disease progression in corals. Coral Reefs (25), 569–576
Williams SD, CS Walter, and EM Muller (2021) Fine scale temporal and spatial dynamics of the Stony Coral Tissue Loss Disease outbreak within the Lower Florida Keys. Frontiers in Marine Science 8