A COMPARISON OF CONSTITUTIVE AND INDUCED IMMUNE RESPONSE IN CORAL COLONIES OF VARIABLE SYMBIONT DENSITIES

by

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ABSTRACT

Scleractinian corals form the basis of diverse coral reef ecosystems. However, corals are in swift decline globally, in part due to rising disease prevalence. Most corals are dependent on symbiotic interactions with single-celled algae (family Symbiodiniaceae) to meet their nutritional needs. Preliminary evidence suggests that suppression of host immunity may be essential to this relationship. To explore potential immunological consequences of symbiotic relationships in corals, we investigated constitutive and induced immune activity in the facultatively symbiotic coral, *Astrangia poculata*. Brown (high symbiont density) and white (low symbiont density) colonies of *A. poculata* were collected from Rhode Island. First, we compared constitutive immune phenotypes between these two groups. Symbiont density was strongly correlated to several of these immune phenotypes; catalase activity and melanin were significantly positively correlated to symbiont density. Next, we investigated potential variation in induced immune response between the two groups. Colonies of *A. poculata* with variable symbiont densities were exposed to a pathogenic challenge. We then measured differences in constitutive and induced immunity using transcriptomic approaches. Preliminary results indicate significant differences in response to immune challenge as a result of variable symbiont density. Our results highlight the complex nature of symbiosis-immune interplay in cnidarians and emphasize the need for nuanced approaches when considering symbiosis.

I. INTRODUCTION

Reef-building (scleractinian) corals are key ecosystem engineers, which create the structural basis of habitat that supports almost a quarter of all known marine life (Knowlton et al., 2010). However, the health of coral reefs worldwide is rapidly deteriorating, partially as a result of anthropogenic climate change (Burke, Reytar, Spalding, & Perry, 2011). Changing environmental conditions such as increasing ocean temperatures, excess nutrients, habitat degradation, and ocean acidification all threaten the health of coral reefs and have triggered mass die-offs of scleractinian corals (Meesters, Pauchli, & Bak, 1997). By far the two largest drivers of coral mortality have been outbreaks of pathogenic disease and coral bleaching events, a phenomenon which involves the breakdown of the relationship between the host coral and algal endosymbiont (T. P. Hughes et al., 2003). As a result of mortality associated with increasing frequency of disease and bleaching events, coral reefs are now considered one of the most endangered ecosystems (Meesters et al., 1997). Widespread loss of live corals has led to an overall decrease in the architectural complexity of the reef environment (Hughes et al., 2003). The loss of coral reefs and subsequent loss of the structural complexity these corals provide is predicted to result in a great loss of biodiversity (Alvarez-Filip, Dulvy, Gill, Cote, & Watkinson, 2009; Pratchett, Hoey, & Wilson, 2014). This will be detrimental to the many ecosystem services that they provide, including coastline protection and support for fishing industries.

Disease has been identified as one of the largest drivers of coral declines (Eddy et al., 2021; Miller et al., 2009). As anthropogenic effects on natural ecosystems have become

more pronounced, so too has the severity and prevalence of epizootic disease outbreaks which have disproportionately impacted corals (Croquer & Weil, 2009; Meesters et al., 1997). Current widespread diseases in the Caribbean include White Band, White Plague, White Pox, and Stony Coral Tissue Loss Disease (SCTLD; E Weil, 2019; Miller et al., 2009). Despite the impact of these diseases, in many cases the causative pathogens remain unknown (Marshall & Baird, 2000). Several pathogens have been associated with the recent outbreak of SCTLD, including *Vibrio coralliilyticus,*(Palmer & Traylor-Knowles, 2012) but an official causative pathogen has not yet been identified. Co-infections with *V. corallillyticus* and SCTLD have also been identified which may suggest that the bacteria play secondary role in lesion progression and disease exasperation (Landsberg et al., 2020; Ushijima et al., 2020). Interestingly, susceptibility to SCTLD and other diseases is highly variable among and within coral species (Weil, 2004). Previous work has suggested differential immune gene expression, as a result of varying symbiont densities, may impact disease susceptibility under pathogen challenge (Lauren E. Fuess, Palacio-Castro, Butler, Baker, & Mydlarz, 2020; Roesel & Vollmer, 2019)

While ecological variation in coral disease susceptibility has been well documented, the mechanisms contributing to this variation remain poorly understood. This is largely due to a gap in knowledge as it pertains to the coral immune response, which was under-studied until recent years. The cnidarian immune response is a three-step system. The immune response is based upon pathogen recognition, signaling pathways, and effector responses (Mydlarz, Fuess, Mann, Pinzón, & Gochfeld, 2016). Corals have a variety of pathogen recognition molecules, such as Toll-like receptors and NOD-like receptors,

which are capable of identifying a diversity of pathogens (Palmer, Bythell, & Willis, 2010). Post-recognition, signaling pathways are activated to appropriate defense mechanisms (Palmer et al., 2010). The end result of these signaling pathways are effector responses, which are activated in order to eliminate the threat (Palmer et al., 2010). Corals are able to employ a diversity of effector responses including antioxidants, antimicrobial peptides, and melanin (Palmer et al., 2010). Preliminary evidence suggests that variation in several components of the coral immune response might contribute to variations in immunity and disease susceptibility (L. E. Fuess, Pinzon, Weil, Grinshpon, & Mydlarz, 2017; Palmer et al., 2010; Traylor-Knowles, 2021). Furthermore, other factors such as symbiotic state may also contribute to variation in immunity, though the connection between these traits is less well understood (L. E. Fuess, Butler, Brandt, & Mydlarz, 2020; Harman, Barshis, Hauff Salas, Hamsher, & Strychar, 2022). This study aimed to further understanding of the effects of variation in symbiotic state on host immunity, using the facultatively symbiotic coral *Astrangia poculata*.

Astrangia poculata as a model species

Astrangia poculata, a temperate coral, is an excellent model system for addressing questions related to coral immunology and symbiosis. *A. poculata* is a hardy, abundant, temperate coral found from as far north as Massachusetts to the Gulf of Mexico (Dimond et al., 2012). They are easily accessible across a wide geographic range; in fact, recent evidence shows that there is a population off the coast of Texas (Bahr, pers. obs.). Furthermore, the abundance of this species provides a distinct advantage over tropical coral species, many of which are endangered and difficult to acquire in large numbers. In addition, *A. poculata* is facultatively symbiotic; colonies demonstrate extreme natural

variation in symbiont density (Sharp, Pratte, Kerwin, Rotjan, & Stewart, 2017). Symbiotic states are broadly classified based on summer color, approximate chlorophyll concentration, and symbiont density ("brown" < 10^6 cells cm^{-2}, "white" 10^4 -10^6 cm^{-2}; (Sharp et al., 2017). We can leverage this immense natural variation in symbiont density to study questions related to immune-symbiosis interplay in cnidarians.

The following chapter is in review in *Biology Letters.*

II. VARIATION IN SYMBIONT DENSITY IS LINKED TO CHANGES IN CONSTITUTIVE IMMUNITY IN THE FACULTATIVELY SYMBIOTIC CORAL, *ASTRANGIA POCULATA.*

A. INTRODUCTION

Scleractinian corals are key ecosystem engineers, which create the structural basis of diverse coral reef systems (Burke et al., 2011). However, the health of coral reefs worldwide is deteriorating, largely due to anthropogenic climate change (Meesters et al., 1997). Changing environmental conditions such as increased ocean temperatures and ocean acidification have led to coral die-offs (T. P. Hughes et al., 2003); global coral reef cover has declined by 50% from 1957 to 2007 (Eddy et al., 2021). The two largest drivers of coral mortality have been disease outbreaks and bleaching events (Croquer & Weil, 2009; Evans et al., 2020; Miller et al., 2009). Previous studies suggest extensive inter- and intraspecific variation in response to disease (Weil, 2004) and propensity to bleaching (Marshall & Baird, 2000). However, while the factors contributing to variation in bleaching susceptibility have been well studied in many coral species (Krueger et al., 2015; Marshall & Baird, 2000), the mechanisms driving variation in coral disease susceptibility largely remain unknown.

The coral immune response consists of pathogen recognition, signaling pathways, and effector responses (Mydlarz et al., 2016). Corals have a variety of pathogen recognition molecules, such as Toll-like receptors and NOD-like receptors, capable of identifying a diversity of pathogens (Palmer & Traylor-Knowles, 2012). Post-recognition, signaling pathways appropriate defense mechanisms and trigger effector responses (Palmer & Traylor-Knowles, 2012). Corals use effector responses such as melanin production,

antioxidants, and/or antimicrobial peptides to eliminate pathogens (Palmer & Traylor-Knowles, 2012). Preliminary evidence suggests that natural variation in several immune components might contribute to variation in disease resistance (L. E. Fuess et al., 2017; Levy et al., 2021; Palmer et al., 2010).

Beyond its role in pathogenic defense, the coral immune system also plays roles in the establishment and maintenance of symbioses (Davy, Allemand, & Weis, 2012; L. E. Fuess et al., 2020; Kvennefors et al., 2010; Mansfield et al., 2017; Mansfield & Gilmore, 2019; Neubauer, Poole, Weis, & Davy, 2016; Rosset et al., 2021; Tivey et al., 2020; V. M. Weis, 2019; V. M. Weis, Davy, Hoegh-Guldberg, Rodriguez-Lanetty, & Pringle, 2008). The onset and maintenance of coral symbiosis with Symbiodiniaceae is theorized to circumvent or modulate host immune response (Davy et al., 2012; Detournay, Schnitzler, Poole, & Weis, 2012; Mansfield et al., 2017; Merselis, Lirman, & Rodriguez-Lanetty, 2018; Meyer & Weis, 2012). Furthermore, modification of immunity may extend beyond establishment of the relationship. In the threatened Caribbean coral *Orbicella faveolata*, which is obligately symbiotic, experimentally manipulated higher Symbiodiniaceae density was linked to negative effects on host immune gene expression (Lauren E. Fuess et al., 2020). Similarly, a study using *Acropora cervicornis*, found a negative correlation between bleaching and disease, suggesting the reduction in symbiont density associated with bleaching might reduce symbiont-associated immune suppression and increase host capacity to respond to disease (Merselis et al., 2018). Still understanding of the prevalence of potential symbiosis-immune trade-offs across cnidarian species, and the effects of natural symbiont density variation (i.e. non-stress related) on these tradeoffs, is poorly understood. To better understand how

Symbiodiniaceae density and immunity might be linked in diverse scleractinian corals, we investigated variation in constitutive immunity among colonies of the facultatively symbiotic scleractinian coral, *Astrangia poculata,* which displays immense natural variability in densities of its symbiont *Breviolum psygmophilum*.

B. METHODS

Sample collection

Astrangia poculata colonies were collected from Fort Wetherill in Jamestown, Rhode Island in April 2021 (41°28′40″ N, 71°21′34″ W) at a depth of 10-15 meters, via SCUBA. Colonies were visually assessed and sorted into either high or low symbiont density groups (termed "brown" or "white" colonies respectively); 10 colonies of each type were collected. Visual assessment of colony color is a reliable method for distinguishing corals with high symbiont density (>10^6 cells cm^{-2}) from those with low symbiont density (10^4-10^6 cells cm^{-2} (Sharp et al., 2017)). It should be noted that we use the terms "brown" and "white" as colonies grouped in the white category are rarely completely aposymbiotic. Following collection, the colonies were returned to Roger Williams University where they were maintained for several weeks in closed systems containing locally sourced seawater and fed three times weekly with frozen copepod feed. Samples were then flash frozen in liquid nitrogen and shipped to Texas State University for analyses.

Protein extraction

Tissue was removed from colonies with extraction buffer (TRIS with DTT, pH 7.8) using protocols outlined by Fuess (L. E. Fuess, Pinzomicronn, Weil, & Mydlarz, 2016). First, tissue was removed and isolated from a fixed surface area (2.14 cm^2) on the flattest portion of the coral for Symbiodiniaceae density calculation. Then, tissue from the remaining fragment was removed and isolated into a separate aliquot. Both aliquots of

tissue extracts were homogenized using a Fisherbrand Homogenizer 150 prior to downstream processing.

The Symbiodiniaceae aliquot was processed using a series of consecutive centrifugation and wash steps. The homogenate was centrifuged at 376 RCF for 3 minutes and the supernatant was removed. The resultant pellet was resuspended in 500µL, and the product was centrifuged again using the same procedure. This step was repeated, and the sample was preserved in 500µL of 0.01% SDS in deionized water, stored at 4C.

The host aliquot was processed to obtain subsamples for protein activity assays and melanin concentration estimation. Following homogenization, 1 mL of the host aliquot was flash frozen, and stored at 20ºC for melanin concentration estimation (see **Melanin** section). The remainder of the host aliquot was centrifuged for 5 minutes at 1301 RCF using an Eppendorf Centrifuge 5804 R. The resulting supernatant (protein enriched cell-free extract) was flash frozen in liquid nitrogen and stored at –80ºC for downstream assays.

Symbiont density

Symbiodiniaceae density was estimated using a standard hemocytometer and Nikon Eclipse E600 microscope. Symbiodiniaceae counts were repeated in triplicate and averaged to calculate symbiont density/tissue area.

Biochemical Immune Assays

A Red660 assay (G Biosciences, St. Louis, Missouri) based on existing methods (Pinzon, Beach-Letendre, Weil, & Mydlarz, 2014) was used to determine sample protein concentration and standardize assays. All immune assays were run in duplicates on 96 well plates using a Cytation 1 cell imaging multi-mode reader with Gen5 software (BioTek).

Prophenoloxidase Cascade Assays (PPO, PO, and MEL)

Total phenoloxidase activity (PPO + PO) and melanin abundance was estimated using previously established methods (L. E. Fuess et al., 2016) adapted to *A. poculata.* For total phenoloxidase activity, 20µL of coral extract were diluted into 20µL of 50 mM phosphate buffered saline (pH 7.0) in a 96 well plate **(**Greiner bio-one, Frickenhausen, Germany)**.** Samples were incubated with 25µL of trypsin (0.1 mg/mL) for 30 minutes at room temperature, allowing for cleavage of PPO into PO. Post-incubation, 30µL of dopamine was added to each well. Absorbance was read every minute for 20 minutes at 490nm. Change of absorbance at the steepest point of the curve was used to calculate total phenoloxidase activity, standardized by protein concentration (L. E. Fuess et al., 2016; Mydlarz & Palmer, 2011).

To estimate melanin concentration, subsampled tissue extracts for the melanin assay were dried on a speed vac (Eppendorf, Vacufuge plus**)** in a tared 1.5mL microcentrifuge tube. Dried tissues were weighed and processed to assess melanin concentration. Two hundred microliters of glass beads (10mm) were added to each tube. Samples were then vortexed for 10 seconds and 400uL of 10M NaOH was added to each tube. Tubes were

vortexed for 20 seconds and incubated in the dark for 48 hours, with a second 20 second vortex occurring after 24 hours. Post-incubation, the tubes were vortexed and then centrifuged at 100 RCF for 10 minutes at room temperature. The resultant supernatant (40µL) was transferred to a ½ well UV plate (UV-STAR, Greiner bio-one, Frickenhausen, Germany). Absorbance was read at 410 and 490nm. We used a standard curve of melanin dissolved in 10M NaOH to calculate mg melanin from absorbance (L. E. Fuess et al., 2016; Mydlarz & Palmer, 2011). Melanin concentration was standardized per mg of dried tissue weight.

Antioxidant Assays

The activity of two coral antioxidants was investigated: catalase (CAT) and peroxidase (POX), following established methods (L. E. Fuess et al., 2016; Mydlarz & Palmer, 2011), adapted to *A. poculata*. Catalase was measured by diluting 5µL of sample with 45µL of 50mM PBS (pH 7.0) in a transparent UV 96-well plate **(**UV-STAR, Greiner bio-one, Frickenhausen, Germany)**.** To initiate the reaction, 75µL of 25mM H_2O_2 was added to each well. Absorbance was read at 240nm every 30 seconds for 15 minutes. Scavenged H_2O_2 was calculated as the change in absorbance at the steepest portion of the curve. A standard curve was used to determine change in H_2O_2 concentration (mM), and results were standardized by protein concentration (L. E. Fuess et al., 2016; Mydlarz & Palmer, 2011).

To measure peroxidase activity, 20µL of sample was diluted in 20µL of 10mM PBS (pH 6.0) in a standard 96-well plate (Costar, Corning, Kennebunk, ME). Fifty microliters of 25mM guaiacol in 10mM of PBS (pH 6.0) was added to each well of the plate. To

initiate the reaction, 20µL of 20mM H_2O_2 was added to each well. Absorbance was read every minute for 15 minutes at a wavelength of 470nm. Peroxidase activity was calculated as the change in absorbance at the steepest portion of the curve, standardized by protein concentration (L. E. Fuess et al., 2016; Mydlarz & Harvell, 2007).

Antibacterial Assay

Antibacterial activity of *A. poculata* samples was assessed against *Vibrio coralliilyticus* (strain RE22Sm; provided by D. Nelson University of Rhode Island), a known coral pathogen (Vidal-Dupiol et al., 2011). Bacterial culture was revived from frozen stock and grown overnight in Luria broth (LB). After 24 hours, 1mL of bacterial culture was diluted in 100mL of mYP30 broth and grown for an additional 48 hours. Prior to assays, the culture was diluted to a final optical density at 600nm of 0.2. To initiate the assay, 140µL of bacterial culture and 60µL of sample, diluted to a standard protein concentration, were combined into wells of a sterile 96-well plate (Costar, Corning, Kennebunk, ME)**.** Sample absorbance was read every 10 minutes at 600nm for 6 hours at 27ºC. Change in absorbance during the logarithmic growth phase of the curve was used to calculate growth rate (L. E. Fuess et al., 2016; Pinzon et al., 2014).

Statistical Analyses

Prior to statistical testing, outliers were identified and removed if necessary, using the 'nooutlier' function in R. Normality was also assessed, and data was transformed as needed; Symbiodiniaceae density was square root transformed. We assessed the effects of symbiont density on each of our immunological metrics using two approaches. First, we

tested for differences in assay activity between colonies grouped as white or brown using a t-test. Second, we used a Pearson correlation test to assess direct correlations between symbiont density and assay activity. T-tests and correlations were run independently for each assay.

C. RESULTS

Statistical analysis revealed a significant association between symbiotic state and host immune phenotypes. Both melanin concentration (t-test, p=0.0004; Figure 1A) and catalase activity (T-test, p=0.048; Figure1B) were significantly higher in brown colonies than white. Furthermore, melanin concentration (Pearson correlation, R=0.64, p=0.003; Figure 1C) and catalase activity (Pearson correlation, R= 0.62, p=0.005; Figure 1D) were significantly positively correlated to symbiont density. No other assays were significantly associated with symbiont state or symbiont density (**Tables 1-2**).

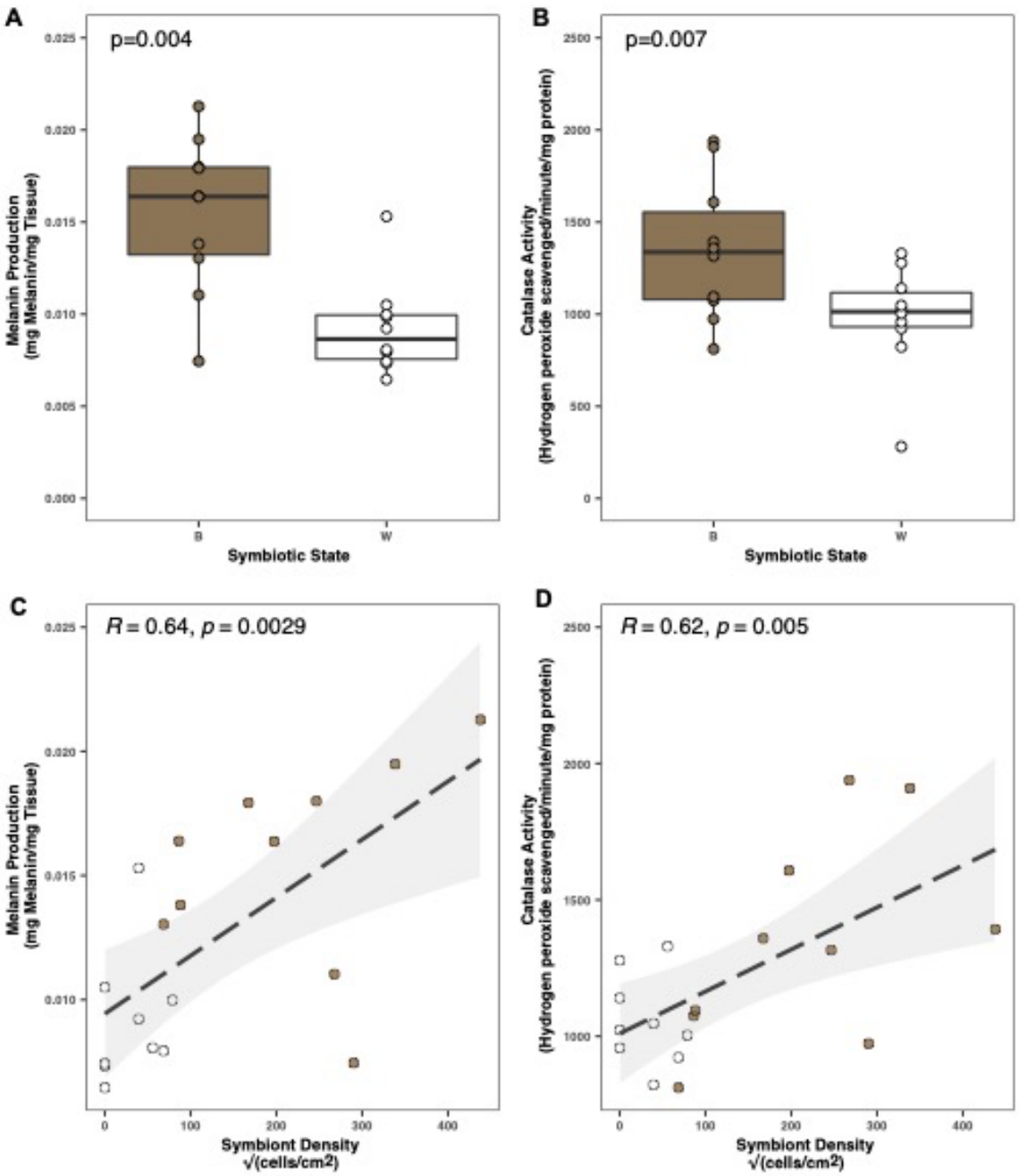

Figure 1. Both symbiont state and symbiont density affect melanin concentration and catalase activity. **A-B:** Box and whisker plots displaying differences in immune parameters between white and brown colonies for melanin **(A)** and catalase **(B). C-D:** symbiont-immune assay correlation results for melanin concentration (**C**) and catalase activity (**D).**

Table 1.1 T-test results for each immunological assay.

Assay	Statistic value	dF	p-value
Peroxidase	-0.696	13.8	0.498
Prophenoloxidase	-0.816	18	0.425
Catalase	2.19	12.5	0.0482*
Antibacterial	1.01	12.9	0.334
Melanin	4.96	11.2	0.0004*

Table 1.2 Pearson-correlation results between assay activity and square-root transformed symbiont density.

Assay	corr. value	dF	p-value
Peroxidase	0.2430729	17	0.316
Prophenoloxidase	-0.0130134	18	0.9566
Catalase	0.6155106	17	0.005026*
Antibacterial	-0.06311574	18	0.7915
Melanin	0.6900038	17	0. 0011*

D. DISCUSSION

Here we used a facultatively symbiotic coral, *Astrangia poculata,* to investigate trade-offs between constitutive immunity and Symbiodiniaceae density in corals. Past studies have suggested trade-offs between the maintenance of symbiotic relationship and immunity in obligately symbiotic corals (Fuess et al., 2020; Merselis et al., 2018). In contrast, our results show no trade-offs between Symbiodiniaceae abundance and constitutive immunity. Instead, we find a positive association between constitutive immunity and Symbiodiniaceae density in *A. poculata*. These findings confirm the complex nature of the relationship between algal symbiosis and immunity in cnidarians and highlight the need for further study of symbiosis-immune interplay in diverse systems.

We document positive correlations between symbiont density and two metrics of constitutive immunity: catalase activity and melanin concentration. Importantly, while both systems function in immunity, they also serve secondary roles in maintenance of coral-algal symbiosis (Merle, Sabourault, Richier, Allemand, & Furla, 2007). While antioxidant activity is important in combating ROS bursts associated with pathogen defense, it is also important in general stress response, including response to thermal stressors (Wall et al., 2018). Symbiont release of ROS is believed to be a cause of thermally induced bleaching, or breakdown of algal symbiosis (Szabó, Larkum, & Vass, 2020). Consistent with this theory, increased antioxidant production is associated with increased resistance to thermal bleaching (Nielsen, Petrou, & Gates, 2018). Similarly, in addition to its roles in encapsulation of pathogens (Palmer & Traylor-Knowles, 2012), melanin may play secondary roles in stress response, including protection of algal

symbionts from UV damage (i.e., symbiont shading; (Palmer, Bythell, & Willis, 2011)). Consequently, observed patterns of higher activity of these two pathways may be indicative of algal symbiont management and proactive stress mitigation mechanisms rather than direct consequences of symbiosis on immunity.

A second hypothesis could explain the observed associations between Symbiodiniaceae density and immunity more generally: resource allocation theory. Resource allocation theory posits that organisms allocate a fixed energetic budget to competing needs (ex: growth, reproduction, and immunity; (Sadd & Schmid-Hempel, 2009)). When energy budgets are fixed, increases in any one category come at the cost of another (i.e. tradeoffs; (Sadd & Schmid-Hempel, 2009)). Consequently, energetic budgets can have significant impacts on resources allocated to immunity. For example, reductions in energy budgets caused by starvation resulted in decreased expression of immune genes and resistance to pathogens in the cnidarian *Nematostella vectensis* (Aguirre Carrión et al., 2022). Indeed, facultative symbiosis may be a natural source of variation in energetic budget; colonies of corals with variable densities of Symbiodiniaceae may vary in their base energetic budget due to increased photosynthetically derived carbon. Past studies have linked increased photosynthetic energy acquisition to increased Symbiodiniaceae density (A. D. Hughes, Grottoli, Pease, & Matsui, 2010; Pupier et al., 2019). Consequently, increased *B. psygmophilum* densities in *A. poculata* may increase a colonies total energetic budget, allowing for greater resource allocation to immunity and explaining elevated catalase and melanin levels in colonies with higher *B. psygmophilum* density.

Regardless of mechanism, these findings add to a growing body of work considering the effects of symbiont density on immunity in cnidarians. Interestingly, previous work in obligately symbiotic corals suggests a negative relationship between symbiont density and immune gene expression (Lauren E. Fuess et al., 2020; Merselis et al., 2018), opposite to this study. A similar pattern was also observed in another obligately symbiotic coral, *Acropora tenuis;* immune gene expression was downregulated to allow for the establishment of symbiosis (Yuyama, Ishikawa, Nozawa, Yoshida, & Ikeo, 2018). However, these previous studies involved obligately symbiotic corals, whereas our results describe patterns in a facultatively symbiotic coral. Variation in symbiont density, and therefore energetic budget, is likely more pronounced in the latter group, affecting our results. Additionally, the past studies applied broad transcriptomics approaches while this study only measured a handful of immune effector responses with dual roles in stress response and symbiosis maintenance. A broader approach might yield different results. Finally, in the Fuess et al. 2020 study, nutrient enrichment was used to artificially manipulate symbiont density (Lauren E. Fuess et al., 2020). Recent findings have suggested that nutrient enrichment may inhibit coral immune responses (PO activity) (Dougan et al., 2020), suggesting this may have confounded results from the previous study. More studies investigating symbiosis-immune interactions in diverse cnidarian species using consistent approaches will be essential in disentangling this nuanced relationship.

In summary, our results highlight a positive association between *B. psygmophilum* density and immune parameters in the temperate coral *A. poculata*, which contrasts with past studies of obligately symbiotic corals. This association is most likely either related to

the dual function of these parameters or a consequence of increased energetic budgets associated with symbiosis. Importantly, our approach only measured a subset of potential effector responses. Future studies incorporating more responses or measures of receptor and signaling activity would improve interpretation of these trends. Additionally, our results are limited to the context of constitutive immunity; further studies considering pathogen response would be informative. Finally, our results are limited to the context of common garden conditions; additional natural studies which highlight the immunological effects of interactions between symbiont density and environmental variation would be informative. Nevertheless, our data provides an important first step in highlighting the nuanced association between immunity and algal symbiosis in scleractinian corals.

III. COMPARISON OF INDUCED IMMUNE RESPONSE ACROSS SYMBIONT STATES IN THE FACULTATIVELY SYMBIOTIC CORAL, *ASTRANGIA POCULATA.*

A. INTRODUCTION

To better explore the relationship between symbiosis and immunity in *Astrangia poculata* in the context of both constitutive and induced immunity, we ran a subsequent experiment combining biochemical and transcriptomic approaches. It is well known that establishment of symbiosis involves immune suppression to allow for assimilation of the symbiont rather than destruction (Davy et al., 2012; V. Weis, Reynolds, deBoer, & Krupp, 2001). Maintenance of this intracellular symbiosis also requires constant modulation by the host immune system (V. M. Weis, 2019). Furthermore, recent research has found that under pathogen challenge, symbiotic state and/or symbiont cell density can impact the host coral immune response to environmental stress and pathogen exposure (Harman et al., 2022; Roesel & Vollmer, 2019; Z. Zhou et al., 2019). However, many of these studies were conducted in obligately symbiotic cnidarians which demonstrate limited natural variation in symbiont density. Here, we leverage *Astrangia poculata*, a temperate facultatively symbiotic coral, to more precisely study the impact of variability in symbiont density on host immunity. Specifically, here we investigate how variability in symbiont density may impact the response of hosts to pathogenic challenge. Colonies of *A. poculata* with variable symbiont densities were exposed to the known coral pathogen, *Vibrio corallillyticus* (Gibbin et al., 2019; Vidal-Dupiol et al., 2011; Z. Zhou et al., 2019). We assayed differences in immunity as a result of immune challenge,

symbiont density, and the combination of these two traits, using both biochemical immune assays and transcriptomic approaches.

B. METHODS

Based on preliminary findings (**Chapter 1**), we designed a second experiment to investigate the effects of symbiotic state on host response to pathogenic stimuli. Cultures of the known coral pathogen *Vibrio coralliilyticus* were obtained from David Nelson at the University of Rhode Island. *V. coralliilyticus* (strain RE22Sm) was maintained in liquid mYP30 media at 27°C in a shaking incubator set to 200RPM. mYP30 media is comprised of 1g of yeast extract (Sigma-Aldrich), 5g of Peptone Primatone (Sigma-Aldrich), 30g of instant ocean salt (Instant Ocean), and DI H_2O (pH 7.5-7.6).

To determine bacterial load in cultures, we generated an OD to CFU standard curve using a SmartSpec Plus Spectrophotometer (BioRad) following standard protocols. First, we created a serial dilution of bacterial stock ranging from 10^1 to 10^{-8}. We then estimated optical density at 600nm for each dilution until unreadable (10^1, 10^{-1}, 10^{-2}, 10^{-3}). The 10^{-8} dilution culture was then streaked on a plate with mYP30 agar and resulting CFUs were counted after 48 hours. These counts were used to create a standard curve of OD to CFU. Next, we developed protocols for heat inactivation of *V. coralliilyticus* by heating 1mL aliquots of culture (1 x 10^7/mL concentration) at 90°C for 5-30 minutes. Aliquots were removed every 5 minutes and streaked on separate mYP30 agar plates. Plates were checked at 24 and 48 hours to verify heat inactivation. Based on this, it was determined that 5 minutes at 90°C was sufficient for inactivation of bacteria.

Colonies of white and brown *A. poculata* were collected from Fort Wetherill, Jamestown, Rhode Island and sent to Texas State by K. Sharp at Roger Williams University. At Texas State, corals were acclimated to the aquarium system following established husbandry protocols (Rotjan et al., 2019) for one month. At the end of this

period, colonies of *A. poculata* were exposed to either heat-inactivated *V. coralliilyticus* (1×10^7 CFU in 100uL of buffer) or a buffer control (filtered sea water) via injection (*n*= 18 per group; roughly divided between symbiotic state). *V. coralliilyticus* was grown overnight in mYP30 media following standard culturing conditions. The optical density of resulting cultures was measured, and cultures were diluted to a final concentration of 1×10^8 CFU using our previously established OD standard curve. Cultures were then aliquoted into individual microcentrifuge tubes (1mL per tube). Each tube was centrifuged at 16000 RCF for 4 minutes. Filtered seawater (FSW) was prepared by filtering through a 0.2um filter. The media was removed, and the sample was then resuspended in 1mL of FSW and heated for 5 minutes at 90ºC in a heat block. Heat inactivation was confirmed via plate streaking. Colonies were injected with 100uL of either heat inactivated bacteria or FSW in four locations using a PrecisionGlide Needle (BD). The samples were then incubated for 4 hours before they were sampled for transcriptomic analyses and the remaining colony was flash frozen for immune assays. Samples preserved for transcriptomic analyses were stored in 1mL of RNAlater (Sigma-Aldrich) for 24 hours at 4 ºC, then stored in -80ºC.

Immunological Assays & Symbiont Density Estimation

Samples flash frozen in liquid nitrogen were analyzed using the biochemical immune assays and symbiont density estimation methods described in **Chapter 1**.

Statistical Analyses- Immune Assays

Differences in immune activity were assessed using parametric multi-factorial testing. First, outliers were identified using a Rosner test and removed. Then, normality was assessed with a wilks-shapiro test. Prophenoloxidase and peroxidase activity were non-

normally distributed and therefore, normalized using a square root transformation. Following transformation, the distribution of the data fit the assumption of normality. Additionally, symbiont density, which is count data, was square root transformed. We then ran three different general linear models (GLM) to assess the impact of symbiont density, treatment, and the combination of the two factors on activity of each measured immune phenotype independently. First, we ran the model: immune activity ~ treatment*symbiont density; no significance was assessed for the interaction of the two terms. We then ran the model: immune activity ~ treatment + symbiont density. Finally, to look at broad scale differences between corals of high and low symbiont density, we ran the model: immune activity ~ treatment*symbiotic state, where symbiotic state was reflective of initial broad classifications of colonies into "brown" and "white" groups.

RNA Extraction

RNA was extracted from sub-samples preserved in RNA-later using the RNAqueous extraction kit (Ambion by LifeTechnologies**)** following manufacturer's protocols with the addition of a bead beating step prior to lysis to separate coral tissue from the skeleton. The sample was added to a tube with the addition 0.5 mm glass beads and the lysis buffer and beat on a bead beater (Fisherbrand Bead Mill 24 Homogenizer) for 1 minute (Wuitchik et al., 2021). Following extraction, samples were qualitatively assessed using the Take 3 plate on the Cytation 1 cell imaging multi-mode reader with Gen5 software (BioTek). Samples with 260/280 ratios between 1.962 - 2.136 and RNA concentrations of > 20 ug/mL were sent to the University of Texas Genomic Sequencing and Analysis Facility for TagSeq library prep and sequencing on NovaSeq with single end, 100 base pair reads. A random subset of an additional 11 samples representative of all treatment

groups and meeting quality metrics were sent to NovoGene for sequencing on an Illumina NovaSeq platform with 150 base pair, paired end reads. These samples were used for *de novo* construction of a reference *A. poculata* transcriptome.

Transcriptome Assembly

A new reference transcriptome for *A. poculata* was constructed using reads generated from the 11 randomly selected corals. Briefly, demultiplexed reads were quality assessed and trimmed using Cutadapt (Martin, 2011). Resulting reads were then used to assemble a *de novo* transcriptome in Trinity following standard protocols (Grabherr et al., 2011). The resulting transcriptome was then filtered using BBSplit (Bushnell, 2014) to separate coral host reads from symbiont reads and generate a host-only reference transcriptome. The resulting transcriptome was annotated against the UniProtKB/Swiss-Prot database using a blastx algorithm from BLAST (Ye, McGinnis, & Madden, 2006) with an evalue cut off of .0001. The results were parsed to isolate the hit with the highest e-value for each annotated transcript.

Read Alignment & Differential Expression Analyses

TagSeq samples were processed and aligned to the reference genome following an open-source pipeline (github.com/z0on/tag-based_RNAseq). First, reads were demultiplexed, trimmed, and quality assessed using a combination of custom Perl scripts and cutadapt (Martin, 2011). Resulting reads were then mapped to the generated reference transcriptome using Bowtie2 (Langmead & Salzberg, 2012) to generate a read count matrix. This matrix was then analyzed using DESeq2 (Love, Huber, & Anders, 2014) to assess for variation in gene expression as a result of our factors of interest, using the following model: gene expression ~ immune treatment + symbiont density + immune

treatment*symbiont density. Resulting differential expression results were further analyzed for gene ontology enrichment as a result of each model term with the Gene Ontology Mann-Whitney U pipeline for R (github.com/z0on/GO_MWU). Significant genes and GO terms were identified as those with an adjusted p-value < 0.1.

C. RESULTS

Immune Activity

Three GLM models were used to analyze the results of each assay. No results were significant when analyzing the interaction of symbiont density and treatment (**Table 2.1**). However, two metrics of immunity were significantly impacted by terms in the additive GLM (**Table 2.2**). Catalase was significantly associated with symbiont density ($p = 0.036$), whereas melanin was significantly different between treatment groups ($p =0.025$). Finally, several immune parameters were also significantly impacted by factors in the interactive GLM assessing treatment and broad classification of symbiont state (**Table 2.3**). Specifically, melanin was significantly different between treatment groups ($p=0.015$), and symbiotic states ($p=0.005$), whereas peroxidase activity was significantly different between symbiotic states ($p=0.024$). No significance was found for the interaction of immune challenge and symbiont density/symbiotic state in any of our models.

Table 2.1 Generalized linear model (GLM) results for each immunological assay, p values reported are for the model: Treatment * SQRT symbiont. Tests were run independently for each assay.

	Treatment				Symbiont Density				Interaction			
Assay	Est.	Std. Err	t value	Pr(>|t|)	Est.	Std. Err	t value	Pr(>|t|)	Est.	Std. Err	t value	Pr(>|t|)
CAT	15.923	147.031	0.108	0.914	0.692	0.911	0.759	0.454	0.699	1.120	0.624	0.537
POX	0.326	0.222	1.469	0.153	0.003	0.001	1.849	0.075	-0.002	0.002	-1.215	0.235
PPO	-0.013	0.206	-0.063	0.950	0.001	0.001	0.432	0.669	0.001	0.002	0.414	0.682
MEL	9.102e-06	8.652e-05	0.105	0.917	9.120e-07	5.375e-07	1.697	0.100	-8.624e-07	6.609e-07	-1.305	0.202
AB	-0.642	0.425	-1.509	0.142	-0.001	0.003	-0.353	0.727	0.004	0.003	1.181	0.247

Table 2.2 Generalized linear model (GLM) results for each immunological assay for the model: Treatment + SQRT Symbiont. Tests were run independently for each assay.

	Treatment				Symbiont Density			
Assay	Est.	Std. Err	t value	Pr(>|t|)	Est.	Std. Err	t value	Pr(>|t|)
CAT	97.685	65.967	1.481	0.1488	1.154	0.525	2.199	0.036*
POX	0.0861	0.102	0.844	0.406	0.001	0.001	1.504	0.144
PPO	0.064	0.090	0.705	0.486	0.001	0.001	1.425	0.164
MEL	-9.188e-05	3.912e-05	-2.349	0.025*	3.415e-07	3.161e-07	1.080	0.288
AB	-0.620	0.522	-1.188	0.244	-0.003	0.004	-0.690	0.496

Table 2.3 Generalized linear model (GLM) results for each immunological assay reported for the model Treatment*Symbiotic State. Test were run independently for each assay.

	Treatment				Symbiotic State				Interaction			
Assay	Est.	Std. Err	t value	Pr(>|t|)	Est.	Std. Err	t value	Pr(>|t|)	Est.	Std. Err	t value	Pr(>|t|)
CAT	156.404	81.842	1.911	0.066	-0.534	126.789	-0.004	0.997	-172.876	163.684	-1.056	0.2993
POX	0.0505	0.113	0.445	0.660	-0.414	0.173	-2.395	0.024 *	0.278	0.227	1.225	0.2310
PPO	0.0767	0.111	0.693	0.494	-0.000	0.175	-0.002	0.998	-0.039	0.224	-0.176	0.862
MEL	-1.056e-04	4.090e-05	-2.582	0.015 *	-1.997e-04	6.516e-05	-3.065	0.005 *	1.392e-04	8.354e-05	1.666	0.10577
AB	-0.139	0.220	-0.631	0.533	0.004	0.417	0.008	0.993	-0.186	0.503	-0.370	0.714

Transcriptome assembly and alignment

De novo construction of a reference transcriptome for *A. poculata* resulted in a host-only transcriptome of 1,154,619 transcripts with an N50 of 332. A total of 326,864 (28.3%) transcripts were annotated by comparison to the UniProt database. Alignment of TagSeq reads to this reference transcriptome yielded an average mapping rate of 75.49%, with an average of 688,248 reads mapped to the host-only transcriptome per sample.

Immune Challenge

No genes were differentially expressed as a result of immune challenge. However, 41 biological process GO terms were differentially enriched as a result of this treatment. Ten terms related to apoptosis and autophagy were significantly differentially enriched in response to immune challenge including: *autophagy* (delta rank=1102, p_{adj}= 0.0412) and *regulation of intrinsic apoptotic signaling pathway by p53 class mediator* (delta rank: -2198, p_{adj} = 0.0396; **Figure 2.1**).

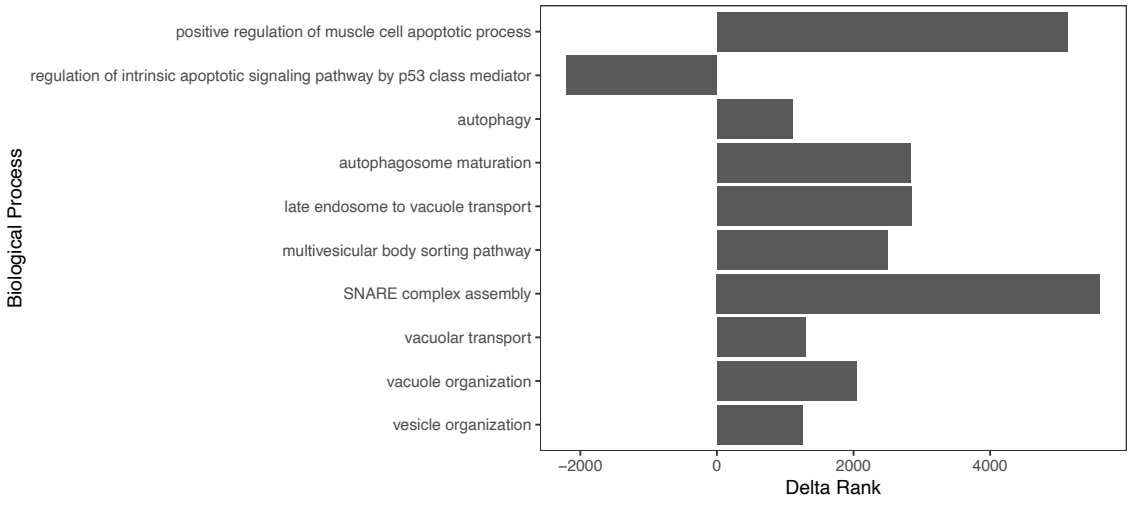

Figure 2.1 GO MWU analysis for terms related to apoptosis and autophagy which were significant differentially enriched between treatment groups (control and immune challenge). Bars indicated delta rank values; positive values are more enriched in immune challenged corals.

Symbiont Density

Symbiont density affected expression of a total of seven unannotated genes. Furthermore, only four biological process GO terms were differentially enriched as a result of symbiont density: *protein-containing complex organization*, *ubiquitin-dependent protein catabolic process via the multivesicular body sorting pathway*, *plasma membrane repair*, and *aminoglycan catabolic process* (**Figure 2.2**).

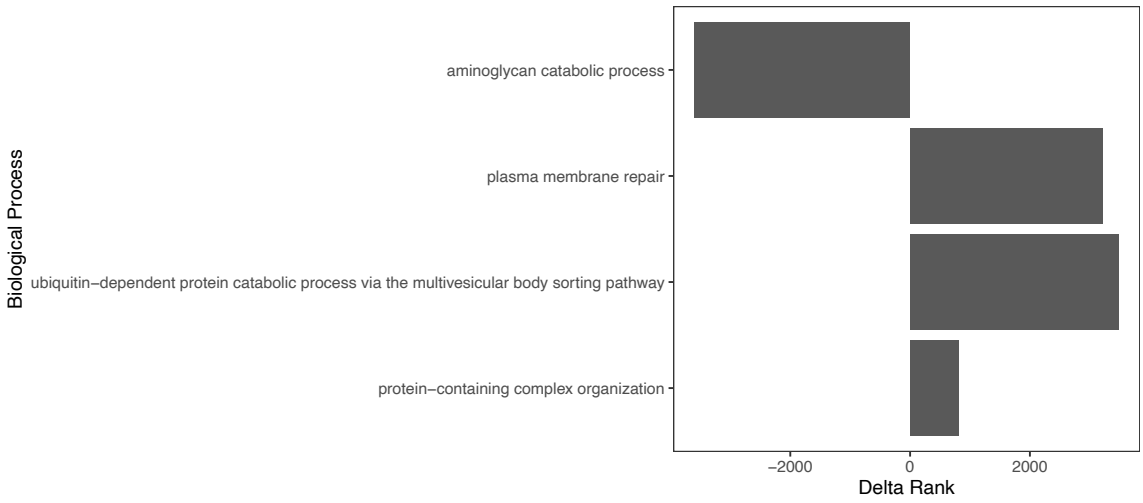

Figure 2.2 Gene ontology enrichment results for the impact of symbiont density on biological processes. Bar magnitude indicates delta rank; positive values are positively associated with symbiont density.

Interaction effect

Six genes were differentially expressed as a result of the interaction between symbiont density and immune challenge. Of these, 3 were annotated, including Rpl29, Bro, and POLDIP2. All six of these genes were positively associated with symbiont density under immune challenge conditions only (positive log-fold change; **Figure 2.3**). A total of 27 biological process gene ontology terms were differentially enriched as a result of this interaction. Of those, six were related to ciliary action, including: *cilium movement*,

microtubule-based movement, *cilium or flagellum-dependent cell motility*, microtubule-based process, *axonemal dynein complex assembly*, and *microtubule bundle formation* (**Figure 2.4**). All these terms followed negative expression patterns (i.e., negatively associated with symbiont density under immune challenge conditions).

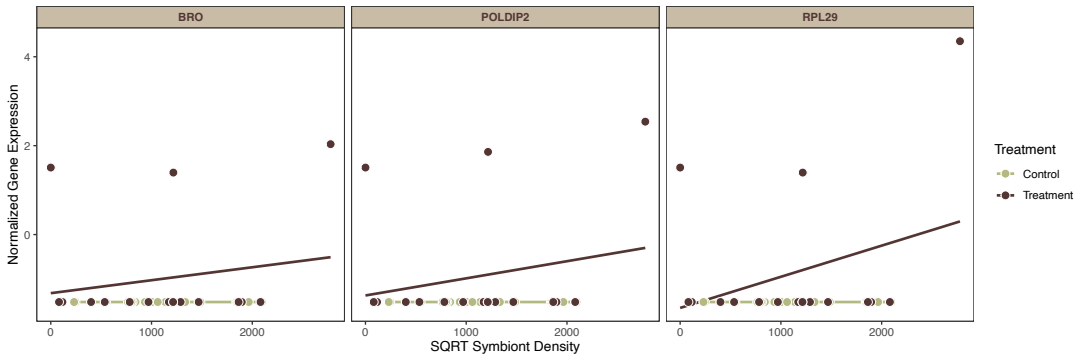

Figure 2.3 Differential expression of three genes (BRO, POLDIP2, and RPL29) involved in ciliary action between control and treatment (pathogen exposed) groups.

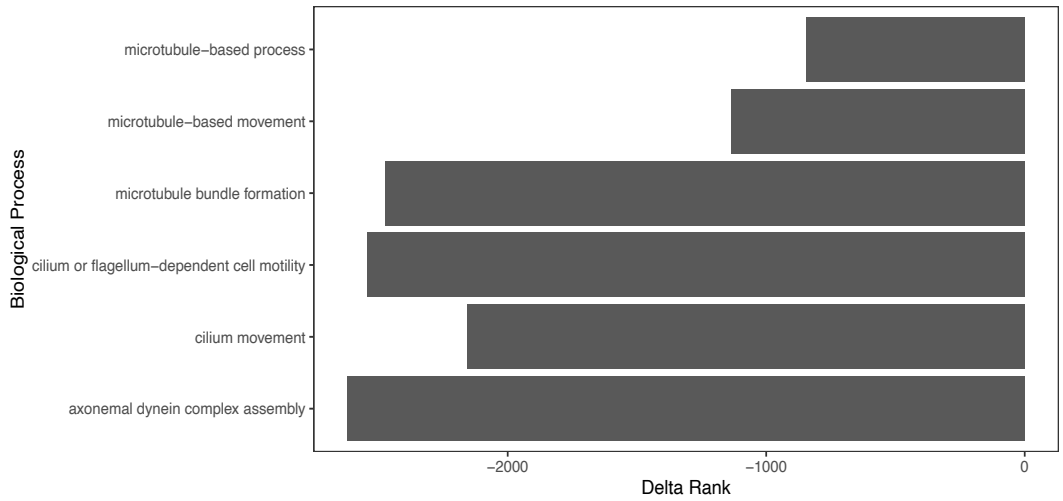

Figure 2.4 Gene ontology enrichment results for the interaction effect (symbiont density * treatment) on biological processes. Bar magnitude indicates delta rank; negative values are positively associated with symbiont density.

D. DISCUSSION

Here we studied the facultatively symbiotic coral, *Astrangia poculata* to further disentangle the relationship between symbiosis and immunity, focusing specifically on induced immune response. Results from my first chapter illustrated a clear difference in the constitutive immune response between coral colonies of varying symbiont density. In this chapter we used expanded analyses to both more comprehensively investigate differences in constitutive immunity between corals of variable symbiont density, and investigate the effects of this variation on induced responses. Our results confirm the findings in our earlier study and highlight important biological processes which may modulate the pathogen response in the facultatively symbiotic coral, *Astrangia poculata.*

Results from our immune assays are congruent with the trends we saw in the first chapter. While there were no significant differences in induced immunity between corals of variable symbiont density, our results suggest that melanin abundance is consistently linked to symbiont density. This is also consistent with recently published independent studies investigating *A. poculata* innate immunity (Harman, Barshis, Hauff Salas, Hamsher, & Strychar, 2022). The lack of observed differences in induced immunity is more likely an artifact of the experimental approach rather than a true lack of difference. Corals were exposed to the heat-killed pathogen for 4 hours which was an optimal time point for capturing differences in gene expression, but less ideal for measuring changes in protein activity, due to the lag between signal detection and induction of protein responses (Mydlarz & Harvell, 2007). Future studies with extended sampling should be used to clarify the effects of variation in symbiont density on induced immunity using protein-based approaches.

Gene expression analyses provided further context regarding differences in constitutive immunity between corals of variable symbiont density. Though no genes were significantly differentially expressed as a result of immune challenge, eight terms related to autophagy were significantly differentially enriched in response to immune challenge. These terms included a general autophagy term and various vacuole assembly processes, which suggests the mounting of an immune response via autophagosomal vacuoles (Downs et al., 2009). A term related to apoptosis was also negatively enriched in relation to immune challenge. This is unsurprising as both apoptosis and autophagy have been previously linked to coral immunity (L. E. Fuess et al., 2017). Specifically, autophagy has been posited to confer disease resistance, whereas apoptotic responses are associated with disease susceptibility (L. E. Fuess et al., 2017). During immune challenge, autophagy may be initiated in order to consume non-essential cell components which can then be used as resources for macromolecule formation and creation of effector molecules (L. E. Fuess et al., 2017; Maiuri, Zalckvar, Kimchi, & Kroemer, 2007). Autophagy is also immune mechanism itself, triggered by receptors such as toll-like receptors. These receptors bind to microbial associated molecular patterns on the cell surface of the pathogen and activate autophagic signaling to eliminate intracellular pathogens (Cooney et al., 2010; L. E. Fuess et al., 2017; Shi & Kehrl, 2008). Autophagy as a whole is a more rapid and efficient pathogen response compared to apoptotic signaling (L. E. Fuess et al., 2017). Apoptosis, or programmed cell death, has also been linked to bleaching and the innate immune response to pathogen exposure (Lesser, 2011; Oakley & Davy, 2018; F. Zhou, Yang, & Xing, 2011). Fuess et al., found that coral colonies that were more susceptible to disease exhibited more reliance upon apoptotic

immune responses than did those that were classified as disease resistant (L. E. Fuess et al., 2017). Furthermore, Tchernov et al., found that an apoptotic response was higher in coral colonies that soon experienced fatality then those that survived the immune challenge (Tchernov et al., 2011). These findings suggest that *A. poculata* mounts a robust immune response to pathogen exposure that is marked by signatures of potential disease resistance.

In addition to noting strong differences in apoptosis and autophagy as a result of immune challenge in *A. poculata,* we also observed differences in ciliary action induction in response to immune challenge between corals of differing symbiont density. Previous studies have illustrated a link between ciliary movement & biogenesis and stress condition such as sediment stress (Erftemeijer, Riegl, Hoeksema, & Todd, 2012). Several studies have found that corals increase ciliary action and slough off mucus sheets to move excess sediment and associated bacteria away from the coral (Erftemeijer et al., 2012). Indeed, ciliary action is essential for the regulation and maintenance of the coral's surface mucosal layer (Brown & Bythell, 2005), which is often regarded as the coral's first layer of defense (Bakshani et al., 2018)**.** Mucopolysaccharides associated with the surface mucus layer can trap or repel bacteria (Rublee, Lasker, Gottfried, & Roman, 1980). Apical cilia provide water flow to sweep mucus and other trapped particles like bacteria off the surface of the coral (Mullen, Peters, & Harvell, 2004). Multiple studies have linked ciliary action to the coral infection response directly (Gavish, Shapiro, Kramarsky-Winter, & Vardi, 2021). Preliminary evidence also suggests important roles of ciliary action in stress response in *Astrangia poculata* specifically (Peters & Pilson, 1985). Here we documented increased ciliary action in response to immune challenge in corals with

lower symbiont density. This suggests that corals with lower symbiont density are more apt to induce essential initial defense mechanisms within the surface mucosal layer than colonies with higher symbiont density. Our findings here may be linked to differences in dynamic energy budget as a result of variable symbiont density. Corals with higher symbiont density may have a greater energetic budget (Muller, Kooijman, Edmunds, Doyle, & Nisbet, 2009) which they can use to sustain costly cellular immune responses (antimicrobial peptides). Consequently, they may be less reliant on initial mechanical defense. Colonies of lower symbiont density, however, may compensate for reduced energetic budget by instead inducing immune-protection responses, preventing further infection, and avoiding costly immune defenses.

Overall, our results emphasize the need for nuanced approaches when evaluating the relationship between symbiosis and immunity in corals. Our immune assay results confirm the previously documented positive relationship between melanin concentration and symbiont density (Harman, Barshis, Hauff Salas, Hamsher, & Strychar, 2022). Gene expression results, suggest that *Astrangia* induces autophagy and suppresses apoptosis in response to immune challenge, which is characteristic of a beneficial immune response. We also demonstrate that corals with lower symbiont density demonstrate stronger induction of ciliary responses in response to immune challenge. This is likely linked to differences in energetic budgets and used as a response to prevent initial pathogen infection/ invasion through the gastrovascular cavity (Gavish et al., 2021). Combined our results provide an important first step to broadly characterizing the *A. poculata* immune response and understanding the relationship between symbiosis and immunity in this important study species.

LITERATURE CITED

Aguirre Carrión, P. J., Desai, N., Brennan, J. J., Fifer, J. E., Davies, S. W., & Gilmore, T. D. (2022). doi:10.1101/2022.06.09.495518

Alvarez-Filip, L., Dulvy, N. K., Gill, J. A., Cote, I. M., & Watkinson, A. R. (2009). Flattening of Caribbean coral reefs: region-wide declines in architectural complexity. *Proc Biol Sci, 276*(1669), 3019-3025. doi:10.1098/rspb.2009.0339

Bakshani, C. R., Morales-Garcia, A. L., Althaus, M., Wilcox, M. D., Pearson, J. P., Bythell, J. C., & Burgess, J. G. (2018). Evolutionary conservation of the antimicrobial function of mucus: a first defence against infection. *npj Biofilms and Microbiomes, 4*(1), 14. doi:10.1038/s41522-018-0057-2

Brown, B. E., & Bythell, J. C. (2005). Perspectives on mucus secretion in reef corals. *MARINE ECOLOGY PROGRESS SERIES, 296*, 291-309. doi:10.3354/meps296291

Burke, L., Reytar, K., Spalding, M., & Perry, A. (2011). *Reefs at Risk Revisited*.

Bushnell, B. (2014). BBMap: A Fast, Accurate, Splice-Aware Aligner.

Cooney, R., Baker, J., Brain, O., Danis, B., Pichulik, T., Allan, P., . . . Simmons, A. (2010). NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. *Nat Med, 16*(1), 90-97. doi:10.1038/nm.2069

Croquer, A., & Weil, E. (2009). Changes in Caribbean coral disease prevalence after the 2005 bleaching event. *Dis Aquat Organ, 87*(1-2), 33-43. doi:10.3354/dao02164

Davy, S. K., Allemand, D., & Weis, V. M. (2012). Cell biology of cnidarian-dinoflagellate symbiosis. *Microbiol Mol Biol Rev, 76*(2), 229-261. doi:10.1128/MMBR.05014-11

Detournay, O., Schnitzler, C. E., Poole, A., & Weis, V. M. (2012). Regulation of cnidarian-dinoflagellate mutualisms: Evidence that activation of a host TGFbeta innate immune pathway promotes tolerance of the symbiont. *Dev Comp Immunol, 38*(4), 525-537. doi:10.1016/j.dci.2012.08.008

Dimond, J. L., Kerwin, A. H., Rotjan, R., Sharp, K., Stewart, F. J., & Thornhill, D. J. (2012). A simple temperature-based model predicts the upper latitudinal limit of the temperate coral Astrangia poculata. *Coral Reefs, 32*(2), 401-409. doi:10.1007/s00338-012-0983-z

Dougan, K. E., Ladd, M. C., Fuchs, C., Vega Thurber, R., Burkepile, D. E., & Rodriguez-Lanetty, M. (2020). Nutrient Pollution and Predation Differentially Affect Innate Immune Pathways in the Coral Porites porites. *Frontiers in Marine Science, 7*. doi:10.3389/fmars.2020.563865

Downs, C. A., Kramarsky-Winter, E., Martinez, J., Kushmaro, A., Woodley, C. M., Loya, Y., & Ostrander, G. K. (2009). Symbiophagy as a cellular mechanism for coral bleaching. *Autophagy, 5*(2), 211-216. doi:10.4161/auto.5.2.7405

E Weil, E. H.-D., M Gonzalez, S Williams, S SuleimánRamos, M Figuerola, T Metz-Estrella. (2019). Spread of the new coral disease "SCTLD" into the Caribbean: implications for Puerto Rico. *34*, 38-43.

Eddy, T. D., Lam, V. W. Y., Reygondeau, G., Cisneros-Montemayor, A. M., Greer, K., Palomares, M. L. D., . . . Cheung, W. W. L. (2021). Global decline in capacity of coral reefs to provide ecosystem services. *One Earth, 4*(9), 1278-1285. doi:10.1016/j.oneear.2021.08.016

Erftemeijer, P. L., Riegl, B., Hoeksema, B. W., & Todd, P. A. (2012). Environmental impacts of dredging and other sediment disturbances on corals: a review. *Mar Pollut Bull, 64*(9), 1737-1765. doi:10.1016/j.marpolbul.2012.05.008

Evans, R. D., Wilson, S. K., Fisher, R., Ryan, N. M., Babcock, R., Blakeway, D., . . . Thomson, D. P. (2020). Early recovery dynamics of turbid coral reefs after recurring bleaching events. *J Environ Manage, 268*, 110666. doi:10.1016/j.jenvman.2020.110666

Fuess, L. E., Butler, C. C., Brandt, M. E., & Mydlarz, L. D. (2020). Investigating the roles of transforming growth factor-beta in immune response of Orbicella faveolata, a scleractinian coral. *Dev Comp Immunol, 107*, 103639. doi:10.1016/j.dci.2020.103639

Fuess, L. E., Palacio-Castro, A. M., Butler, C. C., Baker, A. C., & Mydlarz, L. D. (2020). Increased Algal Symbiont Density Reduces Host Immunity in a Threatened Caribbean Coral Species, Orbicella faveolata. *Frontiers in Ecology and Evolution, 8*. doi:10.3389/fevo.2020.572942

Fuess, L. E., Pinzomicronn, C. J., Weil, E., & Mydlarz, L. D. (2016). Associations between transcriptional changes and protein phenotypes provide insights into immune regulation in corals. *Dev Comp Immunol, 62*, 17-28. doi:10.1016/j.dci.2016.04.017

Fuess, L. E., Pinzon, C. J., Weil, E., Grinshpon, R. D., & Mydlarz, L. D. (2017). Life or death: disease-tolerant coral species activate autophagy following immune challenge. *Proc Biol Sci, 284*(1856). doi:10.1098/rspb.2017.0771

Gavish, A. R., Shapiro, O. H., Kramarsky-Winter, E., & Vardi, A. (2021). Microscale tracking of coral-vibrio interactions. *ISME Communications, 1*(1). doi:10.1038/s43705-021-00016-0

Gibbin, E., Gavish, A., Krueger, T., Kramarsky-Winter, E., Shapiro, O., Guiet, R., . . . Meibom, A. (2019). Vibrio coralliilyticus infection triggers a behavioural response and perturbs nutritional exchange and tissue integrity in a symbiotic coral. *ISME J, 13*(4), 989-1003. doi:10.1038/s41396-018-0327-2

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., . . . Regev, A. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat Biotechnol, 29*(7), 644-652. doi:10.1038/nbt.1883

Harman, T. E., Barshis, D. J., Hauff Salas, B., Hamsher, S. E., & Strychar, K. B. (2022). Indications of symbiotic state influencing melanin-synthesis immune response in the facultative coral Astrangia poculata. *Dis Aquat Organ, 151*, 63-74. doi:10.3354/dao03695

Hughes, A. D., Grottoli, A. G., Pease, T. K., & Matsui, Y. (2010). Acquisition and assimilation of carbon in non-bleached and bleached corals. *MARINE ECOLOGY PROGRESS SERIES, 420*, 91-101. doi:10.3354/meps08866

Hughes, T. P., Baird, A. H., Bellwood, D. R., Card, M., Connolly, S. R., Folke, C., . . . Roughgarden, J. (2003). Climate change, human impacts, and the resilience of coral reefs. *Science, 301*(5635), 929-933. doi:10.1126/science.1085046

Knowlton, N., Brainard, R. E., Fisher, R., Moews, M., Plaisance, L., & Caley, M. J. (2010). Coral reef biodiversity. *Life in the world's oceans: diversity distribution and abundance*, 65-74.

Krueger, T., Hawkins, T. D., Becker, S., Pontasch, S., Dove, S., Hoegh-Guldberg, O., . . . Davy, S. K. (2015). Differential coral bleaching-Contrasting the activity and response of enzymatic antioxidants in symbiotic partners under thermal stress. *Comp Biochem Physiol A Mol Integr Physiol, 190*, 15-25. doi:10.1016/j.cbpa.2015.08.012

Kvennefors, E. C., Leggat, W., Kerr, C. C., Ainsworth, T. D., Hoegh-Guldberg, O., & Barnes, A. C. (2010). Analysis of evolutionarily conserved innate immune components in coral links immunity and symbiosis. *Dev Comp Immunol, 34*(11), 1219-1229. doi:10.1016/j.dci.2010.06.016

Landsberg, J. H., Kiryu, Y., Peters, E. C., Wilson, P. W., Perry, N., Waters, Y., . . . Work, T. M. (2020). Stony Coral Tissue Loss Disease in Florida Is Associated With Disruption of Host–Zooxanthellae Physiology. *Frontiers in Marine Science, 7*. doi:10.3389/fmars.2020.576013

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nat Methods, 9*(4), 357-359. doi:10.1038/nmeth.1923

Lesser, M. P. (2011). Coral Bleaching: Causes and Mechanisms. In Z. Dubinsky & N. Stambler (Eds.), *Coral Reefs: An Ecosystem in Transition* (pp. 405-419). Dordrecht: Springer Netherlands.

Levy, S., Elek, A., Grau-Bove, X., Menendez-Bravo, S., Iglesias, M., Tanay, A., . . . Sebe-Pedros, A. (2021). A stony coral cell atlas illuminates the molecular and cellular basis of coral symbiosis, calcification, and immunity. *Cell, 184*(11), 2973-2987 e2918. doi:10.1016/j.cell.2021.04.005

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol, 15*(12), 550. doi:10.1186/s13059-014-0550-8

Maiuri, M. C., Zalckvar, E., Kimchi, A., & Kroemer, G. (2007). Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nature Reviews Molecular Cell Biology, 8*(9), 741-752. doi:10.1038/nrm2239

Mansfield, K. M., Carter, N. M., Nguyen, L., Cleves, P. A., Alshanbayeva, A., Williams, L. M., . . . Gilmore, T. D. (2017). Transcription factor NF-kappaB is modulated by symbiotic status in a sea anemone model of cnidarian bleaching. *Sci Rep, 7*(1), 16025. doi:10.1038/s41598-017-16168-w

Mansfield, K. M., & Gilmore, T. D. (2019). Innate immunity and cnidarian-Symbiodiniaceae mutualism. *Dev Comp Immunol, 90*, 199-209. doi:10.1016/j.dci.2018.09.020

Marshall, P. A., & Baird, A. H. (2000). Bleaching of corals on the Great Barrier Reef: differential susceptibilities among taxa. *Coral Reefs, 19*(2), 155-163. doi:10.1007/s003380000086

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal, 17*(1). doi:10.14806/ej.17.1.200

Meesters, E. H., Pauchli, W., & Bak, R. P. M. (1997). Predicting regeneration of physical damage on a reef-building coral by regeneration capacity and lesion shape. *MARINE ECOLOGY PROGRESS SERIES, 146*(1-3), 91-99. doi:DOI 10.3354/meps146091

Merle, P. L., Sabourault, C., Richier, S., Allemand, D., & Furla, P. (2007). Catalase characterization and implication in bleaching of a symbiotic sea anemone. *Free Radic Biol Med, 42*(2), 236-246. doi:10.1016/j.freeradbiomed.2006.10.038

Merselis, D. G., Lirman, D., & Rodriguez-Lanetty, M. (2018). Symbiotic immuno-suppression: is disease susceptibility the price of bleaching resistance? *PeerJ, 6*, e4494. doi:10.7717/peerj.4494

Meyer, E., & Weis, V. M. (2012). Study of cnidarian-algal symbiosis in the "omics" age. *Biol Bull, 223*(1), 44-65. doi:10.1086/BBLv223n1p44

Miller, J., Muller, E., Rogers, C., Waara, R., Atkinson, A., Whelan, K. R. T., . . . Witcher, B. (2009). Coral disease following massive bleaching in 2005 causes 60% decline in coral cover on reefs in the US Virgin Islands. *Coral Reefs, 28*(4), 925-937. doi:10.1007/s00338-009-0531-7

Mullen, K. M., Peters, E. C., & Harvell, C. D. (2004). Coral Resistance to Disease. In E. Rosenberg & Y. Loya (Eds.), *Coral Health and Disease* (pp. 377-399). Berlin, Heidelberg: Springer Berlin Heidelberg.

Muller, E. B., Kooijman, S. A., Edmunds, P. J., Doyle, F. J., & Nisbet, R. M. (2009). Dynamic energy budgets in syntrophic symbiotic relationships between heterotrophic hosts and photoautotrophic symbionts. *J Theor Biol, 259*(1), 44-57. doi:10.1016/j.jtbi.2009.03.004

Mydlarz, L. D., Fuess, L., Mann, W., Pinzón, J. H., & Gochfeld, D. J. (2016). Cnidarian Immunity: From Genomes to Phenomes. In S. Goffredo & Z. Dubinsky (Eds.), *The Cnidaria, Past, Present and Future: The world of Medusa and her sisters* (pp. 441-466). Cham: Springer International Publishing.

Mydlarz, L. D., & Harvell, C. D. (2007). Peroxidase activity and inducibility in the sea fan coral exposed to a fungal pathogen. *Comp Biochem Physiol A Mol Integr Physiol, 146*(1), 54-62. doi:10.1016/j.cbpa.2006.09.005

Mydlarz, L. D., & Palmer, C. V. (2011). The presence of multiple phenoloxidases in Caribbean reef-building corals. *Comp Biochem Physiol A Mol Integr Physiol, 159*(4), 372-378. doi:10.1016/j.cbpa.2011.03.029

Neubauer, E. F., Poole, A. Z., Weis, V. M., & Davy, S. K. (2016). The scavenger receptor repertoire in six cnidarian species and its putative role in cnidarian-dinoflagellate symbiosis. *PeerJ, 4*, e2692. doi:10.7717/peerj.2692

Nielsen, D. A., Petrou, K., & Gates, R. D. (2018). Coral bleaching from a single cell perspective. *ISME J, 12*(6), 1558-1567. doi:10.1038/s41396-018-0080-6

Oakley, C. A., & Davy, S. K. (2018). Cell Biology of Coral Bleaching. In M. J. H. van Oppen & J. M. Lough (Eds.), *Coral Bleaching: Patterns, Processes, Causes and Consequences* (pp. 189-211). Cham: Springer International Publishing.

Palmer, C. V., Bythell, J. C., & Willis, B. L. (2010). Levels of immunity parameters underpin bleaching and disease susceptibility of reef corals. *FASEB J, 24*(6), 1935-1946. doi:10.1096/fj.09-152447

Palmer, C. V., Bythell, J. C., & Willis, B. L. (2011). A comparative study of phenoloxidase activity in diseased and bleached colonies of the coral Acropora millepora. *Dev Comp Immunol, 35*(10), 1098-1101. doi:10.1016/j.dci.2011.04.001

Palmer, C. V., & Traylor-Knowles, N. (2012). Towards an integrated network of coral immune mechanisms. *Proc Biol Sci, 279*(1745), 4106-4114. doi:10.1098/rspb.2012.1477

Peters, E. C., & Pilson, M. E. Q. (1985). A comparative study of the effects of sedimentation on symbiotic and asymbiotic colonies of the coral Astrangiadanae Milne Edwards and Haime 1849. *Journal of Experimental Marine Biology and Ecology, 92*(2-3), 215-230. doi:10.1016/0022-0981(85)90096-6

Pinzon, C. J., Beach-Letendre, J., Weil, E., & Mydlarz, L. D. (2014). Relationship between phylogeny and immunity suggests older Caribbean coral lineages are more resistant to disease. *PLoS One, 9*(8), e104787. doi:10.1371/journal.pone.0104787

Pratchett, M. S., Hoey, A. S., & Wilson, S. K. (2014). Reef degradation and the loss of critical ecosystem goods and services provided by coral reef fishes. *Current Opinion in Environmental Sustainability, 7*, 37-43. doi:10.1016/j.cosust.2013.11.022

Pupier, C. A., Fine, M., Bednarz, V. N., Rottier, C., Grover, R., & Ferrier-Pages, C. (2019). Productivity and carbon fluxes depend on species and symbiont density in soft coral symbioses. *Sci Rep, 9*(1), 17819. doi:10.1038/s41598-019-54209-8

Roesel, C. L., & Vollmer, S. V. (2019). Differential gene expression analysis of symbiotic and aposymbiotic Exaiptasia anemones under immune challenge with Vibrio coralliilyticus. *Ecol Evol, 9*(14), 8279-8293. doi:10.1002/ece3.5403

Rosset, S. L., Oakley, C. A., Ferrier-Pages, C., Suggett, D. J., Weis, V. M., & Davy, S. K. (2021). The Molecular Language of the Cnidarian-Dinoflagellate Symbiosis. *Trends Microbiol, 29*(4), 320-333. doi:10.1016/j.tim.2020.08.005

Rotjan, R. D., Sharp, K. H., Gauthier, A. E., Yelton, R., Lopez, E. M. B., Carilli, J., . . . Urban-Rich, J. (2019). Patterns, dynamics and consequences of microplastic ingestion by the temperate coral, Astrangia poculata. *Proc Biol Sci, 286*(1905), 20190726. doi:10.1098/rspb.2019.0726

Rublee, P. A., Lasker, H. R., Gottfried, M., & Roman, M. R. (1980). Production and bacterial colonization of mucus from the soft coral Briarium asbestinum. *Bulletin of Marine Science, 30*(4), 888-893.

Sadd, B. M., & Schmid-Hempel, P. (2009). Principles of ecological immunology. *Evol Appl, 2*(1), 113-121. doi:10.1111/j.1752-4571.2008.00057.x

Sharp, K. H., Pratte, Z. A., Kerwin, A. H., Rotjan, R. D., & Stewart, F. J. (2017). Season, but not symbiont state, drives microbiome structure in the temperate coral Astrangia poculata. *Microbiome, 5*(1), 120. doi:10.1186/s40168-017-0329-8

Shi, C. S., & Kehrl, J. H. (2008). MyD88 and Trif target Beclin 1 to trigger autophagy in macrophages. *J Biol Chem, 283*(48), 33175-33182. doi:10.1074/jbc.M804478200

Szabó, M., Larkum, A. W. D., & Vass, I. (2020). A Review: The Role of Reactive Oxygen Species in Mass Coral Bleaching. In A. W. D. Larkum, A. R. Grossman, & J. A. Raven (Eds.), *Photosynthesis in Algae: Biochemical and Physiological Mechanisms* (pp. 459-488). Cham: Springer International Publishing.

Tchernov, D., Kvitt, H., Haramaty, L., Bibby, T. S., Gorbunov, M. Y., Rosenfeld, H., & Falkowski, P. G. (2011). Apoptosis and the selective survival of host animals following thermal bleaching in zooxanthellate corals. *Proc Natl Acad Sci U S A, 108*(24), 9905-9909. doi:10.1073/pnas.1106924108

Tivey, T. R., Parkinson, J. E., Mandelare, P. E., Adpressa, D. A., Peng, W., Dong, X., . . . Loesgen, S. (2020). N-Linked Surface Glycan Biosynthesis, Composition, Inhibition, and Function in Cnidarian-Dinoflagellate Symbiosis. *Microb Ecol, 80*(1), 223-236. doi:10.1007/s00248-020-01487-9

Traylor-Knowles, N. (2021). Unlocking the single-cell mysteries of a reef-building coral. *Cell, 184*(11), 2802-2804. doi:10.1016/j.cell.2021.05.007

Ushijima, B., Meyer, J. L., Thompson, S., Pitts, K., Marusich, M. F., Tittl, J., . . . Paul, V. J. (2020). Disease Diagnostics and Potential Coinfections by Vibrio coralliilyticus During an Ongoing Coral Disease Outbreak in Florida. *Front Microbiol, 11*, 569354. doi:10.3389/fmicb.2020.569354

Vidal-Dupiol, J., Ladriere, O., Meistertzheim, A. L., Foure, L., Adjeroud, M., & Mitta, G. (2011). Physiological responses of the scleractinian coral Pocillopora damicornis to bacterial stress from Vibrio coralliilyticus. *J Exp Biol, 214*(Pt 9), 1533-1545. doi:10.1242/jeb.053165

Wall, C. B., Ricci, C. A., Foulds, G. E., Mydlarz, L. D., Gates, R. D., & Putnam, H. M. (2018). The effects of environmental history and thermal stress on coral physiology and immunity. *Marine Biology, 165*(3), 56. doi:10.1007/s00227-018-3317-z

Weil, E. (2004). Coral reef diseases in the wider Caribbean. *Coral Health and Disease*, 35-68. doi:10.1007/978-3-662-06414-6_2

Weis, V., Reynolds, W., deBoer, M., & Krupp, D. (2001). Host-symbiont specificity during onset of symbiosis between the dinoflagellates Symbiodinium spp. and planula larvae of the scleractinian coral Fungia scutaria. *Coral Reefs, 20*(3), 301-308. doi:10.1007/s003380100179

Weis, V. M. (2019). Cell Biology of Coral Symbiosis: Foundational Study Can Inform Solutions to the Coral Reef Crisis. *Integr Comp Biol, 59*(4), 845-855. doi:10.1093/icb/icz067

Weis, V. M., Davy, S. K., Hoegh-Guldberg, O., Rodriguez-Lanetty, M., & Pringle, J. R. (2008). Cell biology in model systems as the key to understanding corals. *Trends Ecol Evol, 23*(7), 369-376. doi:10.1016/j.tree.2008.03.004

Wuitchik, D. M., Almanzar, A., Benson, B. E., Brennan, S., Chavez, J. D., Liesegang, M. B., . . . Davies, S. W. (2021). Characterizing environmental stress responses of aposymbiotic Astrangia poculata to divergent thermal challenges. *Mol Ecol, 30*(20), 5064-5079. doi:10.1111/mec.16108

Ye, J., McGinnis, S., & Madden, T. L. (2006). BLAST: improvements for better sequence analysis. *Nucleic Acids Res, 34*(Web Server issue), W6-9. doi:10.1093/nar/gkl164

Yuyama, I., Ishikawa, M., Nozawa, M., Yoshida, M. A., & Ikeo, K. (2018). Transcriptomic changes with increasing algal symbiont reveal the detailed process underlying establishment of coral-algal symbiosis. *Sci Rep, 8*(1), 16802. doi:10.1038/s41598-018-34575-5

Zhou, F., Yang, Y., & Xing, D. (2011). Bcl-2 and Bcl-xL play important roles in the crosstalk between autophagy and apoptosis. *FEBS J, 278*(3), 403-413. doi:10.1111/j.1742-4658.2010.07965.x

Zhou, Z., Zhao, S., Tang, J., Liu, Z., Wu, Y., Wang, Y., & Lin, S. (2019). Altered Immune Landscape and Disrupted Coral-Symbiodinium Symbiosis in the Scleractinian Coral Pocillopora damicornis by Vibrio coralliilyticus Challenge. *Front Physiol, 10*, 366. doi:10.3389/fphys.2019.00366