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THE INFLUENCE OF INTRACELLULAR PHOTOSYMBIOSIS ON THE EVOLUTION AND FUNCTION OF CNIDARIAN IMMUNITY

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by

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DISSERTATION

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Abstract

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Intracellular photosymbiosis with Symbiodiniaceae is facilitated by cnidarian immunity as it is required for symbiont recognition and is subsequently suppressed to maintain stable intracellular symbiosis. To date, it is unclear how the cnidarian-Symbiodiniaceae symbiosis influences immune gene repertoires, immune gene expression, and disease pathology across its independent evolutions. To address these knowledge gaps, my dissertation approaches studying the cnidarian-Symbiodiniaceae symbiosis through a comparative lens to determine how it shapes immune gene evolution and immune responses in divergent symbiotic cnidarian species. In chapter two I survey eight non-symbiotic and seven symbiotic cnidarians proteomes for four families of pattern recognition receptors (PRRs) and find expanded PRR repertoires, a proxy for immune specificity, in symbiotic cnidarians. In chapter three I investigate the influence of photosymbiosis on the disease response of the facultatively symbiotic *Cassiopea xamachana* through survival assays, acidic organelle activity, and gene expression data to find evidence of a trade-off between photosymbiosis and immunity. In chapter four I compare gene expression differences occurring between symbiotic and aposymbiotic animals in facultatively symbiotic cnidarians across two independent evolutions of the cnidarian-Symbiodiniaceae symbiosis and find evidence of large-scale differences in the strategies each species utilizes while hosting symbionts that likely influence how the species respond to anthropogenic stressors. Together, this work approaches studying the cnidarian-Symbiodiniaceae symbiosis through a comparative lens to broaden the field's understanding of the foundational ways in which hosting Symbiodiniaceae intracellularly impacts cnidarians' ability to respond to anthropogenic stressors.

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Chapter 1: Introductory Material

Cnidaria is a basal animal phylum sister to Bilateria. The majority of cnidarians fall within two clades: Anthozoa and Medusozoa (1). These clades are defined by phylogeny and shared life history traits. Anthozoa, consisting of the classes Hexacorallia and Octocorallia, is characterized by the absence of a medusa life stage and a polyp life stage capable of sexual reproduction (2). In contrast medusozoans, consisting of Hydrozoa, Schyphozoa, Cubozoa, and Staurozoa, most often sexually reproduce in a planktonic medusa life stage (3). All cnidarians regardless of clade have simplistic anatomy consisting of two body layers separated by a collagenous matrix known as the mesoglea (4). Despite this simplistic anatomy and their basal position in the metazoan phylogeny, cnidarians have a complex suite of innate immune genes unexpectedly similar to chordates (5,6). As such, studies of cnidarian immunity can inform our understanding of innate immune evolution (5,7). Furthermore, studies of cnidarian immunity are a vital towards understanding the mechanisms and trade-offs of their mutualistic association with photosynthetic dinoflagellates in the family Symbiodiniaceae (8).

Cnidarian Immunity

Cnidarian genomes contain a diverse set of pattern recognition receptors (PRRs) capable of recognizing danger associated molecular patterns (DAMPs) and microbe associated molecular patterns (MAMPs) (5,7,9–11). There are various types of PRRs in cnidarians including Toll-like receptors (TLRs), retinoic acid-inducible gene-I (RIG)-like receptors (RLRs), nuclear oligomerization domain (NOD)-like receptors (NLRs), C-type lectins (CTLs), and scavenger receptors (9,11–14). Briefly, TLRs recognize bacterial cell wall components, viral RNA, and developmental cues, RLRs recognize viral RNA, NLRs recognize a variety of cellular DAMPs,

bacterial cell wall components, and viral RNA, CTLs bind a wide variety of ligands including carbohydrates, proteins, lipids, and inorganic compounds, and scavenger receptors similarly bind a diverse set of ligands including MAMPs and apoptotic cell debris (15–20). Canonical TLRs, RLRs, NLRs, CTLs, and scavenger receptors are all present in anthozoan species (9,11–14). While medusozoans contain a pre-cursor to canonical TLRs as well as NLRs, CTLs, and scavenger receptors (6,21,22). There are a limited number of functional studies characterizing the functions of cnidarian PRRs, with studies on TLRs, RLRs, and CTLs (13,23–25).

The vast majority of functional PRR studies in cnidarians have focused on TLRs. Within Cnidaria there are two types of protein systems capable of functioning as TLRs (7). The first is a system that likely represents the pre-cursor to canonical TLRs, where a transmembrane protein containing an intracellular TIR domain interacts with a separate transmembrane protein with extracellular leucine rich repeats to recognize MAMPs like lipopolysaccharide and activate downstream immune signaling (21). Interestingly, expanded suites of these pre-cursor TLRs have been identified in reef-building corals (26). The second is a canonical TLR, which likely first evolved within Cnidaria (7). These TLRs have been confirmed to play a role in development, MAMP recognition, and activation of proinflammatory immune transcription factors (13).

Functional characterization of cnidarian RLRs is limited to one study, which found the *Nematostella vectensis* RLR capable of binding polyinosinic:polycytidylic acid (poly(I:C)) and long dsRNA. Knockdown of this protein impaired *N, vectensis's* antiviral response, resulting in reduced ability to upregulate RNA interference following exposure of poly(I:C) (Lewandowska et al. 2021). While no functional studies of cnidarian NLRs have been conducted to date, they

have been characterized bioinformatically with extensive expansions in NLRs and unique domain combinations not seen in other animal taxa being reported (10,14,27).

Functional studies of CTLs and scavenger receptors in cnidarians focus on their potential role in recognizing algal symbionts. Multiple studies have found CTLs to be capable of opsonizing symbionts, which is hypothesized to facilitate the establishment of symbiont populations in the cnidarian host (23,25,28). In addition to its symbiont binding capabilities, *Acropora millepora* mannose binding lectin is also capable of binding and agglutinating gram-positive and gram-negative bacteria (23). Similar to CTLs, scavenger receptors have been found to facilitate uptake of algal symbionts, as blocking the scavenger receptor binding site with an inhibitor resulted in reduced symbiont colonization of *Exaiptasia diaphana* (11).

Upon ligand recognition PRRs activate signaling cascades to initiate an organism's response to the recognized DAMP/MAMP. The best studied of these signaling pathways in cnidarians is nuclear factor kappa B (NFκB) activation and signaling (8,29–31). NFκB is an immune transcription factor whose activation can be initiated by various PRRs to elicit a proinflammatory and pro-survival response to immune stimuli (15,32,33). In cnidarians the TLR precursors and canonical TLRs have been shown to be capable of activating NFκB following exposure to LPS (13,21). NFκB signaling has been shown to play a role in various cnidarian stress responses, including heat stress and immune stress (31,34–36).

Cnidarian genomes also contain interferon regulatory factors (IRFs), another immune transcription factor that can act as a co-factor with NFκB to activate an inflammatory response (37,38). IRFs are best known for their namesake induction of the proinflammatory interferon cytokines (39). However, interferons are absent in cnidarians as they did not evolve until the emergence of jawed vertebrates (40). It is unclear the full range of functions these transcription

factors have in cnidarian immunity, as to date there are no functional studies of cnidarian IRFs. They may induce other proinflammatory cytokines or play a role in cell cycle regulation through promoting apoptosis (41,42). Despite the lack of functional investigations into cnidarian IRFs, they have been found to have upregulated expression following a diverse range of stressors including regeneration, bacterial pathogen exposure, and poly(I:C) exposure (24,43,44).

In addition to NFκB and IRF signaling, cnidarians have a suite of more generalized stress response pathways not specific to immunity, including endoplasmic reticulum and mitochondrial unfolded protein responses, activator protien-1 (AP-1) signaling, and forkhead box O (FOXO) signaling (45–48). Together, cnidarian stress response signaling pathways are capable of inducing a wide variety of effector responses. Antimicrobial peptides have been identified in cnidarians as well as the bactericidal protein macrophage expressed gene1/perforin-2 (Mpeg-1/P2) (49–51). While cnidarians lack interferons, they do have other cytokines such as interleukins as well as tumor necrosis factors that can propagate inflammation (52,53). Reactive oxygen species (ROS), nitric oxide production, and autophagy are also often utilized in cnidarian stress responses (34,54,55). Finally, cnidarians are capable of initiating various types of cell death in response to stress, including apoptosis, pyroptosis, and ETosis (56–58).

Cnidarian-Symbiodiniaceae photosymbiosis

The complexity of cnidarian immune systems likely facilitates life history traits such as photosymbiosis with dinoflagellates in the family Symbiodiniaceae. These intracellular mutualisms are very common throughout the phylum with independent evolutions in the classes Hydrozoa, Scyphozoa, Octocorallia, and Hexacorallia (1,59). There are variations within these associations within and between independent evolutions (60–63). This symbiosis is facultative from the perspective of the symbionts, as Symbiodiniaceae can be free living within the water

column or housed within a variety of animal hosts (64–66). In contrast, cnidarian hosts can form facultative, obligate, or a life stage specific mix of facultative and obligate associations with Symbiodiniaceae (67–69). Despite variations in reliance on their symbionts for nutrition and the cell type the symbionts are housed in, cnidarian photosymbiosis with Symbiodiniaceae is broadly characterized by the algal symbionts providing their hosts with the bulk of photosynthates they produce in exchange for nutrients such as nitrogen and the protection of being housed within the hosts (60,61).

In many cases, cnidarian hosts form very specific associations with a single or limited number of symbiont genera (70–73). These preferred Symbiodiniaceae taxa are known as homologous symbionts. The recognition and discernment between homologous and heterologous symbionts likely occur at multiple stages throughout the establishment of intracellular symbiosis. There is growing evidence for both extracellular and intracellular recognition of homologous symbionts by their cnidarian hosts (73,74). As such, many different pattern recognition receptors have been implicated in mediating the uptake of Symbiodiniaceae, including TLRs, CTLs, and scavenger receptors (8,23,25,74). However, currently there is the most support for the role of CTLs in symbiont uptake, as they have been shown to opsonize symbionts across two independent evolutions of the symbiosis (23,25). The transmembrane receptor and intracellular recognition mechanisms are not yet fully described and may vary across the various evolutions of the symbiosis.

Following extracellular recognition, Symbiodiniaceae are phagocytized. If the phagocytized symbiont is incompatible with the cnidarian host it is expelled via vomocytosis (74). However, if a phagocytized symbiont is compatible with the cnidarian host, it will be retained to establish an intracellular niche in a specialized acidic organelle known as the

symbiosome (60,69,74). During the establishment of this intracellular niche the host immune system is suppressed (8,74–76). There are multiple lines of evidence implicating NFκB as a key player in this immune suppression, which persists in symbiont-hosting cells during the maintenance of stable symbiotic associations (30,62,74,75,77,78). Rather than control their intracellular symbiont populations via their immune systems, cnidarians limit their symbionts access to nitrogen (77–82).

Given that cnidarians appear to suppress their immune systems while hosting Symbiodiniaceae it is important to consider this life history trait when investigating cnidarian immune stress responses as the nutrition these animals gain from their symbionts may come at the cost of greater susceptibility to disease (8,62). It is difficult to disentangle the potential trade-offs between symbiosis and immunity in cnidarians that are obligately symbiotic, like reef building corals. Instead, facultatively symbiotic cnidarians can be used to identify how hosting symbionts influences immunity. There are several proposed cnidarian models for studying the Symbiodiniaceae photosymbiosis, the most established of which is the anemone *Exaiptasia diaphana* (69). Recently, there has been growing investment towards developing the upside-down jellyfish, *Cassiopea xamachana*, as a model for studying the cnidarian-Symbiodiniaceae symbiosis through a comparative lens, as its symbiosis is independently evolved from reef building corals and *E. diaphana* (1,68).

Symbiotic cnidarians and anthropogenic change

The trade-offs between symbiosis and immunity in cnidarians are especially pertinent to understand due to the foundational role the cnidarian-Symbiodiniaceae symbiosis plays in coral reef ecosystems. Coral reefs are extremely biodiverse ecosystems that are found in oligotrophic waters. Stony corals serve as the ecosystem engineers of coral reefs, with the three-dimensional

structures their calcium carbonate skeletons create providing a safe habitat for a vast array of marine life (67,83). Biogenesis of these calcium carbonate skeletons is energetically expensive and requires acquisition of photosynthates from Symbiodiniaceae (67). Without the nutrition provided by algal symbionts the coral host is unable to survive for long in its oligotrophic environment (60,84). In recent years coral reef health has been rapidly declining due to disease outbreaks and thermally induced mass bleaching events. Due to anthropogenic forces, these events are increasing in frequency and severity and represent an existential threat to coral reefs (85–87). The potential trade-off between symbiosis and immunity in cnidarians could impact their ability to persist in the face of anthropogenic change. In particular it has widespread implications for the pathology of coral disease, especially ones with complex pathologies impacting both host and symbiont (88,89). To date, it is unclear how the cnidarian-Symbiodiniaceae symbiosis influences immune gene repertoires, immune expression, and disease pathology across its independent evolutions.

To address these knowledge gaps, I utilized a variety of bioinformatic approaches to characterize how the cnidarian-Symbiodiniaceae symbiosis shapes immune gene evolution and cnidarian immune responses. In chapter two, I investigate the PRR repertoires of eight non-symbiotic and seven symbiotic cnidarians and find that symbiosis is associated with expanded PRR repertoires. In chapter three, I leverage the facultatively symbiotic cnidarian, *C. xamachana*, to layer survival assays, physiological assays, and gene expression data from symbiotic and aposymbiotic animals responding to a bacterial pathogen to find evidence of a trade-off between symbiosis and immunity through the mounting of more damaging immune responses in symbiotic animals. In chapter four I compare gene expression differences occurring between symbiotic and aposymbiotic animals of two facultatively symbiotic cnidarian species

spanning two independent evolutions of the cnidarian-Symbiodiniaceae symbiosis to reveal shared and host-specific symbiosis characteristics that may influence each species' ability to respond competently to stress. Together these chapters indicate that the cnidarian-Symbiodiniaceae symbiosis shapes both immune evolution and function across its independent evolutions. By studying this symbiosis and its influence on cnidarian immunity through a comparative lens this works contributes to our understanding of the foundational ways in which hosting intracellular Symbiodiniaceae has impacted cnidarians and their ability to respond to immune stressors.

Chapter 2: Cnidarian pattern recognition receptor repertoires reflect both phylogeny and life history traits

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Abstract

Pattern recognition receptors (PRRs) are evolutionarily ancient and crucial components of innate immunity, recognizing danger-associated molecular patterns (DAMPs) and activating host defenses. Basal non-bilaterian animals such as cnidarians must rely solely on innate immunity to defend themselves from pathogens. By investigating cnidarian PRR repertoires we can gain insight into the evolution of innate immunity in these basal animals. Here we utilize the increasing amount of available genomic resources within Cnidaria to survey the PRR repertoires and downstream immune pathway completeness within 15 cnidarian species spanning two major cnidarian clades, Anthozoa and Medusozoa. Overall, we find that anthozoans possess prototypical PRRs, while medusozoans appear to lack these immune proteins. Additionally, anthozoans consistently had higher numbers of PRRs across all four classes relative to medusozoans, a trend largely driven by expansions in NOD-like receptors and C-type lectins. Symbiotic, sessile, and colonial cnidarians also have expanded PRR repertoires relative to their non-symbiotic, mobile, and solitary counterparts. Interestingly, cnidarians seem to lack key components of mammalian innate immune pathways, though similar to PRR numbers, anthozoans possess more complete immune pathways than medusozoans. Together, our data indicate that anthozoans have greater immune specificity than medusozoans, which we hypothesize to be due to life history traits common within Anthozoa. Overall, this investigation reveals important insights into the evolution of innate immune proteins within these basal animals.

Introduction

Animals sense and interact with microbes through their immune system (90). The majority of knowledge about immune system function stems from studies in vertebrates, which interact with microbes through both an innate and an adaptive immune system (91,92). In contrast, invertebrates rely on innate immunity to detect and respond to microbes. Pattern recognition receptors (PRRs) are key components of innate immunity that detect both danger associated molecular patterns (DAMPs) and microbe associated molecular patterns (MAMPs), activating downstream signaling pathways following this recognition (93). PRRs are drivers of immune specificity in invertebrates, as diverse repertoires of receptors are needed in order to generate a microbe specific immune response (94).

The four best studied families of PRRs are Toll-like receptors (TLRs), retinoic acid-inducible gene I-like receptors (RLRs), nucleotide-binding oligomerization domain-like receptors (NLRs), and C-type lectins (CTLs) (95,96). While it was originally thought that many of these PRR families first arose in vertebrates, studies following the advent of next generation sequencing revealed that TLRs, RLRs, NLRs, and CTLs are present in basal metazoans and thus are evolutionarily ancient (7,9,10,23,97). Studying PRRs in basal taxa informs our understanding of PRR evolution and more broadly immune evolution.

Cnidaria is a basal phylum sister to Bilateria with over 10,000 species spanning three major clades: Anthozoa, Medusozoa, and Endocnidozoa which span a diverse array of life history strategies ranging from sedentary to planktonic to parasitic (1). Despite their primitive morphology, cnidarian genomes are complex and contain a repertoire of innate immune genes unexpectedly similar to mammals (6,98). Due to this, cnidarians are exceptional candidates for investigations into innate immune evolution as they are not only basal, but also have immune

repertoires are not reduced like the nematode model *Caenorhabditis elegans* or derived in function like the arthropod model *Drosophila melanogaster* (98–100). Additionally, common life history traits within Cnidaria have been linked to immunity. Several cnidarian taxa in Anthozoa and Medusozoa form intracellular nutritional symbiosis with algae in the family Symbiodiniaceae, the maintenance of which has been linked to several different PRRs as well as NFkB signaling (8). Similarly, many cnidarians have colonial body plans, and likely have a larger need for allorecognition and thus immune specificity (1,101,102). Therefore, the wide variety of life history traits found within cnidaria has the potential to create a gradient of selective pressures for immune specificity, which likely is reflected in their PRR repertoires.

Toll-like receptors (TLRs) are transmembrane PRRs that are capable of recognizing a wide variety of ligands including bacterial cell wall components, viral RNA, and developmental cues (15,103,104). Canonically, TLRs consist of extracellular leucine rich repeats (LRRs) which bind to DAMPs and MAMPs and an intracellular Toll/interleukin-1 receptor (TIR) domain that activates signal transduction through protein-protein interactions with other TIR domain-containing proteins (15,103,105). Several pathways can be activated by TLRs following ligand engagement, including the MAPK, IFN, and NFkB pathways (15,105). Within Cnidaria, prototypical TLRs have been identified in several anthozoan species, and functional studies indicate that the *Nematostella vectensis* TLR functions in pathogen recognition, activation of NFkB signaling, and development (13,26). Additionally, a medusozoan species lacking prototypical TLRs, *Hydra vulgaris*, is still capable of TLR-NFkB signaling through TLR-like proteins containing a transmembrane domain and an intracellular TIR domain that appear to interact with transmembrane proteins with extracellular leucine rich repeats to perform the function of the prototypical TLR (21).

Retinoic acid-inducible gene I-like receptors (RLRs) are cytosolic PRRs that detect intracellular viral RNA (19,106,107). Mammals have three RLRs: RIG-I, MDA5, and LGP2. All RLRs have a central ATPase containing DExD/H box helicase domain and a C-terminal regulatory domain (19,106). RIG-I and MDA5 also contain CARD domains which in mammals interact with the CARD domain of signaling adaptor MAVS to initiate downstream signaling, activating transcription factors IRF3 and NFkB and ultimately resulting in an antiviral response (32,108). Additionally, RIG-I and LGP2 have a repressor domain (RD) within the C-terminal regulatory domain (19,106). As LGP2 lacks CARD domains, it is unable to initiate antiviral signaling and instead likely acts as a concentration dependent biphasic switch in mammals, positively regulating MDA5 at low concentrations and negatively regulating RIG-I and MDA5 at high concentrations (109–111). Some RLRs with sequences most similar to RIG-I have been identified in the *N. vectensis* genome (9).

Nucleotide-binding oligomerization domain-like receptors (NLRs) are intracellular PRRs capable of recognizing a wide array of DAMPs and MAMPs including reactive oxygen species (ROS) (18), organelle calcium efflux (112), Lipopolysaccharide (113), peptidoglycan (17), and viral RNA (114). Prototypically NLRs contain an N terminal effector domain, a central NACHT/nucleotide binding domain, and C terminal leucine rich repeats. NLRs can activate several innate immune pathways following ligand engagement, including the NFkB, MAPK, interferon and inflammasome assembly pathways (17,18,115). *H. vulgaris* and two stony coral species have been shown to have large NLR repertoires, containing unique domain combinations that are not seen in mammalian NLRs (10,14,27).

C-type lectins (CTLs) are a very diverse protein family that can act as either soluble or transmembrane PRRs (16,94). They are characterized by the C-type lectin domain (CTLD)

which is most well-known for calcium dependent carbohydrate binding but is also capable of binding to proteins, lipids, and inorganic compounds (16). CTLs can activate NFkB as well as the lectin complement pathway (116,117). A bioinformatic study of *N. vectensis* CTLs also found a large repertoire that could not be categorized by the mammalian CTL classification system (12).

Our study aims to build upon the current base of knowledge on cnidarian PRRs by expanding to investigate four PRR types in a phylogenetically diverse group of cnidarians. Previous studies are heavily concentrated in two classes, the anthozoan class Hexacorillia (stony corals and anemones), and two model systems within the medusozoan class Hydrozoa: *H. vulgaris* and *Hydractinia symbiolongicarpus* (7,9,12,14,21,22,26,98). To date, PRRs have not been investigated in cnidarians with a free-swimming adult medusae form, meaning that current studies also lack diversity in terms of life history strategies. Furthermore, only one anemone species, *N. vectensis*, has studies of all four PRR types (9,12,13,27). Thus, we lack knowledge of the number and structure of PRRs in the remaining classes of the phylum and the full PRR repertoires of even the well-studied species.

Within the past couple of years, a wealth of cnidarian genomic resources has become available, particularly in the medusozoan clade, making it possible to investigate PRRs in a far more diverse set of cnidarian species (nine anthozoans and six medusozoans), with strong potential to provide a more detailed picture of PRR and innate immune evolution (118–122). Therefore, we surveyed the proteomes of 15 cnidarians, nine anthozoans and six medusozoans, for putative TLRs, RLRs, NLRs, and CTLs with the hypothesis that medusozoans would have less diverse PRR repertoires. Next, because TLRs, RLRs, NLRs, and CTLs are all capable of activating NFkB signaling in mammals, we investigated the PRR to NFkB pathways in all 15

cnidarian species, as well as the lectin complement pathway to determine if there is a disparity in downstream immune pathway completeness between the anthozoan and medusozoan clades.

Materials and Methods

PRR survey

The proteomes of *Acropora millepora* (123), *Actinia tenebrosa* (122), *Aurelia sp*. (119), *Calavadosia cruxmelitensis* (120), *Cassiopea xamachana* (124), *Clytia hemisphaerica* (118), *Dendronephyta gigantea* (125), *Exaiptasia daiphana* (previously *Exaiptaisa pallida*) (126), *Hydra vulgaris* (6), *Montipora capitata* (127), *Morbakka virulenta* (128), *Nematostella vectensis* (129), *Orbicella faveolata* (130), *Pocillopora damicornis* (131), *Xenia sp*. (121), and sponge *Amphimedon queenslandica* (132) were surveyed for TLRs, RLRs, NLRs, and CTLs using HMMR (133). A summary the clade, class, life history traits, genome assembly size, and predicted proteins for each species can be found in supplementary table 1. All proteomes used were genome based with the exception of *N. vectensis*. This was due to a failure to find the complete prototypical TLR in the genome-based proteome of *N. vectensis*, despite several previous studies reporting its presence in the genome (13). The complete prototypical TLR was also absent in smallest transcriptome shotgun assembly available (NCBI GenBank: HADO000000000.1), so the second smallest transcriptome shotgun assembly (NCBI GenBank:HADN000000000.1) was filtered and used (129). Transdecoder was used to extract the longest open reading frame of each contig and translate it into a predicted peptide sequence (134). Then CD-HIT was used to collapse sequences with a similarity level of 0.85 to limit the number of splice isoforms in the assembly (135). This resulted in a proteome with 42,379 contigs, 14,000 higher than the average number of predicted proteins in the genome-based proteomes used in this study (supplementary table 1) (136). However, PRR numbers are similar

across the genome based proteome of *N. vectensis*, NCBI GenBank: HADO000000000.1, and GenBank:HADN000000000.1 (supplementary table 2) so excess contigs have minimal impact on results.

Queries were made by using Clustal Omega to make an alignment of all human TLRs, RLRs, NLRs, and CTLs respectively (137,138). All sequences that had an E-value less than $10^{-4.9}$ following the HMMR search were then run through pfam to predict protein domains using the batch search tool (139). Non-repeat domains with an individual E-value of less than $10^{-4.9}$ were counted and repeat domains were counted if they had an individual E-value less than 10^{-3}. TMHMM was used to predict transmembrane domains in TLRs and CTLs (140).

TLRs were classified as prototypical if the predicted protein had both a TIR domain, LRR, and transmembrane domain that met our inclusion threshold. Proteins with a TIR domain and a transmembrane domain were classified as TLR-like proteins. Proteins with a DExD/H box helicase, CARD, and C terminal RIG regulatory domain were classified as RIG-I/MDA5-like receptors. Proteins were classified as LGP2-like receptors if they had a DExD/H box helicase, and RIG-I repressor domain (Figure 1). In several proteins the DExD/H box helicase domains had E-values meeting our inclusion threshold but were not considered as a member of the pfam domain clan following post processing. However, given that Cnidaria is a phylogenetically distant and basal phyla, these domains were counted given the context of the surrounding domains which always included the RIG-I repressor domain. NLRs were classified as prototypical if the predicted protein contained at a minimum both the NACHT domain and LRRs and as NLR-like if they contained at a minimum the NACHT domain. CTLs were divided into 3 groups. Proteins with the CTLD and a transmembrane domain, proteins with CLTD and a pfam domain indicative of extracellular localization, and proteins with the CTLD and no additional

domains indicative of where they may be localized (Figure 1). Pfam domains considered to be indicative of extracellular localization included cysteine rich secretory domain (141), CUB (142), F5/8 (143), ShK (144), Von Willebrand factors (145), thrombospondin (146), trefoil (147), Fibrinogen □/□ terminal globular domain (148), NIDO (149), PKD (150), coagulation factor Xa inhibitory domain (151), U-PAR/Ly6 (152), complement Clr like EGF (153), and Xlink (154).

The total number of TLRs, RLRs, NLRs and CTLs in each species were then used as input for ancestral state reconstructions. The phylogenetic tree used for this analysis was created using Orthofinder (155). Ancestral state reconstructions based on maximum likelihood (ML) were made using the fastAnc function in the R package phytools. Phytools was also used to visualize the ML ancestral state reconstructions (156).

Relationship between PRR repertoires and clade and life history traits

Principal component analysis was run in R and visualized with the R package ggfortify (157). The input of the PCA analysis was the total number of PRRs in each PRR type per species. To test for associations between total PRR number and clade, intracellular algal symbiosis, coloniality, and mobility, generalized linear models were run in R. Because there is considerable overlap between anthozoan species, symbiotic species, colonial species, and sedentary species, five individual models were tested (total PRRs~trait, family = quasipoisson) (Supplementary table 1).

Downstream immune pathway completeness

NFκB

BLASTp was used to identify NFκB and IKBA in all species included in the study (158). Human sequences of p100, p105, and IKBA were used as queries (138). The top 5 best hits to each query were then run through pfam to predict domains using the same inclusion thresholds as the PRRs. Because the *N. vectensis* NF□B is known to be truncated only the Rel DNA binding domain and Rel dimerization domain were required for a cnidarian protein to be considered NF□B (29). Multiple ankyrin repeats were required for a cnidarian protein to be considered IKBA.

PRR to NF□B pathways

Cnidarian proteomes were searched for members of the PRR to NF□B KEGG pathways using BLASTp and a human query (138,158,159). The cnidarian protein with the highest E-value to each query was then blasted against the human proteome (GCA_000001405) (138). If the human query and best human hit following reciprocal blast were the same protein, the protein was counted as present in the cnidarian.

Lectin complement pathways

Because complement consists 5 mosaic protein families that likely did not expand until teleosts, we used pBLAST to search for representatives of the C3, Factor B/C2, MASP, and C6 families (158,160). Human sequences for all members of a given family were used as queries (138). Each protein family has a unique domain composition so the top 5 best hits to each query were run through pfam using the batch search option to predict domains (139). Proteins were considered members of the C3 family if they contained multiple macroglobulin domains, CUB, and C345c. Proteins were considered members of the Factor B/C2 family if they contained sushi repeats, von Willebrand factors, and a serine protease. Proteins were considered members of the MASP family if they contained 2 CUB domains, sushi repeats, and serine protease. Proteins

were considered members of the C6 family if they contained TSP, low density lipoprotein receptor domain class A, MACPF, and sushi repeats (160).

Results

PRR survey

The number of prototypical TLRs and TLR-like proteins vary across the species surveyed from 0 to 24. No prototypical TLRs were found in any of the six medusozoan species and in two anthozoans, *E. daiphana* and *Xenia sp*. (Figure 2). In contrast, two anthozoans have expansions in prototypical TLRs, *D. gigantea* and *A. millepora*, with four prototypical TLRs found in *D. gigantea* and ten in *A. millepora*. TLR-like proteins with a TIR domain and a transmembrane domain were found in all species except for *C. hemisphaerica* and *M. virulenta* (Figure 2, supplementary Table 3). Based upon ancestral state reconstruction, one TLR was present in the common ancestor shared by medusozoans and anthozoans (figure 3A). Relative to this ancestral cnidarian and other anthozoans, stony corals (*A. millepora, M. capitata, P. damicornis, O. faveolata*) have expansions in TLRs, specifically TLR-like proteins (Figure 2, Figure 3A).

RIG-I/MDA5-like receptors were found in the proteomes of all nine anthozoan species (Figure 2). These proteins all contained both the C terminal regulatory domain and the RIG-I C terminal repressor domain (supplementary Table 4). Two species of anemone, *A. tenebrosa* and *E. daiphana*, have expansions in RLRs relative to both other anthozoans and the common ancestor shared by medusozoans and anthozoans (Figure 3B). These expansions are largely due to the high number of RIG-I/MDA5-like receptors in these species (Figure 2). *A. queenslandica,* the sponge outgroup*, Xenia sp., A. tenebrosa, A. millepora,* and *M. capitata* all contained proteins with domains characteristic of LGP2 (Figure 2). In *A. millepora* and *M. capitata*, these

proteins had an RNA dependent RNA polymerase domain in addition to the canonical LGP2 domain organization (supplementary Table 4). No RLRs were found in any of the six medusozoan species (Figure 2).

Diverse arrays of NACHT containing proteins were found in the majority of cnidarian species surveyed. Prototypical NLRs containing NACHT and LRR domains were present in the sponge *A. queenslandica* and all nine anthozoan species but absent in all medusozoan species (Figure 2). No NACHT containing proteins were found in two medusozoan species, the Staurozoan *C. cruxmelitensis* and the Cubozoan *M. virulenta* (Figure 2). The majority of anthozoan species were found to have expanded NLR repertoires relative to medusozoans. However, only a smaller subset of anthozoan NLR repertoires are expanded relative to the common ancestor shared by medusozoans and anthozoans (Figure 3C).

Anthozoan NLR repertoires are not only generally larger (Figure 3C), but also more diverse in terms of domain composition (supplementary Table 5). Excluding LRRs 35 different Pfam domains were found in combination with NACHT across the 15 cnidarian species. Several of these domains are associated with immunity, including Dzip3/hRUL138-like HEPN nuclease (161), caspase recruitment domain (CARD) (162), death domain (DD) (163), ZU5 (164), glycosyl transferase (165), NB-ARC (166), WD-40 repeats (167), and Toll/interleukin receptor (TIR) domain (168). Excluding LRRs, the Dzip3/hRUL138- like HEPN domain was the most common domain found in combination with NACHT in *C. hemisphaerica, Xenia sp., D. gigantea, A. tenebrosa, E. daiphana, A. millepora, M. capitata, P. damicornis, and O. faveolata*. Anthozoans show large expansions of proteins with Dzip3/hRUL138- like HEPN domain fused to NACHT, which ranged from 17 proteins in *O. faveolata* to 83 in *D. gigantea*. TIR was the most common domain found with NACHT in *C. xamachana* and DD was most common domain

found with NACHT in *H. vulgaris*. Domains associated with transposable elements were found with NACHT in two species, *H. vulgaris* and *M. capitata*. A protein model with NACHT and the hAT family C terminal dimerization domain was found in *H. vulgaris* while *M. capitata's* proteome contains a protein with NACHT and an endonuclease reverse transcriptase domain and a protein with NACHT and integrase (supplementary Table 5).

All of the cnidarians in this study have expanded repertoires of CTLs relative to the sponge outgroup (Figure 2, Figure 3D). Two medusozoan species that are planktonic as adults, *M. virulenta* and *Aurelia sp*., had the fewest CTLs of the cnidarian species (Figure 2, Figure 3D). Additionally, anthozoans have expanded CTL repertoires relative to both medusozoans and the common ancestor shared by medusozoans and anthozoans (Figure 3D). The species with the most predicted CTLs were *A. tenebrosa* and *E. daiphana*, two closely related sea anemones (Figure 2, Figure 3D).

Across all 15 cnidarians, 70 different protein domains were found in combination with CTLD. The two clades differed in the domains most commonly found with CTLD. In six out of the nine anthozoan species the epidermal growth factor domain (EGF) was the most common domain found in conjunction with CTLD. The concanavalin A-like lectin domain was most commonly found with CTLD in four of the six medusozoans and two of the nine anthozoans. In the remaining medusozoans, *C. hemisphaerica* and *M. virulenta*, Von Willebrand factors and cysteine rich secretory domains respectively were most commonly found in conjunction with CTLD. Other common domains fused to CTLD were immunoglobulin, fibronectin, MAM, CUB, cysteine rich scavenger receptor, Kazal-type serine protease inhibitor, and PAN. Proteins with the domain organization of mannose binding lectin (MBL) (CTLD, collagen) were found in *C. xamachana* and *C. hemisphaerica.* As with the NLRs, the CTL search yielded surprising domain

combinations. Reverse transcriptase domains were found in combination with CTLD in four species, *M. virulenta*, *H. vulgaris, E. daiphana* and *M. capitata*, and integrase and CTLD were found in *M. capitata*. Additionally, *M. capitata* and *P. damicornis* had predicted proteins with the MAC/Perforin domain in addition to CTLD (supplementary Table 6).

Relationship to clade and life history traits

Our results indicate the presence of a divide between the two cnidarian clades in PRR number across all four PRR types. Principle component analysis resulted in the medusozoans and a single anthozoan, *N. vectensis*, grouping tightly across both PC1 and PC2. The anthozoans excluding *N. vectensis* group relatively tightly across PC1, driven largely by CTLs and NLRs, but show more variance across PC2. The separation of the anthozoans across PC2 is most likely due to the expansions in prototypical TLRs and TLR-like proteins found in stony corals and the expansion of RIG-I/MDA5-like proteins in *A. tenebrosa* and *E. daiphana* (Figure 4A). A generalized linear model found significant associations between the total PRR number and clade ($p = 0.0001$). Total PRR number was also associated with ability to host intracellular algal symbionts ($p=0.038$), colonial animals ($p = 0.033$), and sedentary animals ($p=0.014$) (Figure 4B-E).

Downstream immune pathway completeness

Given the differences in PRR numbers across the two cnidarian clades, we then investigated the completeness of the pathways downstream of TLRs, RLRs, NLRs, and CTLs that lead to master immune regulator NFκB (Figure 5). All species' proteomes contained at least one homolog of NFκB. Stony corals and sponge outgroup *A. queenslandica* contain NFκB

homologs most similar in domain composition to the mammalian NFκB queries (p100, p105), containing the Rel binding domain, Rel dimerization domain, Ankyrin repeats, and the death domain (Figure 5C). Anthozoans *E. daiphana* and *A. tenebrosa*, have seemingly lost the death domain in their NFκB, which contains the Rel binding domain, Rel dimerization domain, and ankyrin repeats (Figure 5C). Notably, anthozoans *N. vectensis*, *D. gigantea*, *Xenia sp*., and all of the medusozoan species have NFκB homologs that have lost ankyrin repeats and the death domain (Figure 5C).

In addition to lacking RIG1/MDA5, prototypical NLRs and prototypical TLRs, medusozoans have slightly less complete PRR to NFκB pathways when compared to anthozoans (Figure 5A-B). Notably, IKKγ (NEMO) is absent in the majority of species, although the majority of species. contained at least one IKK (supplementary Table 7). IRAK1/4, TRIF, RIP1, RIP2, MAVs, and CASP10 were missing in all cnidarian species (Figure 5A-B). In the case of CASP8, CASP10, and CARD9 the best reciprocal best blast hit was a slightly different caspase or caspase recruitment protein (supplementary Table 7).

Homologs of MASP, C2, and C3 were found in the majority of species in this study (Figure 6). Two medusozoans, *C. cruxmelitensis* and *H. vulgaris* lacked MASP homologs. Notably, all anthozoans had proteins with the domain structure characteristic of the C2/Bf family and C3 family while *C. hemisphaerica* and *H. vulgaris* lacked C2/Bf proteins and *Aurelia sp.* and *C. xamachana* lacked proteins in the C3 family. However, both the *Aurelia sp.* and *C. xamachana* proteomes contained proteins with all of the characteristic C3 family domains except for the C34c domain (supplementary Table 8). No proteins matching the C6-9 protein family's domain structure were found in any of the species (Figure 6).

Discussion

PRR survey

Our study builds upon previous studies of cnidarian PRRs to give a higher resolution picture of PRR evolution within the phylum. We show a clear split in PRR number between two major cnidarian clades, Anthozoa and Medusozoa. Most notably medusozoans lack prototypical TLRs, all RLRs, and prototypical NLRs, while anthozoans possess prototypical PRRs. Further, we show that within Anthozoa extensive expansions of NLRs and CTLs are present in soft corals as well as what has been previously described in stony corals and anemones. In some cases, our results vary slightly from previous studies in the exact number of PRRs identified, due to differences in methodology including queries and search algorithms used, inclusion thresholds, genomic resources, and treatment of splice isoforms (10,12,26,27,169). These slight differences do not impact the overall findings of this study, including expanded PRR repertoires in the majority of anthozoans relative to medusozoans and a lack of prototypical PRRs in medusozoans not previously reported outside of Hydrozoa (21).

The presence of prototypical TLRs in two anthozoan classes, Octocorallia and Hexacorallia, and the absence of prototypical TLRs in medusozoans, sponges (170,171), ctenophores (172), and placozoans (173) suggests that within Metazoa prototypical TLRs first appear in the ancestral anthozoan and have been secondarily lost in *Xenia sp*. and *E. daiphana* (126). Anthozoan prototypical TLRs likely are similar in function to the *N. vectensis* TLR which has been shown to play a role in pathogen recognition, NFkB signal transduction, and development (13). Despite their lack of prototypical TLRs, it is likely that *Xenia sp*., *E. daiphana*, and medusozoans are capable of TLR-NFκB signaling through TLR-like proteins, as studies in *H. vulgaris* have indicated (21).

The full mechanism by which transmembrane LRR proteins interact with TLR-like proteins is unknown; however, if multiple transmembrane LRRs are capable of interacting with a single TLR-like protein this pathogen recognition system could provide a versatile source of immune specificity in cnidarians. This study provides further support to previous findings of a lineage specific expansion in TLR-like proteins in stony coral species. This expansion is hypothesized to be involved in distinguishing mutualistic or commensal microorganisms from parasitic microorganisms within the coral holobiont and mounting an appropriate immune response (7,26,174) . This potential function of TLR-like proteins is particularly vital to understand in corals as microbiome composition is thought to influence resistance to both disease and thermal stress, two existential threats to coral reefs (175,176)

The presence of the C terminal RIG-I repressor domain indicates that the RIG-I/MDA5-like receptors in anthozoans are more similar to RIG-I than MDA5. This supports previous findings that *N. vectensis* RIG-I/MDA5-like receptors group more closely with vertebrate RIG-I than MDA5 (9). The presence of LGP2-like proteins in *A. queenslandica*, *Xenia sp*., *A. tenebrosa*, *A. millepora*, and *M. capitata* contradicts the hypothesis that RIG-I is the most evolutionarily ancient RLR and supports the hypothesis that LGP2 is the first RLR to emerge (9,177). The fact that LGP2 is not consistently present in anthozoans indicates either that it is commonly secondarily lost due to low selective pressure or that it has evolved independently several times. Additionally, It is unclear what function LGP2 has in the absence of RIG-I and MDA5; however, the presence of an RNA dependent RNA polymerase domain in the *A. millepora* and *M. capitata* LGP2-like receptors indicates that their LGP2-like receptors most possibly amplify dsRNA targets for RNA interference and do not function like vertebrate LGP2 (110,111,178).

The presence of prototypical NLRs in *A. queenslandica* and all nine anthozoan species indicates that prototypical NLRs have been secondarily lost in Medusozoa. However, LRRs appear to not be necessary for DAMP recognition in medusozoan NLRs, as an NLR in *H. vulgaris* was shown to be upregulated in response to both lipopolysaccharide and flagellin (10). Thus, NLR-like proteins may still be a source of immune specificity in cnidarians. As in previous studies, we found expansions of NLRs within anthozoan species, indicating that NLRs are a substantial source of immune specificity within the clade (14,27). Gene expression studies indicate that NLRs are upregulated in response to immune stressors and modulate apoptosis and immunity (14,34,179). Our data support the link between cnidarian NLRs and apoptosis as many of the effector domains found in cnidarian NLRs including CARD (162), ZU5 (164) , DD (163), NBARC (166), and WD-40 repeats (167) are associated with apoptotic signal transduction.

Because NLRs are involved not only in pathogen recognition but also in self/altered-self/non-self-recognition, traits such as coloniality and ability to form nutritional algal symbiosis may be linked to expansions in NLRs (8,180). A recent study indicates that in *E. daiphana*, a symbiotic anemone, microalgae are taken into the cell largely indiscriminately and the decision to retain these microalgae as symbionts or expel them likely occurs intracellularly (74). As intracellular PRRs, NLRs are great candidates for modulating interactions between the algal symbionts and the immune system of their cnidarian hosts during the establishment of symbiosis, a hypothesis with some support from transcriptomic studies (76). Currently, knowledge of cnidarian NLRs stems from both bioinformatic and gene expression studies (10,14,27,34,179) as this family of PRRs has yet to be functionally studied within the phylum. Functional studies of cnidarian NLRs are needed, as our results and previous studies indicate that anthozoans have invested in large and diverse NLR repertoires (14,27).

The expansion of CTLs across all 15 cnidarian species relative to the sponge outgroup aligns with previous findings of diverse and large CTLD containing protein repertoires in invertebrate species (12,94). Despite this diversity and the potential for cnidarian CTLs to greatly contribute to immune specificity, functional studies have only been conducted on homologs of mannose binding lectin (MBL), the activator of the lectin complement pathway (23,181). These studies indicate that MBL homologs in two corals, *P. damicornis* (181) *and A millepora* (23) are capable of binding to both bacteria and Symbiodiniaceae leading to the hypothesis that lectin/glycan interactions are a mechanism of recognition during symbiont infection. This hypothesis has found some support from transcriptomic studies (28,75). The ability of cnidarian lectins to interact with MASP and activate the lectin-complement pathway has yet to be investigated. Both of the cnidarian lectins for which we have functional studies and the majority of cnidarian species in this study lack CTLs with the collagen helix domain characteristic of MBL (23,181). However, it is possible that cnidarians are capable of lectin-complement activation despite lacking collagen domains as studies have shown that CTLs lacking collagen domains are still able to interact with MASP (182,183).

CTLs in cnidarians are potentially a large source of immune specificity which to date has been understudied. Based on their large and diverse CTL repertoires, cnidarians are likely utilizing CTLD containing proteins for a variety of functions. Transcriptomic and proteomic studies indicate that CTLs are involved in coral wound healing (184) and disease response (185,186) in addition to their hypothesized role in mediating symbiosis with Symbiodiniaceae (23,28,75,181). Thus, further functional studies of cnidarian CTLs are warranted and should focus both on highly conserved CTLs and on novel cnidarian CTLs with the potential to shed light on disease processes.

Relationship to clade and life history traits

There is a divide in number of PRRs and downstream immune pathway completeness between Medusozoa and Anthozoa. There are several life history traits common in the anthozoan clade that could explain a greater need for immune specificity and thus this division (1). Three of these life history traits, intracellular algal symbiosis, coloniality, and sedentary lifestyle, we found to have a significant association with total number of PRRs. While intracellular algal symbiosis occurs in both Medusozoa and Anthozoa, there are far more symbiotic anthozoan genera (8,61). The establishment and maintenance of intracellular algal symbionts is a complex process, with many cnidarian species hosting several species of algae, and thus it likely requires immune specificity (8). Similar to intracellular algal symbiosis, there are colonial organisms in both Medusozoa and Anthozoa, but they are far more common in Anthozoa. Colonial invertebrates require allorecognition systems to distinguish self-tissues from conspecific tissues, which may necessitate a more diverse repertoire of PRRs (101,102). Animals that spend the majority of their lifespan motile are far more common in the medusozoan clade. In contrast, anthozoan species are largely sedentary, often residing in microbe rich environments like estuaries and coral reefs (1). As sedentary animals are unable to avoid antigen accumulation through movement, they likely also have a greater need for immune specificity (187).

There are several other life history traits that may be associated with immune specificity in cnidarians that we were unable to test due to either a lack of data or low sample size. Number of mutualistic and commensal bacterial symbioses is almost certainly factor in the amount of immune specificity a given cnidarian has, however this information is not available for many of the species in this study (188). It is also possible that anthozoans and medusozoans have a

similar need for immune specificity and simply employ different methods to meet this demand. Medusozoans could rely more heavily on other pattern recognition receptor types, such scavenger receptors, rather than TLRs, RLRs, NLRs, and CTLs to provide their immune specificity (20). Other possible sources of immune specificity not reflected in PRR number include increased substitution rates and post translational modifications (189,190). With future studies and increased resolution of genomic and proteomic resources we hope that this study can be used as a basis for linking life history to mechanisms of immune specificity.

Downstream immune pathway completeness

Our study indicates a complex history of NFkB within Cnidaria, as ankyrin repeats appear to have been secondarily lost at least three times within the phylum. This C-terminal inhibitory domain prevents NFκB from trafficking to the nucleus and must be removed via proteolysis in order for NFκB to bind to DNA. This regulatory function may not be under strong selective pressure within Cnidaria. There are several functional studies on cnidarian NFκB proteins, including *N. vectensis* Nv-NFκB, which is truncated and does not include ankyrin repeats (29,31). These studies show that *E. daiphana*'s NFκB binding specificity is more similar to both human NFκB p50 and Nv-NFκB than c-Rel and RelA, despite the Nv-NFκB having a similar domain organization to c-Rel and RelA (31). Because ankyrin repeats appear to have been independently lost, it is unclear if soft coral and medusozoan NFκB share similar binding specificity to Ep-NFκB, Nv-NFκB, and human NFκB p50. However, several studies in both medusozoans and anthozoans show that NFκB is responsive to pathogen exposure (21,31,34,179).

Consistent with previous studies, we found that while cnidarians have the majority of proteins in PRR to NFκB pathways they are missing some components found in mammalian PRR to NFκB pathways (31,98). However, the absence of these components does not mean that cnidarians are incapable of PRR to NFκB signaling (21,31). Cnidarians can compensate for missing pathway components either by proteins upstream of the missing component interacting directly with proteins further downstream or through proteins that are not homologous to mammalian pathway members but are able to functionally replace them (10,191).

While cnidarians likely retain PRR to NFκB signaling through these mechanisms, these missing proteins still indicate potential fundamental differences between cnidarian and mammalian innate immune pathways. Functional replacements may not be regulated or regulate immune pathways in the same manner as their mammalian counterparts. For example, although it appears as though cnidarians have a functional replacement for RIPs, this functional replacement likely is not regulated in the same manner as RIPs because the adaptor proteins RIPs interact with in the decision to promote pro-life NFκB signaling or cell death signaling are also absent (10,192). The lack of RIPs in cnidarians could result in a greater propensity for cell death signaling over pro-life NFκB signaling, a hypothesis that could explain disease phenotypes in white syndrome coral diseases (193). The absence of MAVs from the RLR-NF□B pathway is notable, as it is unclear how RLR antiviral signaling occurs in the absence of this key adaptor protein, however interactions with other CARD domain containing proteins may mediate RLR signaling in cnidarians (32,108).

No proteins in the C6 family were found in any of the 15 cnidarian species in this study, consistent with previous findings in *N. vectensis*, *E. daiphana* and reef building corals (28,126,160). This indicates that cnidarians are unable to form the membrane attack complex and

instead use complement for opsonization through C3 (160). While opsonization of microbes by C3 has not been directly shown in cnidarians, transcriptomic studies in anthozoans show that complement signaling is responsive to bacterial pathogens (28,35). Scyphozoans *C. xamachana* and *Aurelia sp*. lack the complete domain structure of C3. However, because the domain they are missing, C345c mainly functions in interacting with C6 family proteins, this protein may still be able to opsonize (194).

Conclusions

As a whole, our data indicate that anthozoans have greater immune specificity than medusozoans, with expansions of NLRs and CTLs providing the majority of this specificity. We hypothesize that a greater immune specificity in anthozoans is needed due to life history traits common within the clade, such as being sedentary, having a colonial body plan, and hosting a complex microbiota that includes intracellular algal symbionts. More broadly, our data indicate that studying cnidarian PRRs can give insight not only into where within Metazoa prototypical PRRs arose but also how basal prototypical PRRs function and the systems by which DAMPS and MAMPs were recognized prior to the emergence of these prototypical PRRs. Further investigations into medusozoan immunity would likely provide a greater understanding of non-prototypical pattern recognition systems. The ecological threat coral diseases pose has led to a wealth of knowledge on anthozoan immune responses (195). Placing these studies in an evolutionary context could give further information as to how basal prototypical PRRs function and more broadly how innate immunity evolved.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

MAE and BAD conceived of the project. Data collection and bioinformatic analysis was conducted by MAE. MAE, BAD, and LDM wrote the manuscript.

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Chapter 2 Figures:

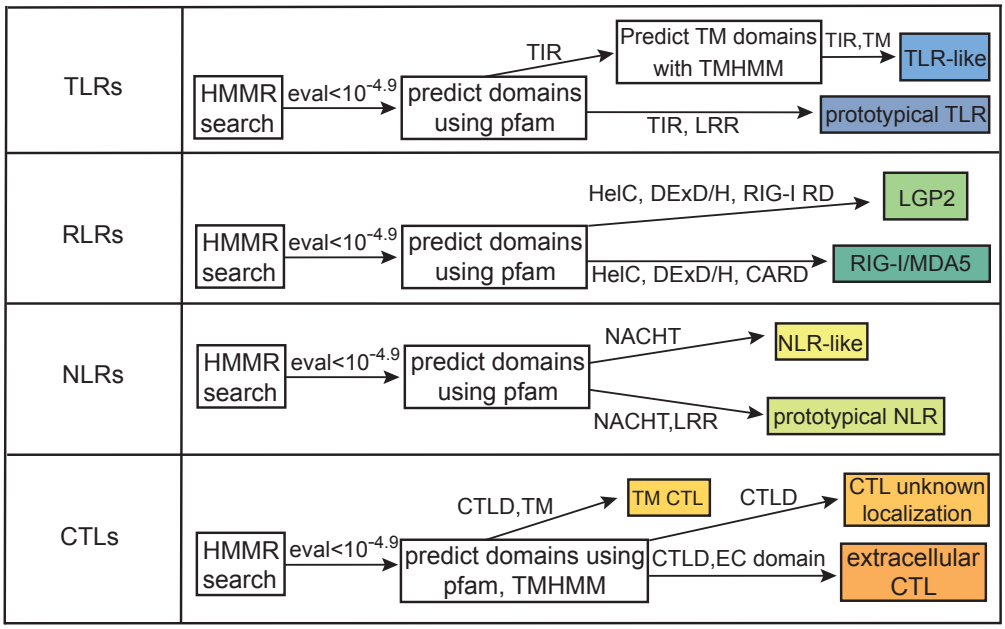

Figure 1: Flow chart of methodology used to identify PRRs. Shown is the minimum domain requirements for a protein to be classified in the various categories as well as homology requirements based upon E-value.

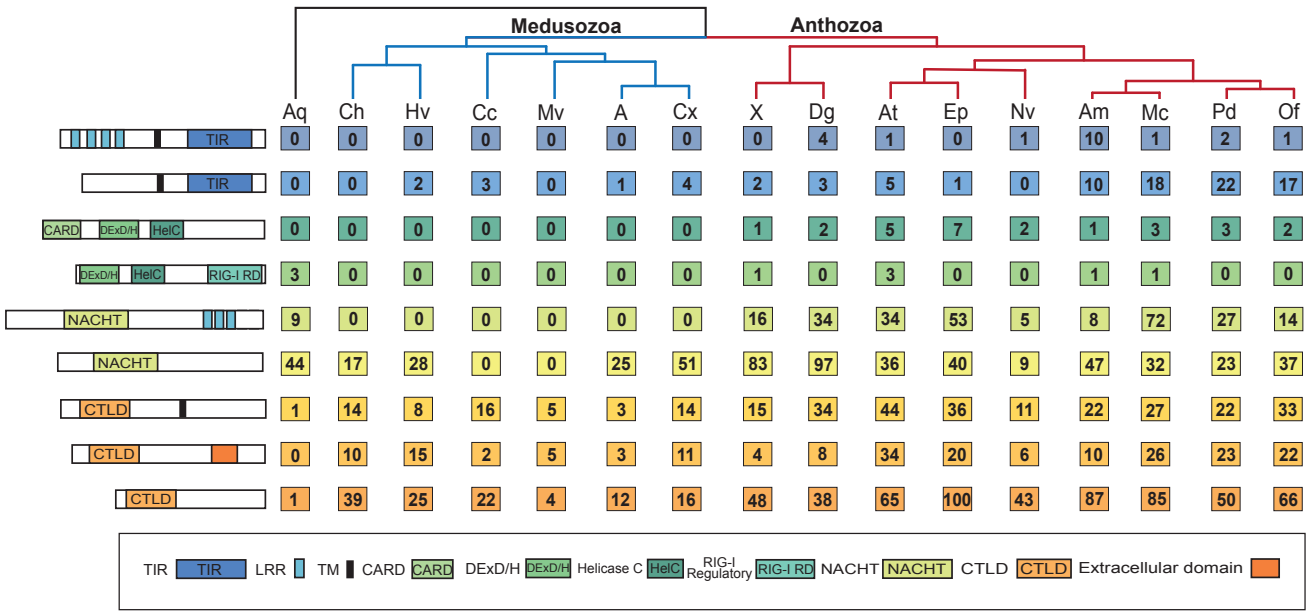

Figure 2
Number of PRRs of each type found in each species. Far left shows schematics of the minimum domain composition for each PRR type, from top to bottom: prototypical TLR, TLR-like, RIG-I/MDA5-like, LGP2-like, prototypical NLR, NLR-like, transmembrane CTL, extracellular CTL, CTL with unknown localization. To the right of the schematics is the corresponding number of each PRR type found in each species' proteome. Species are grouped by phylogeny, with anthozoans in red, medusozoans in blue, and the sponge outgroup in black. Species abbreviations: Aq: *Amphimedon queenslandica*, Ch: *Clytia hemisphaerica*, Hv: *Hydra vulgaris*, Cc: *Calavadosia cruxmelitensis*, Mv: *Morbakka virulenta*, A: *Aurelia sp.*, Cx: *Cassiopea xamachana*, X: *Xenia sp.*, Dg: *Dendonephthya gigantea*, At: *Actinia tenebrosa*, Ep: *Exaiptasia daiphana*, Nv: *Nematostella vectensis*, Am: *Acropora millepora*, Mc: *Montipora capitata*, Pd: *Pocillopora damicornis*.

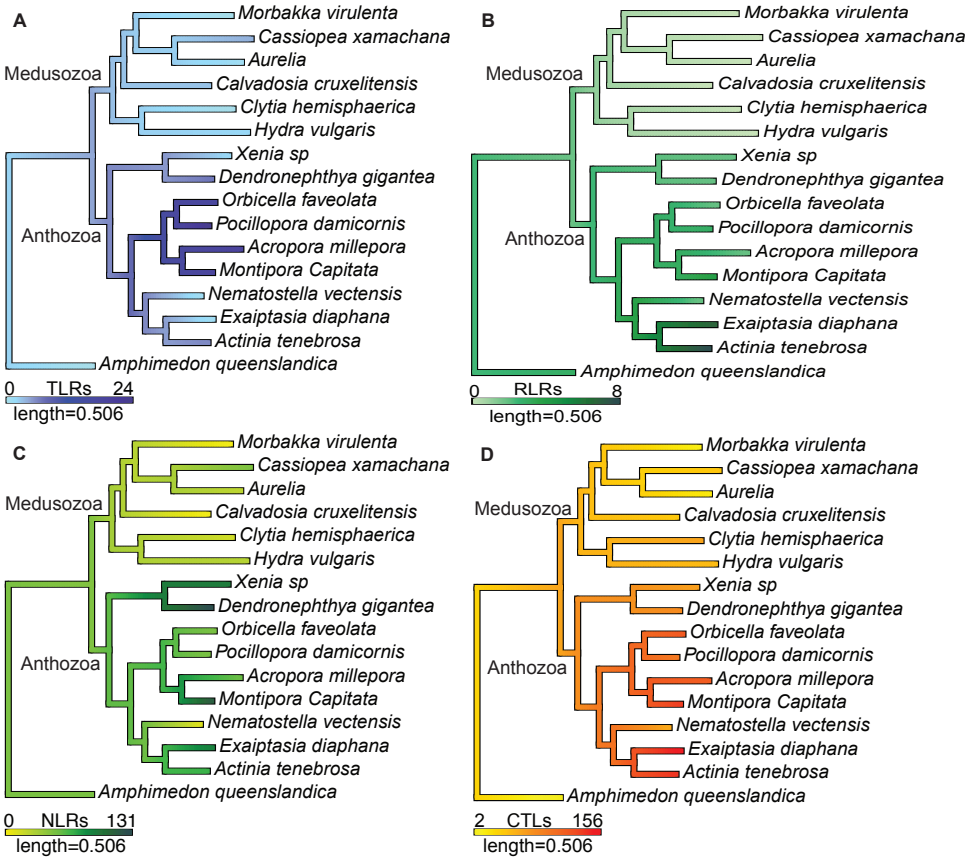

Figure 3
Ancestral state reconstructions of the number of (**A**) prototypical TLRs and TLR-like proteins, (**B**) RIG-I/MDA5-like receptors and LGP2-like receptors (**C**) prototypical NLRs and NLR-like proteins (**D**) CTLs

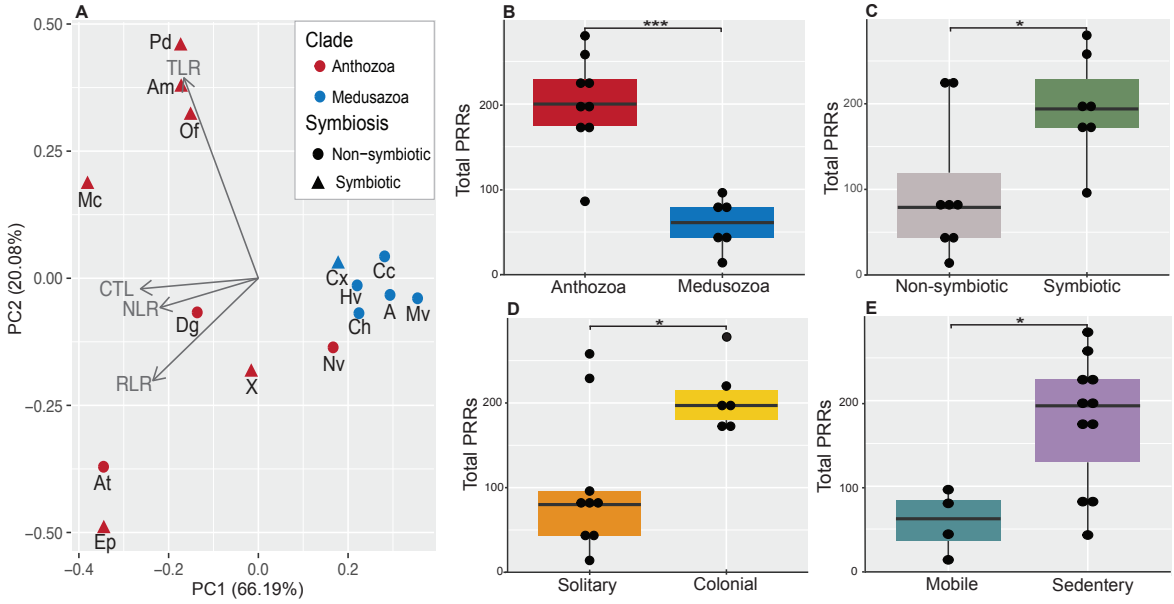

Figure 4
(**A**) Principal component analysis of total number of PRRs in each category, red points indicate anthozoan species, blue points indicate medusozoan species, triangular points indicate symbiotic species, and circular points represent non-symbiotic species. Species abbreviations: Aq: *Amphimedon queenslandica*, Ch: *Clytia hemisphaerica*, Hv: *Hydra vulgaris*, Cc: *Calavadosia cruxmelitensis*, Mv: *Morbakka virulenta*, A: *Aurelia sp.*, Cx: *Cassiopea xamachana*, X: *Xenia sp.*, Dg: *Dendonephthya gigantea*, At: *Actinia tenebrosa*, Ep: *Exaiptasia daiphana*, Nv: *Nematostella vectensis*, Am: *Acropora millepora*, Mc: *Montipora capitata*, Pd: *Pocillopora damicornis*. (**B-E**) Boxplots of total PRR number separated by (**B**) Clade, (**C**) Symbiotic status, (**D**) Colonial status, and (**E**) motility. *** indicates a p-value less < 0.001 and * indicates a p-value < 0.05 resulting from a generalized linear model. Points represent individual species' total PRR numbers binned into groups of 10.

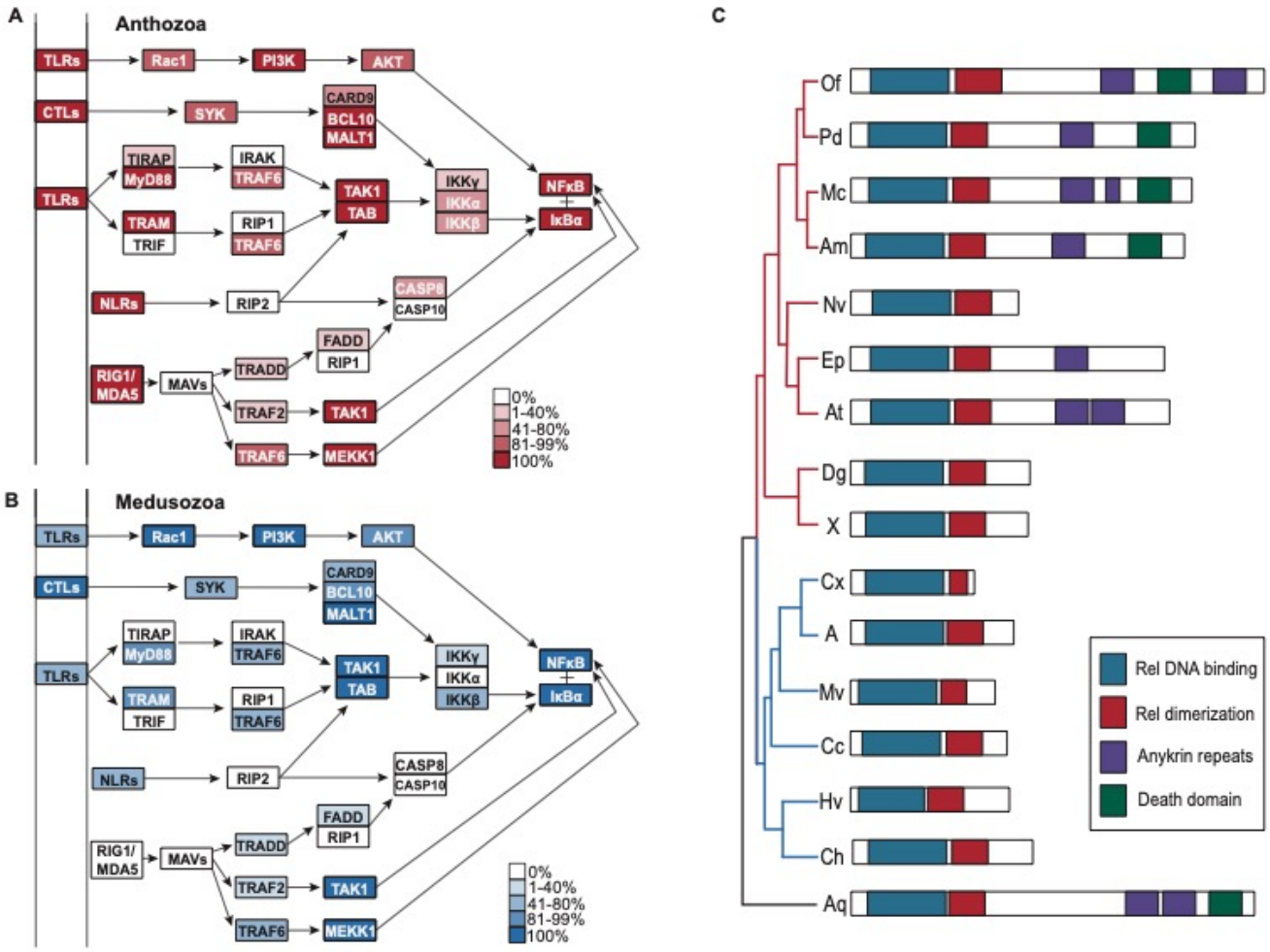

Figure 5
(**A-B**) PRR-NFκB pathways modified from Kegg. Opacity of the boxes represents the percentage of (**A**) Anthozoans or (**B**) Medusozoans with that pathway component. (**C**) schematic of the domains found in NFκB in each species, grouped by phylogeny. Medusozoan species are indicated by the blue phylogenetic tree branches. Anthozoan species indicated by the red phylogenetic tree branches.

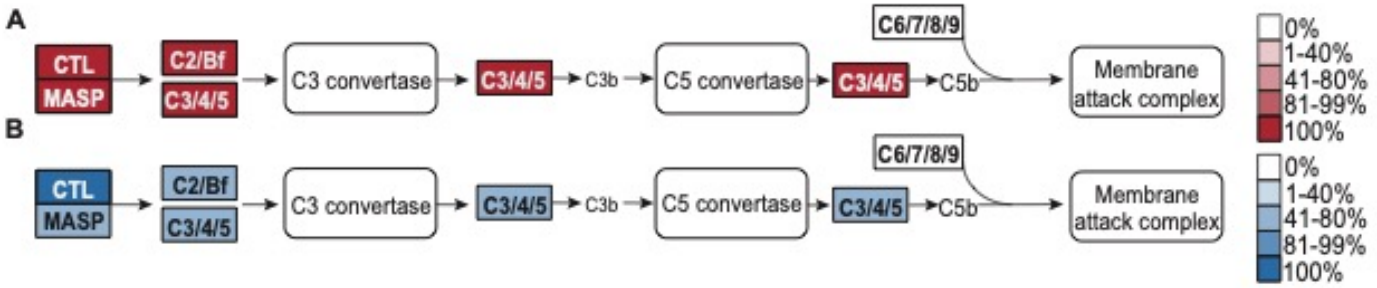

Figure 6
(**A-B**) Lectin-complement pathway modified from Kegg pathway. Opacity of the boxes represents the percentage of (**A**) Anthozoans or (**B**) Medusozoans with that pathway component.

Chapter 3: Trade-off between photo-symbiosis and innate immunity influences cnidarian's response to pathogenic bacteria

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Abstract

Mutualistic relationships with photosynthetic organisms are common in cnidarians, which form an intracellular symbiosis with dinoflagellates in the family Symbiodiniaceae. The establishment and maintenance of these symbionts is associated with the suppression of key immune factors. Due to this, there are potential trade-offs between the nutrition cnidarian hosts gain from their symbionts and their ability to successfully defend themselves from pathogens. To investigate this potential trade-off, we utilized the facultatively symbiotic polyps of the upside-down jellyfish *Cassiopea xamachana* and exposed aposymbiotic and symbiotic polyps to the pathogen *Serratia marcescens*. Symbiotic polyps had lower probability of survival following *S. marcescens* exposure. Gene expression analysis 24 hours following pathogen exposure found that symbiotic animals mounted a more damaging immune response, with a stronger inflammatory and reactive oxygen species response resulting in disruptions to the protein folding environment in the endomembrane system. Underlying this more damaging immune response may be differences in constitutive and pathogen-induced expression of immune transcription factors between aposymbiotic and symbiotic polyps rather than broadscale immune suppression during symbiosis. Our findings indicate that hosting symbionts limits *C. xamachana's* ability to survive pathogen exposure, indicating a trade-off between symbiosis and immunity that has potential implications for coral disease research.

Introduction

Throughout the metazoan phylogeny several taxa have evolved photo-symbiosis or mutualistic relationships with photosynthetic organisms (196). In these mutualisms the symbionts provide their hosts with photosynthates which often account for the bulk of the hosts'

nutrition in exchange for nutrients such as nitrogen and the protection of being housed within the host(60,197). Photo-symbiosis is common throughout the cnidarian phylogeny, with the vast majority of symbiotic cnidarians forming an intracellular symbiosis with dinoflagellates in the family Symbiodiniaceae(1,60). This symbiosis is best known for its vital role in coral reef ecosystems, as the nutrition provided by the symbionts often allows the cnidarian hosts to live in oligotrophic environments that would otherwise be uninhabitable for them if they relied upon heterotrophy alone(67,198).

The extent to which the cnidarian host is reliant upon their symbionts for nutrition varies. This symbiosis can be obligate, as seen in tropical reef building corals, facultative, as seen some anemone and soft corals, or both depending on the life stage, as seen in symbiotic scyphozoans (61,67,69) . While all algal symbionts are housed intracellularly in a specialized acidic organelle called the symbiosome, the cell type in which the symbionts reside is variable (60,61,69). This is likely due to the complex evolutionary history of the cnidarian-algal symbiosis, which has independently evolved several times (1,77,199). Members of the classes Hexacorallia and Octocorallia, which account for the vast majority of symbiotic cnidarians, house their symbionts in the gastrodermis (60,69). This is in contrast to the more distantly related scyphozoans whose symbionts pass through the gastrodermis to mobile cells called amebocytes in the mesoglea (61).

The complete mechanisms of symbiosis establishment and maintenance within cnidarians are still unknown and may vary across the independently evolved symbioses (8,69). Recognition of the algal symbionts likely occurs via pattern recognition receptors, though many different classes of these receptors have been implicated (8). However, there is evidence in soft and hard corals that lectins opsonize the symbionts prior to phagocytosis (23,25,69,121). Following phagocytosis, non-compatible symbiont species are expelled from the symbiont-hosting cells via

vomocytosis while compatible symbionts are retained to establish their intracellular niche (74) The establishment of compatible symbionts is strongly associated with the suppression of the cnidarian hosts' innate immune system (8,76,78,200). Studies in indicate that this immune suppression likely occurs via the suppression of the master immune regulator and transcription factor NFkB or through the suppression of pathways upstream of NFkB (30,74,75). This immune suppression persists in the symbiont-hosting cells in order to retain their symbionts (30,76,78).

Because symbiotic cnidarians suppress their immune systems while maintaining populations of intracellular symbionts, there is a potential trade-off between the nutrition these animals get from their symbionts and their ability to respond to pathogens. With recent increases in frequency and severity of coral disease outbreaks, it is pertinent to understand the how hosting symbionts influences cnidarians' ability to defend against pathogens (87–89,201). Aposymbiotic *E. diaphana* have been shown to be less susceptible to *Serratia marcescens* infection relative to their symbiotic counterparts (202). This species has also shown marked differences in transcriptional response between symbiotic and aposymbiotic animals in response to *Vibrio coralliilyticus* (35). However, it has not been established if immune suppression and the subsequent trade-off between symbiosis and immunity is shared across independent evolutions of the cnidarian-algal symbiosis, given the differences in the symbiont housing cell type. Therefore, we tested the outcomes and responses of aposymbiotic and symbiotic Cassiopea sp. to the known cnidarian pathogen *S. marcescens*. Cassiopea are benthic jellyfish that are facultatively symbiotic in their polyp life stage (68). This facultative symbiosis can be leveraged to disentangle the role of symbiosis in pathogen induced stress. We found that symbiotic Cassiopea, similar to *E. diaphana*, are more susceptible to bacterial infection relative to their aposymbiotic counterparts. To further investigate the mechanisms of this tradeoff between

symbiosis and immunity we measured their acidic organelle activity and gene expression following pathogen exposure. These data give more insights into the trade-offs between symbiosis and immunity in cnidarians by identifying core shared responses and phenomena across the independently evolved symbioses.

Methods

Animal Husbandry

Cassiopea polyps were obtained from Dallas Children's Aquarium (Dallas, TX 75210) and maintained at 27˚C in 35 ppt artificial seawater (ASW). Polyps were fed artemia nauplii and given water changes twice per week. Aposymbiotic polyps were generated by maintaining animals in the dark for a minimum of two months. Polyps were considered aposymbiotic when they had fewer than 10 symbionts visible via a dissecting microscope. To confirm that our aposymbiotic polyps had vastly reduced symbiont populations relative to symbiotic polyps, we imaged 10 polyps from each symbiotic state under an eGFP filter using Zeiss imager Z2 microscope and quantified the mean fluorescence of the bell of each polyp in ImageJ (version 1.53t) by subtracting the mean background fluorescence from the mean total fluorescence. A T-test was used to confirm a significant reduction in symbiont density (figure 1A, $p=2.12*10^{-7}$).

Survival analysis and quantification of acidic organelle activity

Polyps were fed artemia nauplii 48 hours prior to the start of all experiments. *S. marcescens* was cultured at 30˚C for 24 hours in a general artificial seawater media (203). The bacteria were pelleted, washed with 0.2 μm filtered artificial seawater, and diluted down to a concentration of 10^8 CFU/mL prior to placing the polyps into the solution. Survival experiments

had four treatment groups, symbiotic controls, aposymbiotic controls, symbiotic exposed, and aposymbiotic exposed and were run at an ambient temperature of 27˚C. Polyp mortality of each group was collected every 24 hours following the start of the experiment. Mortality was defined as a polyp with the complete loss of bell structure. Differences in survival between treatment groups were tested using a Kaplan-Meier survival analysis using the R package survival and visualized with the R package survminer (204–206).

Acidic organelle activity was measured following 24 and 72 hours of exposure to the respective treatments by incubating polyps in the dark in a solution of 5µL/mL lysotracker red (Invitrogen L7528) in 0.2 µm filtered ASW for 30 minutes. Following the incubation period animals were washed three times in 0.2 µm filtered ASW before being mounted onto slides and imaged at 5x under an RFP filter using a Zeiss imager Z2 microscope. Mean florescence of the bell of each polyp was quantified using image j by subtracting the mean background fluorescence from the mean total fluorescence. A two-way ANOVA (mean fluorescence ~ symbiotic status*treatment) was used to test for differences in acidic organelle activity between treatment groups at 24 and 72 hours.

RNA Extractions and Sequencing

30 polyps non-clonal polyps per treatment group were exposed to their respective treatment conditions for 24 hours. Polyps were then pooled in groups of 5 for a total of 6 replicates per treatment group, placed into 600 µL of 1x DNA/RNA shield (Zymo: R1100) and flash frozen in liquid nitrogen. Samples were homogenized and RNA was extracted using the Zymo RNA miniprep plus kit (R1057). Extracted RNA was sent to Novogene Co. LTD. Following quality control 18 samples proceeded to polyA tail capture and cDNA library

preparation. The cDNA libraries were then sequenced on an Illumina NovaSeq 6000 using 150 bp paired-end sequencing.

Data Analysis

Raw reads were trimmed using Fastp (version 0.23.3) and mapped to the gene models of the *C. xamachana* draft genome using Salmon (version 1.9.0) (124,207,208). The corresponding proteome was annotated using both eggNOG mapper (version 2.1.12) and STRING (taxon identifier STRG0A63JRD) (209,210). All previously identified *C. xamachana* PRRs were identified in the proteome using BLAST (158,211). Tximport was used to format the reads and correct for biases in length and GC content (212). An outlier analysis was performed through a principal component analysis using PCAtools and a cluster dendrogram from the WGCNA R package, following these analyses 3 samples were excluded from analysis (supplemental figure 1) (213,214). Following the exclusion of outliers, a second principal component analysis was done (214). The loadings for PC1 were then used as a variable for gene ontology (GO) enrichment analysis using GOMWU (215).

Differentially expressed genes were called using DESeq2 with the following model: ~symbiotic status + treatment + treatment*symbiotic status (216). The appropriate contrasts in DESeq2 were used to separate DEGs from the following comparisons: symbiotic controls vs aposymbiotic controls, aposymbiotic exposed vs aposymbiotic controls, symbiotic controls vs symbiotic exposed, and symbiotic exposed vs aposymbiotic exposed. The log fold change for each of these comparisons was then used for rank-based GO enrichment analysis using GOMWU (217).

Signed gene co-expression networks were detected using WGCNA with a soft power of 9 and a minimum module size of 150 (supplemental figure 2) (213). Modules were then correlated to treatment and symbiotic status while each contig was correlated to treatment. Of the modules significantly correlated to any given trait or combination of traits the most significantly correlated modules to a given trait or traits were tested for functional enrichments. If no enrichments were found, the second most correlated module for a given trait was then tested for functional enrichments. Functional enrichments were tested for using STRING, against the background of all contigs with a mean expression greater than 10 in the experiment (210).

Results

Survival analysis and acidic organelle activity

Symbiotic polyps are significantly less likely to survive exposure to *S. marcescens* at a concentration of 10^8 CFU/mL relative to their aposymbiotic counterparts (Kaplan Meier survival analysis p=0.0067) (Fig 1B). Only 5.9% of symbiotic polyps survived exposure to the pathogen, as compared to 53.8% of aposymbiotic polyps. Acidic organelle activity did not significantly vary between any of the treatment groups 24 hours following *S. marcescens* exposure (Fig 1C). However, it significantly increased 72 hours following *S. marcescens* exposure (two-way ANOVA, p=0.038), with no significant effect of symbiosis on this increase (Fig 1D).

Gene expression Analysis

An average of 11.8 million reads per replicate mapped to the *C. xamachana* gene models for an average mapping rate of 55.14 (supplemental file 1). Three replicates were removed based on hierarchical clustering and an initial principal component analysis (supplemental figure 1).

This resulted in 15 samples, four symbiotic control replicates, four symbiotic pathogen exposed replicates, four aposymbiotic control replicates, and three aposymbiotic pathogen exposed replicates, for downstream analysis. After filtering genes with low expression in the dataset the count data consisted of 16,989 expressed genes.

A principal component analysis showed distinct grouping of the replicates by treatment group on the first two principal components (figure 2A). Notably, the symbiotic control replicates group most closely with aposymbiotic pathogen exposed replicates across the first principal component (PC1), which accounts for 27.65% of the variation within the dataset (figure 2A). The loadings of PC1 were then used as input for rank-based gene ontology enrichment analysis to identify processes significantly associated with PC1. Thirty-two biological process GO terms were significantly (padj<0.01) positively associated with PC1, including ion homeostasis, regulation of autophagy, reactive oxygen species metabolic process, innate immune response, and secretion (supplemental file 2, figure 2B).

Four comparisons were made during differential gene expression analysis, falling into two categories: symbiotic state comparisons and pathogen response comparisons (figure 3A). Symbiotic state comparisons test for differences between the symbiotic states within the same treatment groups, comparing 1. aposymbiotic controls and symbiotic controls and 2. aposymbiotic pathogen exposed and symbiotic pathogen exposed. Pathogen response comparisons test the response of each symbiotic state to S. marcescens, comparing 3. aposymbiotic controls and 4. aposymbiotic pathogen exposed, and symbiotic controls and symbiotic pathogen exposed. There were 1157 DEGs between aposymbiotic controls and symbiotic controls, 442 of which were annotated (supplemental file 3). Of these 1157 genes, 230

were differentially expressed in both symbiotic state comparisons, regardless of exposure to *S. marcescens* (figure 3B).

Significant downregulation of nuclear-factor kappa B subunit 1 (NFkB) and members of the heat shock protein family occurred in symbiotic controls relative to aposymbiotic controls (figure 4). Several other genes associated with immunity were significantly upregulated in symbiotic animals relative to their aposymbiotic counterparts, including nitric oxide synthase 1 (NOS1), MBL associated serine protease 1 (MASP1), interferon regulatory factor 1 (IRF1), and two C-type lectins (figure 4). Additionally, the antioxidant superoxide dismutase 2 (SOD2) was upregulated in symbiotic animals (figure 4). Several genes involved in transport were also significantly upregulated in symbiotic animals between both symbiotic state comparisons, including gamma-aminobutyric acid receptor gamma-2 (GABRB2), gamma-aminobutyric acid receptor rho-2 (GABRR2), solute carrier family 23 member 2 (SLC23A2), and NPC intracellular cholesterol transporting 11B (NPC2) (figure 4). Ranked gene ontology enrichment analysis found 157 GO terms significantly up and downregulated in the symbiotic controls relative to aposymbiotic controls (figure 5A, supplemental file 4). Several of these significantly upregulated enrichments in symbiotic controls are associated with ion transport and the nervous system (Fig 5A). Lipid catabolism, the spliceosomal complex, and mitotic cell cycle were all downregulated in symbiotic controls relative to aposymbiotic controls (figure 5A).

When the aposymbiotic and symbiotic pathogen exposed replicates were compared there were 799 differentially expressed genes, 418 of which were annotated (supplemental file 3). Symbiotic polyps exposed to *S. marcescens* significantly upregulated PTEN induced kinase 1 (PINK1), mitogen activated kinase kinase 3 (MAP2K3), transcription factor JunD (JUND), laccase domain containing 1 (LACC1), nitric oxide synthase 1 (NOS1), peroxidasin (PXDN),

and superoxide dismutase 1 (SOD1) relative to aposymbiotic polyps exposed to the same bacteria (figure 4). Rank based gene ontology enrichment analysis found further evidence for a strong immune effector response in symbiotic polyps relative to aposymbiotic polyps following pathogen challenge with the GO terms immune effector process, secretion, and reactive species metabolic process all upregulated (figure 5A, supplemental file 4). A total of 338 GO terms were significantly differentially expressed in this comparison.

The aposymbiotic polyps exposed to *S. marcescens* differentially expressed 746 genes relative to aposymbiotic controls, 442 of which were annotated (supplemental file 3). Many of these genes have functions in innate immunity like integrin subunit beta 1 (ITGB1), several interferon regulatory factor homologs, a TLR-like receptor, LACC1, NOS1, and macrophage expressed 1 (MPEG1) (figure 4). Rank based gene ontology enrichment analysis found 414 differentially expressed GO terms, with several processes enriched in pathogen exposed aposymbiotic polyps relative to control polyps, including the upregulation of phagocytosis, secretion, response to cytokine, regulation of autophagy, regulation of reactive oxygen species biosynthetic process, and intracellular pH reduction (figure 5B, supplemental file 4).

Symbiotic polyps differentially expressed 1270 genes following pathogen exposure relative to symbiotic controls, 773 of which were annotated (supplemental file 3). There were 191 genes overlapping between the aposymbiotic pathogen response comparison and the symbiotic pathogen response comparison, including the upregulation of an IRF1 paralog, LACC1, NOS1, SOD1, and MPEG1 (figure 4). Additionally, symbiotic polyps upregulated PXDN, MAP2K3, NADPH oxidase 3 (NOX3), caspase 10 (CASP10), catalase 2 (CAT2), and PINK1 in response to *S. marcescens* (figure 4). This comparison had 515 GO enrichments. Similar to the aposymbiotic response to the pathogen, symbiotic polyps upregulated

phagocytosis, secretion, response to cytokine, regulation of autophagy, regulation of reactive oxygen species biosynthetic process, and intracellular pH reduction (figure 5B, supplemental file 4). However, symbiotic polyps' response to *S. marcescens* also involved the upregulation of response to oxygen radical, negative regulation of autophagy, and several terms indicating endoplasmic reticulum stress resulting in an unfolded protein response including ERAD pathway, response to endoplasmic reticulum stress, and response to unfolded protein (figure 5B).

Signed weighted gene co-expression networks were created using a minimum module size of 150, resulting in 23 modules of co-expressed genes. Of these 20 modules, 6 had significant correlations to treatment, 5 had significant correlations to symbiotic status, and 2 had significant correlations for treatment and symbiotic status (supplemental figure 3). The module most negatively correlated to symbiosis ($r= -0.9$, $p=5*10^{-6}$) had STRING enrichments for terms related to assembly of collagen fibrils, disulfide bonds, and peptidase activity (figure 6, supplemental file 5). The module with the highest positive correlation to symbiosis ($r= 0.92$, $p=8*10^{-7}$) did not have any significant STRING enrichments, however the next highest module positively correlated to symbiosis ($r= 0.59$, $p=0.02$) was enriched for several terms related to transport, including cation channel activity and voltage-gated ion channel activity as well as terms related to cell adhesion (figure 6, supplemental file 5). The module most negatively correlated to *S. marcescens* exposure ($r= -0.81$, $p=3*10^{-4}$) was enriched for terms related to the nitrogen metabolism, gene expression, and protein translation. In contrast, the module most positively correlated to *S. marcescens* exposure ($r= 0.93$, $p=7*10^{-7}$) was enriched for the innate immune system, endomembrane system, lysosome, and SLC-mediated transmembrane transport. The red module was negatively correlated to both symbiosis ($r= -0.81$, $p=3*10^{-4}$) and *S. marcescens* exposure ($r= -0.63$, $p=0.01$), and was enriched for cell cycle, DNA repair, and

cytoskeleton organization. Finally, the turquoise module was positively correlated to both symbiosis (r= 0.67, p=0.007) and pathogen exposure (r= 0.76, p=0.001) and had some similar enrichments to the black module such as lysosome, endosome, and innate immune system. The turquoise module is also enriched for ROS and RNS production in phagocytes, exocyst, and the distal axon (figure 6, supplemental file 5).

Discussion

The lower survival of symbiotic polyps following *S. marcescens* exposure indicates the presence of a trade-off between the nutritional benefit of hosting symbionts and immunocompetence in *C. xamachana*. We found that hosting Symbiodiniaceae alters both constitutive and pathogen induced *C. xamachana* gene expression. Symbiosis in *C. xamachana* altered constitutive expression of metabolism, ion transport, and innate immunity. When exposed to *S. marcescens*, symbiotic *C. xamachana* upregulated a stronger reactive oxygen species and immune effector response disrupting protein homeostasis in the endomembrane system likely leading to low survival rates. We hypothesize that differences in constitutive and pathogen induced expression of proinflammatory transcription factors drives symbiotic polyps' greater susceptibility to bacterial pathogens.

In comparing the two control groups of our study we found several notable differences in the constitutive expression of symbiotic and aposymbiotic *C. xamachana*. Several genes involved in transport and metabolism were differentially expressed in symbiotic animals relative to their aposymbiotic counter parts. This is a common signature of the cnidarian-algal symbiosis, however interestingly some of the expression patterns found in our study are opposite what has been reported in studies of symbiotic anthozoans. For example, GLUL, which is responsible for

the removal of ammonia by converting it to glutamine, is downregulated in symbiotic Cassiopea but upregulated in symbiotic *E. diaphana* and overexpressed in the symbiont hosting cells of *Stylophora pistillata* and *Xenia sp*. (77,79,121,218). This, along with the lack of enrichments for the upregulation of nitrogen metabolism both in the DEG analysis between control groups and the WGCNA modules most correlated to symbiosis indicates that *C. xamachana* may not alter their nitrogen metabolism while hosting symbionts unlike other symbiotic cnidarians (78–80). However, symbionts in *C. xamachana* likely are still nitrogen limited (82,219,220). There is evidence that unlike symbiotic anthozoans, *C. xamachana* limit symbiont access to dissolved inorganic nitrogen through their microbiome rather than host gene expression, possibly contributing to the high nutrient tolerance of the genus (219,220).

We found extensive evidence for the constitutive differential regulation of ion transport while in a symbiotic state, with both the ranked gene ontology enrichment analysis between control groups and the WGCNA module positively correlated to symbiosis having enrichments involving the nervous system and transmembrane ion transport. Similar expression patterns have been observed in *E. diaphana*, with many of the upregulated genes associated with ion transport (KCNA2, GAABRR2, CNTNAP4 in our study) functioning to decrease membrane excitability (79,221–223). Several intracellular protozoan parasites cause similar changes to their host's ion transport often to prevent the host cell from producing nitric oxide (224–226). Symbiodiniaceae may employ similar strategies while residing intracellularly, as high levels of host derived nitric oxide have been shown to lead to symbiosis breakdown (227,228). However, nitric oxide synthase is upregulated in symbiotic controls relative to aposymbiotic controls, so the changes in expression of these ion transporters may serve a different function in the maintenance of symbionts.

Similar to both *Xenia* and stony corals, we found evidence of lectins playing an important role in the cnidarian-algal symbiosis (23,25,121,229). Two C-type lectins were upregulated in symbiotic controls relative to aposymbiotic controls, one transmembrane CTL and one extracellular CTL. This is notable CTLs have been shown to be capable of binding to Symbiodiniaceae, indicating a potential role in facilitating the preferential uptake of symbionts by cnidarian host cells (23,25). C-type lectins can potentially activate MASP, which was upregulated in symbiotic *C. xamachana* relative to aposymbiotic *C. xamachana* regardless of treatment (230). Upregulation of MASP has been found as a response to symbiont colonization in *E. diaphana* along with upregulation of other members of the lectin-complement pathway (35,231). These components of the complement pathway were not differentially regulated in our study, as they are not present in in *C. xamachana*, so MASP in *C. xamachana* may have functions other than complement activation (211).

Notably, we found that NFkB is downregulated in symbiotic controls relative to aposymbiotic controls, indicating that symbiotic *C. xamachana* suppress the immune factors under the transcriptional control of NFkB. This same expression pattern has been found in symbiotic anemones (30,75). Additionally, there is evidence that stony corals negatively regulate NFκB pathways while hosting symbionts, indicating that NFkB downregulation is a common characteristic of the cnidarian-algal symbiosis across independent evolutions of the trait (76,78). However, we did not find evidence of large-scale immune suppression in symbiotic animals. This, along with the symbiotic control replicates grouping with the aposymbiotic pathogen exposed replicates across PC1 in the PCA indicates that immune suppression due to hosting symbionts unlikely to be driving differences in survival rates between symbiotic states as has been hypothesized in other symbiotic cnidarians (8). Rather, survival differences between

symbiotic and aposymbiotic C. xamachana could be due to the higher constitutive expression of IRF1 and IRF4 in symbiotic animals, a phenomenon that hasn't been reported in other symbiotic cnidarians (79). IRFs are transcription factors with various immune functions that include activating the expression of a proinflammatory immune response (39,232). IRF expression is both constitutive, providing basal levels of defense against microbes, and inducible, following danger associated molecular pattern (DAMP) recognition (233–235). IRF1 can be activated by toll-like receptors (TLRs) and while *C. xamachana* do not have prototypical TLRs, they have TLR-like proteins similar to what is found in hydra (38,211,236). One of these TLR-like proteins was upregulated in symbiotic controls relative to aposymbiotic controls and upregulated in response to *S. marcescens* regardless of symbiotic state. This pattern recognition receptor could be responsible for the constitutive activation of IRF1 and IRF4 in a symbiotic state and the pathogen induced activation of IRF1 and IRF4. Interestingly, *C. xamachana* seem to have two paralogs of IRF4, one of which has higher constitutive and induced in aposymbiotic animals and the other, along with IRF1, has higher constitutive and induced expression in symbiotic animals. The differential regulation of these transcription factors may underly why symbiotic animals produced a more extreme immune response following *S. marcescens* exposure.

The pathogen response of symbiotic *C. xamachana* shared some similarities with aposymbiotic *C. xamachana*. Regardless of symbiotic state, *C. xamachana* polyps upregulated both upstream pro-inflammatory immune signaling and immune effectors such as MPEG1, a bactericidal protein (50,237). However, there was a slight difference in the types of pro-inflammatory transcription factors upregulated in response to *S. marcescens* between the symbiotic and aposymbiotic animals. Symbiotic animals significantly increasing expression of IRF1, one paralog of IRF4, and NFkB whereas aposymbiotic polyps upregulated IRF1 and both

paralogs of IRF4. Additionally, IRF1 and IRF4 expression in symbiotic polyps was significantly higher when comparing symbiotic and aposymbiotic pathogen exposed replicates. These differences in the regulation of pro-inflammatory transcription factors may explain why while both symbiotic states are synthesizing reactive oxygen species and a mounting a pro-inflammatory innate immune response, symbiotic polyps are upregulating these processes at a greater magnitude than aposymbiotic polyps (38,238). The GO enrichments differentially expressed between symbiotic and aposymbiotic polyps exposed to the pathogen indicate that the high expression levels of ROS production and inflammation is likely resulting endoplasmic reticulum stress in symbiotic polyps (239,240). Thus, symbiotic polyps are accumulating more cellular damage generated by their immune response.

Our gene expression data indicate that symbiotic polyps are experiencing disruptions to endomembrane system homeostasis and the protein folding environment within the cell, likely due to the accumulation of ROS (241). Symbiotic polyps upregulated both the ERAD pathway as well as an unfolded protein response. These pathways both work to remove unfolded proteins from the ER and are indicative of disruptions to protein homeostasis (242). In response to the accumulation of misfolded and/or unfolded proteins the UPR reduces protein synthesis and upregulates chaperonins to attempt to refold or repair the misfolded proteins (240,242). Any proteins unable to be folded are degraded via autophagy or the ERAD pathway (239,240,242). If the cell is unable to correctly repair or refold its proteins or the misfolded proteins accumulate and are not able to be degraded, the UPR transitions into a cell death pathway (239,243). Given symbiotic polyps have considerably more disruptions to protein homeostasis at 24 hours following *S. marcescens* exposure, they likely are transitioning to cell death pathways sooner than aposymbiotic polyps, resulting in lower survival.

Differential expression of genes associated with autophagy is a common component of cnidarian immune response, particularly at relatively early timepoints following pathogen exposure or in disease resistant corals species who aren't as severely impacted by a given pathogen (34,244). In the context of innate immunity, autophagy is cytoprotective and anti-inflammatory, counteracting the damage that secreted inflammatory factors can cause to mitochondria and the endomembrane system (245). Both symbiotic and aposymbiotic polyps significantly changed their regulation of autophagy and had increased acidic organelle activity following exposure to *S. marcescens*. However, given the signatures of higher reactive oxygen species production and ER stress in symbiotic polyps it is likely that upregulation of autophagy is insufficient to counteract the damage caused by their immune response, resulting in the negative regulation of autophagy and transition to cell death pathways (243,245).

Conclusions

Together, our data demonstrate that there is a tradeoff between photosymbiosis and immunocompetence in *C. xamachana*. Underlying this tradeoff is the differential regulation of immune transcription factors both constitutively and in response to *S. marcescens* exposure rather than broadscale immune suppression in symbiotic animals. Symbiotic *C. xamachana* mount a stronger and more damaging inflammatory and reactive oxygen species response following pathogen exposure, resulting in greater disruptions to cellular homeostasis and ultimately in decreased survival rates. The tradeoff between symbiosis and immunity seems to be shared across independent evolutions of the cnidarian-algal symbiosis, as *E. diaphana* are also more susceptible to *S. marcescens* in a symbiotic state (202). With the expanding threat of disease to coral reef ecosystems, this tradeoff could be a major factor in coral disease

susceptibility and dysbiosis via bleaching or nutrient pollution. The cost of hosting symbionts should be investigated further and potentially incorporated into the paradigms of coral disease research (201,246).

Ethics

This study did not require ethical approval from an animal welfare committee.

Data accessibility

Microscopy images, survival data, all shell scripts, and all R code used will be available upon publication in the Data Dryad Repository. Shell scripts and R code are also available on GitHub. All raw sequence data and associated sample metadata will be available in the NCBI SRA database (BioProject ID PRJNA1077944) upon publication. Publicly available data used in this study include the draft *C. xamachana* genome gene models (https://mycocosm.jgi.doe.gov/Casxa1/Casxa1.home.html).

Authors' contributions

M.A.E.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, software, resources, visualization, validation, writing-original draft, writing-review & editing. K.B. methodology, software, writing-review & editing. E.B. software, writing-review & editing. R.B. formal analysis, writing-review & editing. B.D. conceptualization, methodology, writing-review & editing. L.D.M. conceptualization, funding acquisition, supervision, writing- review & editing.

Conflict of Interest Declaration

The authors declare no conflicts of interest.

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Chapter 3 Figures

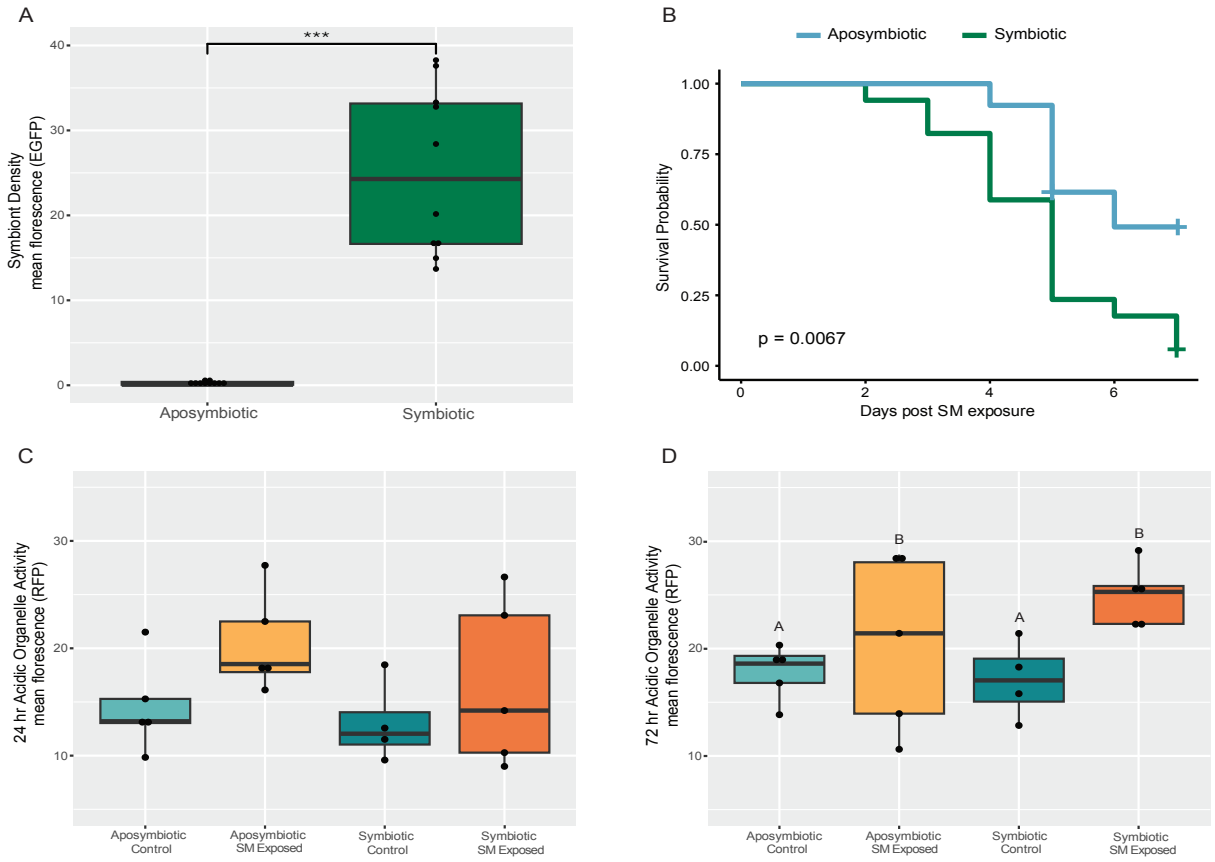


Figure 1. (A) Difference in symbiont density measured as mean eGFP florescence between symbiotic and aposymbiotic polyps. Points represent individual polyp's mean eGFP florescence. *** indicates a p-value < 0.001. (B) Kaplan Myer survival plot comparing survival probability between symbiotic and aposymbiotic polyps following exposure to 10^8 CFU of S. marcescens. Crosses indicate points where individuals lived past the end of S. marcescens exposure. C-D Boxplots of acidic organelle activity measured as mean RFP florescence at (C) 24 hours and (D) 72 hours following S. marcescens exposure. A and B represent groups with a p-value < 0.05 resulting from a two-way ANOVA and Tukey post-hoc test. Points represent individual polyp's mean RFP florescence.

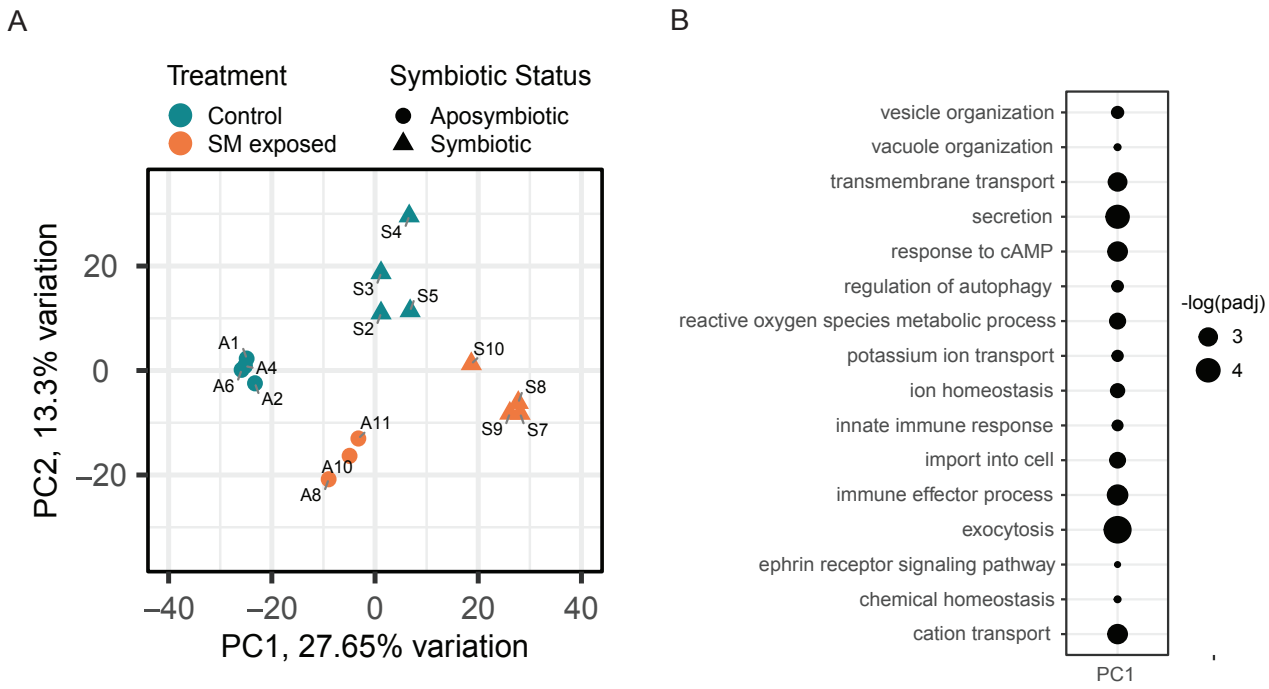


Figure 2. (A) Principal component analysis of rlog normalized count matrix. Teal points indicate control replicates, orange points indicate pathogen exposure replicates, circular points indicate aposymbiotic replicates, and triangular points indicate symbiotic replicates. (B) Rank-based gene ontology enrichments significantly positively associated with PC1. Size of each point is reflective of the -log adjusted p-value of its corresponding term.

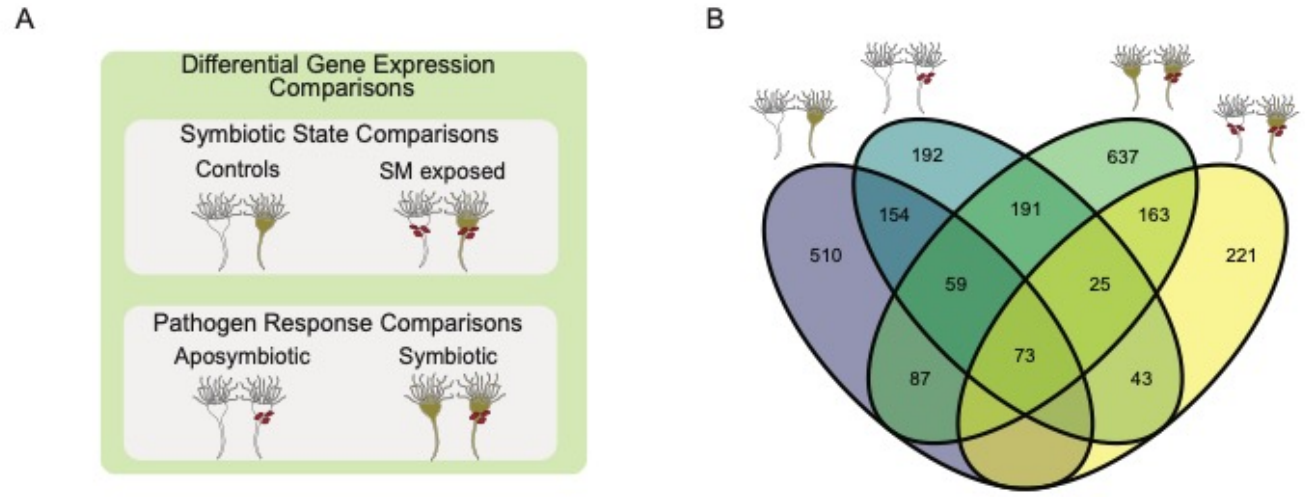


Figure 3. (A) Schematic of the 4 comparisons made during differential gene expression analysis separated into symbiotic state comparisons and pathogen response comparisons. (B) Venn diagram of overlapping differentially expressed genes between all differential gene expression comparisons. Specific comparisons indicated by polyp icons.

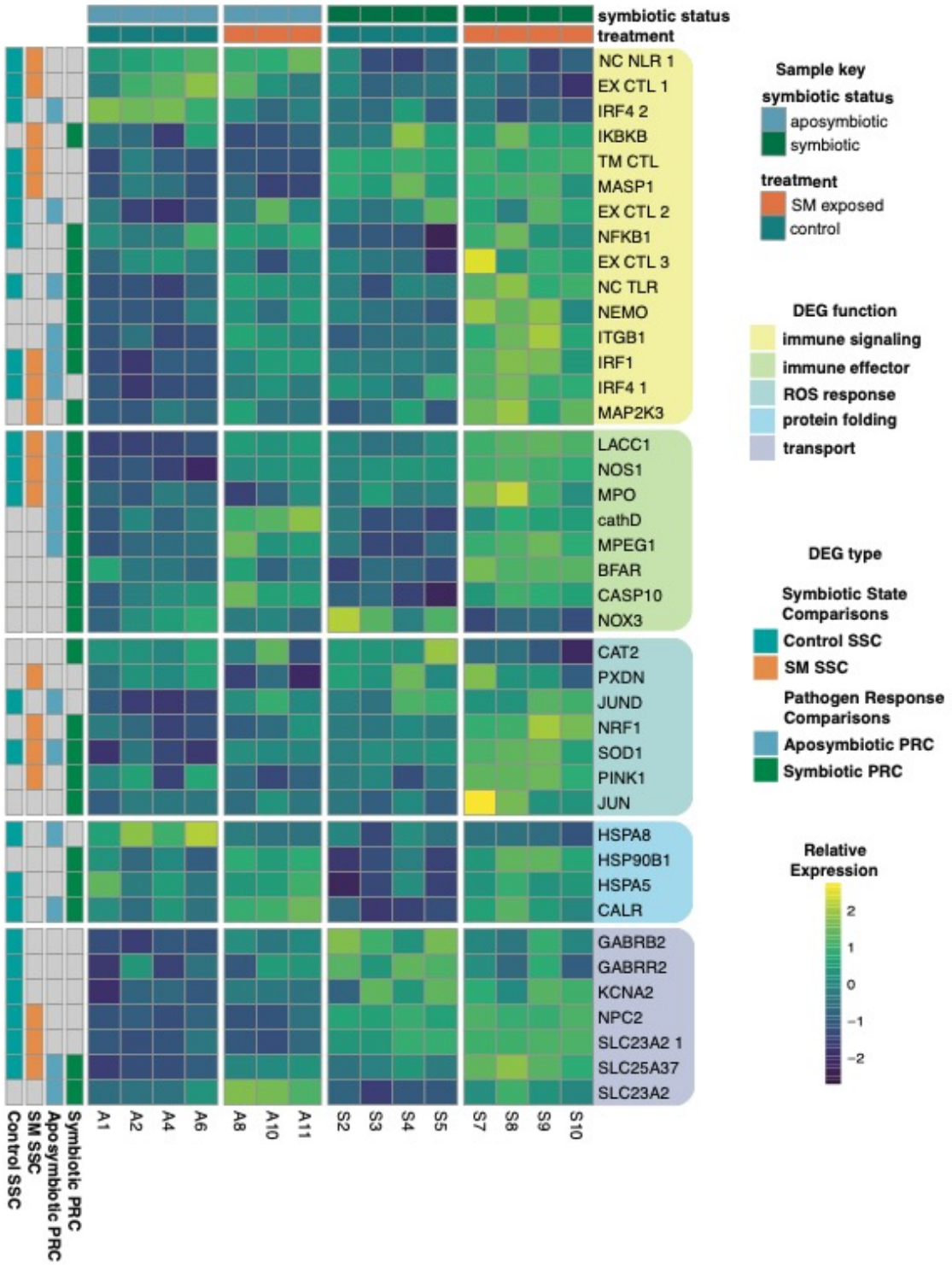


Figure 4. Heat map of select DEGs using relative rlog expression. Column key indicates symbiotic status/treatment of each section. Row key indicates which comparison(s) the gene is differentially expressed in. Rows are grouped by DEG function. PRR abbreviations: NC NLR: non-canonical NLR, EX CTL: extracellular C-type lectin, TM CTL: transmembrane C-type lectin, NC TLR- non-canonical TLR.

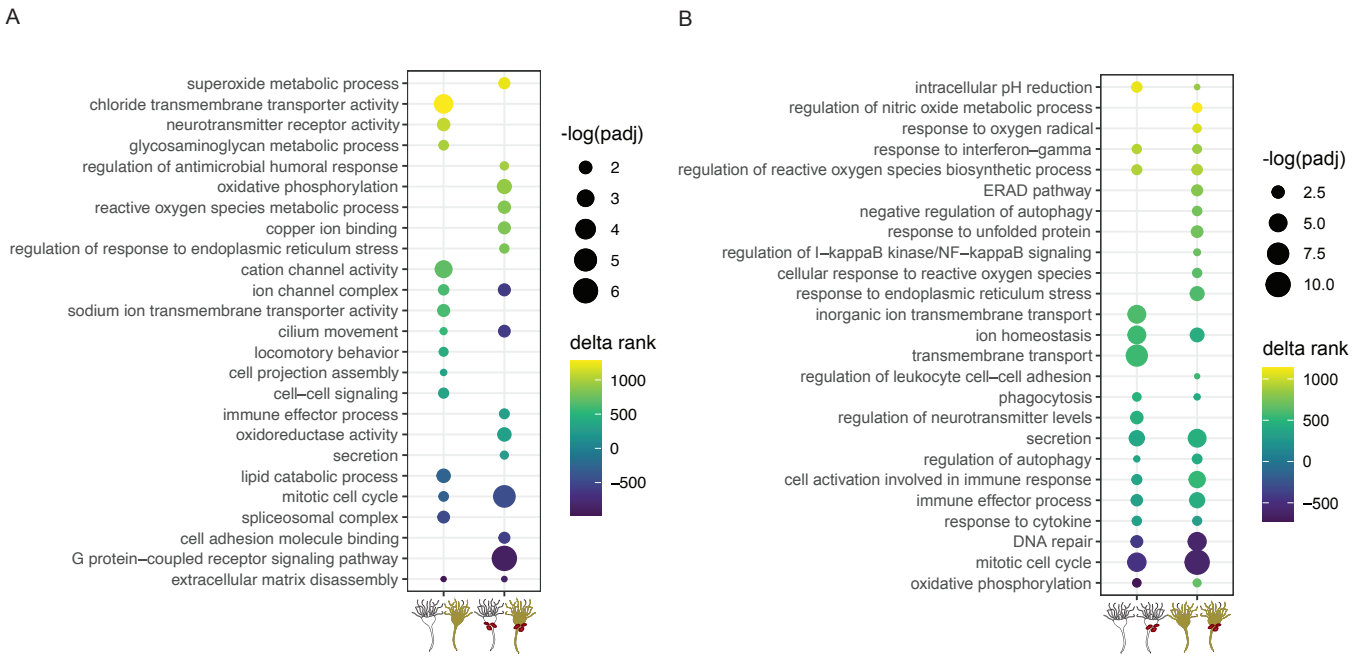


Figure 5. Curated set of rank-based gene ontology enrichments significantly differentially expressed in (A) symbiotic state comparisons and (B) pathogen response comparisons. Color of each point indicates the delta rank of its corresponding term while size indicates the –log adjusted p-value.

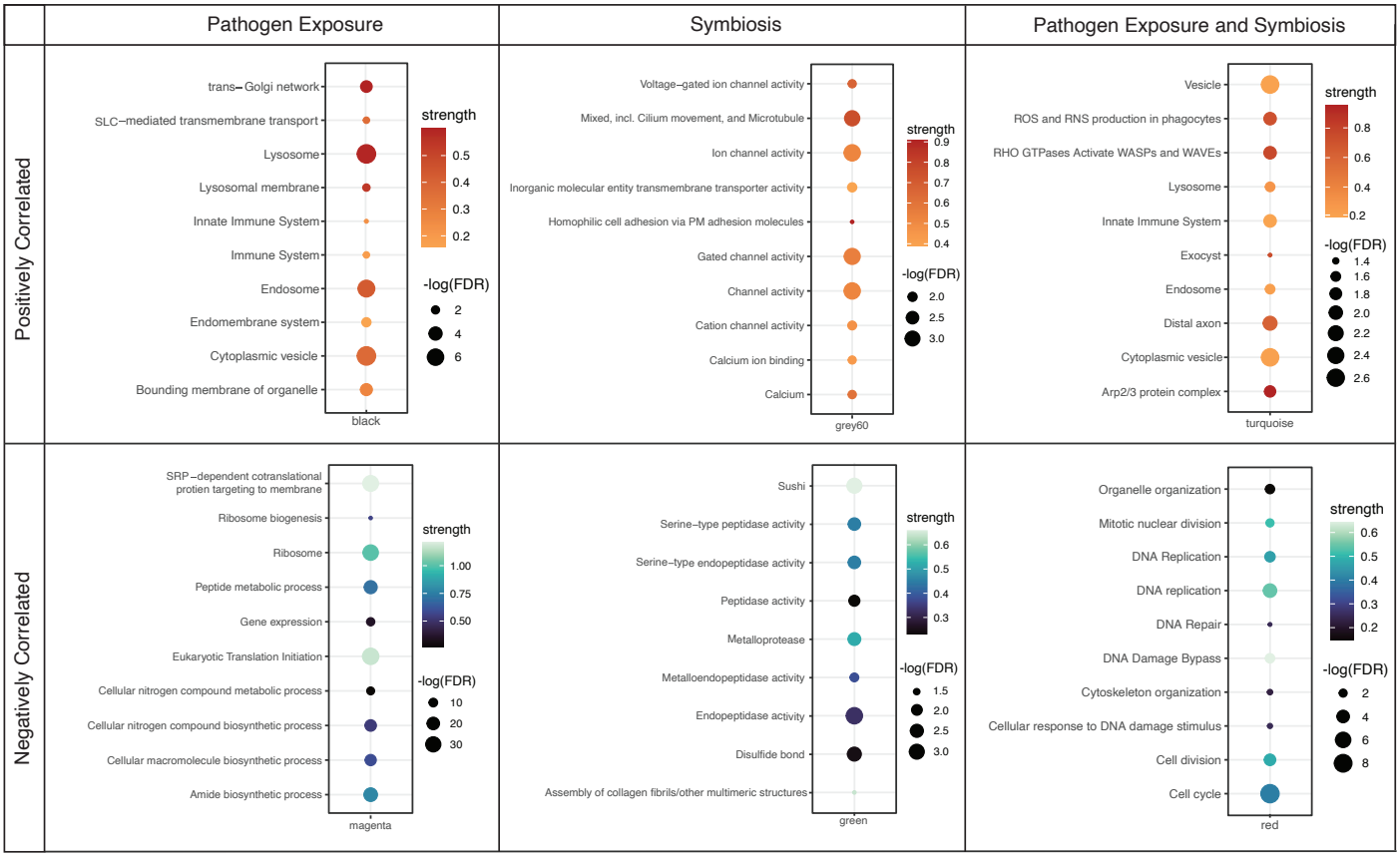
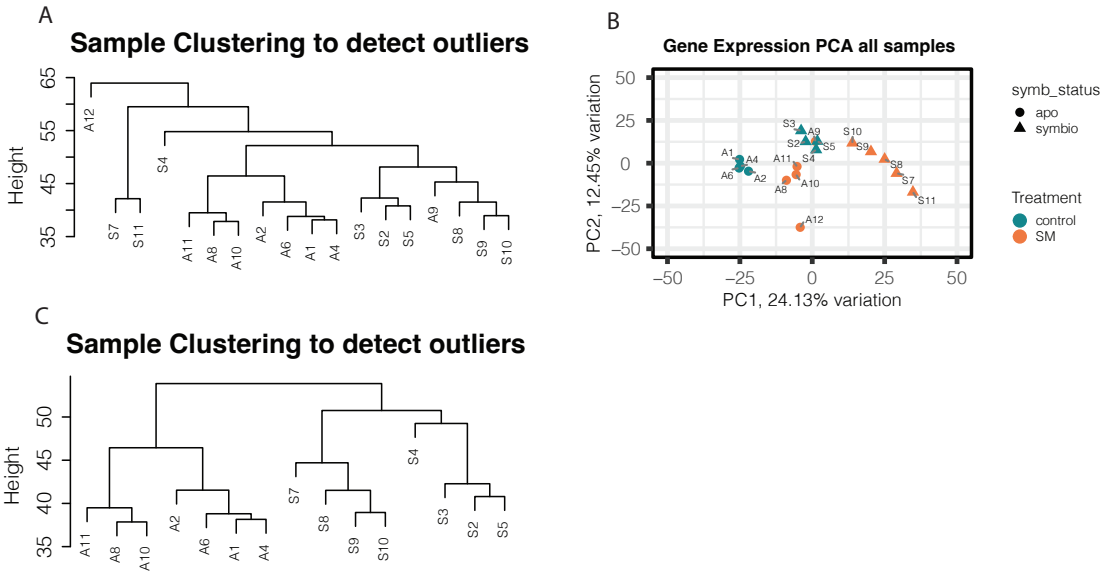
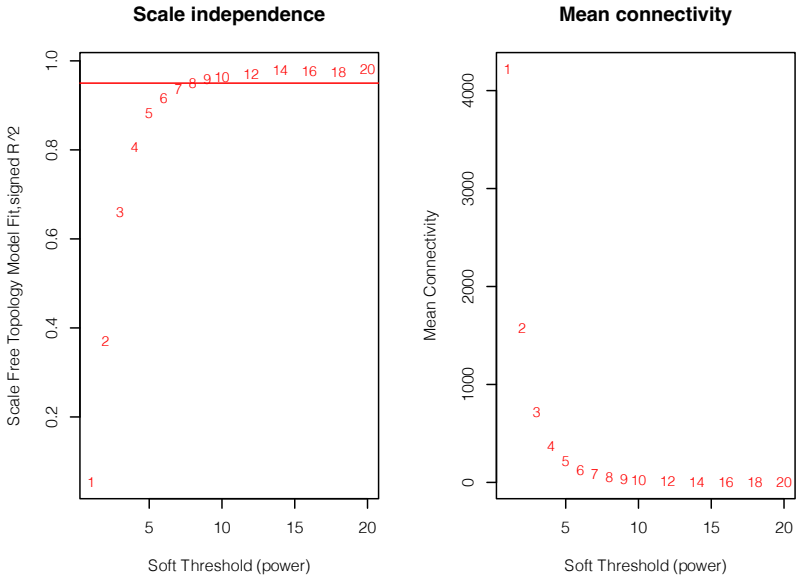


Figure 6. STRING enrichments of WGCNA modules significantly correlated to symbiosis, pathogen exposure, or symbiosis and pathogen exposure. Color of each point corresponds with the strength of the enrichment term and size of each point indicates the –log false discovery rate of the enrichment term.

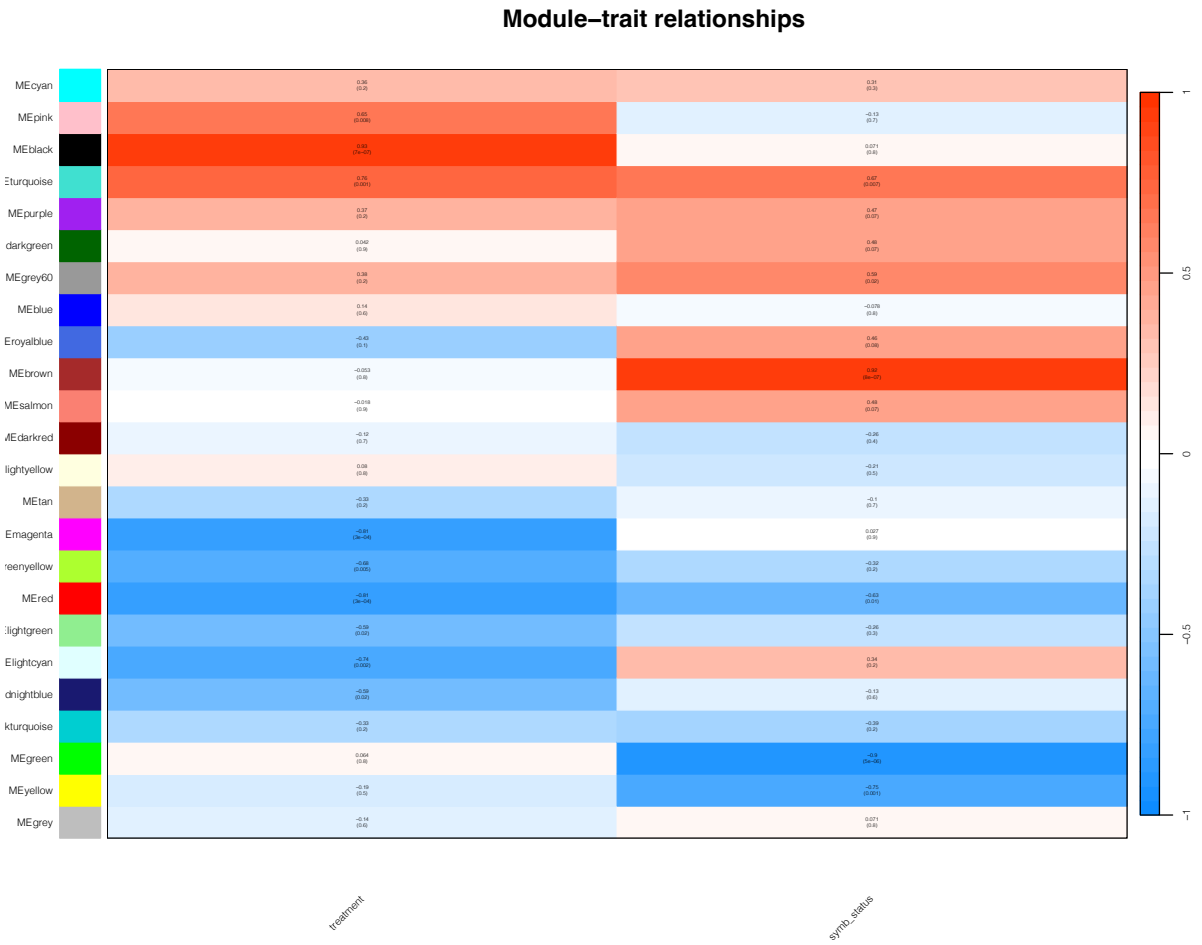
Chapter 3 Supplementary Information



Supplementary Figure 1: Outlier analysis (A) cluster dendrogram of all samples (B) PCA of all samples (C) cluster dendrogram following removal of A9, A12, and S11



Supplementary Figure 2: WGCNA soft power selection (A) scale independence (B) Mean connectivity



Supplementary Figure 3: WGCNA-module trait relationships. Heatmap of Pearson correlations between module eigengene expression and treatment/symbiotic state

Chapter 4: Investigating facultative cnidarian photosymbiosis through a comparative lens using differential gene expression

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Abstract

Photosymbiosis with dinoflagellates in the family Symbiodiniaceae has independently evolved several times in Cnidaria. There are variations in the characteristics of the cnidarian-Symbiodiniaceae symbiosis across its independent evolutions that likely influence how the cnidarian hosts react to different anthropogenic stressors. As such investigating the cnidarian-Symbiodiniaceae symbiosis through a comparative lens can help identify host and taxa specific characteristics as well as define core characteristics of the photosymbiosis that occur across independent evolutions. Therefore, we compared differences in gene expression between symbiotic and aposymbiotic animals in two facultatively symbiotic cnidarians, *Exaiptasia diaphana* and *Cassiopea xamachana* spanning two independent evolutions of the cnidarian-Symbiodiniaceae symbiosis. We found that both species upregulated processes related to ion transport and the nervous systems while hosting symbionts, potentially to provide their symbionts with key nutrients for photosynthesis. Downregulation of lipid metabolism and upregulation of cytoskeletal elements also occurred in symbiotic animals in both species. However, our data provides evidence that the cnidarian-Symbiodiniaceae symbiosis functions very differently in *C. xamachana* and *E. diaphana*. *C. xamachana* showed no evidence of utilizing host gene expression to limit their symbiont's access to nitrogen for population control, whereas *E. diaphana* displayed large changes to nitrogen metabolism while in a symbiotic state. *C. xamacha*na may instead shift in their microbiome to limit their symbiont's nitrogen, contributing to their relatively high tolerance to nutrient stress. The two species also differentially regulate stress response orthogroups in opposite directions during symbiosis, possibly influencing each species' tolerance to various anthropogenic stressors.

Introduction

Various genera of photosynthetic dinoflagellates in the family Symbiodiniaceae form close mutualistic relationships with a wide variety of animal taxa (247). In these photo-symbiotic associations Symbiodiniaceae gain protection and nutrients from their hosts in exchange for providing their hosts with photosynthates (60,197). These mutualisms are present in various invertebrate phyla including Porifera, Cnidaria, Platyhelminthes, and Mollusca (64). Across these phyla, there are a wide variety of strategies hosts employ in acquiring and housing their symbionts. Symbiodiniaceae can be acquired vertically, horizontally or through predation of other symbiotic animals and housed intracellularly or extracellularly depending on the animal taxa (248–250).

Of the various mutualisms Symbiodiniaceae form, the best studied is their intracellular symbiosis with cnidarians. This is due to the cnidarian-Symbiodiniaceae symbiosis being the foundation of coral reef ecosystems, allowing reef-building corals to thrive in otherwise uninhabitable oligotrophic environments (67,198). While photo-symbiosis is best studied in reef building corals and the closely related anemone *Exaiptasia diaphana*, a diverse array of phylogenetically distant cnidarians form mutualisms with Symbiodiniaceae (1,60). The cnidarian-Symbiodiniaceae symbiosis has independently evolved multiple times (1,77). Regardless of these independent evolutions, Symbiodiniaceae are housed intracellularly within cnidarians, though there is variation by taxa of the cell type utilized to house the symbionts (60,61,69). The majority of symbiotic cnidarians, members of Hexacorallia and Octocorallia, house their symbionts in gastrodermal cells (60,69). Whereas Scyphozoans use amebocytes located in their mesoglea to house their symbionts (61,220).

The full mechanisms of symbiont recognition in cnidarians are still unknown and may vary across the independent evolutions of the symbiosis (8,69). Symbiont recognition is often very specific, as many cnidarians have preferred species of Symbiodiniaceae with which they form associations (71,73,251). There likely are multiple stages of recognition occurring both extracellularly and intracellularly, as preferred symbionts are both more likely to be phagocytized and retained to establish their intracellular niche (73,74). During establishment key host immune factors are downregulated and the symbionts are retained in an acidic membrane-bound organelle known as the symbiosome (8,30,69,74,75). Maintaining symbiont populations intracellularly is associated with suppression of immunity in symbiont hosting cells (30,76,78). As such, cnidarians must control their symbiont populations via means other than their immune systems. This is achieved via limiting the symbionts' access to nitrogen (78–82). While the limitation of symbionts' access to nitrogen occurs across independent evolutions of the cnidarian-Symbiodiniaceae symbiosis, the members of the holobiont responsible for this limitation may vary by taxa (82,219,220).

Housing symbionts influences how cnidarians respond to and tolerate various stressors, including elevated temperatures, starvation, pathogens, and nutrient pollution (36,252,253). The different strategies cnidarians employ for housing and maintaining their symbiont populations across independent evolutions may influence stress tolerance. For example, the symbiotic jellyfish *Cassiopea xamachana* primarily digest their symbionts during heat stress rather than primarily expelling them as seen in reef-building corals. This difference in bleaching strategies, which may be influenced by the symbiont housing cell type, could contribute to *C. xamachana's* relatively high tolerance to heat stress (254). It is therefore pertinent to take a comparative approach to investigating the cnidarian-Symbiodiniaceae symbiosis, as identifying host or taxa

specific symbiosis characteristics could contribute to a better understanding of which strategies are more amenable to surviving anthropogenic stressors. Additionally, this comparative approach could help identify how cnidarian life history strategies and the length of co-evolution between cnidarian host and symbiont influence host/taxa specific symbiosis characteristics. Furthermore, comparative studies can be utilized to identify core characteristics of the cnidarian-Symbiodiniaceae symbiosis that occur across independent evolutions (82).

Therefore, we utilized gene expression data from two highly divergent facultatively symbiotic cnidarians, *E. diaphana* and *C. xamachana*, spanning two independent evolutions of the cnidarian-Symbiodiniaceae symbiosis (1). Both species have a strong affinity for a single Symbiodiniaceae genus, with *E. diaphana* preferentially hosting the genus *Brevolium* and *C. xamachana* preferentially hosting *Symbiodinium* (73,251). However, both species are capable of forming stable symbiosis with both *Symbiodinium* and *Brevolium* (72,255,256). These species also house their symbionts in different cell types and have dissimilar life history traits (61,69). As such, comparing gene expression changes between symbiotic and aposymbiotic animals between these two species can help identify candidates for host/taxa-specific characteristics and core characteristics of the cnidarian-Symbiodiniaceae symbiosis.

Methods

C. xamachana RNAseq dataset

Symbiotic *C. xamachana* polyps were obtained from the Dallas Children's Aquarium. All polyps were kept in artificial seawater with a salinity of 35 ppt at 27˚C, fed Artemia nauplii twice per week, and received 50% water changes following every feeding. Symbiotic polyps were kept on a 12:12 light:dark cycle while aposymbiotic polyps were generated by being maintained in the

dark for a minimum of two months. Dark-treated polyps were assessed under a light dissecting microscope and were considered aposymbiotic when they contained less than 10 visible symbionts. To confirm this method resulted in two polyp populations with drastic differences in symbiont densities, 10 polyps from the aposymbiotic population and 10 polyps from the symbiotic population were mounted onto wet mount slides and imaged at 5x under an eGFP filter to detect symbiont autofluorescence using a Zeiss imager Z2 microscope. Mean eGFP fluorescence was used as a proxy for symbiont density and measured in the bell of each polyp using ImageJ (version 1.53t) by subtracting the mean background fluorescence from the mean total florescence (257). This found an average 124-fold difference in mean fluorescence between symbiotic and aposymbiotic polyps, with aposymbiotic polyps having an average mean fluorescence of 0.20 and symbiotic polyps having an average mean fluorescence of 25.26.

Thirty non-clonal polyps per symbiotic state were sampled towards the end of the daily light cycle and pooled into groups of 5 for a total of 6 replicates per treatment group. Replicates were placed into 600 µL of DNA/RNA shield (Zymo: R1100) and then immediately flash frozen. The Zymo RNA miniprep plus kit was used for RNA extraction following tissue homogenization in 1x DNA/RNA shield. Quality control, polyA tail capture, cDNA library preparation was done by Novogene Co. LTD prior to sequencing on an Illumina NovaSeq 6000 using 150 bp paired-end sequencing.

E. diaphana RNAseq dataset

The *E. diaphana* dataset utilized in this study was produced by Baumgarten et al. 2015 and was downloaded from NCBI SRA (BioProject PRJNA261862) using SRAtoolkit (258). A brief summary of their methods is as follows: the CC7 clonal *E. diaphana* line were maintained

at 25˚C in a 12:12 light:dark cycle and fed *Artemia* nauplii twice per week. Aposymbiotic animals were generated with alternating treatments of cold-shock and diuron. Following these treatments animals were maintained in the light for at least a month then tested for the absence of symbionts via fluorescence stereomicroscopy. The symbiotic animals in the replicates used in this study were generated by infecting aposymbiotic individuals with a strain of *Brevolium minutum* and sequenced 30 days following infection. Samples from four biological replicates (individual CC7 anemones) per symbiotic state were collected for RNA sequencing at the midpoint of the 12h light cycle. RNA was extracted using a phenol-chloroform extraction protocol and mRNA was isolated using Dynabeads oligo$(dT)_{25}$. Following quality control and cDNA library prep samples were sequenced on an Illumina HiSeq2000 using 101 paired-end sequencing (126).

Read Processing

Raw reads were trimmed with Fastp (version 0.23.3) before being mapped to their respective gene models using Salmon (version 1.9.0) (124,126,207,208). The corresponding protein models for both species were annotated using eggNOG mapper (version 2.1.12) (209). Orthologous genes between the two species were identified using Orthofinder (version 2.5.2-0) (155). A third facultatively symbiotic cnidarian species, *Astrangia poculata*, was included in the Orthofinder input to improve ortholog identification (259).

Differential gene expression analysis

Tximport was used to generate a count matrix and correct for biases due to GC content and length (212). First, a count matrix of individual gene expression was generated for each

species, with all genes being included regardless of if they received an annotation from eggNOG. Next, differential gene expression analysis was run individually on each species using DESeq2 (216). Log2 fold change output of DESeq2 was used as input for rank-based gene ontology (GO) enrichments associated with housing symbionts in each species using GO-MWU (260). The results of each species individual GO enrichment results were then compared to identify shared significantly differentially expressed GO terms between the two species.

Differential orthogroup expression analysis

An additional a count matrix of summarized orthogroup expression was generated using Tximport as in Beavers et al. 2023 (88). Differential orthogroup expression between symbiotic states in each species was called using an individual DESeq2 analysis for each species. Overlapping differentially expressed orthogroups (DEOGs) were identified and annotated using a representative *E. diaphana* gene model that was a member of each orthogroup. Overlapping DEOGs being differentially expressed in the same direction between species and overlapping DEOGs being differentially expressed in opposite directions between species were tested for STRING enrichments using a STRING annotation of the *E. diaphana* gene models (organism ID STRG0A90ZHD) (210).

Results

In the *C. xamachana* dataset an average of 12 million reads per sample (56.2%) mapped to the reference gene models while the *E. diaphana* had an average of 6.5 million reads mapped per sample (51.64%) (supplemental file 1). One *E. diaphana* sample was excluded from analysis due to a paired end read being unavailable on the NCBI SRA. This resulted in eight *C. xamachana*

samples, four aposymbiotic and four symbiotic, and seven *E. diaphana* samples, three aposymbiotic and four symbiotic. After filtering genes and orthogroups with low expression *C. xamachana* had count data consisting of 17,086 expressed genes and 9,327 expressed orthogroups. *E. diaphana* had count data consisting of 19,806 expressed genes and 12,015 expressed orthogroups.

Differential gene expression analysis

There was a log scale difference in differentially expressed genes (DEGs) with symbiosis between the two species. *C. xamachana* had 898 DEGs while *E. diaphana* had 9710 (supplemental file 2). Because of this log-scale difference, rank-based gene ontology enrichments were performed on each species individually to summarize the biological processes, molecular functions, and cellular components with significant differences in expression between the symbiotic states.

In *C. xamachana* 52 biological processes, 26 molecular functions, and 28 cellular components were differentially expressed between symbiotic and aposymbiotic animals (supplemental file 3). The majority of the top ten most upregulated biological processes and molecular functions in symbiotic *C. xamachana* were related to the nervous system and/or ion transport (Figure 1A,1C). This trend was also seen in the top ten upregulated cellular components, which included “main axon”, “dendritic shaft”, “synaptic membrane”, and “postsynapse” (supplemental figure 1). There was more variation in the most highly downregulated GO terms for each category in *C. xamachana*. Highly downregulated GO terms include “RNA processing”, “lipid catabolic process”, “cell cycle phase transition”, and “spliceosomal complex” (Figure 1A,1C, supplemental figure 1).

E. diaphana differentially expressed 661 biological processes, 137 molecular functions, and 121 cellular components between symbiotic and aposymbiotic animals (supplemental file 3). There was more variation in the types of biological processes and molecular functions most upregulated in *E. diaphana* relative to *C. xamachana*. Terms related to GTPase activity and signal transduction, cell cycle regulation, gated ion channels, and microtubule motor activity were all among the top ten most upregulated biological processes and molecular functions (figure 1B,1D). Several nervous system related biological process and molecular function GO terms were also upregulated, including the gamma-aminobutyric acid signaling pathway (Figure 1D, supplemental file 3). Many of the 10 most significantly downregulated GO terms in these categories were related to nitrogen metabolism, including “cellular amide metabolic process”, “peptide metabolic process”, and “ammonium transmembrane transporter activity”. “Antioxidant activity” and “oxidoreductase activity acting on peroxide” were also among the top 10 most significantly downregulated molecular functions (Figure 1C, 1D). While not in the top ten most significantly downregulated biological processes, both “response to interferon beta” and “immune effector response” were significantly downregulated with symbiosis (supplemental file 3).

A total of 49 GO terms were significantly differentially expressed between symbiotic states across both species, 22 biological processes, 14 molecular functions, and 13 cellular components (Figure 2, supplemental figure 2). Both species upregulated terms related to ion transport and the nervous system in a symbiotic state (figure 2). Additionally, both species upregulated terms related to cilium assembly, cilium movement, and actin filament-based processes (figure 2A). Interestingly, the species expressed GO terms related to cell cycle regulation differently while in a symbiotic state, with *E. diaphana* upregulating them and *C.*

xamachana downregulating them (Figure 2A). Similarly, the term “condensed chromosome” was upregulated in *E. diaphana* but downregulated in *C. xamachana* (supplemental figure 2). The term “amino acid activation” followed the same trend. Terms related to lipid metabolism were downregulated in symbiotic animals in both species (Figure 2A).

Differential orthogroup expression analysis

Similar to the differential gene expression analysis the differential orthogroup expression analysis found log scale differences in the number of differentially expressed orthogroups (DEOGs) between the two species. *C. xamachana* had 674 DEOGs between symbiotic states whereas *E. diaphana* had 6676 (Figure 3A, supplemental file 4). There were 276 overlapping DEOGs between the two species (Figure 3A). Of these 276 over half (53.3%) were being differentially expressed in opposite directions (Figure 3B). These oppositely regulated DEOGs between *C. xamachana* and *E. diaphana* had STRING enrichments for “metabolism of amino acids and derivatives”, “FAD binding”, “cellular amino acid metabolic process”, and “anion binding” (Figure 3C). The remaining 46.7% of the overlapping DEOGs were differentially expressed in the same direction across the two species and had STRING enrichments for “collagen formation”, “extracellular matrix organization”, and “disulfide bond” (Figure 3D).

Within the 276 overlapping DEOGs between the two species were 47 orthogroups with a GO annotation for “response to stress”, 63% (30/47) of which were being differentially expressed in opposite directions. This group included orthogroups annotating as antioxidants like SOD1 and PRDX5, immune transcription factors IRF1 and IRF4, a heat shock protein Hsp60, and DICER1. Interestingly, the expression patterns of IRF and IRF4 are inversed between the species, with one IRF orthogroup up and down regulated in each species (Figure 4A-B). The overlapping DEOGs also included 102 orthogroups with a GO annotation of “metabolic process”

88 of which also had an annotation for “nitrogen compound metabolic process”. The majority of the orthogroups (57/88) with the “nitrogen compound metabolic process” GO annotation were being expressed in different directions between the two species. On such orthogroup is GLUT, which was downregulated with symbiosis in *C. xamachana* and upregulated with symbiosis in *E. diaphana* (Figure 4C-D). There were some instances where different orthogroups annotating as the same gene were differentially regulated in each species, indicating that genes with similar functions were changing their expression similarly across species though each species was utilizing different orthogroups for that function. Both species upregulated different orthogroups annotating as MASP and GABA receptors (supplemental file 4).

Discussion

Our results indicate that hosting symbionts induces large changes in host gene expression across independent evolutions of the cnidarian-Symbiodiniaceae symbiosis. We found that regardless of species, ion transport and nervous system processes were upregulated in animals hosting Symbiodiniaceae while lipid metabolism was largely downregulated. However, our results suggest major differences in the cellular and molecular mechanisms utilized by *C. xamachana* and *E. diaphana* to maintain intracellular symbiont populations. Specifically, we find evidence for differences in the strategies these species utilize to limit their symbionts’ access to nitrogen, as well as opposing patterns of differential regulation in cell cycle and stress response genes during symbiosis.

Although *C. xamachana* and *E. diaphana* both exhibited large changes in gene expression between symbiotic states, there was a log-scale difference in DEGs and DEOGs between the two species. This likely is not due to differences in read depth or mapping rates

between the species as *E. diaphana* on average had a lower number of reads per sample and lower mapping rates than *C. xamachana*. Rather, it is likely that this log-scale difference in differential expression is due to differences in experimental design between the two datasets used in this study. Overall, the *E. diaphana* dataset had less variance than the *C. xamachana* dataset, as it utilized a single clonal line infected with a single symbiont strain and had a more stringent definition of aposymbiotic animals. In contrast, the *C. xamachana* dataset utilized pools of individuals from the same population housing symbionts of unknown species in the genus *Symbiodinium* and aposymbiotic animals had extremely small numbers of symbionts still present in their tissue. These incongruencies between the datasets could result in the *E. diaphana* dataset having much higher power to detect differences between symbiotic and aposymbiotic animals. Another possible contributing factor to the log-scale differences in differential gene expression is the length of time between symbiont infection and sequencing in each species. The symbiotic *E. diaphana* were sequenced just thirty days post symbiont infection, whereas the symbiotic *C. xamachana* population had been hosting symbionts for over a year at the time of sequencing. As such, the more recently established *E. diaphana* symbiosis may cause greater changes in gene expression relative to the *C. xamachana* dataset (126).

Despite the large differences in scale of differential expression between the two datasets, we found multiple lines of evidence indicating both species upregulate ion transport and genes related to the nervous system when in a symbiotic state. *C. xamachana* seem to induce stronger changes in these processes with symbiosis relative to *E. diaphana*. However, the number and strength of ion transport and nervous system related GO enrichments that occur in both species across independent evolutions of the cnidarian-Symbiodiniaceae symbiosis indicates that they play a vital role in stable symbiosis management. The exact function these processes play in

maintaining stable symbiont populations intracellularly is unclear, though they may help symbionts escape host immune effector responses or provide the symbionts with key nutrients for symbiosis (227,261,262).

Symbiodiniaceae, like closely related intracellular protozoan parasites, could induce changes in host ion transport to decrease membrane excitability and prevent host cells from mounting a nitric oxide response (224,226–228,263). Alternatively, changes in ion transport could serve to provide the symbionts with key elements needed for photosynthesis. *Symbiotic C. xamachana* and *E. diaphana* both significantly upregulated potassium transport, which is a metal required for photosynthesis (262). Potassium deficiency results in decreases to photosynthesis and growth in plants, algae, and cyanobacteria (262,264,265). Additionally, it can be protective against reactive oxygen species production and photooxidative damage in plants (266). Similarly, chloride transmembrane transporter activity was upregulated in both species possibly due to chloride's role in photosynthetic oxygen evolution (267). Calcium, copper, and zinc transporters were also upregulated genes or orthogroups in both species. These ions can also facilitate photosynthesis, though copper and zinc levels must be balanced to prevent toxic effects (261,268–273). Even processes specific to the nervous system, like the upregulation of GABA receptors in both species and the upregulation of the GABA signaling pathway in *E. diaphana*, could be linked to symbiosis. While GABA is best known as an inhibitory neurotransmitter in animals, it is metabolized differently in plants and is accumulated in response to certain stressors due to its ability to enhance photosynthesis, reduce reactive oxygen species generation, and activate antioxidants (274). If Symbiodiniaceae are capable of metabolizing GABA in the same way as land plants, cnidarian hosts may be supplying their symbionts with GABA to enhance photosynthesis and reduce concentrations of ROS (274).

Both *C. xamachana* and *E. diaphana* altered expression of lipid metabolism while hosting symbionts. Although the two species showed opposite regulation of lipid oxidation during symbiosis, lipid metabolism and lipid catabolism were downregulated in symbiotic animals regardless of species. This trend aligns with previous studies on the cnidarian-Symbiodiniaceae symbiosis that found decreases in lipid catabolism to be indicative of increased energy stores that characterize a stable symbiosis with a host's preferred symbiont (275,276). Additionally, we found evidence for the differential regulation of the sphingosine rheostat in both species. The final enzyme in the sphingolipid degradation pathway, SGPL1, was a DEOG shared between the two species. This enzyme degrades sphingosine-1-phosphate attenuating its pro-survival, pro-cell proliferation signal (277). The sphinogsine rheostat has been found to be altered by the presence of Symbiodiniaceae in *E. diaphana*, with increased sphingosine-1-phosphate concentrations in symbiotic anemones shifting the rheostat towards cell survival and proliferation (278,279). *C. xamachana's* downregulation of SGPL1 is in line with this pro survival shift in the rheostat while hosting symbionts (277). In contrast, the upregulation of SGPL1 in *E. diaphana* may contradict previous studies on the sphingosine rheostat activity during symbiosis in this species. However, population-based differences in the extent to which sphingosine-1-phosphate concentrations increase during symbiosis have reported. As such the clonal line of *E. diaphana* (CC7) used in this study may experience less extreme shifts in the sphingosine rheostat as a result of hosting Symbiodiniaceae through the upregulation of SGPL1 (278).

Terms related to the cytoskeleton, like "microtubule bundle formation" and "actin-filament-based process" were upregulated with symbiosis regardless of species. Additionally, the shared DEOGs being expressed in the same direction in both species were enriched for terms

related to the extracellular matrix. This could be due to the increased need for intercellular and intracellular trafficking due to the presence of Symbiodiniaceae derived photosynthates in the cnidarian hosts (60). Additionally, these photosynthates change the osmotic balance in symbiont housing cells causing them to increase in volume during the day, requiring cytoskeletal and extracellular matrix changes (280). It is unlikely that upregulation of cytoskeletal terms is due to changes in cell cycle regulation during symbiosis in both *C. xamachana* and *E. diaphana*, as the two species largely regulated cell cycle related GO terms in opposite directions. Generally, *E. diaphana* upregulated terms related to the mitotic and meiotic cell cycles while *C. xamachana* downregulated terms related to mitosis. The exception to this trend was the upregulation of "negative regulation of cell cycle process" in *E. diaphana* and downregulation of the same term in *C. xamachana*. Upregulation of the cell cycle is commonly seen in symbiotic cnidarians relative to their aposymbiotic counterparts (78,256,281). As such, the majority of these processes being downregulated in *C. xamachana* is unusual. Differences in cell cycle regulation between *C. xamachana* and other symbiotic cnidarians may be due to the major differences in their life cycles. This study uses *C. xamachana* polyps, which are not capable of sexual reproduction and are only able to strobilate when hosting symbionts (68,282). Strobilation is the energetically expensive process, so symbiotic *C. xamachana* polyps may invest the photosynthates gained from their symbionts into energy stores to be able to regenerate following ephyra production rather than into cell proliferation and growth (68,72).

We found multiple lines of evidence indicating that nitrogen metabolism is not regulated the same way during symbiosis in *C. xamachana* and in *E. diaphana*. Specifically, in line with prior research we found ample evidence of altered nitrogen metabolism, specifically altered ammonium transport in the most highly differentially regulated GO terms of *E. diaphana*, while

these terms were not significantly differentially regulated at all in *C. xamachana* (79–81). While the absence of these GO enrichments could be due to the differences in power between the two gene expression datasets, the presence of these terms in the top 10 most significantly differentially regulated *E. diaphana* GO terms and their absence in *C. xamachana* indicate at the very least that *C. xamachana* modulates its nitrogen metabolism to a much lesser extent while hosting symbionts relative to *E. diaphana* and likely other symbiotic anthozoans (77–79,121). This is notable, as there is ample evidence that cnidarians limit their intracellular symbiont populations through nitrogen limitation, specifically through recycling ammonium via the glutamine synthase/glutamate synthase (GS/GOAT) system (79,81). Interestingly, despite the lack of transcriptomic changes to *C. xamachana's* nitrogen metabolism during symbiosis, there is evidence that their symbionts are deprived of inorganic nitrogen in hospite (82,219,220). This may be due to shifts in symbiotic *C. xamachana's* microbiome towards ammonium oxidizing and nitrite reducing bacteria, a strategy that may be made possible by their symbionts being hosted in cells in the mesoglea rather than in cells in the gastrodermis (219). *C. xamachana's* utilization of its microbiome to limit its symbiont's access to nitrogen rather than the alteration of host nitrogen metabolism may explain why its symbiont cell densities are the least responsive to ammonium relative to other cnidarians in clade Anthozoa (82,219). Thus, it is likely that *C. xamachana's* strategy conveys greater resilience to nitrogen nutrient pollution (219).

Differences between *C. xamachana* and *E. diaphana* in stress response orthogroup expression could similarly lead to differences in tolerance to various stressors. Unlike previous studies we found no significant downregulation of NFκB in either species, with NFκB actually being upregulated in the *E. diaphana* dataset (30,75,253). This could be due to the amount of time the anemones had been infected with symbionts prior to sequencing or regulation of NFκB

post transcriptionally as protein and transcript concentrations do not necessarily indicate transcription factor activity (253). Unlike *E. diaphana*, *C. xamachana* downregulated NFκB at a nearly significant level. Instead of significant downregulation of NFκB, we found that both species upregulated and downregulated an orthogroup of a different immune transcription factor family, interferon regulatory factor (IRF) (39). While *C. xamachana* and *E. diaphana* did not change the expression of the same IRF orthogroup, this suggests that IRFs may also play a role in symbiotic cnidarians shifting their immune expression to accommodate intracellular symbionts.

Despite changes in immune transcription factor expression in both species, only *E. diaphana* had some evidence via gene ontology enrichments of mass downregulation of immune effectors. As there is evidence in *C. xamachana* and *E. diaphana* of a tradeoff between symbiont derived nutrition and the ability to survive bacterial pathogens, it is notable that neither species showed evidence of drastic downregulation of immunity in symbiotic animals relative to aposymbiotic animals (253). Given that well over half of the DEOGs with a "stress response" annotation were being expressed in opposite directions between the two species, the mechanism of greater pathogen susceptibility in symbiotic animals may be different between the two species. As *C. xamachana* upregulate orthogroups PRDX5 and SOD1 while *E. diaphana* downregulate them, symbiotic *C. xamachana* may have constitutively higher levels of ROS and thus be more prone to ROS damage during a stress response than symbiotic *E. diaphana*.

Overall, across two independent evolutions of the cnidarian-Symbiodiniaceae symbiosis we found evidence that ion transport and nervous system processes are upregulated in symbiotic animals, potentially to provide Symbiodiniaceae with key nutrients for photosynthesis. *C. xamachana* and *E. diaphana* also similarly regulated lipid metabolism and cytoskeletal elements

in symbiotic animals relative to their aposymbiotic counterparts. Despite these similarities, our data provides evidence that the cnidarian-Symbiodiniaceae symbiosis functions very differently across independent evolutions of the trait. Specifically, *C. xamachana* do not seem to utilize host gene expression to limit their symbionts' access to nitrogen, potentially contributing to their high tolerance to nutrient pollution. *C. xamachana* and *E. diaphana* also largely differentially regulate stress response orthogroups in opposite directions while hosting symbionts, which could contribute to differences in various stress tolerances between the two species.

Chapter 4 Figures

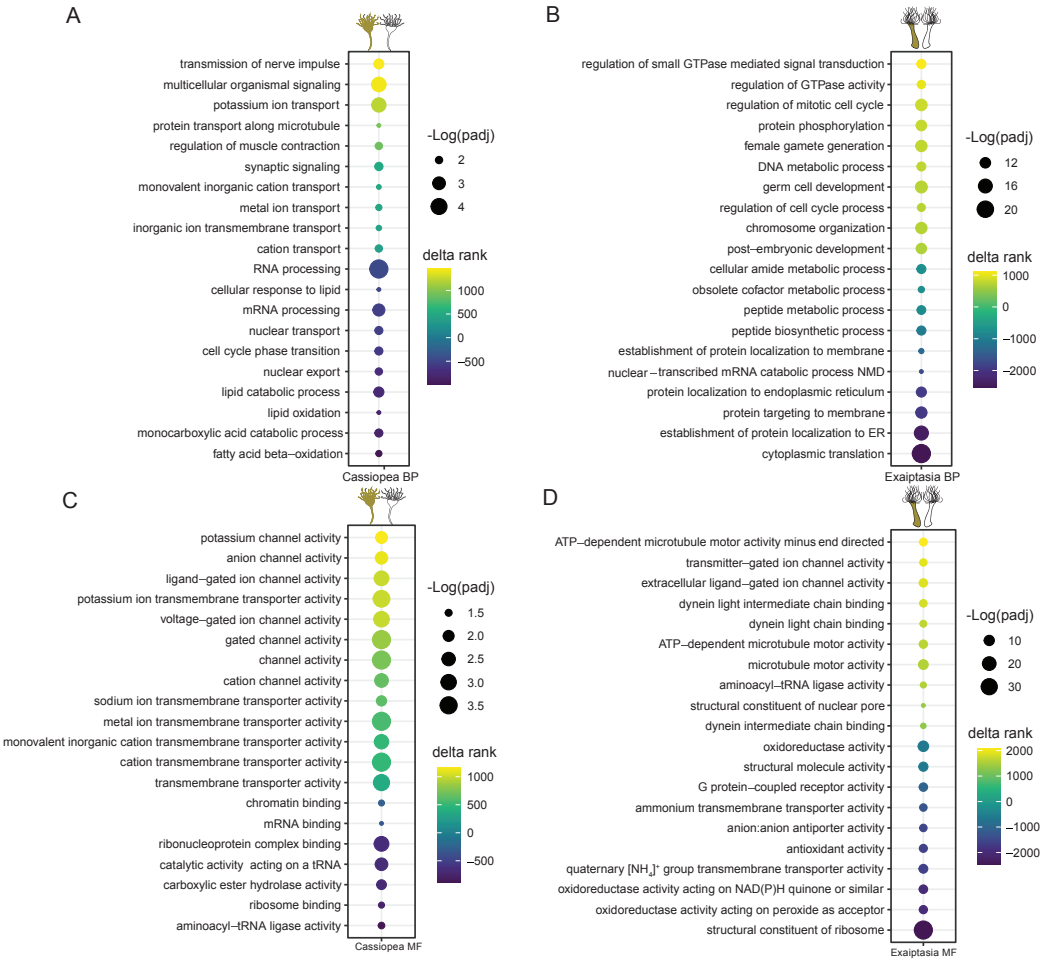


Figure 1: Most significantly up and down regulated GO terms in symbiotic animals relative to aposymbiotic animals. (A-B) Most significantly differentially regulated biological processes with symbiosis in A) *C. xamachana*, B) *E. diaphana*. (C-D) Most significantly differentially regulated molecular functions with symbiosis in C) *C. xamachana* D) *E. diaphana.*

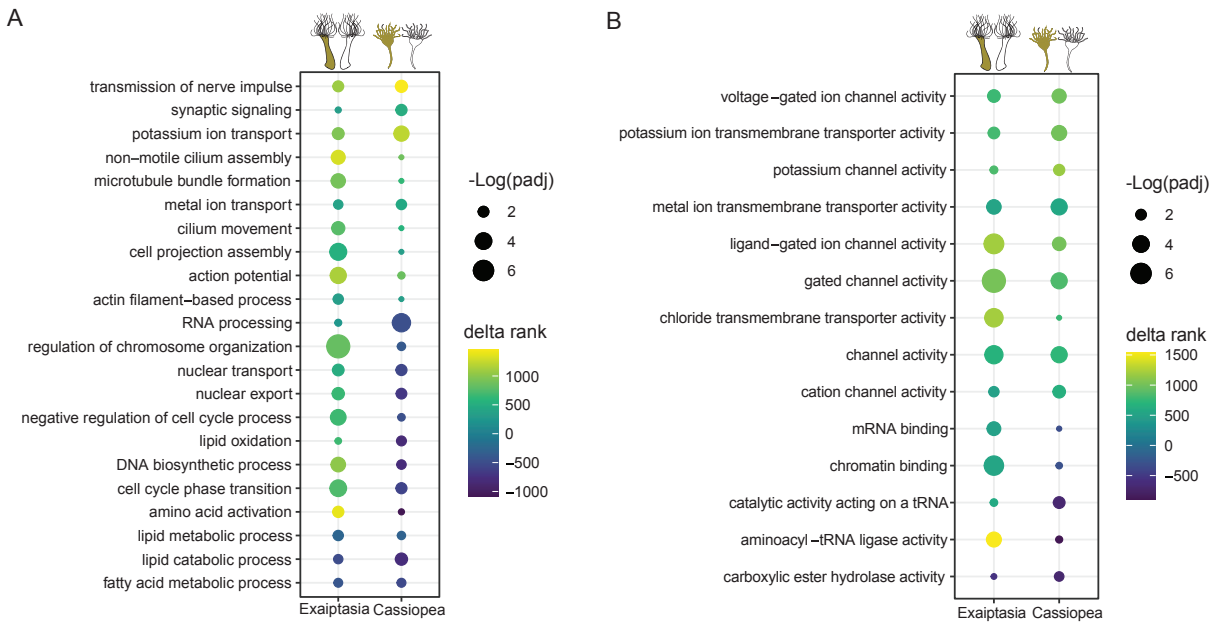


Figure 2: GO terms in the categories A) biological process and B) molecular function differentially regulated in symbiotic animals relative to aposymbiotic animals in both species.

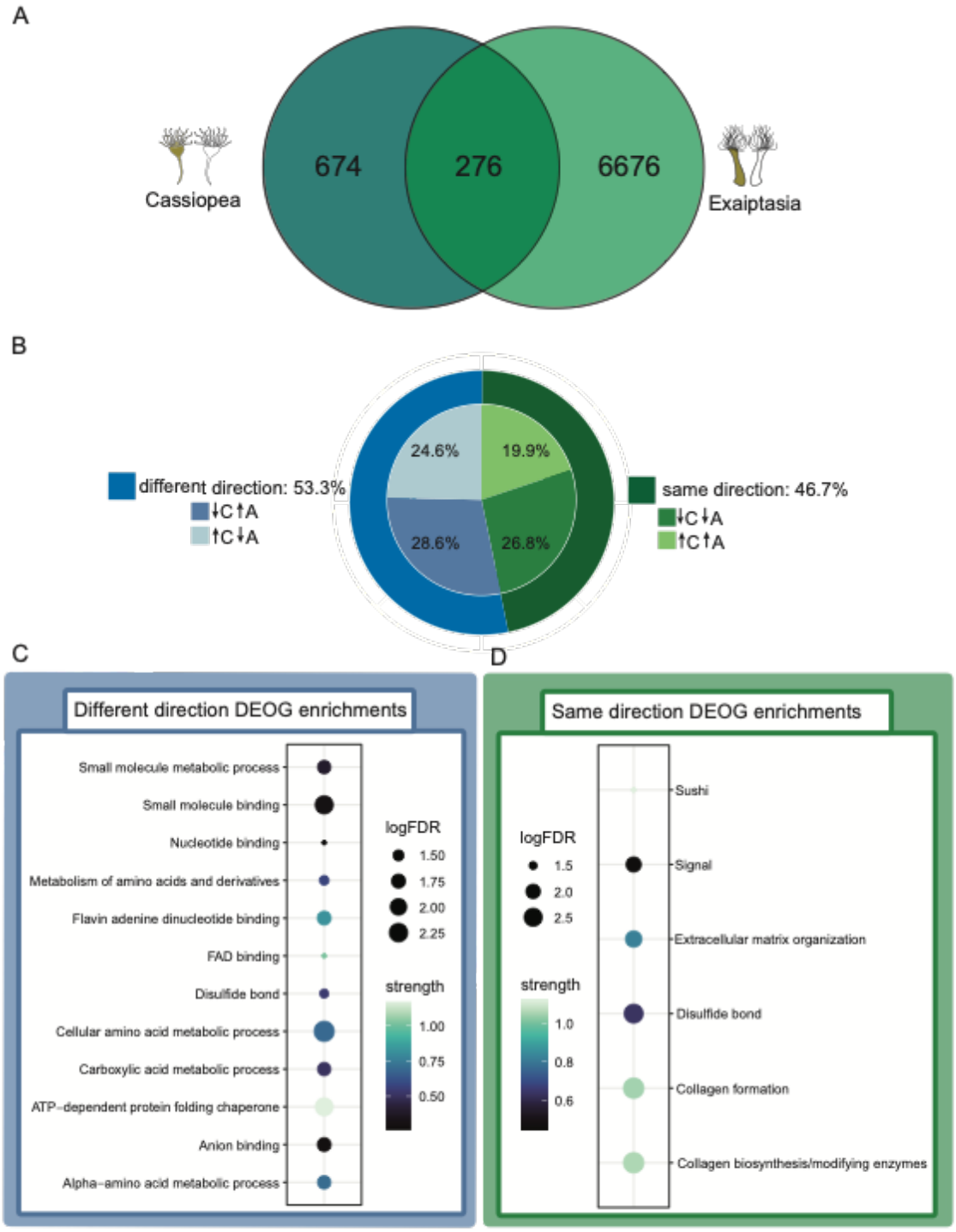


Figure 3: Overview of composition of the shared DEOGs between *C. xamachana* and *E. diaphana*. A) DEOG number and overlapping DEOGs between *C. xamachana* and *E. diaphana* B) directionality of overlapping DEOGs between the species. C) STRING enrichments for DEOGs being expressed in opposite directions during symbiosis in each species D) STRING enrichments for DEOGs being expressed in the same direction in both species during symbiosis

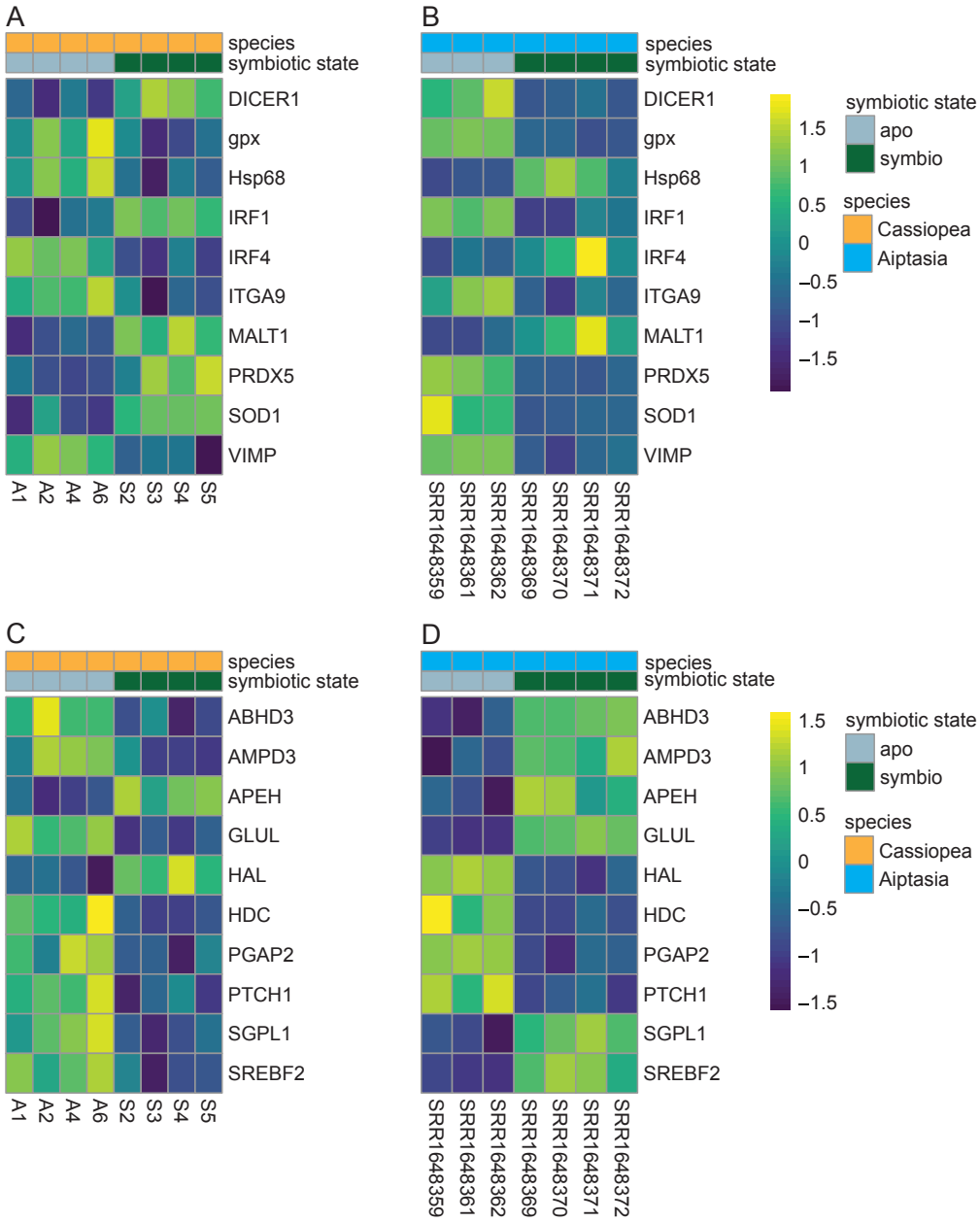
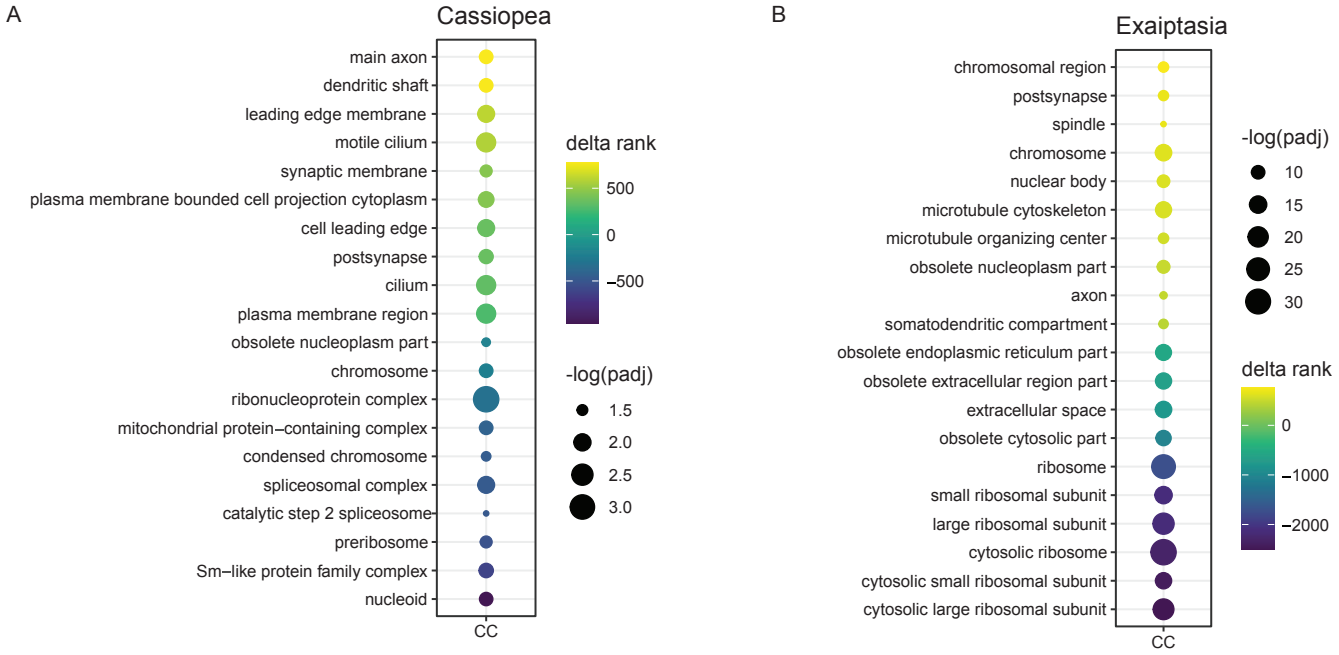
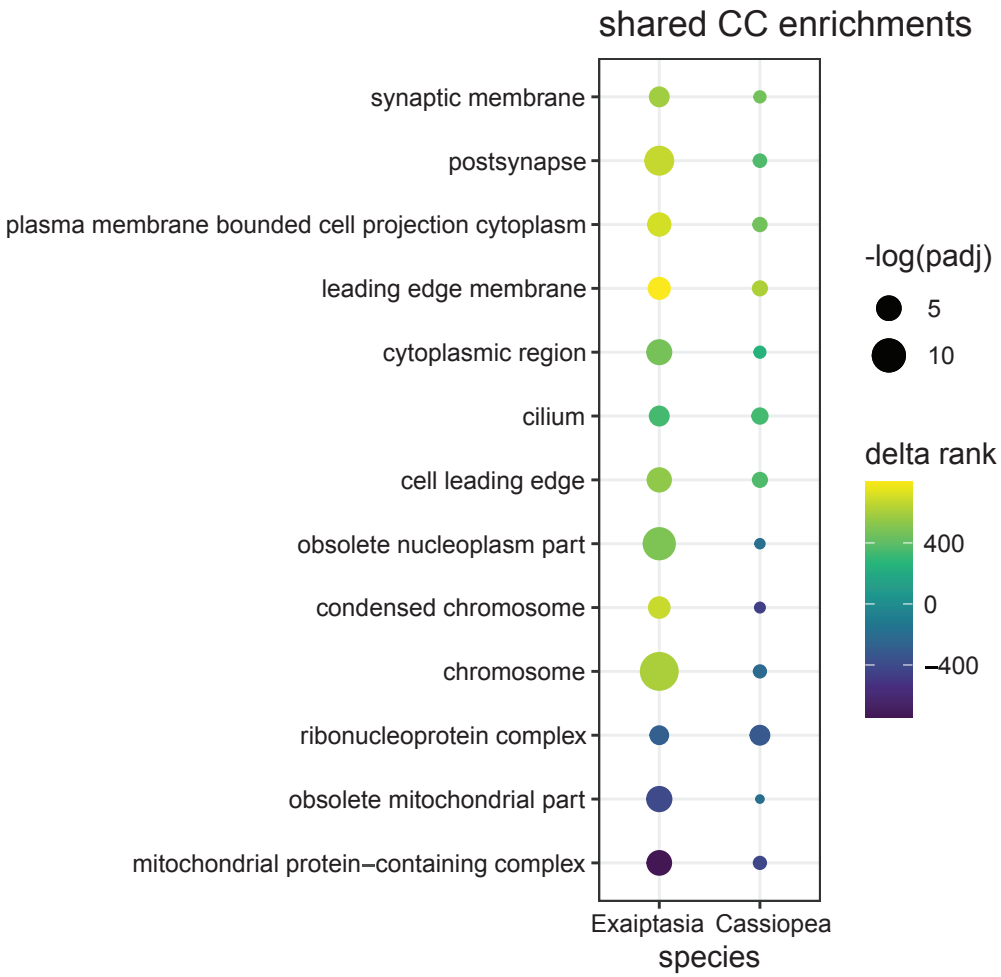


Figure 4: Heat maps depicting relative rlog expression of selected shared DEOGs in each species. (A-B) stress response DEOGs in (A) *C. xamachana* and (B) *E. diaphana*. (C-D) metabolism DEOGs in (C) *C. xamachana* and (D) *E. diaphana*

Chapter 4 Supplementary Figures



Supplementary figure 1: top 10 up and down regulated cellular component GO terms in (A) *C. xamachana* and (B) *E. diaphana*



Supplementary figure 2: shared cellular component (CC) GO terms differentially regulated during symbiosis in *E. diaphana* and *C. xamachana*

Chapter 5: Conclusion

In this dissertation I present three distinct research chapters contributing to the understanding of the ways in which the Cnidarian-Symbiodiniaceae photosymbiosis influences immune gene family expansions and immune function. In chapter one I surveyed the proteomes of fifteen species across the cnidarian phylogeny. This work represents the first characterization of innate immune gene repertoires in three classes within clade Medusozoa: Scyphozoa, Cubozoa, and Staurozoa. The breadth and diversity of this study gave new insights into the evolution of innate immune genes, specifically giving higher resolution as to where in the metazoan phylogeny canonical TLRs first arose. Because I surveyed four families of PRRs across a diverse set of taxa, this work also serves as a resource for the field. Cnidarian PRRs often are misannotated or unannotated by traditional genome or transcriptome annotation methods, likely due to the high amount of repeat motifs within them and their unusual domain combinations. The curated lists of PRRs I created can help alleviate this problem in the fifteen species I used and their close relatives. My third chapter serves as an example for how this resource can be utilized. I was able to identify *C. xamachana* PRRs in my gene expression data and found that TLR-like receptors and CTLs are both upregulated in response to gram-negative bacteria.

My second chapter was able to link key life history traits, including symbiosis, with expanded PRR repertoires, likely indicative of a greater need for immune specificity. Furthermore, through my use of the curated *C. xamachana* PRRs in my third chapter I was able to show that the some of the same families implicated in mediating anthozoan symbiosis, CTLs and TLRs, are differentially expressed in the independently evolved *C. xamachana* symbiosis.

This work builds upon previous studies that focused on determining the level of coevolution that occurs between cnidarian hosts and their symbionts, as well as previous studies working to discern the stages of symbiont recognition and the PRRs responsible for that recognition.

In my third chapter I show that tradeoffs between symbiosis and immunity occur across independent evolutions of the cnidarian-Symbiodiniaceae symbiosis. Through the coupling of survival analyses and gene expression data I provided a potential mechanism for this trade off. Contrary to previous hypotheses, my data did not support broadscale immune suppression as the mechanism for this tradeoff, rather it seems that animals are more prone to extreme, damaging inflammatory immune responses while housing symbionts. As a trade-off between the nutrition acquired from Symbiodiniaceae and immunity occurs across independent evolutions of the cnidarian-algal symbiosis, it should be both researched further such that it can be incorporated into the paradigms of coral research. This trade-off implies that researchers should use caution when using symbiont density as a metric for coral health with the assumption that higher symbiont densities are more beneficial. Instead, it may be beneficial to establish a range of healthy symbiont densities at which corals have adequate nutrition at minimalized cost to their immune function. Additionally, my work can help contextualize why some diseases that cause host and symbiont disfunction and dysbiosis, like Stony Coral Tissue Loss Disease (SCTLD), are so devastating. Further investigation into the trade-off between symbiosis and immunity in cnidarians is vital as understanding they ways in which hosting symbionts influences cnidarian's immune responses and susceptibility to various diseases is paramount to mitigating the mounting ecological threat of coral disease.

My work has helped advance *C. xamachana* as a model for studying the cnidarian-Symbiodiniaceae symbiosis through a comparative lens. In my third and fourth chapters I was

able to identify shared traits of this mutualism that occur across independent evolutions. In my fourth chapter I show large-scale differences in the ways in which *C. xamachana* alters its gene expression while hosting symbionts relative to *E. diaphana*. Broadly my work indicates that the cellular and molecular mechanisms of these independently evolved symbioses may be more different that previously thought. Through the addition of gene expression data of symbiotic and aposymbiotic *C. xamachana*, I built upon previous studies on nutrient transfer within the *C. xamachana* symbiosis to show that *C. xamachana* do not show gene expression signatures of limiting their symbionts access to nitrogen. Instead, it is likely that this function is provided by shifts in the bacterial communities of symbiotic animals which may explain *C. xamachana's* high nutrient tolerance. This work lays the foundation for identifying shared and host/taxa specific mechanisms of the cnidarian-Symbiodiniaceae symbiosis and determining how these mechanisms interact with anthropogenic stressors to influence symbiotic cnidarian species' ability to persist in the Anthropocene.

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