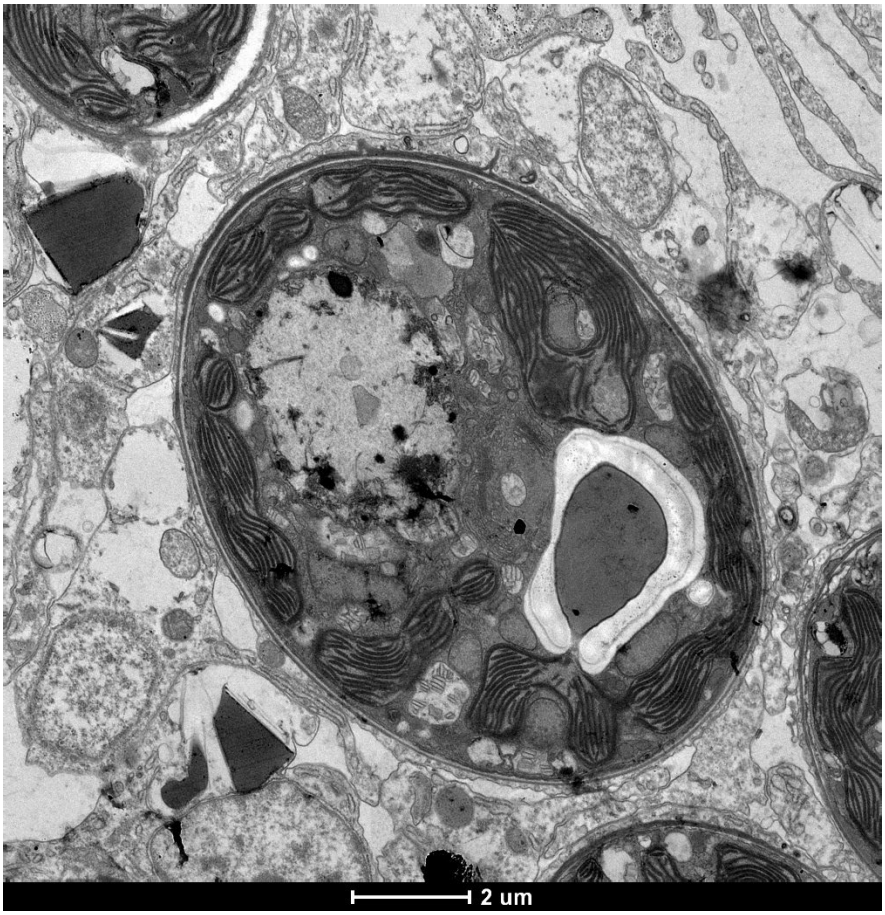
A holistic time-series analysis of stony coral tissue loss disease



A holistic time-series analysis of stony coral tissue loss disease

Final Report

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Management Summary (300 words or less)

The causative agent for SCTLD is still unidentified while studies have suggested a range of different potential etiological agents. Therefore, this project aimed to take a holistic approach to determine the etiology of SCTLD. However, an important aspect of this project would be using multiple analyses on the same set of samples, so they are directly comparable. Utilizing laboratory transmission experiments with naïve coals infected with diseased corals collected from SCTLD endemic zones in Florida, samples were taken at a time points before and after exposure to disease as well as various points during infection. This allows us to look at how this disease manifests over time under controlled laboratory conditions. For each timepoint, each sample taken was split into sub-samples for different processing protocols that will be used for transcriptomics, proteomics, metabolomics, microbiome, histological, and transmission electron microscopy (TEM) analysis. This project has resulted in over 300 samples taken with approximately 60 samples for each type of analysis mentioned that have all been processed. This is a multi-phase project, with this project being the first phase focused on sample processing and the next fiscal year focusing on data analysis. The dataset derived from this project will be a valuable resource/framework for future disease responses as well as serve as a comparative dataset for other analyses.

Executive Summary (max 1 page)

As stony coral tissue loss disease (SCTLD) continues to spread throughout the Caribbean and continues to kill off corals in Florida, the causative agent responsible for this malady is still unidentified. Because the causative agent is still unknown, the development of more targeted treatments and feasible diagnostic tools is severely hampered. However, the various analyses conducted on a wide variety of sample sets have not positively identified a causative agent for SCTLD. This may be, in part, due to the variability between reefs and coral colonies over time and location as well as the potential complexity of this disease, which may involve multiple agents and environmental stressors. Therefore, the goal of this project was to take a holistic approach and begin processing a standardized set of SCTLD samples for a wide variety of analyses. The strength of this approach is that all the datasets generated from each sample can be directly compared to one another.

To reduce potential 'background noise' and environmental variables, this project uses closed, aquarium systems as well as the use of naïve healthy corals that were obtained from areas before SCTLD had arrived and subsequently kept in captivity. This ensured we could control any environmental variables such as temperature, nutrient input, and microbes entering the system. Further, the use of naïve corals reduced the possibility of SCTLD-associated microbes associated with healthy fragments before exposure to disease. Lastly, these naïve corals were infected in the laboratory using diseased corals, but samples were taken at various time points to try to capture the transition from a healthy to a disease state in our future analyses. Samples were taken at five timepoints: pre-experiment (T1), 48 h from the start of the experiment (T2), 7 days post-T2 or the first gross signs of disease (T3), 7-days post T3 or before full fragment mortality (T4), and 7-days post T4 or before full fragment mortality (T5). For every experimental tank, there was also a control tank with only naïve healthy corals under similar conditions except for a healthy fragment instead of a diseased fragment.

At each timepoint, a fragment in the experimental tank as well a control tank was split into five subsamples and preserved for multi-omics extractions, chemical extraction, histology, transmission electron microscopy (TEM), and extractions for immunological assays. This project (FY 2023-2024) focused on the processing of these >300 samples in preparation for the analyses that are planned for next phase (FY 2024-2025). All the samples have been processed and will be analyzed for this funding period and analysis will begin during the next funding period.

1. TABLE OF CONTENTS

Required headings include:

- Background/Introduction: Brief summary of the project and why it is needed. Include a description of what work will be performed and/or completed with the DEP Funding.

- Methods: Must include detailed qualitative standards (who, how, when, where), and quantitative standards (minimum and maximum services provided).
- Results:
- Discussion and Management Recommendations: What is the management relationship to the results? What benefits have been provided by the Project?

2. BACKGROUND

Florida's Coral Reef is currently experiencing a multi-year disease-related mortality event, that has resulted in massive die-offs in multiple coral species. Approximately 21 species of coral, including both Endangered Species Act-listed and the primary reef-building species, have displayed tissue loss lesions which often result in 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 southwest past the Marquesas in the Lower Florida Keys. The best available information indicates that the disease outbreak is continuing to spread west and throughout the Caribbean.

Previous studies, including those by our research group, confirmed SCTLD to be an infectious disease (1–3). However, the exact etiological agent has yet to be identified. Several bacterial groups are associated with disease (4–6) while broad-spectrum antibiotics are mostly effective against disease lesions (1, 7–9). Although, it is unclear if bacteria are initiating disease or are playing more of an opportunistic role. For example, the bacterial pathogen *Vibrio coralliilyticus* may be causing coinfections with SCTLD that exacerbate lesion progression, but pure cultures of this microbe are unable to consistently initiate disease (10). This was an important and concerning revelation that further complicates the situation, but it allowed researchers to rule out this pathogen as a primary agent, although it remains a threat to corals.

A recent study had identified viral-like particles (VLPs) within coral tissue that were associated with the microalgae symbionts, which appeared to resemble filamentous RNA viruses (11). The authors of that study suggested they belonged to the viral family Alphaflexiviridae based on morphology, but they did not have supporting sequencing data. Also, these VLPs were observed in both diseased and apparently healthy corals, so no connection between these VLPs and disease has yet to be established. There have been other described viruses associated with corals and their symbionts unrelated to SCTLD (12–14). Viruses typically outnumber bacteria in a 10:1 ratio in marine systems, so their mere presence does not provide strong evidence for their role as a primary pathogen.

The presence of these VLPs could be latent infections that manifest during host stress, which was observed in the previous viral studies (12–14). Alternatively, they could also be an opportunistic infection that occurs after a primary agent infects, or SCTLD may be polymicrobial disease that requires infection with multiple pathogens. The latter two scenarios are observed with other coral diseases with the primary and secondary infections of *Montipora* white syndrome in Hawaii (15) and the polymicrobial black band disease (16, 17), respectively.

This project is building upon the current work of our research group, which is focusing on identifying the viruses and microbes associated with diseased corals, determining if they are specific to SCTLD lesions, and establishing if there are any associations between potential pathogens and the start of SCTLD lesions through time.

This is a multi-phase project with Phase I focusing on sample collection and the beginning of sample processing. Phase II will focus on samples analysis.

Project Goals and Tasks:

The goals of this project are to 1) create a comprehensive dataset from DNA, RNA, proteins, and metabolites extracted from SCTLD samples taken over time, and 2) document the cellular pathologies of SCTLD over time using histology and transmission electron microscopy (TEM). The data generated from this project will provide a comprehensive view of what is occurring during a SCTLD infection over time, as well as generate samples and data that can be used by other research groups for future analyses.

The project proposed here is a multi-phase project; this is Phase I of the project (FY 2023 – 2024) that will only focus on sample collection and processing. Analysis of the samples will be conducted in Phase II of the project (FY 2024-2025), pending availability of funds. If further funding is available and a potential agent(s) is identified, then Phase III will focus on establishing a causal relationship between the agent and the onset of SCTLD (FY 2025 – 2026).

- **Task 1a:** Required reporting deliverables (UNCW).
- **Task 1b:** Collect various sample types over the course of a time series experiment for SCTLD from in vitro infections using non-naïve corals (UNCW, SMS).
- **Task 3:** Extract naïve coral samples for metabolomic analysis.
- **Task 4:** Process naïve coral samples for histology.
- **Task 5:** Process naïve coral samples for TEM.
- **Task 6:** Sequence DNA samples from Task 2 for microbiome analysis.
- **Task 7:** Process samples for time series of healthy corals from DRTO for TEM.
- **Task 8:** Process samples of acute Orbicella tissue loss lesions for TEM.

The outcomes of this project will be incorporated into an on-going coral disease response effort which seeks to improve understanding about the scale and severity of the coral disease outbreak on Florida's Coral Reef, identify primary and secondary causes, identify management actions to remediate disease impacts, restore affected resources, and ultimately prevent future outbreaks. As such, collaboration amongst partners is encouraged when appropriate to avoid duplication of efforts and ensure alignment of needs. Coordination with other Principal Investigators is recommended and required, as appropriate.

3. METHODS

3.1. Task 1 - Coral Experiments

This project utilized diseased corals collected directly from the field and apparently healthy corals exposed during laboratory transmission experiments (see **Supplementary File S1** for metadata on corals used). Multiple sample types were taken over time (described below) to observe any changes between pre-exposure, initial disease, early-stage disease, and late-stage disease (a total of 5 timepoints per replicate). We transmitted SCTLD to apparently healthy corals obtained from the Key West nursery collected before the arrival of SCTLD (~2017), likely making them naïve to SCTLD.

After collection in 2017, these naïve were housed at the Smithsonian Marine Station (SMS) under strict biosecurity protocols overseen by Drs. Ushijima and Paul (see below). The SMS is currently housing naïve colonies of *M. cavernosa* (n=4) and *O. faveolata* (n=1) that are large enough for our planned experiments, as well as additional smaller fragments that can be used for controls. These naïve corals will be exposed to diseased corals collected from the field at permitted locations in the Florida Keys using a hammer and chisel (Collection permit FKNMS-2022-049). Additional transmission experiments will be conducted from field-collected corals from the Key West nursery or corals of opportunity provided to us by the NOAA FKNMS. While these additional corals will not be naïve, they would provide an important comparison of naïve corals (described above) versus healthy corals surviving in endemic zones in Florida. This could reveal if there are specific biomarkers or host traits associated with each group of corals.

All corals and experimental tanks will be kept in seawater pumped from offshore that is initially filtered down to 0.22 µm into storage containers and kept out of direct sunlight. Within the storage containers, the water is circulated 24/7 through a 20 µm-pore filter, activated carbon filter, and a UV-sterilizer. The seawater is again filtered down to 0.22 µm prior to use. All closed-system mothertanks housing the naïve coral colonies at the SMS use this clean seawater but are also recirculated through an activated carbon and UV-sterilizer to maintain biosecurity. Therefore, any seawater used in these experiments should be free of most or all viable bacteria and infectious viral particles from the ocean.

Each experimental replicate will consist of pre-exposure samples, and samples from both an experimental tank (with a diseased donor fragment) and a control tank (with a naïve donor fragment; outlined in **Figure 1**). Our goal is to use a separate diseased colony per experimental replicate; diseased coral species used will depend entirely on colonies with active lesions available from the field. Each experimental tank will consist of one diseased fragment (*Diseased Colony 1* in **Figure 1**) and four naïve fragments from the same colony (*Naïve Colony 2* fragments depicted in **Figure 1**). I.e., four naïve fragments are exposed to disease at the same time. The control tank will consist of four naïve fragments from the same naïve colony as in the experimental replicate (*Naïve Colony 2* in **Figure 1**) and one naïve fragment from a different colony (*Naïve Colony 3* in **Figure 1**).

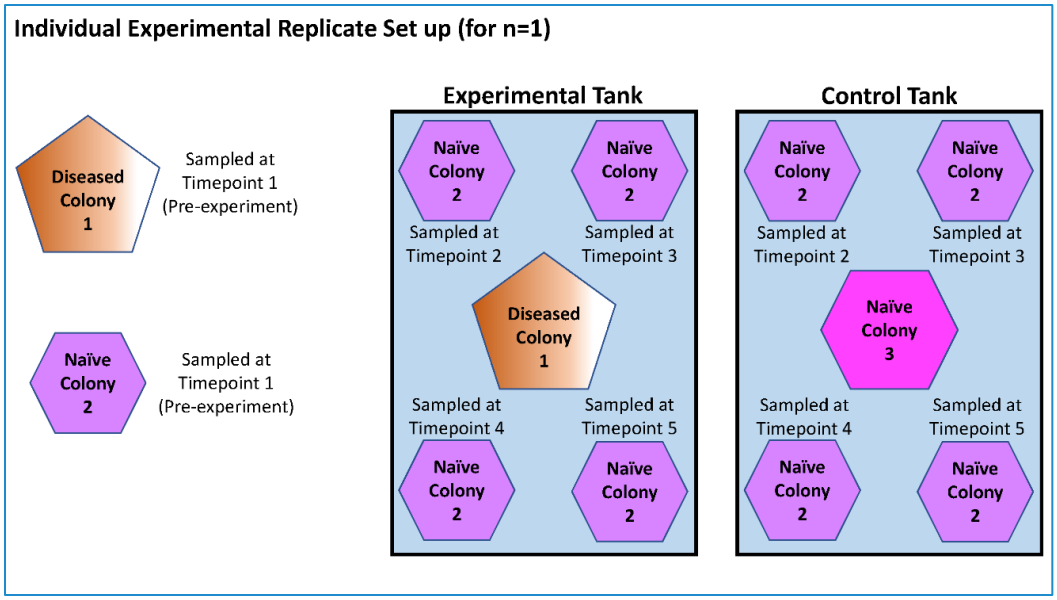

Figure 1. *Schematic of an individual experimental replicate for the proposed time-series experimental set up. Each experimental replicate will consist of an experimental tank, where fragments of naïve, apparently healthy corals will be exposed to a fragment with SCTLD, and a control tank, where fragments of naïve healthy corals from the same colony will be exposed to a naïve, apparently healthy coral from a different colony. With the controls included, there will be a total of 10 different samples per replicate per sample type.*

Naïve and diseased colonies will be fragmented for experiments using a diamond blade masonry saw. These healthy fragments will be fragmented into *medium* sized fragments approximately 4 x 4 cm. Healthy corals will be allowed at least 10 days to heal after fragmentation before use in this experiment. After healing and before experimental set up, one fragment of diseased and one fragment of healthy coral will each be split into four samples using a band saw for histology, TEM, metabolomics, and multi-omics (see *Sample Types* section below). This will serve as *Timepoint 1* (pre-experiment) for this experiment. Control/healthy corals will be cut before any experimental/diseased corals and the saw will be thoroughly cleaned after each coral fragment.

The healthy and diseased fragments will then be arranged in an experiment and control tank as depicted in **Figure 1**. After 48 h of exposure, another experimental and control fragment will be taken for *Timepoint 2*, and each split into four samples like the previous timepoint. After 7 days or the first signs of disease in the experimental tank (whichever occurs first), a third experimental control and experimental fragment will be processed for *Timepoint 3*. This will be repeated after 7 days for *Timepoint 4* and then again for *Timepoint 5*. During this experiment, coral fragments will be photographed daily, and tanks will have partial water changes every other day. Biosecurity will be maintained throughout these experiments with control fragments always being processed first, different tools used for different tanks, and the use of sterilized seawater (see description above).

For each experimental replicate there will be five different time points where samples will be taken:

- *Timepoint 1*: Pre-experiment
- *Timepoint 2*: 48 h post exposure to disease
- *Timepoint 3**: 7-days post timepoint 2 to disease or when first gross disease signs are observed (tissue loss or bleaching).
- *Timepoint 4**: 7-days post timepoint 3
- *Timepoint 5**: 7-days post timepoint 4

*For Timepoints 3-5, an infected fragment will be preferentially selected for sampling over an apparently healthy fragment in the experimental tanks. Timepoints 4 & 5 may be adjusted depending on the progression rates of disease. However, each time an experimental fragment is sampled, a corresponding control fragment will also be sampled.

There are multiple contingencies if disease transmission does not match up with the sampling scheme described above:

- If a recipient coral exposed to disease begins to develop disease signs before Timepoint 2 (48 h post exposure), then that coral will be processed (i.e., the different samples taken) as well as the corresponding control fragment and samples from each of the other replicates to keep sampling consistent.
- If a coral develops disease before subsequent timepoints, then that fragment and each corresponding control fragment will be processed.
- If disease transmission is faster for some replicates than others, then the sampling scheme will be modified to fit the rate of disease progression.

3.2. Task 2 - Multi-Omics Extractions

3.2.1. RNA / DNA Extraction protocol:

All 61 samples were extracted using the Zymo BIOMICS DNA/RNA Mini Prep Kit, following manufacturer's protocol, with the following modifications: Sample preparation; Approximately 50 mg of coral tissue was cut from the frozen corals using forceps, scapels, and dissecting scissors, and immediately placed into a DNAse/RNAse free 2mL tube with 750 uL of DNA/RNA shield and on ice. The samples were then lysed using a high-speed bead beater at 27 Hz for 3 minutes, twice. The samples were centrifuged, the supernatant was placed into a new 2mL tube, and an equal volume of DNA/RNA lysis buffer was added to the supernatant. From here, all steps taken followed the manufacturer's instructions. After the elution step, each sample was analyzed using a Nanodrop, Qubit, and finally a Fragment Analyzer at the Cornell Biotech Facility. Each sample's DNA concentration, RNA concentration, and RNA Quality number is shown on the Excel sheet.

3.2.2. Protein Extraction Protocol and Mass Spectrometry:

Using an ethanol-cleaned razor blade, a 3 x 3 x 3 mm chip of coral, including the coral polyps and 3 mm into the skeleton, was taken to maintain sampling consistency. All surfaces were cleaned between samples. The samples were placed in bullet blender tubes with metal beads. To each sample, 200 µL of S-Trap lysis buffer (recipe below) was added immediately. Samples were then vortexed and stored in a -80°C freezer until

further processing. Samples were processed in a bullet blender at setting 12 for 3 rounds of 2 minutes each in a cold room to ensure they remained cold. Note: Samples contained protease inhibitors and were in SDS to denature proteins, rendering them inactive. Following blending, samples were centrifuged at 10,000 rcf for 10 minutes at room temperature to avoid SDS precipitation. 100 µL of the supernatant was transferred to a particle-free tube. A BCA protein quantification assay was conducted on all samples. If a sample contained less than 100 µg of protein, a new sample was prepared using the same method. All samples were run through an S-trap, and then submitted for Data-Independent acquisition (DIA) Mass Spectrometry.

3.3. Task 3 - Metabolomics

Stony coral tissue loss disease-affected corals were collected on SCUBA and maintained separately in temperature-controlled raceways. Healthy coral replicates that were collected from the Key West nursery ahead of the disease front were each cut into multiple fragments that, could be collected for analysis as snapshots of the pathogenesis of SCTLD. Upon initial exposure, and as the disease transmitted and progressed, fragments of each coral were collected at successive intervals consistent across replicates. Samples for metabolomics analysis were harvested into whirl-paks and immediately transferred to the -80 °C freezer. Whirl-paks were opened and placed upright into large glass beakers in the lyophilizer (Labconco Freezone 6). Samples typically required 24-36 hours to completely dry at -40 °C and 100 to 150 x 10^{-3} mb. Once dry, samples were returned to the - 80° C to await extraction.

All 61 metabolomics samples were extracted from October 3 – Nov. 27, 2023. Samples were removed from the whirl-Pak and always handled with sterile forceps. Dimensions were determined to the 0.1 mm with digital calipers, and images were taken from a consistent distance for surface area determination via ImageJ software. Finally, samples were transferred to a prewashed (MeOH 3x), preweighed 100 ml beaker, and dry weight was recorded to nearest milligram.

Extractions were batch processed by time series collection date, with two solvent controls processed in tandem with each of the five timepoints. Each sample was extracted in 80 ml of 2:2:1 EtOAc: MeOH: H2O (3x over 24 hours including one overnight extraction). With each solvent change, samples were sonicated at 42 kHz (Bransonic 1510) for 1 minute and left to extract at room temperature (23 °C).

Unfiltered supernatant was concentrated via rotary evaporation until solvents were removed (40 °C @ 10-150 x 10^{-3} mb, Buchi Rotavapor R-300). In leu of filtration, concentrate was resuspended in 40 ml of 3:1 MeOH: H2O and centrifuged in 50 ml conical (Falcon) at 4500 x g for 15 minutes. Clarified supernatant was then returned to a clean round bottom flask and concentrated. Final concentrate was transferred in 1:1 EtOAc: MeOH to a 7 ml glass scintillation vial and dried in vacuo at 40 °C (ThermoSci Savant SPD121P SpeedVac Concentrator). Any remaining water was frozen to -80° C and removed via lyophilization.

A 5 mg aliquot of each sample was transferred with a micro spatula into 1.5 ml microcentrifuge tubes (VWR Cat # 20170-038) and stored in the -20°C freezer. Spatula was wiped clean between samples with 95% EtOH on a Kimwipe (3X). Solvent controls were left in their 7 ml vials for processing.

3.4. Task 4 - Histology

A total of 61 samples fixed with 20% Z-fix (excluding one dead sample [0523T281]) for histological examinations in the time series transmission experiment conducted at SMS during 17 May 2023 – 27 June 2023 were transported to FWRI-FWC on 8 September. The external surface of fixed samples was photographed for tissue-loss lesions during 12 – 17 September 2023 by using a digital camera (JENOPTIK GRYPHAX, Jena, Germany) attached to a dissecting microscope (LEICA M125, Wetzlar, Germany). The presence or absence of mesenterial filaments protrusion at the surface of the epidermal tissue and heterotrich ciliates (*Halofolliculina*) infection in the skeletal tissue were also examined simultaneously.

On 11 October 2023, the decalcification process, using 10% EDTA solution, began on all 61 samples fixed for histological examination. Prior to decalcification, the samples presumed to potentially possess tissue-loss lesions (i.e., recipients, apparently healthy corals exposed to disease [ED] and donor, diseased corals from the field [DD]) were enrobed with agarose to preserve the tissue integrity of fragile lesion margins. All other samples (recipients, apparently healthy corals exposed to healthy control corals [HC] and pre-experiment naïve apparently healthy corals [HH]) were not enrobed in agarose because they were presumed to not have lesions. All of the samples completed decalcification on 11 December 2023 and then both gross external and internal surface views were reexamined with a dissecting microscope and photographed. Any tissue abnormalities of the samples observed at this step were noted; for example, some samples exhibited protrusion of mesenterial filaments, and both oral and aboral views are shown (Figure 1, Figure 2A).

The samples were grossed and then further processed into histological specimen slides. On 23 January 2024, all 61 samples received from laboratory transmission experiments were sectioned for histological slides. Four slides were completed from each sample, with two slides stained with hematoxylin and eosin (H&E) and two slides stained with thionin. One slide set of each pair of stains per sample was sent to Dr. Esther Peters' lab at GMU and the other set was retained at FWRI.

An Excel file was created on Google Drive for communication among LH, EP, and YK about histology slide interpretation.

External close-up photomicrographs of post-fixed samples prior to processing for histology slides will be useful when interpretating the slides. All three of LH, EP, and YK have access to these photographs.

The histological specimen slides will be observed and interpreted. An excel sheet outlining all of the samples processed for histology was provided with this report **Supplementary File S6** (*S6 TASK_4_FINAL_REPORT_DELIVERABLE.xlsx*).

3.5. Task 5, 7, 8 - Transmission Electron Microscopy (TEM)

Samples were fixed using a recipe originally from Thierry Work to standardize fixation across experiments for comparative analyses (11). Samples were fixed in a combination of 2.5% glutaraldehyde and 2% paraformaldehyde in Instant Ocean (pH 8, 35ppt) and kept at 4°C. Samples from the Dry Tortugas (Task 7) were fixed in 2.5% glutaraldehyde in filtered sea water. Samples were then decalcified in 10% EDTA (pH 7) shaking at room temperature until the skeleton dissolved. Coral tissue was then cut into 1 mm^3 chunks. Samples were rinsed 3 times for 15 minutes each with 0.35 M sucrose in a

0.1 M sodium cacodylate buffer solution. Samples were post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 hours at room temperature. Samples were rinsed twice for 15 minutes each with 0.1 M sodium cacodylate buffer. Samples were then dehydrated with a series of ethanol solutions (50, 70, 95, 100, 100%) for 15 minutes each. Samples were then added to a 1:1 mixture of Spurr's resin with 100% ethanol for 1 hour. Samples were then embedded in 100% Spurr's resin overnight. Fresh 100% Spurr's resin was added to samples and samples were put into a 70 °C vacuum oven overnight. Then 90 nm sections were cut with a diamond knife and placed onto a 0.25% formvar coated copper grid. Sections were stained with UranyLess for 5 minutes, rinsed with ultrapure water, stained with lead citrate for 5 minutes, rinsed with ultrapure water, and allowed to dry overnight. Sections were then imaged using a FEI Tecnai Spirit Bio Twin TEM at UNCW's Richard Dillaman Bioimaging Facility. The status of all corals processed for TEM are recorded in **Supplementary File S7** (*S7 Coral TEM Sample Status.xlsx)*, which is provided with this report.

3.6. Task 6 - Microbiome Sequencing

Extracted DNA from 61 samples was received at UF from USDA for microbiome analysis. For each sample, we successfully characterized the bacterial community through 16S rRNA gene libraries (V4 region). After quality-filtering, there were 5,078 - 136,511 sequencing reads per sample (average = 47,829 reads per sample). Raw sequencing reads are publicly available in NCBI under BioProject PRJNA1120034 (https://www.ncbi.nlm.nih.gov/bioproject/1120034). A total of 12,418 microbial taxa were detected from these 61 samples. Analysis of the microbiome libraries will continue in the next fiscal year.

4. RESULTS

All results and data files were provided with this report and uploaded to the DataOne portal.

4.1. Task 1a - Required reporting deliverables (UNCW).

4.1.1. Naïve coral transmission experiments

Naïve corals collected from the Key West Nursery before the arrival of SCTLD as well as diseased corals from the Florida Keyes, which are summarized in **Supplementary File S1** (*S1 Coral Collected for Task 1.csv*).

Each of the four sample types collected from the fragments were for subsequent multi-omics extraction/analysis (Task 2), metabolomics (Task 3), histology (Task 4), and TEM (Task 5). Samples were saved in 20% Z-fix for histology (at room temperature), a glutaraldehyde/paraformaldehyde mixture for TEM (at 4 °C), or flash frozen at -80 °C for metabolomics and meta-omics analysis. These samples were taken prior to the experimental start and at the timepoints indicated above. All RNA and protein extraction products (from Task 2) were saved for other potential analysis types like microbiome analysis (Task 6), host metatranscriptomics, metagenomics, or other analyses that were subsequently processed. All TEM resulting images were saved in a digital format and all histological slides will be digitized in sharable formats. The major analysis of these

samples will be conducted in Phase II of this project (Fiscal year 2024 – 2025). A complete outline of all samples collated from the naïve coral transmission experiment can be found in **Supplementary File S2** (*S2 Samples Collected for Task1.xlsx*).

4.1.2. All report files

4.1.2.1. All report files will be provided as a link to OneDrive folder "Ushijima et la. DEP Final Report 2024 (for submission)" as well as uploaded to the DataOne Portal.

4.1.2.2. The files for the corresponding tasks are listed below:

4.1.2.2.1. **Task 1a:** Required reporting deliverables (UNCW).

1) Copy of DAC presentation; PDF file *6.5.2024 Ushijima et al. SCTLD Time Series Project.pdf*
2) Supplementary File S1 - list of corals used; Excel sheet *S1 Coral Collected for Task 1.csv*
3) Supplementary File S2 – list of samples collected; Excel sheet *S2 Samples Collected for Task 1 .xlsx*

4.1.2.2.2. **Task 2:** Process samples from naïve corals and extract RNA and proteins for a multi-omics analysis (USDA).

1) Supplementary File S3 – list of RNA, DNA, and protein extractions; Excel sheet *S3 Task_2_time_series_samples_list_RNA_DNA_protein.xlsx*

4.1.2.2.3. **Task 3:** Extract naïve coral samples for metabolomic analysis (SMS, GT, UNCW).

1) Supplementary File S4 – list of extracts; Excel file *S4 Time series extractions_Task3.xlsx*
2) Supplementary File S5 – table of annotations; Excel file *S5 Time Series_Jun24_deliverables_Annotation Table.xlsx*

4.1.2.2.4. **Task 4:** Process naïve coral samples for histology and begin digitizing histological slides (FWC, GMU).

1) Supplementary File S6; table of histology samples; Excel file *S6 TASK_4_FINAL_REPORT_DELIVERABLE.xlsx*
2) Photos of post-fixed coral samples; in zipped folder *Task4_post-fixed_macrophotos.zip*

4.1.2.2.5. **Task 5:** Prep naïve coral samples for analysis with TEM and image at least 15 corals (UNCW).

1) Supplementary File S7; table of TEM samples; Excel sheet *S7 Coral TEM Sample Status.xlsx*
2) Representative TEM images of at least 15 corals; zipped folder *Time Series TEM Photos.zip*

4.1.2.2.6. **Task 6:** Sequence DNA samples from naïve coral for microbiome analysis (UF).

1) The raw data files have been provided with this report Supplementary File S8; Excel sheet *S8 051624_GE7495-A1-12+B1-12+E8-12_EvaGreen_Deliverable.xlsx*
2) , Supplementary File S9; Excel sheet *S9 051624_GE7495-C1-12+D1-12+F1_EvaGreen_Deliverable.xlsx*
3) Supplementary File S10; Excel sheet *S10 051624_GE7495-E1-7_EvaGreen_Deliverable.xlsx*

4.1.2.2.7. **Task 7:** Process samples for time series of healthy corals from DRTO and image at least 10 samples on TEM (TXST, MOTE, UNCW). This is part of a separate, complementary project.

1) Supplementary File S7; table of TEM samples; Excel sheet *S7 Coral TEM Sample Status.xlsx*
2) Representative TEM images of at least 10 corals; zipped folder *DRTO Series TEM Photos.zip*

4.1.2.2.8. **Task 8:** Process samples of diseased *O. faveolata* with a potentially new coral disease observed in the Florida Keys (NSU, UNCW).

1) Supplementary File S7; table of TEM samples; Excel sheet *S7 Coral TEM Sample Status.xlsx*
2) Representative TEM images of embedded corals; zipped folder *Orbicella Acute TL Photos.zip*

4.2. Task 1b - Collect various sample types over the course of a time series experiment for SCTLD from in vitro infections using non-naïve corals.

4.2.1. Endemic coral transmission experiments

Due to the widespread bleaching in 2023 and 2024, the collection of corals has been significantly delayed and the location of enough replicates of diseased corals and finding colonies large enough has been unable to be completed. Task 1b could not be completed during the course of this project period and therefore would not be charged for during invoicing.

4.3. Task 2 – Process naïve coral samples and extract DNA, RNA, and proteins for a multi-omics analysis.

We successfully extracted high quality DNA from 61 samples and safely shipped it to the University of Florida for Task 6 completion. The total DNA was eluted in 50 µL of H_2O, and ranged from 50ng/µL to 200 ng/µL. From the same samples, we successfully extracted RNA from 61 samples, with concentrations ranging from 40 ng/uL to 200 ng/µL depending on species. Fragment analysis determined that the RNA was High Quality and un-degraded with an average RNA Quality Number (RQN) of 8.0. We are

prepared for High Throughput Illumina Sequencing for the time series samples. In collaboration with the University of Washington, we extracted a minimum of 100 µg of protein from 61 coral samples. Each sample has run through the mass spectrometer and is pending analysis (**Figure 2**).

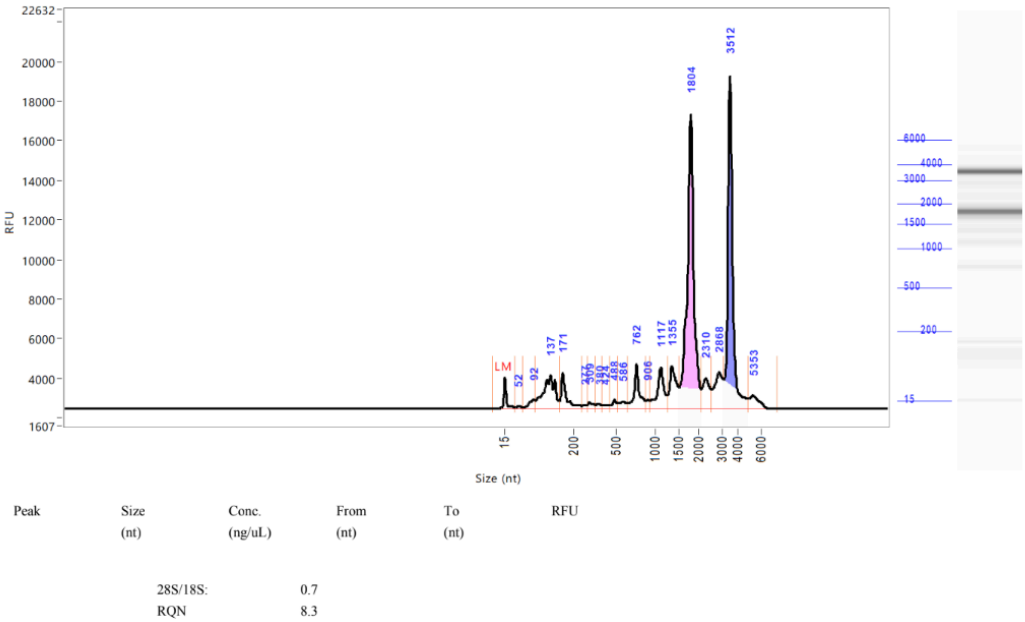

Figure 2. *Representative Snapshot of the Fragment Analysis of each sample featuring RNA Quality Number (RQN). For this analysis, the samples were diluted to 5ng/µL.*

4.4. Task 3 – Extract naïve coral samples for metabolomic analysis.

All extractions performed at SMS are outlined in **Supplementary File S4** (*S4 Time series extractions_Task3.xlsx*) that was provided with this report. Exploratory statistical analysis was performed on positive mode data acquired from LC-MS/MS to visualize metabolome shifts across time and between experimental and control recipient groups. Alpha diversity plots of both control and diseased *Montastraea cavernosa* (MCAV) recipient fragments were plotted over 5 time points to monitor changes to feature diversity over time (**Figure 3**). Both healthy and diseased recipient MCAV fragments showed no statistically significant groups by timepoint. However, there seems to be a trend in the MCAV recipients exposed to SCTLD where feature diversity decreases 48 hours after start of exposure and increases at later time points. Alpha diversity plots were generated in qiime2.

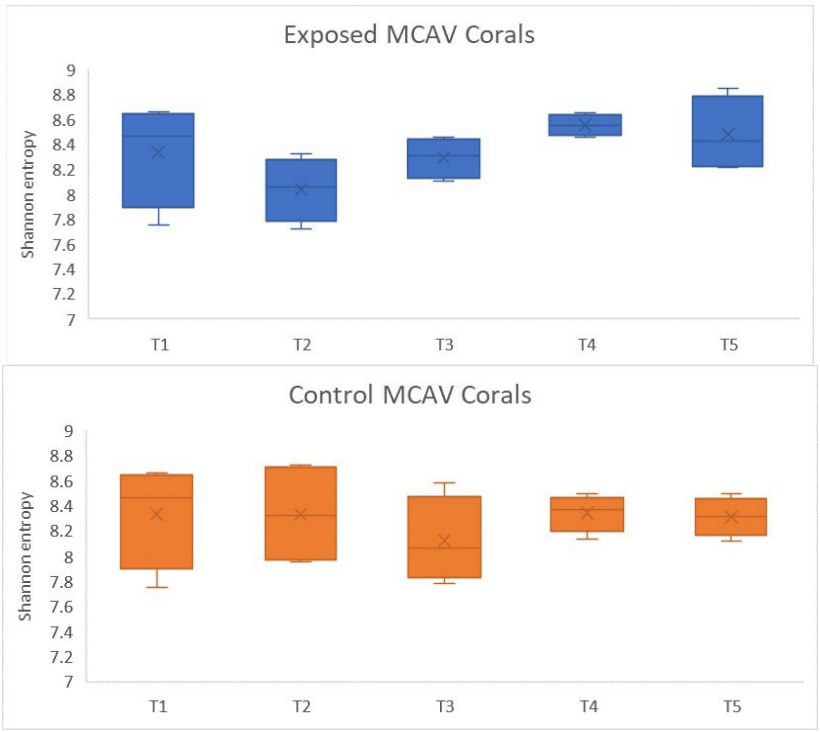

***Figure 3**. Alpha diversity of diseased and control recipient MCAV corals over experimental time points*

The blank subtracted and log transformed quant tables were used to create principal component analysis (PCA) plots comparing healthy and diseased MCAV recipient fragments over time points 2, 3, 4, and 5 (**Figure 4**). The largest difference between metabolomes of control and experimental groups was captured at time point 2, where two distinct clusters can be observed. This observation is interesting because time point 2 fragments were sampled at 48 hours after exposure – before any visual signs of disease were present. Another trend captured by the PCA is that the intragroup variability of the SCTLD exposed coral fragments seems to increase over time. PCA plots were generated with the statistical analysis [one factor] module in MetaboAnalyst.

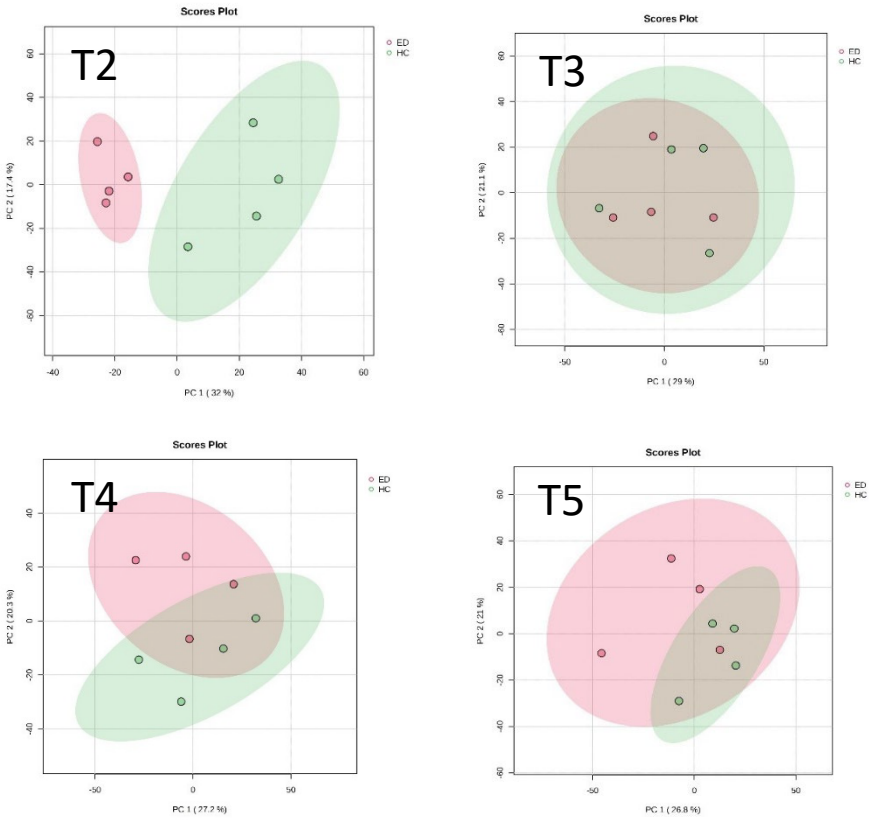

Figure 4*. PCA of healthy and diseased MCAV fragments over exposure time points*

Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) was performed to confirm that the metabolome of SCTLD exposed corals sampled at time point 2 is different from all other time points (**Figure 5)**. sPLS-DA allows the user to restrict the number of components and features included in analysis to reveal sample components driving differences between groups. sPLS-DA was performed using blank subtracted and log transformed data, and parameters were set to 5 components and 10 variables per component. Groups were assigned based on time point and exposure vs control designations; controls were grouped into 4 distinct time points (HC_T2, HC_T3, HC_T4, HC_T5), exposed were grouped into 4 distinct time points (ED_T2, ED_T3, ED_T4, ED_T5), and pre-experimental was grouped (HH_T1). The analysis reveals metabolome

overlap of all groupings except ED_T2, further confirming that something unique is being detected at time point two in SCTLD exposed fragments. It is possible that inducible defense is driving the differences captured at this time point, and features that are driving these differences are currently being annotated. sPLS-DA analysis was performed in statistical analysis [one factor] module in MetaboAnalyst.

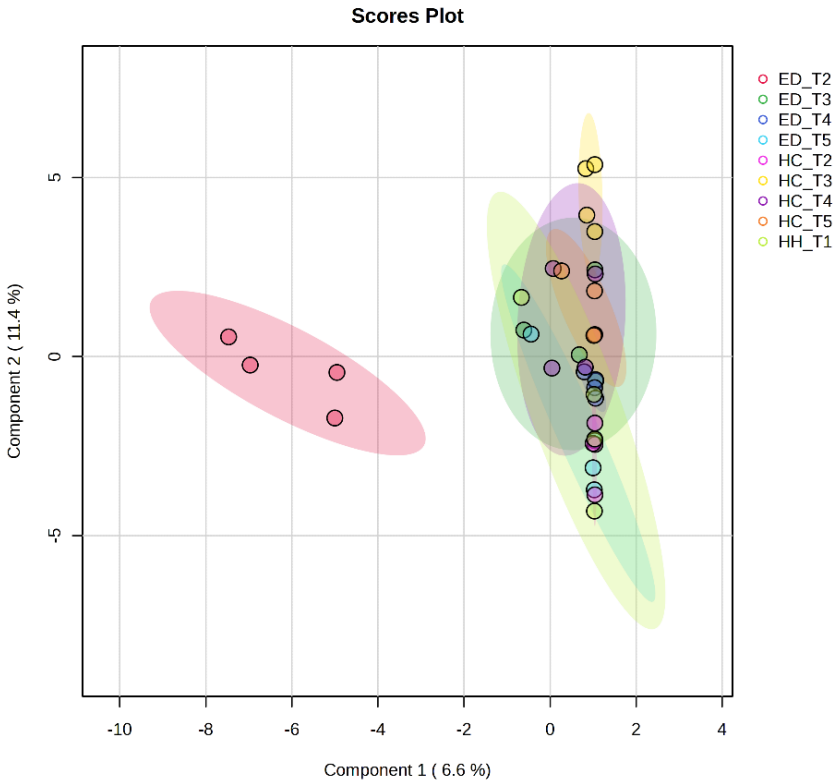

***Figure 5**. sPLS-DA reveals clustering of exposed time point 2 coral fragments compared to all other recipient groupings*

Similarity percentage (SIMPER) analysis was performed in PRIMER to identify additional features driving differences between groups assigned for sPLS-DA analysis. SIMPER revealed 267 features responsible for driving differences between time point two exposed corals and to date nearly a quarter of those features have been annotated. Lipids make up nearly all annotated features with signatures from both host and symbiont metabolomes being identified. The symbiont derived lipids include monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), diacylglyceryl-O-(N,N,N-trimethyl)-homoserine and diacylglyceryl-hydroxymethyltrimethyl-β-alanine (DGTSA). The host derived lipids include acylcarnitine, phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS). All proposed annotations can be found in the excel document "Time Series_Jun24_deliverables_Annotation Table.xlsx" included with this report.

An Upset Plot was generated in UpsetR Shiny Plot to identify features that are present or absent in the MCAV groupings generated for sPLS-DA analysis (**Figure 6**). MCAV fragments exposed to SCTLD and sampled at time point 2 (ED_T2) does not contain 42 features that were present in all other groupings. The ED_T2 grouping also contains 11 features that were absent in all other groupings. All features unique and absent in ED_T2 can be found in the attached excel document **Supplementary File S5** (*S5 Time Series_Jun24_deliverables_Annotation Table.xlsx)* included with this report. The annotation of these features will be attempted.

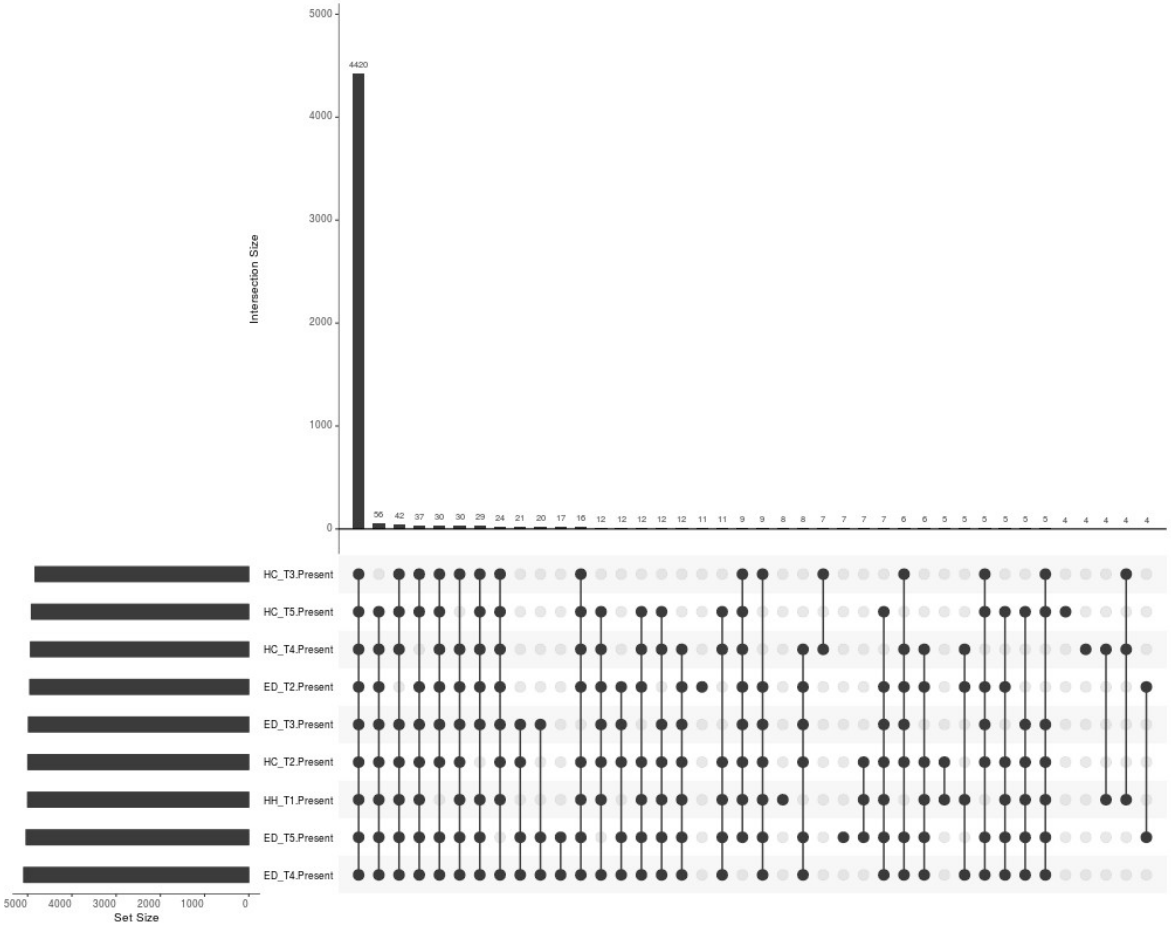

Figure 6. *An Upset Plot generated in UpsetR Shiny Plot to identify features that are present or absent in the MCAV groupings generated for sPLS-DA analysis.*

4.5. Task 4 – Process naïve coral samples for histology.

Prior to processing the histology slides in post-fixed samples, gross characteristics of the samples were identified with the aid of dissecting microscope. Tabulated numbers of grossly identified characteristics of tissue-loss, protrusion of mesenterial filaments, thickening of mesenterial filaments, presence of endolithic fungi-algae and *Halofolliculina* infection are shown in Table 1.

Tissue-loss lesions confirmed (Figure 7, Figure 8A, B) over the time course of this study is shown in Table 2. The lesion noticed in post-fixed samples among ED specimens

was as early as 8 d in MCAV and 41 d in OFAV (Table 2). Among the post-fixed samples submitted for histological study, tissue-loss was confirmed only in the ED (25 – 43.8%) and DD (87.5 – 100%) specimens (Table 1).

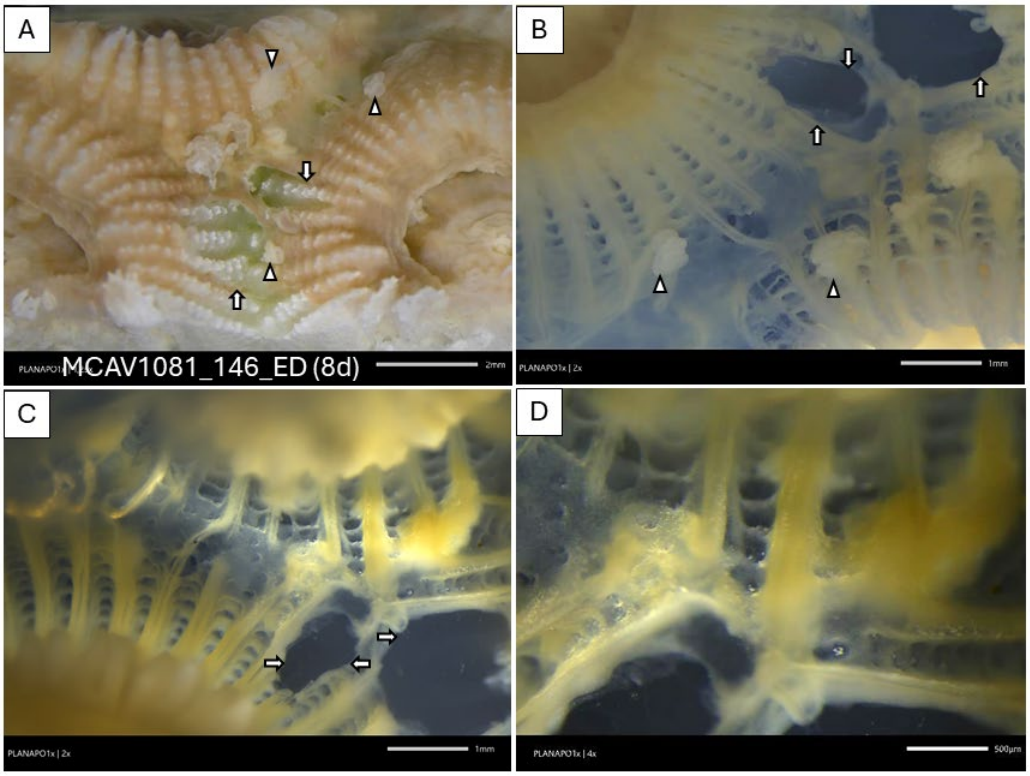

Figure 7. *MCAV_1081_0523TS146, recipient apparently healthy coral sample collected 8d after first exposed to disease (ED). (A) External oral surface view of fixed tissue before decalcification; arrowheads indicate protrusion of mesenterial filaments and arrows indicate the tissue-loss areas. (B) External oral surface view of the same area as (A) after decalcification. Note the lysed-like appearance of the coenenchyme at the tissue-loss areas (arrows) and the protruded mesenterial filaments (arrowheads). (C) Post-decalcified tissue of internal aboral surface view, reverting the image of (B). Note the tissue-loss areas (arrows) emerged adjacent to aggregated, irregularly shaped mesenterial filaments, and the attenuated coenenchyme. (D) Higher magnification of (C).*

Mesenterial filaments protrusion at the external oral surface (Figure 7A, B, Figure 8A) was grossly detected in MCAV in seven ED samples (37.5%), but none was detected in HH, DD, and HC samples. For CNAT, the protrusion was observed only in the DD samples (12.5%) (Table 1). The OFAV specimens never displayed protruded mesenterial filaments at the oral surface.

Mesenterial filament thickening was apparent when the internal view of the sample was visible at the aboral side as well as at the oral surface side after completion of the decalcification process. Mesenterial filament thickening was characterized by aggregated, irregularly shaped filaments (Figure 8B), and polyp formation through extra-tentacular budding between mature polyps (Figure 8C, D). Thickening of the filaments was found in

MCAV (all health conditions) and OFAV (all health conditions except for HH), but not in CNAT (DD) (Table 1).

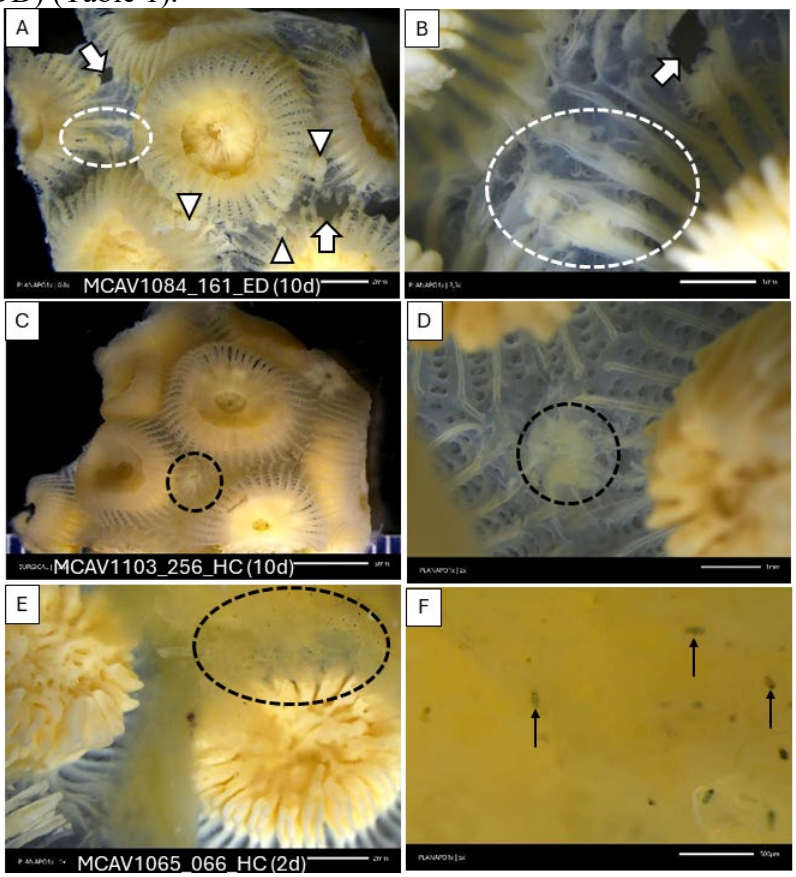

Figure 8. *Post-fixed and post-decalcified coral tissues were examined under a dissecting microscope prior to processing for histology. (A) External oral surface view of MCAV_1084_0523TS161, a recipient apparently healthy coral sample collected 10d after first exposed to disease (ED); arrowheads indicate protrusion of mesenterial filaments and arrows indicate the tissue-loss areas. (B) Internal aboral surface view, reverting the image of (A). Note the tissue-loss areas (arrow) emerged adjacent to aggregated, irregularly shaped mesenterial filaments and the attenuated coenenchyme (dotted white circle). (C) External oral surface view of MCAV_103_0523TS256, a recipient apparently healthy coral sample collected 10d after first exposed to apparently healthy coral (HC); the dotted black circled area indicates a newly developed polyp. (D) Internal aboral surface view, reverting the image of (C); note the thickened mesenterial filaments would develop into a polyp. (E) Internal aboral surface view of MCAV_1065_0523TS066, a recipient apparently healthy coral sample collected 2d after first exposed to apparently healthy coral (HC); the dotted black circled area includes endolithic fungi-algae containing Halofolliculina infection. (F) High magnification of (E) showing individual Halofolliculina cells (arrows).*

A high prevalence of endolithic fungi-algae (Figure 8E, F) was noticed when the internal view was visible in the recipient coral samples (ED, HC, and HH) of MCAV (75 – 100%) and OFAV (50 – 100%) (Table 1).

Halofolliculina (Figure 8E, F) was confirmed only in two MCAV specimens (one DD and HC each) on the lateral and aboral side of skeletal tissue (Table 1).

Table 1. *Post-fixed and post-decalcified samples collected during the time series coral transmission experiment sent to FWC-FWRI for histological examination. All samples were examined under a dissecting microscope for gross external conditions prior to slide processing. The parameters recorded included tissue-loss, mesenterial filaments protrusion, mesenterial filaments thickening (aggregated, irregularly shaped filaments, but also newly budded polyps, when viewed internally after decalcification completion), endolithic algae-fungi infection (viewed internally after decalcification completion), and Halofolliculina infection (viewed both before and after the decalcification from aboral side and internally). The number in parentheses indicates the condition prevalence of the species and sample code (type). Sample code: donor, diseased corals from the field [DD]; pre-experiment naïve apparently healthy corals [HH]; recipients, apparently healthy corals exposed to disease [ED] recipients; apparently healthy corals exposed to healthy control corals [HC].*

Species	Sample code	N	Tissue-loss	Mesenterial filaments protrusion	Mesenterial filaments thickening	Endolithic algae-fungi	Halo-folliculina
CNAT	DD	8	7 (87.5)	1 (12.5)	0 (0)	5 (62.5)	0 (0)
MCAV	DD	1	1 (100)	0 (0)	1 (100)	0 (0)	1 (100)
OFAV	DD	2	2 (100)	0 (0)	2 (100)	2 (100)	0 (0)
MCAV	HH	4	0 (0)	0 (0)	4 (100)	4 (100)	0 (0)
MCAV	ED	16	7 (43.8)	6 (37.5)	8 (50)	12 (75)	0 (0)
MCAV	HC	20	0 (0)	0 (0)	12 (60)	15 (75)	1 (5)
OFAV	HH	1	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)
OFAV	ED	4	1 (25)	0 (0)	2 (50)	2 (50)	0 (0)
OFAV	HC	5	0 (0)	0 (0)	1 (20)	4 (80)	0 (0)

***Table 2**. Number of time-series samples collected over time (d) and processed for histological analysis. The number in parentheses indicates tissue-loss confirmation in post-fixed specimens. Sample code: donor, diseased corals from the field (DD); pre-experiment naïve apparently healthy corals (HH); recipients, apparently healthy corals exposed to disease (ED) recipients; apparently healthy corals exposed to healthy control corals (HC).*

Spec	Sample code	N	0d	2d	5d	8d	10d	12d	17d	27d	36d	41d
CNAT	DD	8	5(4)		1(1)					1(1)	1(1)	
MCAV	DD	1								1(1)		
OFAV	DD	2								1(1)	1(1)	
MCAV	HH	4	4									
MCAV	ED	16		4		4(1)	1(1)	4(2)	1(1)		2(2)	
MCAV	HC	20		4		4	1	5	2		3	1
OFAV	HH	1	1									
OFAV	ED	4		1		1		1				1(1)
OFAV	HC	5		1		1		1			1	1
TOTAL		61	10(4)	10	1(1)	10(1)	2(1)	11(2)	3(1)	3(3)	8(4)	3(1)

4.6. Task 5 – Process naïve coral samples for TEM.

The status of all corals processed for TEM are recorded in *S7 Coral TEM Sample Status.xlsx*, which is provided with this report. Results are observational only as quantitative measurements still must be taken. Viral-like particles (VLPs) are observed in each health state (naïve and diseased). VLPs are also observed regardless of timepoint. Analysis of these samples are planned for the next phase of this project (FY 24-25). Preliminary photos of imaged coral fragments (*Time Series TEM Photos.zip*) for this task are available in the DataOne page for this project or a link was provided with the report to FL DEP.

4.7. Task 6 – Sequence DNA samples from Task 2 for microbiome analysis.

Analysis of the microbiome libraries will continue in the next fiscal year. We also successfully quantified the abundance of the coral pathogen *Vibrio coralliilyticus* using droplet digital PCR (ddPCR) using our previously designed assay (Ushijima et al 2020). Most samples had a few copies of the *vcpA* gene per ng of DNA, but one sample had 43 copies per ng DNA. Analysis of this data will continue in the next fiscal year. The raw data files have been provided with this report **Supplementary File S8** (*S8 051624 GE7495-A1-12+B1-12+E8-12 EvaGreen Deliverable.xlsx),* **Supplementary File S9** *(S9 051624_GE7495-C1-12+D1-12+F1_EvaGreen_Deliverable.xlsx), and* **Supplementary File S10** *(S10 051624_GE7495-E1-7_EvaGreen_Deliverable.xlsx)*.

4.8. Task 7 – Process samples for time series of healthy corals from DRTO for TEM.

The status of all corals processed for TEM are recorded in *S7 Coral TEM Sample Status.xlsx*, which is provided with this report. Results are observational only as quantitative measurements still must be taken. VLPs are observed in samples regardless of health state and timepoint. Symbiodiniaceae have more cellular debris when compared

to the Time Series Corals. Analysis of these samples are planned for the next phase of this project (FY 24-25). Preliminary photos of imaged coral fragments (*DRTO Series TEM Photos.zip*) for this task are available in the DataOne page for this project or a link was provided with the report to FL DEP.

4.9. Task 8 - Process samples of acute *Orbicella* tissue loss lesions for TEM.

The status of all corals processed for TEM are recorded in *S7 Coral TEM Sample Status.xlsx*, which is provided with this report. Results are observational only as quantitative measurements still must be taken. Tissue is darker near the basal body wall when compared to the surface body wall. This can be difficult to observe prior to decalcification. Analysis of these samples are planned for the next phase of this project (FY 24-25). Preliminary photos of imaged coral fragments (*Orbicella Acute TL Photos.zip*) for this task are available in the DataOne page for this project or a link was provided with the report to FL DEP.

5. DISCUSSION AND MANAGEMENT RECOMMENDATIONS

5.1. Discussion

The collective goal of this project was to distribute all samples taken from an SCTLD transmission to naïve coral fragments. The controlled transmission experiments provided us with samples of naïve coral fragments before exposure to SCTLD, right after exposure to SCTLD, as well as at multiple time points after exposure to disease and during disease progression. This experiment had a powerful paired design with each genet of naïve coral infected with SCTLD having a counterpart only exposed to healthy corals that were kept in an identical aquarium system. Thereby, for each sample taken, it had a corresponding healthy control in addition to samples of the healthy naïve corals taken before the start of the experiment as well as the diseased coral collected from the field. Further, at each sampling, the coral fragment was split into 5 representative samples for the various analyses that will be run. Therefore, this was an extensively controlled SCTLD transmission experiment that will have an extensive number of controls and comparability between samples.

Analysis was not the focus of this phase of the study; however, we have accomplished the preliminary processing of all the naïve coral samples, so they are ready for analysis. This included RNA, DNA, and total protein extractions for transcriptomics, microbiome sequencing, and proteomics analysis; chemical extractions for metabolomics; embedding for histology and TEM; and lastly a sample was also sent to Dr. Lauren Fuess (Texas State University) for various immunological assays. The latter (immunological assays) was not originally part of the proposal for this phase; however, the analysis of these results will be in the next phase part of Dr. Fuess' SOW (FY 24-25). As can be seen with the various data files submitted with this report, the preliminary processing for all these analyses was completed this past year. The only data and results that were presented are only preliminary, but full analysis will be the focus on the next phase of the project (FY 24-25). Therefore, no solid conclusions can be made at this stage of the project. Any interpretations of the current data may not represent the larger picture after more analysis is completed in the next phase of the project.

5.2. Management Recommendations

As this phase of the project was only in its sample collection and processing phase, there are currently no results-based management recommendations. However, we recommend the continuation of the sample processing for multiple reasons.

1) This project will create a comprehensive dataset of naïve healthy corals, which would be incredibly important for investigations of future disturbances as a comparative dataset. This would also be valuable for disturbances other than diseases, because of the holistic approach this project is taking.
2) These experiments were carried out under controlled conditions in aquaria, which reduces any variation from environmental conditions. While environmental samples are important, being able to examine datasets collected under controlled conditions would allow us to more accurately observe underlying changes that could be confounded otherwise. The project results will eventually be a curated dataset that will be made publicly available to all researchers.
3) The data collected here will contain comparable *objective* (DNA, RNA, and protein sequences) and *subjective* (histology and TEM) datasets. While still individually important, in combination, these datasets will provide the "bigger picture" on what is occurring with the response of each coral fragment to the various treatments.

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